FACTORS INFLUENCING FRUITING BODY INITIATION OF *AGARICOMYCETES*

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FACTORS INFLUENCING FRUITING BODY INITIATION OF AGARICOMYCETES

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To my family,

my mother *Adilakshmi Devi* and father *Venkata Reddy*,

my sisters *Shoba* and *Madhavi*,

my late grandmother *Chenchamma*,

my brother in law *Venugopal Reddy* and my nephew *Anand Reddy*,

my father in law *Mohan Reddy* and mother in law Srilakshmi,

my wife *Sridevi Reddy*,

and my son *Avin Reddy*

and to my teachers,

Prof. Dr. Ursula Kües

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Content

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Summary

Macroscopic multicellular fruiting bodies of *Agaricomycetes*, commonly referred to as mushrooms, are the most complex developmental structures which exist in the kingdom of fungi. Fruiting bodies serve in production and distribution of the sexual spores, i.e. of the meiotic basidiospores. Fruiting body and basidiospore formation is a costly process and must thus thoroughly be regulated in order to occur in nature at the right time under the correct environmental conditions. Factors which determine the fruiting process are addressed in this study.

In this study, observations are reported on fruiting body development of species of the frequently fruiting-body-autolysing morphological form group of Coprini (inkcaps, formerly known as the now rejected family *Coprinaceae*) and of related genera of non-autolysing dark-spored *Psathyrellaceae*, the litter decay fungus *Agaricus xanthodermus*, the weak tree pathogen *Schizophyllum commune* and other *Agaricomycetes* on the grounds of the North Campus of the University of Göttingen in 2012 to 2016. Fruiting of the fast growing Coprini occurred at several different months in the year under control of rainfall and temperature, mainly in the autumn months, but especially for *Coprinellus* species also in spring to early summer. Many of the Coprini, such as *Coprinus comatus* from the *Agaricaceae* and many species of the genus *Coprinopsis*, as well as *Lacrymaria lacrymabunda* and various *Panaeolus* and *Psathyrella* species from the *Psathyrellaceae* fruited on grassland, even under higher nitrogen input by animal feces in accordance to ecological descriptions of fungi as coprophilous species (dung fungi) and ammonia fungi. *Coprinopsis* and *Psathyrella* species were comparably often observed in fresh wood chip beds and species of the genus *Coprinellus* were particularly often interlinked with solid wood and also living trees. Perennial fruiting bodies of *S. commune* occurred on twigs and branches of likely weakened shrubs and trees of various broadleaf species and they was observed on mostly still barked deadwood. A colony of *A. xanthodermus* fruited repeatedly over the years underneath a Douglas fir with, in the year 2015, mushroom flushes with infections caused by a conidiogenous ascomycete identified as *Hypomyces odoratus*. Mycelia were isolated from the mycopathogen and from collected host fruiting bodies. The mycopathogens were shown in the laboratory to attack host mushrooms and those of other *Agaricus* species while fruiting bodies of *Pleurotus ostreatus* were largely resistant. Growing and grown mycelium of *A. xanthodermus* and of *P. ostreatus* showed degrees of resistancy, unlike mycelium of *C. cinerea*.

Repeated observations on fruiting body production in nature on several distinct places allow for fungal species and genera crude deductions on growth substrates, life styles and also environmental regulation of fruiting body production. Isolated strains in the laboratory can be used to address fungal behaviour under defined conditions. A new technique is presented for sterile isolation of basidiospores propelled off at maturity from basidia out of fruiting bodies. It makes use of their attraction to electrostatic charged lids of plastic Petri dishes. Germination of spores leads to monokaryotic mycelia which can be mated for the formation of a new dikaryon whereas parental dikaryotic mycelium can be obtained by dissection of protected inner cap and stipe tissues and tissue transfer onto media with antibacterial compounds.

Isolated fertile dikaryotic mycelia with two parental genomes are usually needed in the laboratory for research on fruiting body development. Unmated monokaryons are usually sexually sterile unless they carry mutations, for example, in the mating type loci. One of such self-fertile mutants is strain AmutBmut of the model fungus *C. cinerea*. The strain has previously been further mutated for isolation of mutants with defects in fruiting body formation. A specific defect in the first step of fruiting body formation in one of such mutants

was reported to be caused by a missense mutation in the essential gene *cfs1* in a codon for a putative membrane anchor of a potential cyclopropane fatty acid synthase. In this study, a bacterial acid pH stress test by heterologous gene expression in *Escherichia coli* was applied to confirm the enzymatic function. However, the test appears to be very sensitive to inaccuracies in experimental handling difficult to avoid although in some experimental series the results supported the predicted function. Results obtained on gene *cfs1* remained therefore in the end still unclear. Proteomics analysis proofed that protein Cfs1 was successfully overexpressed in *E. coli* strain BL21(DE3) which in the future might be used in development of a more reliable functional test for the protein.

Other *Agaricomycetes* with published genomes as *C. cinerea* carry copies of *cfs* genes. This genetic situation is different from genes for galectins, small secreted galactose-binding lectins expressed at fruiting body initiation of strain AmutBmut and implicated earlier to confer lectin-bridging in hyphal aggregation. Gene inventories of other sequenced *Agaricomycetes* revealed that very few other species (e.g. *Heterobasidion irregulare*, *Laccaria bicolor*) carry any galectin genes. It was therefore concluded that the galectin genes cannot present essential functions in hyphal aggregation in fruiting body development of *Agaricomycetes*, in accordance to a meanwhile established function as defense proteins against nematodes presented in the literature of the *C. cinerea* galectins. Similar restricted distributions of genes were observed variably over some unrelated species of *Agaricomycetes* from gene inventories for other small secreted proteins (simple lectins, membrane pore-forming lectins and membrane pore-forming hemolysins, some of which are in the meantime also proven to have defense functions) that were reported previously in the literature to be expressed in individual species at initation or during fruiting. Also these were then considered as non-essential for fruiting.

Further gene inventories are presented in this study for genes in the thiamin (vitamin B1) biosynthesis pathway of *Agaricomycetes*. Most species of the *Agaricomycetes* are thiamin-auxotrophs which is due to the loss of the *thi5* gene for HMP (hydroxymethyl pyrimidine) phosphate synthase. Loss of *thi5* explains why supplementation of thiamin to media can enhance the fruiting body formation of *S. commune* as reported in the literature. In own first experiments, addition of thiamin to growth media of *C. cinerea* enhanced mycelial growth speed in particular in the defined basidiomycete standard medium (BSM) and may have positively also affected fruiting frequencies in YMG/T complete medium. Analyses of effects of thiamin on growth and fruiting deserve more work in the future.

Zusammenfassung

Makroskopische vielzellige Fruchtkörper von Agaricomyceten, allgemein einfach auch nur als „Pilze“ bezeichnet, sind die komplexesten Entwicklungsstrukturen, die im Reich der Pilze existieren. Fruchtkörper dienen zur Produktion und Verteilung der sexuellen Sporen, d. h. der meiotischen Basidiosporen. Die Bildung von Fruchtkörpern und Basidiosporen ist kostspielig und muss daher sorgfältig reguliert werden, um in der Natur zum richtigen Zeitpunkt unter den richtigen Umgebungsbedingungen stattzufinden. Diese Arbeit behandelt Faktoren im Prozess der Fruchtkörperbildung.

Zuerst werden in dieser Studie Beobachtungen berichtet zur Fruchtkörperentwicklung von Arten der morphologischen Formgruppe von Coprini mit oftmals autolysierenden Fruchtkörpern (Tintlinge, früher bekannt als die jetzt verworfene Familie *Coprinaceae*) und verwandter Gattungen von nicht autolysierenden dunkelsporigen *Psathyrellaceae*, sowie von dem Streu abbauenden Pilz *Agaricus xanthodermus*, von dem schwachen Baumpathogen *Schizophyllum commune* und von anderen auf dem Gelände des Nordcampus der Universität Göttingen in den Jahren 2012 bis 2016 beobachteten Agaricomyceten. Fruchtkörperbildung bei den sich schnell entwickelnden Coprini erfolgte in verschiedenen Monaten eines Jahres, abgängig von Niederschlag und Temperatur. Coprini fruchteten hauptsächlich in den Herbstmonaten, aber insbesondere *Coprinellus*-Arten auch im Frühjahr bis Frühsommer. Viele Coprini, wie *Coprinus comatus* aus der Familie *Agaricaceae* und diverse Arten der Gattung *Coprinopsis*, sowie *Lacrymaria lacrymabunda* und verschiedene *Panaeolus*- und *Psathyrella*-Arten aus der Familie der *Psathyrellaceae* produzierten auf Grünland Fruchtkörper, u.a. einige auch unter einem höheren Stickstoffeintrag durch tierische Fäkalien im Einklang mit einer Einstufung der Pilze als koprophile Arten (Mistpilze) und „ammonia fungi“. *Coprinopsis*- und *Psathyrella*-Arten wurden vergleichsweise häufig in frischen Hackschnitzelbetten beobachtet, Arten der Gattung *Coprinellus* wurden besonders häufig im Zusammenhang mit Massivholz und auch lebenden Bäumen gefunden. Mehrjährige Fruchtkörper von *S. commune* traten an Zweigen und Ästen offenbar geschwächter Sträucher und Bäume verschiedener Laubbaumarten auf und wurden auf meist noch berindetem Totholz beobachtet. Eine Kolonie von *A. xanthodermus* fruchtete im Laufe der Jahre wiederholt unter einer Douglasie, wobei im Jahr 2015 wiederholt Infektionen auf Fruchtkörpern auftraten, die durch einen als *Hypomyces odoratus* identifizierten konidiogenen Ascomyceten verursacht wurden. Mycelien wurden von dem Mykopathogen und aus Fruchtkörpern des Wirtes isoliert. Im Labor wurde gezeigt, dass die mykopathogenen Isolate Fruchtkörper dieses Wirtes und solche anderer *Agaricus*-Arten befallen, während Fruchtkörper von *Pleurotus ostreatus* weitgehend resistent waren. Wachsendes und gewachsenes Myzel von *A. xanthodermus* und *P. ostreatus* zeigten im Gegensatz zum Myzel von *C. cinerea* gute Resistenz.

Wiederholte Beobachtungen zur Fruchtkörperproduktion in der Natur an verschiedenen Orten ermöglichen erste Rückschlüsse zu Substraten für Wachstum von Pilzenarten und -gattungen, zu Lebenszyklen und zur Regulierung der Fruchtkörper-produktion durch Umweltfaktoren. Isolierte Stämme können dann im Labor verwendet werden, um das Pilzverhalten unter definierten Bedingungen zu untersuchen. Eine neue Technik zur sterilen Isolierung von Basidiosporen wird vorgestellt, die von Basidien aus reifen Fruchtkörpern heraus abgeschossen werden. Die Methode nutzt die Anziehung der Sporen durch elektrostatisch aufgeladene Deckel von Kunststoff-Petrischalen. Keimung von Sporen führt dann zu monokaryotischen Mycelien, die zur Bildung eines neuen Dikaryons gepaart werden können. Elterliches dikaryotisches Mycel kann hingegen durch Sezieren von innerem geschützen Hut- und Stielgewebe und Transfer von Gewebestücken auf Medien mit antibakteriellen Verbindungen erhalten werden.

Isolierte fertile dikaryotische Mycelien mit zwei Elterngenomen werden im Labor normalerweise für die Erforschung der Fruchtkörperentwicklung benötigt. Nicht verpaarte Monokaryonen sind normalerweise sexuell steril, es sei denn, sie tragen Mutationen, wie beispielsweise in den Kreuzungstypgenen. Eine solche selbstfertile Mutante ist der Stamm AmutBmut des Modellpilzes *C. cinerea*. Von diesem Stamm wurden in früheren Arbeiten Mutanten mit Defekten in der Fruchtkörperbildung isoliert. Der Defekt in einer solchen Mutante basiert auf einer Missense-Mutation im Gen *cfs1* in einem Codon für einen mutmaßlichen Membrananker einer potenziellen Cyclopropan-Fettsäure-Synthase, die für den ersten Schritt der Fruchtkörperbildung essentiell ist. In dieser Arbeit wurde ein bakterieller Essigsäure-pH-Stresstest durch heterologe Expression des Genes in *Escherichia coli* angewendet, um die enzymatische Funktion zu bestätigen. Obwohl in einigen Versuchsreihen die erhaltenen Ergebnisse die vorhergesagte Funktion unterstützten, scheint der Test jedoch sehr empfindlich gegenüber schwer vermeidbarer Ungenauigkeiten bei der experimentellen Handhabung zu sein. Die mit dem Gen *cfs1* erhaltenen Ergebnisse des Testes blieben daher am Ende in der Aussage offen. Proteomanalyse konnte aber zeigen, dass das Protein Cfs1 im *E. coli*-Stamm BL21 (DE3) erfolgreich überexprimiert wurde und in der Zukunft zur Entwicklung eines zuverlässigeren Funktionstests für das Protein verwendet werden kann.

Andere Agaricomyceten mit publizierten Genomen als *C. cinerea* tragen enenfalls Kopien von *cfs*-Genen. Diese genetische Situation unterscheidet sich von Genen für Galectine, das sind kleine sekretierte Galactose-bindende Lectine, die bei der Initiierung der Fruchtkörper des Stammes AmutBmut exprimiert werden und früher mit einer möglichen Funktion über Lektinbrückenbildung bei der Hyphenaggregation im Fruchten in Verbindung gebracht wurden. Geninventuren anderer sequenzierter Agaricomyceten zeigten, dass nur sehr wenige andere Arten (z. B. *Heterobasidion irregulare*, *Laccaria bicolor*) Galectin-Gene tragen. Es wurde daher der Schluss gezogen, dass die Galectin-Gene keine wesentlichen Funktionen bei der Hyphenaggregation bei der Fruchtkörperentwicklung von Agaricomyceten aufweisen können, weiter unterstützt durch Berichte in der Literatur über eine inzwischen etablierte Funktion der *C. cinerea*-Galectine als Abwehrproteine gegen Nematoden. Ähnliche begrenzte Verteilungen von Genen über jeweils wechselnde wenige nichtverwandte Arten von Agaricomyceten wurden in weiteren Inventuren gefunden für andere kleine sekretierte Proteine (einfache Lektine, membranporenbildende Lektine und membranporenbildende Hämolysine, wobei bei einigen inzwischen ebenfalls auch Abwehrfunktionen nachgewiesen wurden), die zu Beginn oder während der Fruchtkörperbildung von ihren Wirten produziert werden. Auch diese wurden daher dann als nicht essentiel für die Fruchtbildung eingeordent.

Weitere Geninventuren in dieser Arbeit wurden für Gene im Thiamin (Vitamin B1) -Biosyntheseweg von Agaricomyceten durchgeführt. Die meisten Arten von Agaricomyceten sind Thiamin-Auxotrophe, was auf den Verlust des *thi5*-Gens für die HMP (Hydroxymethylpyrimidin)-Phosphatsynthase zurückzuführen ist. Der Verlust von *thi5* erklärt Literaturberichte, warum Zugabe von Thiamin zu Wachstumsmedien die Fruchtkörperbildung von *S. commune* verbessern kann. In eigenen Experimenten verbesserte die Zugabe von Thiamin zu Wachstumsmedien von *C. cinerea* die Myzelwachstumsgeschwindigkeit, insbesondere auf dem definierten Basidiomyceten-Standardmedium (BSM), und verbesserte möglicherweise auch die Frequenz des Fruchtens des Pilzes auf YMG/T-Vollmedium. Weitere Arbeiten über Auswirkungen von Thiamin auf Wachstum und Fruchten sollten in der Zukunft zur weiteren Klärung durchgeführt werden.

Chapter 1

Agaricomycetes and their Fruiting bodies: General Introduction

This chapter provides a general introduction into the ecology of fruiting body development of *Agaricomycetes* and related processes.

Contributions: Kiran Lakkireddy (KL) defined and arranged the specific subjects addressed in this chapter, collected and read relevant literature, observed and photographed mushrooms in nature and selected the collection of photos for the chapter. KL prepared drawings, wrote the draft of the chapter and interacted with Ursula Kües (UK) in discussions to finalize the writing of this chapter. UK is acknowledged for input of detailed knowledge on the subjects of the chapter, for providing literature, optimizing structure and writing of the chapter, and for the supply of Fig. 21. Shanta Subba kindly supplied Fig. 23. Weeradaj Khonsuntia cooperatively shared collected literature.
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Chapter 1

Agaricomycetes and their Fruiting Bodies: General Introduction

Fungi are eukaryotes but are distinct from both the kingdoms of plants and animals and form an own kingdom. They occur in every environment on earth and play very important roles in most ecosystems. Fungi have important biogeochemical roles in the biosphere and they degrade plant materials including wood and other natural products and also abiotic materials such as concrete and stone (Gadd 2007). Mycologists are constantly progressing significant advances in species discovery. Based on high-throughput sequencing methods, the worldwide number of fungal species is nowadays estimated as 5.1 million (Blackwell 2011). The kingdom of *Fungi* is divided into at least eight phyla with the *Ascomycota* and the *Basidiomycota* forming the subkingdom of *Dikarya* (Hibbett et al. 2007; Bauer et al. 2015; Spatafora et al. 2016) and possibly the *Entorrhizomycota* with a few smut-like teliosporic species as a third new phylum of the Dikarya (Bauer et al. 2015; Zhao et al. 2017). Ascomycetes and basidiomycetes are characterized by sexual reproductive phases with specific cells (asci and basidia, respectively) undergoing karyogamy and meiosis for genetically recombined meiospore production (phase of life cycle traditionally referred to as teleomorphs) and possibly by asexual phases which form by mitosis genetically identical mitospores (phase in subsidiary cycles traditionally referred to as anamorphs) (Kendrick 2011). For sexual reproduction, filamentous species in both main phyla of the *Dikarya* (*Ascomycota* and *Basidiomycota*) can form multicellular fruiting bodies, the larger of which are commonly known as mushrooms. Mushrooms are spore-bearing fruiting bodies of fungi of macroscopic size which can easily be seen by the naked eye and be picked by hand. Fruiting bodies are produced above or below ground in soil or on suitable substrates of mostly plant origin (Chang and Miles 1992). The number of mushrooms on Earth is estimated as 140,000 species. Most of the mushrooms forming species are basidiomycetes (Hawksworth 2001). The *Basidiomycota* represent the second largest phylum of the kingdom of *Fungi* (Kirk et al. 2001). *Basidiomycota* are either yeasts or, more often, filamentous fungi composed of hyphae (except for yeasts) and reproduce sexually via the formation of specialized club-shaped cells called basidia. The basidia carry usually four meiospores called basidiospores (Kendrick 2011).

The *Basidiomycota* are divided into three major groups (subphyla) which are called *Pucciniomycotina* (rusts), *Ustilaginomycotina* (smuts) and *Agaricomycotina* (many of which form mushrooms) (Bauer et al. 2006; Hibbett et al. 2007) although a most recent phylogenetic study suggests that the *Wallemiomycetes* might form a class of an own subphylum *Wallemiomycotina* (Zhao et al. 2017). The *Agaricomycotina* enclose 20,000 species which are described and which represent 68% of the known *Basidiomycota*. Based on various phylogenetic studies, the *Agaricomycotina* include the classes of (possibly) the *Wallemiomycetes* (one genus with mycelial mitosporic fungi) and *Genimibasidiomycetes* (with yeast-like species) as basal clades and the classes of the *Dacrymycetes* (jelly fungi named so by the jelly-like consistency of their fruiting bodies), the *Tremellomycetes* (groups of yeasts and fungi with mushrooms of also gelatinous and rubbery-like consistency), and the *Agaricomycetes* which mostly form sizable fruiting bodies (open mushrooms, bracket fungi, puffballs, resupinate species and others) which may have hymenia with basidia for basidiospore production on lamellae, ridges or pores, or which may have a basidia-producing hyphal tissue locked within a closed mushroom. Fleshy mushrooms may last only for a few days while fruiting bodies in other species may be firm and durable (Hibbett et al. 2007, 2014; Kües and Navarro-González 2015; Nguyen et al. 2015; Zhao et al. 2017; Fig. 1 and 2 – species in these and other figures in this chapter have been observed and photographed by the

Fig. 1 Examples of fleshy lamellate fruiting bodies of different shapes, sizes and colours. **A.** *Amanita strobiliformis* (06.09.2014), **B.** *Hygrocybe virginea* (12.11.2011), **C.** *Parasola plicatilis* (11.11.2011), **D.** *Coprinus comatus* (28.09.2011), **E.** *Hygrocybe conica* (10.07.2012), **F.** *Baeospora myosura* (19.09.2014), **G.** *Hygrophoropsis aurantiaca* (12.11.2011), **H.** *Rickenella fibula* (11.11.2011), **I.** *Hypholoma fasciculare* (14.09.2014), **J.** *Psathyrella conopilus* (11.10.2014), and **K.** *Flammulina velutipes* (25.01.2014). Mushrooms were found on the grounds of the North Campus of the University of Göttingen (**A-C**, **E-I**, **K**) and on the cemetery of Weende-Dorf (**D**, **J**).

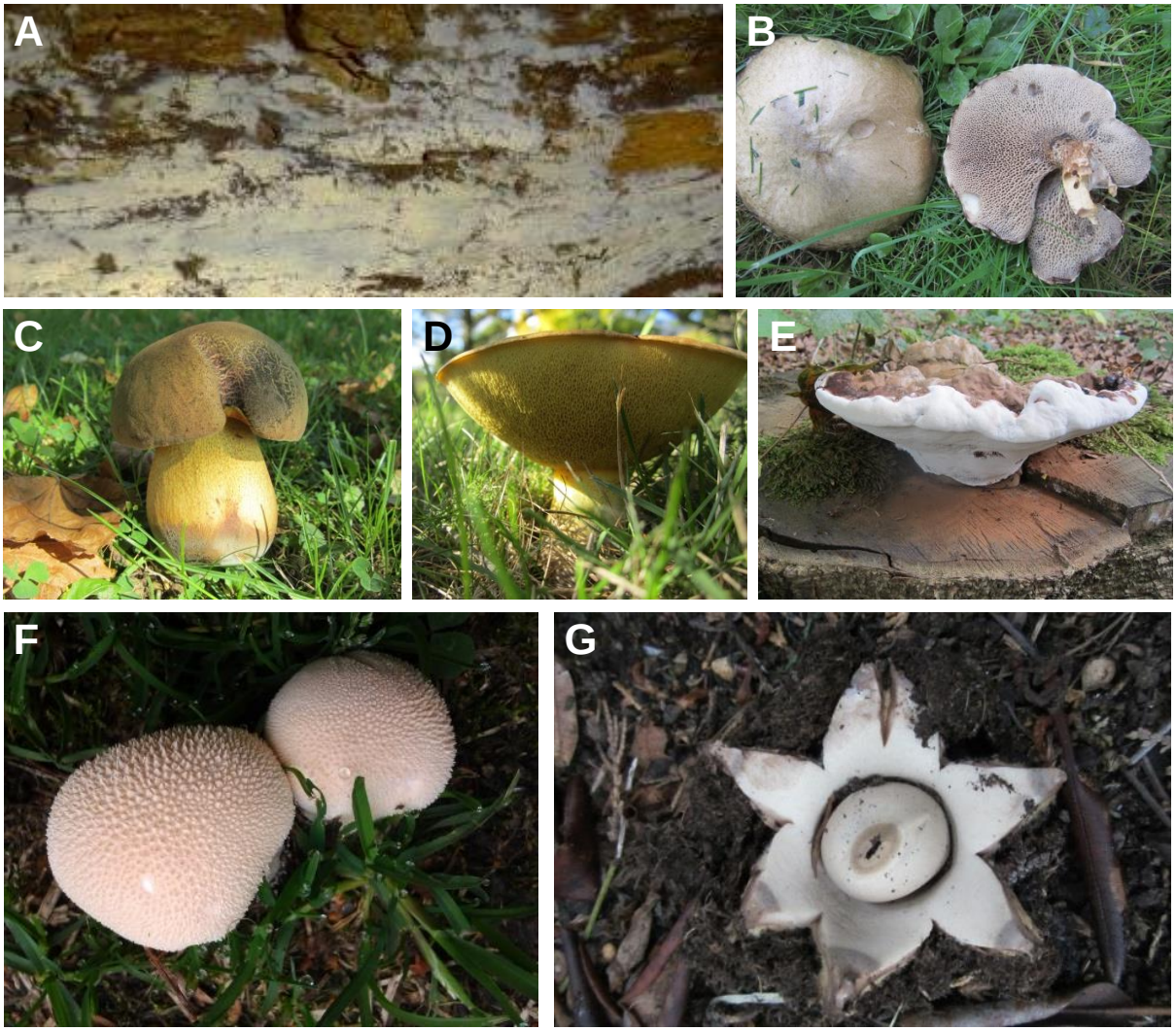

Fig. 2 Examples of a crusteous fruiting body, of porous fleshy and durable mushrooms of different shapes, sizes and colours and of gasteroid mushrooms of different shapes. **A.** Unidentified basidiomycete (29.09.2013), **B.** *Ceriporia reticulatus* (29.09.2014), **C.** *Neoboletus luridiformis* (14.09.2014), **D.** *Suillus bovinus* (27.09.2013), **E.** *Ganoderma applanatum* (06.09.2014), **F.** *Lycoperdon perlatum* (22.09.2013), and **G.** *Geastrum rufescens* (30.09.2014). Mushrooms were found on the grounds of the North Campus of the University of Göttingen (**B**-**E**), on the cemetery of Weende-Dorf (**F**, **G**) and in the Schlossgarten Karlsruhe (**A**).

author of the PhD thesis and determined by using the field guides of Breitenbach and Kränzlin 1986, 1991, 1995) and especially for the Coprini by using the *Coprinus* website of Kees Uljé (http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm). The development of fruiting bodies in the *Agaricomycetes* is the most complex process known in the fungal kingdom (Ohm et al. 2010; Stajich et al. 2010; Kües and Navarro-Gonzaléz 2015). Not only for this reason, numerous species of the *Agaricomycetes* have been placed in an important position in fungal research.

The *Agaricomycetes* contain a wide variety of fungi in terms of life style (Fig. 3 to 5). Some mushrooms are wood decayers (white rot fungi – examples are *Armillaria mellea*, *Auricularia auricula-judae*, *Ganoderma applanatum*, *Heterobasidion irregulare*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor*, and *Tremella*

Fig. 3 Mushrooms of wood-decaying species.
A. Jelly fungus *Auricularia auricula-judae* (08.10.2014), **B.** *Kuehneromyces mutabilis* (19.11.2011), **C.** *Pleurotus ostreatus* (05.11.2014), **D.** *Gloeophyllum sepiarium* (21.10.2014), **E.** *Trametes versicolor* (01.11.2011), **F.** *Pycnoporus cinnabarinus* (15.10.2013), **G.** *Schizophyllum commune* (23.11.2011), **H.** *Laetiporus sulphureus* (01.08.2014), and **I.** *Pholiota squarrosa* (15.11.2011). Mushrooms were found on the North Campus of the University of Göttingen (**A**, **C**, **D**, **F**, **H**) and in the forest vegetation of the Billinghäuser Schlucht in Göttingen (**B**, **E**, **G**, **I**).

Fig. 4 Saprotrophic mushrooms on other substrates.
A. *Coprinellus plagioporus* (16.10.2014), **B.** *Psathyrella conopilus* (11.09.2011), **C.** *Psathyrella multipedata* (26.10.2014), **D.** *Lacrymaria lacrymabunda* (09.11.2014), **E.** *Psathyrella tephrophylla* (06.11.2011), **F.** *Psathyrella spadiceogrisea* (05.11.2016), **G.** *Tubaria hiemalis* (02.01.2012), and **H.** *Baeospora myosura* (15.10.2013). Mushrooms were found on the grounds of the North Campus of the University of Göttingen (**A**, **B**, **E**-**H**) and on the cemetery of Weende-Dorf (**C**, **D**).

Fig. 5 Mushrooms of the mycorrhizal species *Suillus bovinus* (marked by arrows in A.) found underneath an *Abies homolepis* (Nikko fir) tree on the grounds of the North Campus of the University of Göttingen (27.09.2013).
Mushrooms shown in **B.** are those seen in the lower left corner of **A.**

mesenterica; brown rot fungi – examples are *Fomitopsis pinicola*, *Postia placenta*, and *Serpula lacrymans*), some are plant litter decomposers [e.g. *Agrocybe cylindracea* (*Agrocybe aegerita*), *Coprinellus disseminatus*, *Inocybe erubescens*, *Psilocybe cubensis*, and *Psilocybe cyanescens*] and others can grow on compost or dung (such as *Agaricus bisporus* and *Coprinopsis cinerea*). Some can associate with plants - mycorrhizal species as symbionts (forming ectomycorrhizae - for example *Amanita phalloides*, *Amanita muscaria*, *Boletus edulis*, and *Laccaria bicolor*; forming orchid mycorrhizae – for example *A. mellea* and *Mycena orchidicola*) and others can be plant pathogens (strong to moderate pathogens – examples are *A. mellea*, *G. applanatum*, *H. irregulare*, *Inonotus obliquus*, *Moniliophthora perniciosa*, and *T. versicolor*; weak pathogens – examples are *Laetiporus sulphureus* and *Schizophyllum commune*) (Rasmussen 2002; Hibbett 2006; Lakkireddy et al. 2017 and Chapter 3 – Part II; de Mattos-Shipley et al. 2016). The borders between different life styles can be fluent, e.g. between white-rot and brown-rot (Riley et al. 2014; Floudas et al. 2015), between litter and wood degraders (Navarro-Gonzaléz 2008), between saprotrophs and plant pathogens, between saprotrophs and mycorrhizal species, between mycorrhizal species and pathogens (Martos et al. 2009; Muszynska et al. 2011; de Mattos-Shipley et al. 2016; Hibbett 2006). There are furthermore more rare life styles. Some mushroom species can have mutualistic relationships with insects (e.g. *Leucoagaricus gongylophorus*) (Aylward et al. 2013; de Mattos-Shipley et al. 2016), or with algae to form lichens (e.g. *Dictyonema glabratum* and *Thanatephorus* species) (Lücking et al. 2014; Diederich et al. 2014), and an increasing number of reports exist of *Agaricomycetes* as endophytes with potential effects on their hosts in plant-growth promotion and defence against pathogens and pests (Franken et al. 2012; Martin et al. 2015; Murata et al. 2015; Robles et al. 2015; Solis et al. 2016). Others can be mycotrophic [(e.g. *Asterophora lycoperdoides*, *Enteloma abortivum*, and *Squamanita* species) (Ditmar 1809; Redhead et al. 1994; Czederpiltz et al. 2001; Mondiet et al. 2007) or, as a species from the *Tremellomycetes*, *Tremella mesenterica* (Zugmaier et al. 1994)]. Some of the *Agaricomycotina* are also pathogens to humans and animals (such as the yeast *Cryptococcus neoformans*, also from the *Tremellomycetes*) or may be opportunistic infective in immunosuppressed individuals (such as the filamentous *S. commune*) (Hibbett 2006; Chowdhary et al. 2013).

The functions of a fruiting body for a fungal species are the production of sexual spores and via karyogamy and meiosis the recombination of the genetic material within

populations (Kües and Navarro-González 2015). In addition, as ecological functions, mushrooms can be food sources for slugs, ants, birds and insects (beetles and termites) but also for various species of mammals (e.g. squirrels and mice) and humans (Keller and Snell 2002; Witte and Maschwitz 2008; Simpson 2000; Mueller and Gerardo 2002; Fig. 6 and 7). However, mushrooms eaten by some types of animals might be toxic to others and to humans, or also vice versa (Beug and Shaw 2009; Elliott 1922; Michelot and Melendez-Howell 2003, Sabotic et al. 2012; Spiteller 2008). Mushrooms with toxic effects to humans are commonly called toadstools. The most poisonous mushroom toxins to humans are produced by the death cap (*A. phalloides*), the fly agaric (*A. muscaria*) and the deadly fibrecap (*I. erubescens*) (Bresinsky and Besl 1990; de Mattos-Shipley et al. 2016; Hibbett 2006). While the red colour with the white spots (reflecting in matured fruiting bodies rests of the young mushroom´s veil) on the cap of the fly agaric is known to every child (Fig. 8A), many poisonous mushrooms do not show principle colour differences as compared to edible mushrooms (compare e.g. Fig. 8 and 9) but they are more likely to indicate different odors (and perhaps tastes) to alert fungivores (Sherratt et al. 2005). The threats which such mushrooms present to humans are often indicated in their common names (see legend to Fig. 8) whereas names of edible species are often innocuous (see legend to Fig. 9).

Some mushrooms are completely toxin-free (for humans), but are usually considered non-edible by bad (e.g. *Tylopilus felleus*, the bitter bolete) or missing taste (e.g. the amethyst deceiver *Laccaria amethystina*) and by hard texture (e.g. the turkey tail *T. versicolor*) even though for some of these, there might be societies in some countries who also eat such species usually ignored by others for such reasons (Boa 2004). Moreover, while toxins of mushrooms might be harmful to humans, toxins can also have good medicinal effects if well applied. Indeed, many mushrooms, both toxic and non-toxic species, might not be considered for human food supply but are recognized organisms in ethnobotany of many countries for their potential in conferring good health and curing of illnesses. Particularly East-European and East-Asian cultures make traditionally, but also in modern medicine, use of mushrooms for health purposes (Kües et al. 2004, 2015; Wasser 2011). Among, the white rot *G. applanatum* is a highly recognized medicinal species in East-Asia of which also well established cultivation techniques exist (Zhou et al. 2012; Kües et al. 2015). Some other of the above mentioned mushrooms also produce medicinally relevant metabolites of different nature such as a range of specific secondary metabolites and polysaccharides (*A. auricula-judae*, *I. obliquus*, *P. cubensis*, *P. cyanescens*, *S. commune*, *T. versicolor*). Some of these produce hallucinogens, drugs that cause hallucinations, such as the magic mushrooms (*P. cubensis* and *P. cyanescens*) (de Mattos-Shipley et al. 2016; Hibbett 2006). Recent studies have presented preliminary results promising to treat obsessive-compulsive disorder, alcohol and smoking by psilocybin which naturally occurs in magic mushroom species (Carhart-Harris et al. 2016). Many other mushrooms have also great potential for production of useful bioactive metabolites and they are sources for drugs which show pharmacological effects (Lindequist et al. 2005). Generally, it can be assumed that the rich pharmacological potential that mushrooms offer is by far not exploited (Wasser 2011).

The edible mushrooms of the *Agaricomycetes* with their unique nutrient profiles have long been a part of the human diet, going back to ancient times (Jo Feeney et al. 2014; Kües and Liu 2000; Wasser 2011). Many mushrooms are an excellent source of nutrients and contain valuable amounts of proteins, vitamins, fiber, and minerals. Among the so far mentioned species, *A. auricularia-judae*, *A. bisporus*, *A. cylindraceae*, *A. mellea*, *B. edulis*, *C. cinerea*, *P. ostreatus*, *T. mesenterica*, and *S. commune* can be named as known edible species. Some of these are collected from the wild such as the ectomycorrhizal species (*B. edulis*) and tree pathogens (*A. mellea*), some are worldwide cultured on broad scale (*A. bisporus* and *P. ostreatus*), some are cultivated as speciality mushrooms (*A. cylindraceae*),

Fig. 6 Examples of lamellate mushrooms eaten by animals.
A. *Agaricus xanthodermus* (30.08.2014), **B.** *Agaricus campestris* (30.08.2014), **C.** *Lacrymaria lacrymabunda* (30.08.2014), **D.** *Coprinopsis picacea* (29.09.2013), **E.** *Coprinopsis atramentaria* (27.09.2011), **F.** *Coprinellus domesticus* (11.09.2011), **G.** *Coprinellus disseminatus* (13.09.2015), **H.** *Lepiota castaneidisca* (22.09.2013), **I., J.** *Lactarius deliciosus* (06.09.2014, (08.09.2014), **K.** *Hypholoma subericaeum* (29.09.2013), **L.** *Pholiota jahnii* (08.10.2014), **M.** *Pleurotus ostreatus* (28.07.2014), **N.** *Russula rosea* (30.08.2014), and **O.** *Russula exalbicans* (19.07.2011). Mushrooms were found on the North Campus of the University of Göttingen (**E-G**, **I**, **J**, **L-O**), on the cemetery of Weende-Dorf (**A-C**, **H**) and in the Schlossgarten Karlsruhe (**D**, **K**).

Fig. 7 Examples of poroid mushrooms eaten by animals.
A. *Boletus longicurvipes* (06.09.2014), **B.** *Ceriporia reticulatus* (29.09.2014), **C.** *Suillellus luridus* (09.07.2012), **D., F.** *Suillellus queletii* (09.07.2012), **E.** *Boletus splendidus* (11.07.2012), and **G.** *Xerocomus chrysenteron* (09.07.2012). All mushrooms were found on the grounds of the North Campus of the University of Göttingen.

others are particularly grown in several East-Asian countries including China (*A. auricularia-judae* and *T. mesenterica*) or only in specific countries such as Thailand (*C. cinerea* and *S. commune*) (Bao 2004; Kües et al. 2007; Rühl and Kües 2007). Cultivation of mushrooms tackles two major challenges, supplying enough food for the growing human population and reducing waste and environmental pollution when using agricultural and forestry wastes as growth substrates (de Mattos-Shipley et al. 2016). The modern cultivation techniques are helpful to produce huge quantities of various cultivated mushrooms and they can be marketed throughout the year, independently from growth seasons in the wild. Commercial mushroom cultivation is however restricted to the saprotrophic species living on wood, straw and other plant litter. Not surprisingly therefore, the two most popular cultivated mushrooms in the

Fig. 8 Selection of mushrooms known to be poisonous to humans.
A. *Amanita muscaria* (fly agaric, 03.11.2015), **B.** *Leucocoprinus birnbaumii* (flowerpot parasol, 03.08.2012), **C.** *Russula emetica* (vomiting russula, 22.09.2013), **D.** *Hygrocybe conica* (witch´s hat, 10.07.2012), **E.** *Clitocybe phyllophila* (frosty funnel mushroom, 26.10.2016), **F.** *Agaricus xanthodermus* (yellow-staining mushroom; 03.09.2015), **G.** *Amanita phalloides* (death cap, 20.09.2015), **H.** *Amanita strobiliformis* (warted amanita, 10.07.2016), **I.** *Paxillus involutus* (poising pax, 15.10.2016), **J.** *Pholiota squarrosa* (shaggy scalycap, 31.10.2016), **K.** *Entoloma sinuatum* (lead poisoner, 08.09.2014), **L.** *Hypholoma fasciculare* (sulfur tuft, 27.10.2016), **M.** *Boletus splendidus* (satan´s bolete, 11.07.2012), **N.** *Boletus rhodoxanthus* (11.07.2012), **O.** *Caloboletus calopus* (bitter beech bolete, 30.08.2014), and **P.** *Suillellus luridus* (lurid bolete, 10.07.2012). Mushrooms were found on the grounds of the North campus of the University of Göttingen (**D**-**P**; **B**: in a flowerpot in a student´s office), in the forest vegetation of the Göttingen Klausberge (**A**), and in the Schlossgarten Karlsruhe (**C**).

world are the button mushroom (*A. bisporus*) and the oyster mushroom (*P. ostreatus*) which easily grow and fruit on compost and straw and wood debris, respectively (Rühl and Kües 2007; Sanchez 2010). Mycorrhizal fungi in contrast are very difficult or impossible to cultivate on commercial scale. If at all, mycorrhizal species might specifically be inoculated onto susceptible tree species for outdoor planting (Kües and Martin 2011).

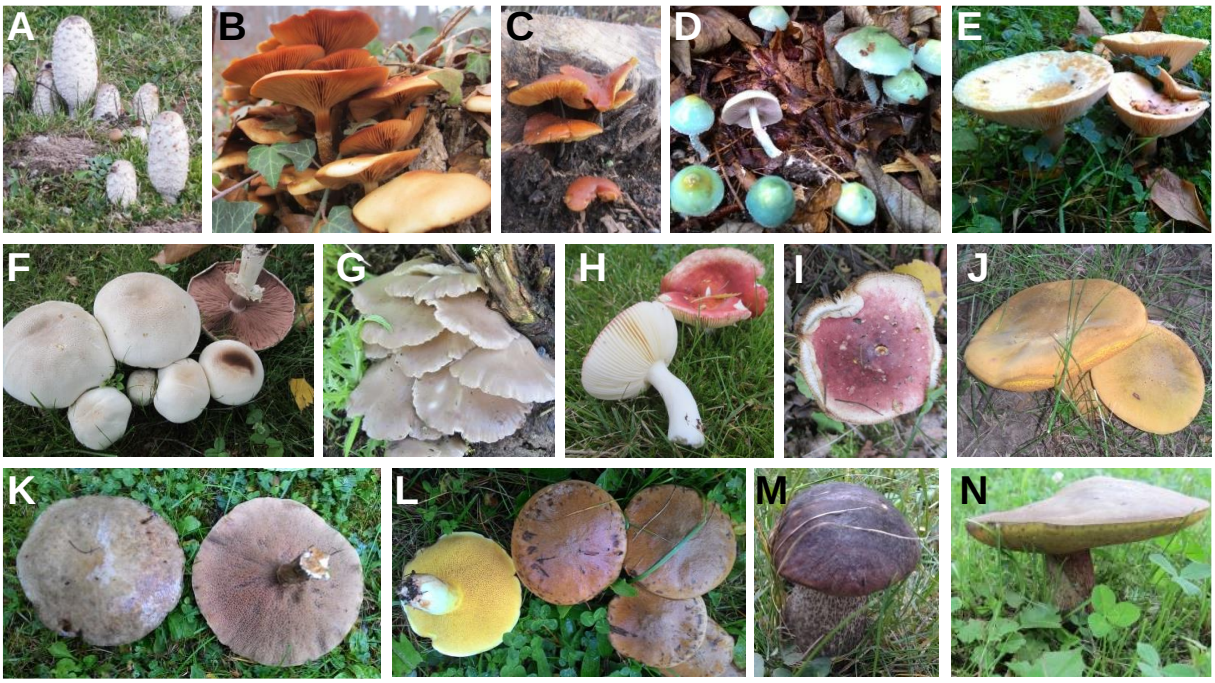

Fig. 9 Selection of edible mushrooms.
A. *Coprinus comatus* (lawyer wig, 05.11.2011), **B.** *Kuehneromyces mutabilis* (sheathed woodtuft, 19.11.2011), **C.** *Flammulina velutipes* (velvet foot, 24.11.2015), **D.** *Stropharia aeruginosa* (verdigris agaric, edibility in dispute, 24.10.2016), **E.** *Lactarius deliciosus* (red pine mushroom, 18.10.2016), **F.** *Agaricus campestris* (field mushroom, 30.08.2014), **G.** *Pleurotus ostreatus* (oyster mushroom, 11.06.2014), **H.** *Russula exalbicans* (19.09.2015), **I.** *Russula rosea* (rosy russula, 25.09.2015), **J.** *Suillus variegatus* (velvet bolete, 09.07.2016), **K.** *Suillus bovinus* (bovine bolete, 27.10.2016), **L.** *Boletus reticulatus* (21.10.2016), **M.** *Leccinum scabrum* (birch bolete, 22.09.2013), and **N.** *Chalciporus piperatus* (peppery bolete, 25.06.2012). Mushrooms were found on the grounds of the North Campus of the University of Göttingen (**A**, **C**-.**E**, **G**-**L**, **N**), in the forest vegetation of the Billinghäuser Schlucht in Göttingen (**B**), on the cemetery of Weende-Dorf (**F**), and in the Schlossgarten Karlsruhe (**M**).

Outside, one may not to have to go far to find mushrooms in nature. They occur in gardens, parks and other public areas with lawns, plant beds, shrubs and trees (such as on the grounds of the North-Campus of the University of Göttingen, Fig. 1 to 12), on meadows (Fig. 11), particularly species-rich on cemeteries (such as on the cemetery in Weende-Dorf, Fig. 12), and of course in forests (Fig. 13). In nature, many species of fungi produce fruiting bodies that can only be observed for a few days in order to decay quickly upon spore release such as for example many of the inkcaps, a morphological group traditionally referred to as the Coprini (see e.g. Figs. 1D, 4A, 6D-G, 9A, 10, 11, 12E, 13E, 14, 15). Mushroom production may change from year to year. When a mushroom flourishes depends highly on climatic conditions and the time of fruiting depends on the geographical location (Boddy et al. 2014). Global warming changes climatic conditions and it directly has effects on distribution of fruiting patterns of mushrooms over the year, on general fungal productivity (mycelial growth and fruiting), on amounts of sporocarp production and on fruiting body sizes. Saprotrophic species thereby react more sensitive to climate changes as compared to mycorrhizal species (Büntgen et al. 2012; Sato et al. 2012; Gange et al. 2011; Ágreda et al. 2016). On the other hand, mycorrhizal species react generally more sensitive to soil disturbances and nutrient and pH changes than the saprotrophs (Arnolds 1991; Newbound et al. 2012).

Fig. 10 Coprini from the *Psathyrellaceae* on wood chips underneath shrubs and trees. **A.** *Coprinellus marculentus* (04.09.2014), **B.** *Coprinellus impatiens* (22.08.2015), **C.** *Coprinopsis ephemeroides* (28.10.2015), **D., E.** *Coprinopsis macrocephala* (13.06.2012, 23.09.2011), **F.** *Coprinopsis stercorea* (29.09.2013), **G.** *Coprinopsis lagopus* (22.10.2016), **H.** *Coprinopsis cothurnata* (17.10.2016), **I.** *Psathyrella conopilus* (18.07.2012), **J.** *Psathyrella candolleana* (18.09.2012), **K.** *Psathyrella marcescibilis* (24.10.2015), **L.** *Psathyrella gracilis* (26.10.2014), **M.** *Psathyrella spadiceogrisea* (22.10.2016), **N.** *Lacrymaria lacrymabunda* (18.09.2012), **O., P.** *Panaeolus antillarum* (30.10.2015, 25.09.2015). Mushrooms were found on the grounds of the North Campus of the University of Göttingen (**A-E**, **G-P**) and in the Schlossgarten Karlsruhe (**F**). Wood chips were spread underneath trees of *Prunus domestica* (plum, **B**), *Acer saccharum* (sugar marple, **D**, **E**, **I**), *Quercus rubra* (red oak, **G**, **H**), *Acer platanoides* (Norway marple, **K**), and *Sorbus americana* (American nountain ash, **P**) and may represent these species; others were of unknown source.

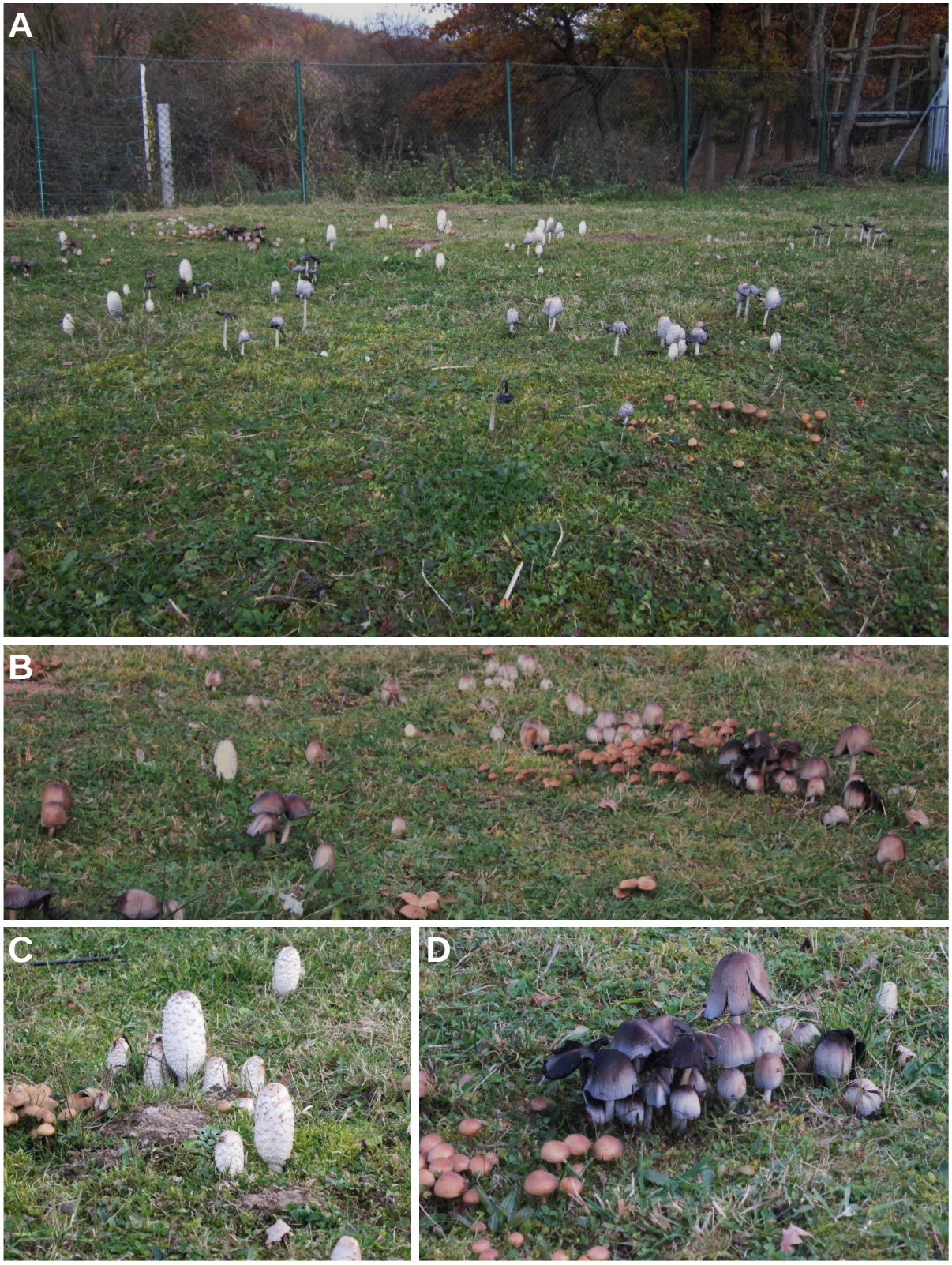

Fig. 11 Mushrooms on a nitrogen-rich meadow at the Tierklinik on the North-Campus of the University of Göttingen on which a flock of sheep was kept over the summer with their feces and urine fertilizing the ground (05.11.2011).
Note that the mushrooms are from inkcap species (*Coprinus comatus*, *Coprinopsis atramentaria*) and a non-autolysing dark-spored *Lacrymaria* species which all belong to the *Psathyrellaceae* and might ecologically be ammonia fungi as documemented for other *Psathyrellaceae* (Sagara 1975; Soponsathien 1998; Suzuki 2006, 2009).

Fig. 12 Selection of mushrooms observed on the cemetery in Weende-Dorf.
A. *Agaricus xanthodermus* (26.10.2014), **B**. *Lepista panaeolus* (09.11.2014), **C.** *Parasola plicatilis* (22.09.2013), **D**. *Psathyrella almerensis* (28.09.2014), **E**. *Coprinellus micaceus* (26.10.2014), **F**. *Panaeolus sphinctrinus* (22.09.2013), **G.** *Panaeolus campanulatus* (22.09.2013), **H**. *Mycena pura* (30.08.2014), **I.** *Pluteus pouzarianus* (26.10.2014), **J.** *Mycena epipterygia* (22.09.2013), **K.** *Mycena filopes* (22.09.2013), **L**. *Hypholoma sublateritium* (28.09.2014), **M**. *Entoloma sepium* (26.10.2014), **N**. *Inocybe fastigiata* (28.09.2014), **O**. *Amanita rubescens* (28.09.2014), **P**. *Hygrophorus marzuolus* (09.11.2014), **Q**. *Lactarius vellereus* (26.10.2014), **R**. *Lactarius vietus* (30.08.2014), **S**. *Paxillus involutus* (26.10.2014), **T**. *Hygrophorus russula* (04.09.2014), and **U**. *Russula silvicola* (04.09.2014). [For other examples, see Fig. 1D, J; Fig. 2F, G; Fig. 4C, D; Fig. 6A-C, H].

Fig. 13 Selection of fungi from the forest.
A. *Auricularia auricula-judae* (06.02.2016), **B**. *Schizophyllum commune* (23.11.2011), **C**. *Armillaria solidipes* (19.11.2011), **D**. *Clitocybe phyllophila* (03.11.2015), **E**. *Coprinopsis picacea* (01.11.2011), **F**. *Panaeolus cyanescens* (19.10.2011), **G**. *Panaeolus retirugis* (03.11.2011), **H**. *Lycoperdon pyriforme* (01.11.2011), **I**. *Fomes fomentarius* (06.02.2016), **J**. *Oxyporus populinus* (06.02.2016), **K**. *Trametes versicolor* (06.02.2016), **L**. *Ganoderma lucidum* (06.02.2016), **M**. *Ischnoderma benzoinum* (06.02.2016), **N**. *Trichaptum abietinum* (06.02.2016), and **O**. *Daedalea quercina* (06.02.2016). Mushrooms were found in the forest vegetation of the Klausberge (**A**, **D**, **H**-**O**) and the Billinghäuser Schlucht (**B**, **C**, **E**-**G**) in Göttingen. [For other examples, see also Fig. 3B, E, G and I; Fig 8A; Fig. 9B].

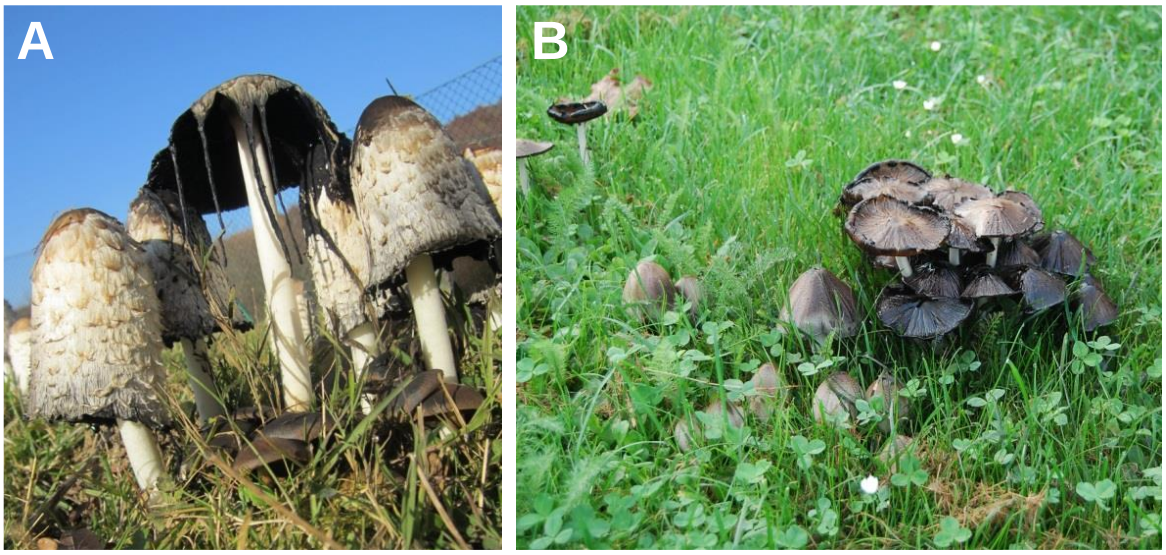

Fig. 14 Autolysing caps of A. the type species *Coprinus comatus* (09.11.2011) of the newly defined genus *Coprinus* from the *Agaricaceae* and B. *Coprinopsis atramentaria* (28.09.2011) from the *Psathyrellacae*.
Mushroom groups are from the meadow shown in Fig. 11.

The visible fruiting bodies of most fleshy species appear normally only for a few days in a year and the presence of invisible mycelia below ground or within a substrate must not be ignored (Watling 1995). In the global regions which uphold soil humidity throughout the year, mushroom yields are positively interrelated to temperature and rainfall (Büntgen et al. 2012; Sato et al. 2012). In nature, mushroom development and fruiting conditions are however poorly understood due to the fact that most of the life cycle of a fungus is hidden below ground or in a substrate, further due to possible changes in ecology and life style and also due to the limited space and time at which mushrooms occur. In order to come to solid conclusions on fruiting conditions of a species, long-term observations over wide areas are required. Only few studies have been done in the wild which are enough extensive for such conclusions. However, the extensive work by Büntgen et al. (2012) and Boddy et al. (2014) came to interesting conclusions regarding the ecology of distinct groups of basidiomycetes: The mycorrhizal fungi in an association of plants can resist climate changes better than saprotrophic fungi. The mycorrhizal mushrooms are for example better protected from high temperature by their host plants which also provide photosynthates to their symbionts (Högberg et al. 2008; Martin et al. 2008; Smith and Read 2008; Kohler et al. 2015). Mycorrhizal species therefore fruit more reliably than saprotrophs in times, numbers, and mushroom sizes (Büntgen et al. 2012; Boddy et al. 2014).

In this work, much attention is given to the inkcaps, the Coprini of the former very large genus *Coprinus* which were defined in the former and now dismissed family *Coprinaceae* by their ability upon maturation to quickly autolyze their caps in order to release their brown to black spores in liquid droplets to the ground (Fig. 14). The liquid which is stained blackish by the spores has been used in medieval age as ink for writing – hence the common name inkcaps (Buller 1931). However, common morphology, habit of spore release and preferential growth on nitrogen-rich dung, composts, and humus was found to not be of expected monophyletic origin. Nowadays therefore upon molecular characterization, the Coprini are separated into four distinct genera which distribute over two families of *Agaricomycetes*. The genus *Coprinus* as newly defined with its type species *Coprinus comatus* (Fig. 14 and 15) belongs to the *Agaricaceae* whereas the genera *Coprinopsis*,

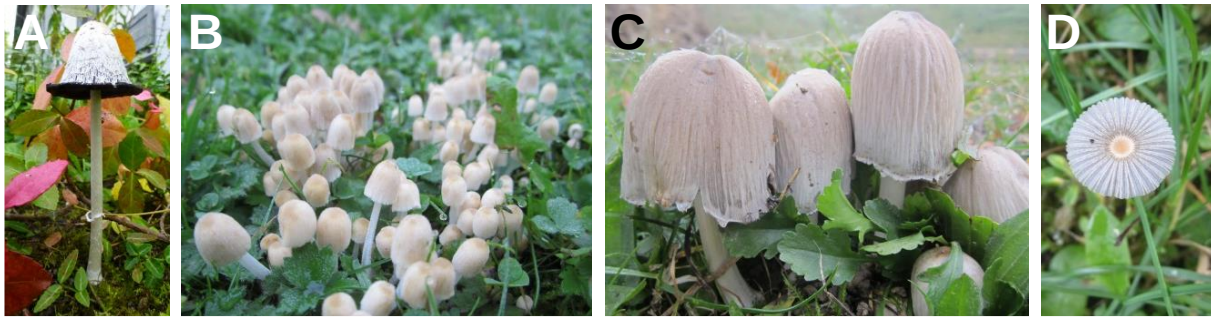

Fig. 15 The former genus *Coprinus* divides now into four different genera distributed over two distinct families (*Agaricaceae* and *Psathyrellaceae*).
A. *Coprinus comatus* (29.09.2013), **B.** *Coprinellus disseminatus* (14.10.2014), **C.** *Coprinopsis atramentaria* (14.10.2014), and **D.** *Parasola plicatalis* (19.09.2016) as examples for species of the four genera (after Redhead et al. 2001). Mushrooms were found **A.** in the Schlossgarten Karlsruhe and **B.-D.** on the grounds of the North Campus of the University of Göttingen.

Coprinellus and *Parasola* (Fig. 15) are included within the *Psathyrellaceae* (Redhead et al. 2001). Occurrence of fruiting bodies of such species on the grounds of the North Campus of the University of Göttingen was recorded over several years (Figs. 1D, 4A, 9A, 10A-E, G, H, 11, 14, 15B-D). Temperature and humidity values were available for the area and fruiting behavior of several species was thus correlated with basic climate conditions (Chapter 3 – Part I of this thesis). For the first time, detailed environmental conditions for fruiting of some inkcaps in nature are thus available. However, environmental observations included more extensively also fruiting of the weak pathogenic model fungus *S. commune* on different hosts (Fig. 16; see also Lakkireddy et al. 2017 and Chapter 3 – Part II) and of the agaric *Agaricus xanthodermus* (Fig. 17; Lakkireddy et al. 2016, 2020 – Chapter 4) while fruiting of many other species has also been recorded on the grounds of the North Campus, although mostly more randomly on the base whenever fruiting bodies were fortuitously noticed. Whenever a species was detected and a camera was at hand, photos were taken for own records. A great number of these were used for the figures in this chapter to document the species richness of mushrooms in the area, as well as the richness in their shapes and their life styles. Furthermore, for proper documentation of experiments undertaken in the laboratory with mushrooms collected in the wild (see Chapters 4 and 5) it was crucial to photograph the individuals used in the further studies.

Changes in climate (temperature, rainfall) are not the only factors that in a human-influenced world can have influence on fruiting of *Basidiomycetes*. Intensive agriculture alters the chemoecological conditions of the soil. A likely example is given in Fig. 11 with the many inkcaps that grew in a meadow fertilized over the summer 2011 by the feces and urine of a flock of sheeps. The species possibly belong like other inkcaps to the ammonia fungi which are specifically adapted in fruiting to high levels of ammonia (Sagara 1975; Soponsathien 1998; Suzuki 2006, 2009). That inkcaps like to fruit in nitrogen-rich environments was also deduced from other observations. The mushroom *C. disseminatus* for example were often observed to grow with the stinging-nettle *Urtica dioica* and other *Urtica* species and *Gleochoma hederacea* (ground ivy) (Fig. 18), plants which tell the ecologists that the soil is recently be disturbed, humid and nitrogen-, phosporus- and calcium-rich (Ellenberg 1986; Falkengrem-Gerup 1995). Other human-influenced habitats are those in parks and gardens with wood chips (Fig. 10). Navarro-Gonzaléz (2008) noted before that wood chips from chipped twigs, branches and stems are an ideal growth habitat for various inkcaps. Chopped wood chips offer unique environmental conditions in that there is good aeration between the

pieces of chopped wood. Wood pieces in contact support uptake of water with solved nitrogen sources from the soil (Philpott et al. 2014). For the same reasons, humidity in wood particles and of the air in between wood parcticles might be better kept (Venner et al. 2011; Cirelli et al. 2016; Fentabil et al. 2016) and there is possibly an increased and more over the time balanced temperature originating both from microbial activities as well as from solar

Fig. 16 The weak pathogen *Schizophyllum commune* on living trees and shrubs on the grounds of the North Campus of the University of Göttingen.
A. *Cotoneaster moupinensis* (Moupin cotoneaster, 08.11.2013)**, B.** *Malus* x *zumi* (golden hornet crabapple, 06.02.2016), **C**. *Malus angustifolia* (southern crabapple 06.02.2016), **D**. *Crataegus monogyna* (common hawthorn, 06.02.2016), **E**. *Acer caudatum* (Ukurundu maple, 06.02.2016), **F**. *Alnus glutinosa* (alder, 06.02.2016),**G**. *Juglans mandschurica* (Manchurian walnut, 10.02.2016), **H**. *Euanymus sanguineus* (spindle tree, 06.02.2016), **I**. *Lonicera maximowiczii* (06.02.2016), **J**. *Lonicera korolkowii* (Sakhalin honeysuckle, 06.02.2016), **K**. *Syringa wolfii* (wolf´s lilac, 06.02.2016), **L**. *Viburnum burejaeticum* (Manchurian viburnum, 15.02.2016).

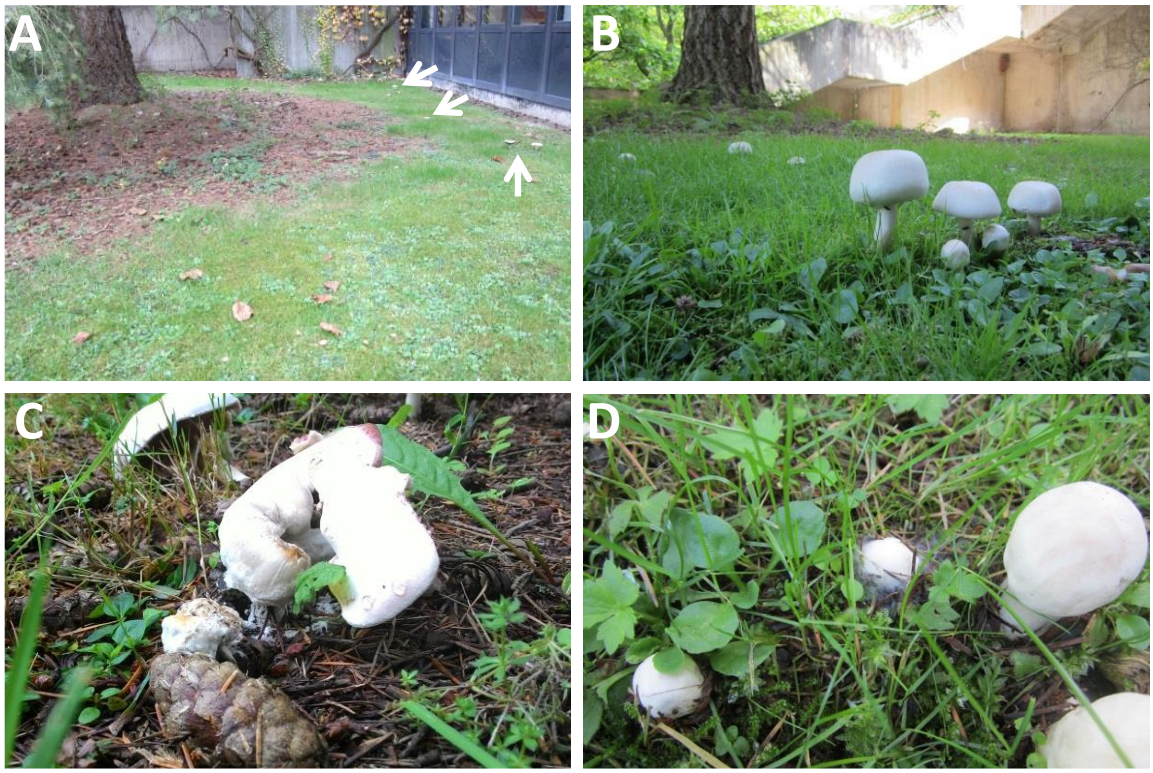

Fig. 17 *Agaricus xanthodermus* underneath a Douglous fir (*Pseudotsuga menziesii*) on a meadow next to building Büsgenweg 5 on the grounds of the North Campus of the University of Göttingen.
A. and **B.** Views on tree with mushrooms underneath (09.11.2014; 28.08.2014), **C.** a crippled fruiting body (01.09.2015), and **D.** a primordium infested with a cobweb-inducing mycoparasite (18.09.2015).

irradiation combined with insulation effects by the loosely arranged wooden material (Vellinga 2008; Goldin and Hutchinson 2015). These are all conditions which various inkcaps tend to like while many other fungi have problems with a higher substrate temperature. Furthermore, because usually branches with barks, bast and sapwood are chopped by gardeners and conservationists in arboristic tree care the material provides easy accessible nutrients like carbon and possibly including also nitrogen (in particular also when leguminoses are involved; Navarro-Gonzaléz 2008) for the fungi (see Fig. 10 and Fig. 18E-G) or, alternatively, the excess of accessible carbon helps the fungi to make use of nitrogen and phosphate from the soil beneath and fix it into its biomass (Homyak et al. 2008; Hannam et al. 2016; Rinne et al. 2017). Furthermore, wood chips tend to suppress growth of understory plant communities (Miller and Seastedt 2009; Wolk and Rocca 2009), giving possibly more free space to the fungi. It is further possible that the inkcaps also participate in degradation of lignocellulose of the vented wood chips unlike of the compact wood into which they usually do not grow likely by lack of much oxygen (Navarro-González 2008). Exceptions might be some weak plant-pathogenic species associated with roots and stem-bases of weakened trees (Navarro-González 2008; Fig. 19). Also in this study, inkcaps were variously be observed on wood chips (Fig. 10 and 18E-G) but also on roots and stem bases of diverse broadleaf trees (Fig. 19), supporting for a range of species that they are opportunistic weak pathogens to trees (further information in Chapter 3 – Part I).

Commercially cultivated *A. bisporus* mushrooms are regularly threaten in culture by diverse biological hazards and pathogens such as such as viruses, bacteria and fungi. Viruses

and the microbial pathogens cause major crop loss in commercial cultivations. Fungal pathogens on *A. bisporus* are commonly ascomycetes. Different fungal mycopathogens may attack vegetative mycelium or at any stage during the development of the mushrooms. Main ascomycete pathogens of *A. bisporus* are *Lecanicillium fungicola* which triggers dry bubble

Fig. 18 Coprini underneath shrubs in community with sting-nettles (*Urtica* species) and and *Glechoma hederaceae* (ground ivy).

A., **B.** *Coprinellus disseminatus* and *Coprinellus domesticus* underneath a *Prunus incisa* (Fuji cherry) tree (24.09.2015), **C.**, **D.** *C. domesticus* underneath another *P. incisa* tree, (09.11.2014), and **E.-G.** *C. disseminatus* underneath *Sambucus nigra* (elder) fertilized before with wood chips from cutting down a group of shrubs (16.10.2012). All mushrooms were observed on the grounds of the North Campus of the University of Göttingen.

Fig. 19 Species of the *Psathyrellaceae* on roots and stem bases of diverse broadleaf trees. **A.-C.** *Coprinellus xanthothrix* (**A**, **B**: 12.05.2012; **C**: 17.07.2012) on a cut elm tree (*Tilia cordata*) which already occurred when the stump was still living and regularly produced many shoots (Navarro-Gonzaléz 2008). **D.-G.** *Coprinellus micaceus* as another species fruiting on the same stump (10.10.2012). **H.-K.** An *Acer pseudoplatanus* (sycamore) stump on which *C. xanthothrix* (29.08.2012) and *Coprinellus disseminatus* occured (11.09.2015). **L.**, **M.** *C. disseminatus* on *Prunus sargentii* (Sargent´s cherry; 12.11.2015), **N.**, **O.** *C. micaceus* on *Sambucus nigra* (elder; 12.11.2015), **P.**, **Q.** *Coprinellus truncorum* on *Cladrastis lutea* (Kentucky yellowwood; 01.07.2013), **R.**, **S.** *Psathyrella candolleana* on *Phellodendron amurense* (Amur cork tree; 05.11.2011), and **T.-U.** *Coprinellus domesticus* (05.11.2011) and **V.-W.** *C. xanthothrix* (19.07.2012) on stumps of unknown trees. All mushrooms were found on the North Campus of the University of Göttingen.

disease of fruiting bodies, *Cladobotryum dendroides* which causes cobweb disease and *Mycogone pericosa* which gives rise to wet bubble disease (Berendsen et al. 2010; McKay et al. 1999; Kouser and Shah 2013). Depending on the pathogenic species and the developmental stages of mushrooms at attack, the type of disease symptoms vary. Initiation of fruiting body development might be suppressed by a pathogen. Once development started, outcomes of the different fungal infections might for example appear as partial disruption of stipe and cap tissues or as smaller or larger necrotic lesions in the cap (North and Wuest 1993; Largeteau et al. 2010). The route of infections in commercial cultivations might originate from contaminated soil or spawn. The pathogens might enter into mushroom casing or spawning in the form of spores or mycelium why high hygienic standards are mandatory to prevent the unwanted infections in culture (Adie et al. 2006; Soković and van Griensven 2006; Szumigaj-Tarnowska et al. 2015). Application of different fungicides is a potential way to control the fungal pathogens on mushroom farms worldwide. Europe and some other countries however have banned the use of hazardous chemicals. A major challenge for mushroom growers is thus to control the diseases with a limited range of chemicals or nochemicals. Another problem linked to the use of chemicals is the potential development of fungicide resistances among pathogen populations, in addition to a potential emergence of new pathogens from the outside (Grogan 2008).

While knowledge and studies on fungal infestations on mushrooms in commercial cultures are understandably broadly available, observations on fungal pathogens on fruiting bodies in nature are on the other hand sparse. However, fruiting bodies of basidiomycetes in nature (Fig. 17) can be also be infected by the same or other ascomycetes than mushrooms in culture. Where described, ascomycete infections are characterized by ramified conidiophores typical of verticillium-like anamorphs. Verticillate conidiophores with whorls and phialides give rise to the phialoconidia as asexual spores. Conidiophores and spores help to identify potential mycopathogens (Gray and Morgan-Jones 1980; Zare and Gams 2008). In this work, drum-stick shaped healthy and diseased young mushrooms of *A. xanthodermus* were observed underneath a Douglas fir (*Pseudotsuga menziesii*) in a meadow on the North Campus of the University of Göttingen on the 1st of September 2015. The surface of deformed stipes and mis-shaped parts of caps appeared covered with white fluffy mycelium indicating a cobweb-type disease on the fruiting bodies (Lakkireddy et al. 2016, 2020 – Chapter 4; Fig. 17). Mycopathogenic strains were isolated from infested material brought into the laboratory. Isolates had a verticilliate condiophore morphology which together with molecular ITS sequences identified them as *Hypomyces odoratus* (the anamorph name is *Cladobotryum mycophilum*). Furthermore, infection studies were performed in the laboratory with vegetative mycelium and fruiting structures of different basidiomycetous species proving the pathogenicity of these ascomycetes (Lakkireddy et al. 2020 – Subchapter 4.2). To fulfil for proof of its pathogenic character Koch´s postulate that an isolated microorganism must be able to cause disease when introduced into a healthy organism (Koch 1876), similar infection tests on *A. xanthodermus* structures in nature using a new flush of primordia and young mushrooms underneath the Douglas-fir in the meadow on the North Campus of the University of Göttingen (18th of September 2015) were unfortunately unsuccessful because mushroom hunters collected all inoculated young mushrooms overnight from the meadow together with any of the other non-inoculated structures (Fig. 20).

As indicated already above, mushrooms are in nature not only threatened by different types of diseases but also by a multitude of animals (slugs, insects, small mammals; see Fig. 6 and Fig. 7 and Lakkireddy and Kües 2017 - Chapter 5) and, as seen here by the example of *A. xanthodermus*, also by mushroom hunters. Activities of mushroom collectors were regularly noted on the grounds of the North Campus of the University of Göttingen, particularly regarding boletes which grew beneath birches and different types of pines. Many

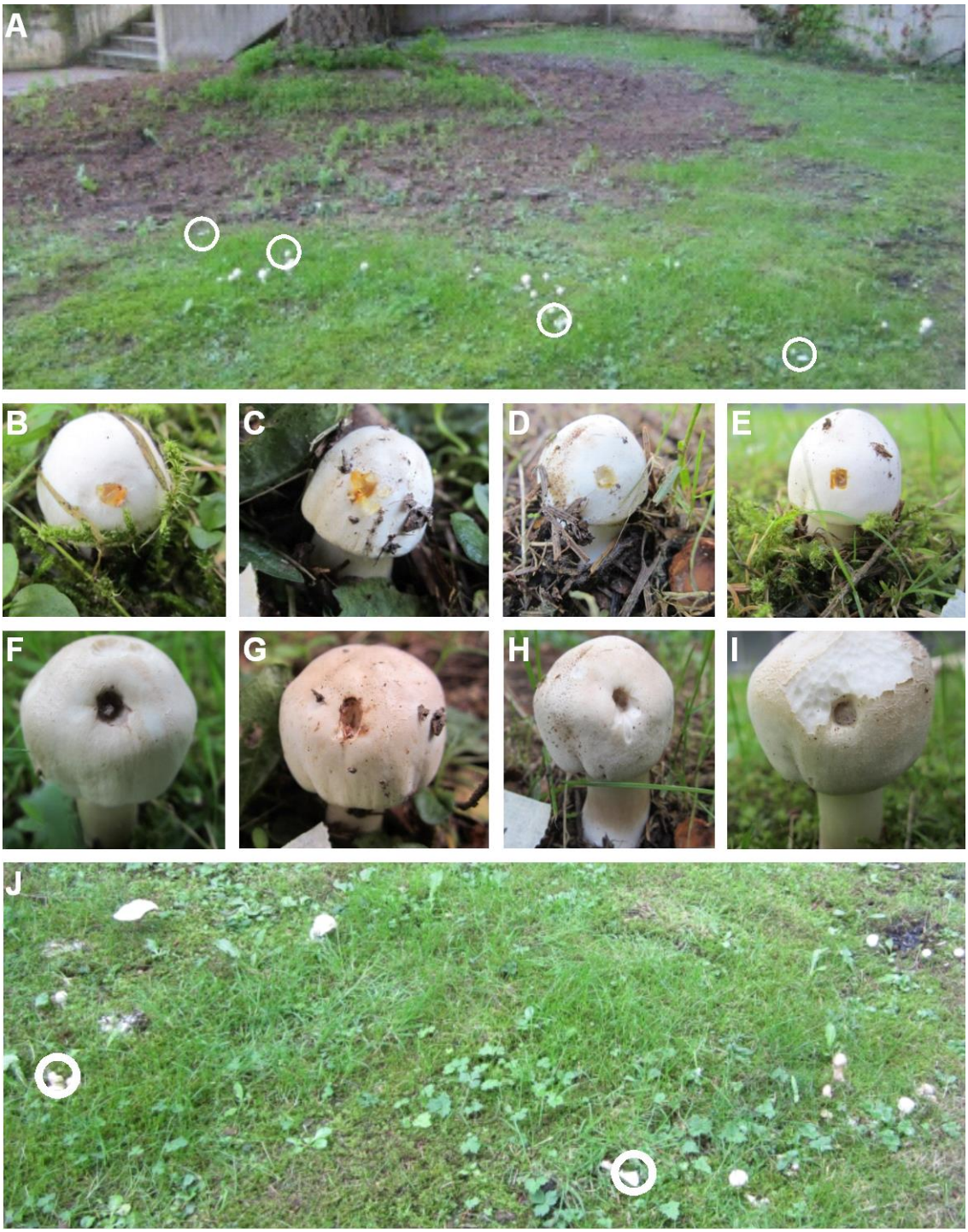

Fig. 20 Infection test of isolated mycopathogens on *Agaricus xanthodermus* primordia in nature.

A.Young primordia (ca. 3 cm in diameter) underneath the Douglas fir (*Pseudotsuga menziesii*) next to the building of Büsgenweg 5 on the grounds of the North Campus of the University of Göttingen. White circles mark positions of primordia which were inoculated with small agar blocks with mycopathogen isolates A1, B1, and C1 (see Lakkireddy et al. 2020 – Subchapter 4.2), respectively and as a control with a sterile agar piece (from left to right). Primordia are photographed directly upon inoculation into small cuts within the caps (**B.-E.**, 18.09.2015) and two days later when cap diameters were ca. 6 cm (**F.-I.,** 21.09.2015): A1 (**B.**, **F.**), B1 (**C.**, **G.**), B1 (**D.**, **H.**), control (**E.**, **I.**). **J.** At the next day (22.09.2015), nearly all young mushrooms were cut away by a mushroom hunter. Encircled are places of cut inoculated mushrooms.

times, mushrooms were cut and then dismissed when finding them not to be an edible species or possibly too grub-infested. Moreover, observations on occurrence of mushrooms in lawns and meadows were regularly affected by maintenance activities of university gardeners when these cut with their lawn cutter machinery the grass and with it any mushroom structures in it (Fig. 12 in Chapter 3 – Part I). For work on mushroom development independent on any unwanted influence from the outside, mushrooms are therefore better brought into the lab where also pure strains of the species can be isolated by suitable microbiological techniques. In such manner, it was thus possible by my colleague Weeradej Khonsuntia with mushrooms of *A. xanthodermus* collected from the wild on 26th of October 2016 to unambiguously demonstrate that the isolated *H. odoratus* strains of this study will infect mushrooms of the original host and cause disease, in addition to that own infection experiments were performed with isolated mycelium of *A. xanthodermus* and mushrooms and mycelia of other Agaricaceae (Lakkireddy et al. 2020 – Subchapter 4.). Last but not least, isolation of mycelial cultures from mushrooms from the wild is also precious for potential commercial cultivation of edible species (Kües and Liu 2000; Rühl and Kües 2007; Sanchez 2010) and by the many other beneficial properties of *Agaricomycetes* which can be exploited in medicine (Xu et al. 2010; Jo Feeney et al. 2014; Kües and Liu 2000; Wasser 2011) and other fields of biotechnology (Pointing 2001; Eibes et al. 2015; Cohen et al. 2002; Degenkolb and Vilcinskans 2016; Hofrichter et al. 2010; Kües 2015a; Masran et al. 2016; Schmidt et al. 2011; Schmidt-Dannert 2016; Wösten et al. 2015).

The typical life cycle of *Agaricomycetes* is heterothallic (Fig. 21). It starts with meiotic basidiospores with one (1n) or two identical haploid nuclei (2 x 1n) which germinate on a suitable substrate into primary mycelia called homokaryons (x 1n) or monokaryons when they specifically contain only one haploid nucleus per hyphal cellular segment (1n). Primary mycelia of heterothallic species are sterile, i.e. they cannot form fruiting bodies with meiotic basidiospores. Monokaryons are compatible with each other for formation of a secondary mycelium, the dikaryon, when they differ in their mating type. Fusion of such two compatible monokaryons leads then to the fertile dikaryon in which two genetically distinct haploid nuclei are brought together within its cells (1n + 1n). This is because in the *Basidiomycetes* the different monokaryotic hyphae fuse but usually not directly after their plasmogamy also the two distinct nuclei by karyogamy. A typical dikaryon is fertile. It will proliferate for mycelial growth until under defined environmental conditions outer signals induce the developmental pathway of fruiting body formation. In the developing fruiting bodies on the fertile dikaryons, karyogamy proceeds in the originally dikaryotic basidia (1n + 1n) which results in a single diploid nucleus (2n). Meiosis follows directly after and this results in four haploid recombined nuclei (4 x 1n). Basidia are specialized cells produced either by the hymenia covering lamella, ridges or pores of mushrooms or internally of fruiting bodies within the gleba (the inner fruiting body tissue) of gasteroid species. After karyogamy and meiosis, typical basidia form at their upper apex on sterigmata each four sexual basidiospores into which the four haploid meiotic nuclei will individually migrate. In addition, a postmeiotic mitosis can occur either within the basidia prior to nuclear migration or after nuclear migration within the basidiospores. As a result, a basidiospore may harbor two identical haploid nuclei (2 x 1n) unless one set of daughter nuclei does not move also into the basidia or when one set of nuclei degenerates again (Kües 2000; Kües and Navarro-Gonzaléz 2015).

Principally, basidiospores and fruiting body tissues are potential sources for mycelium isolation from mushrooms harvested in nature. Germination of basidiospores targets at isolation of monokaryons, in contrast to outgrowth of mycelium from tissues of mushrooms that serves isolation of their parental dikaryons (Fig. 21; Ainsworth 1995). Isolation of spores and of sterile mycelia from wild mushrooms collected from nature can both be very difficult due to their manifold contaminations by bacteria and other fungi and due to the common

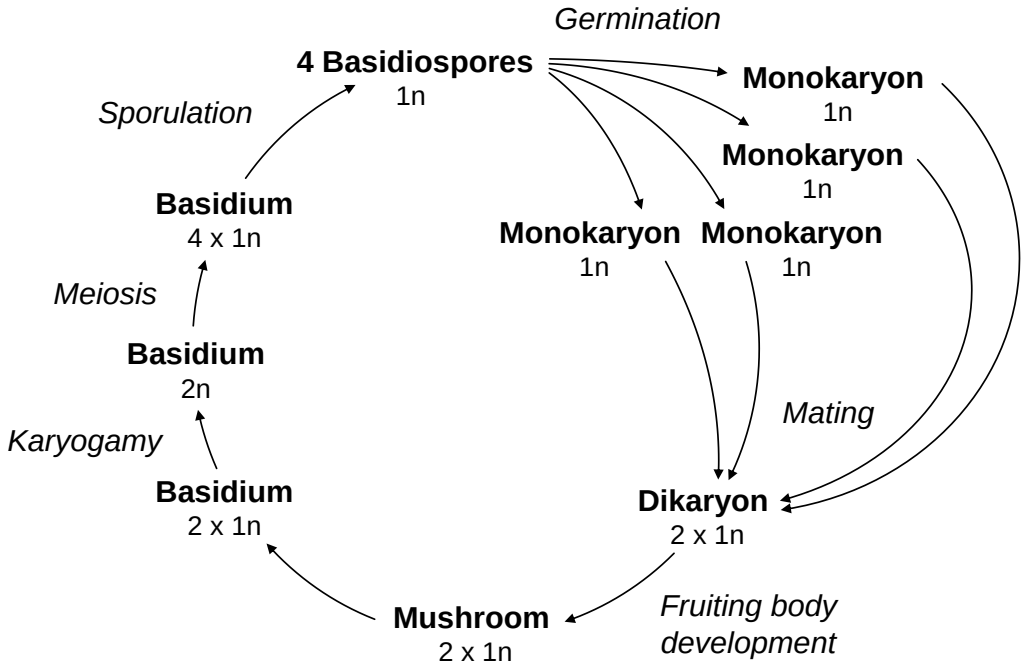

Fig. 21 Schematic presentation of the life cycle of a typical heterothallic mushroom-forming basidiomycete.

Main developmental events are indicated as well as nuclear number and nuclear status in cells (1n = haploid; 2n = diploid), respectively. For mating, fusing monokaryons must differ at least in the mating type genes. Accordingly, the two haploid nuclei in resulting dikaryotic cells are genetically distinct as are the four haploid products in the basidia after meiosis. For tetrapolar species with two distinct mating type loci (*A* and *B*), only two possible functional combinations exist between sister monokaryons (i.e. *A1B1*x *A2B2*; *A1B2*x*A2B1*) germinated from spores of a same basidium which can mate to form a fertile dikaryon (Kües 2015b; this situation is indicated by the arrows in the figure). For simplification of the presentation, a possible post-meiotic mitosis in either basidia or basidiospores resulting in 2 x 1n homokaryotic basidiospores and a homokaryotic mycelial state of more than one nucleus per cell are not further considered here.
(The scheme was kindly provided by Prof. Dr. Ursula Kües.)

occurrences of small animals within and on the structures (beetles, flies and other insects and their larvae, mites, nematodes, slugs – see Fig. 6A, G, K and M and Lakkireddy and Kües 2017 - Chapter 5). Collecting young and thus by age less exposed mushrooms can help to reduce the risk of both microbial and animal contaminations (Ainsworth 1995).

The success of isolating a dikaryotic mycelium from vegetative mushroom tissues depends further on size and consistency of a collected fruiting body. If fresh and of sufficient size, there is a good chance to get sterile tissue from stipes or caps by semi-sterile surgery of their inner parts (Ainsworth 1995). However, after placing isolated inner mushroom tissues on sterile agar medium, the outgrowth of dikaryotic mycelia can be hindered by other faster growing microorganisms, be it bacteria or be it other fungi. Addition of suitable mixtures of bacteriocides to media can supress bacterial growth and addition of the microtubule-binding fungicide benomyl possibly kills sensitive *Ascomycetes* but usually, unwanted fungal growth is still an unavoidable obstacle (Schuytema et al. 1966; Snelling et al. 1996; Lodge et al. 2004). Isolation of sterile dikaryotic mycelium from inner cap tissue of an *A. xanthodermus* fruiting body (Lakkireddy et al. 2020 – Subchapter 4.2) and from inner stipe tissues of

different *Coprinellus* mushrooms and *Psathyerella condolleana* (Chapter 3 – Part I) are examples of success in obtaining pure mycelial cultures in this study. However, in frame of this PhD study as an estimate over the time, in only about an estimated 20 % of attempts sterile mycelia were obtained from tissue surgery of fruiting bodies collected from the wild (own unpublished observations).

Basidiospores are routinely harvested from mushrooms as spore prints. Spores can be collected while placing mushrooms directly on a suitable sheet (such as paper or aluminium foil) or mushrooms might be fixed into the lids of Petri-dishes e.g. by agar, petroleum jelly (Vaseline) or other suitable gluing agents or be positioned above the surfaces by toothpicks or glassrods. Basidiospores when mature drop down by gravity out of the mushrooms onto the sheets or into the bottoms of the Petri-dishes. However, this will also occur to any contaminating fungal spores or bacteria which happen to also fall out of the mushrooms and this is the reason why spore prints often are not clean enough for easy isolation of sterile germlings from the spores (Choi et al. 1999; Lodge et al. 2004).

Basidiospores are however special because typically they are ballistospores which at maturity are forcefully catapulted off from their sterigmata by the fast hygroscopic formation of Buller´s drop at the hilar appendix of a spore and by the fusion of Buller´s drop with a hygroscopic liquid droplet generated in an adaxial dent of the spore (Buller 1909, 1922; McLaughlin et al. 1985; Webster and Davey 1985; Webster et al. 1989; Ingold 1992; Money 1998; Pringle et al. 2005; Fig. 22). It is this fusion that provides the energy which is just sufficient for the spore to propel off from their sterigmata into the middle of the air space in between hymenia on neighbouring lamellae and ridges or into the inner of a pore. Then, when all energy for flight is used up, the spores normally fall out from the caps by gravitational forces (Ingold 1957, 1992; Webster et al. 1989; Pringle et al. 2005; Money and Fischer 2009; Noblin et al. 2009; Fischer et al. 2010a). However, mushroom basidiospores are naturally also electrically charged. Likely, the electrical load arises at least partially from the fast spore flight in combination with the braking effect mediated by the viscosity of the air (Saar and Paramasto 2014). Such charging of ballistospores is widely distributed in the *Agaricomycetes* (Saar and Paramasto 2014; Lakkireddy and Kües 2017 - Chapter 5). Accumulated spore charges lead to that in horizontal electrical fields the spores drift sidewards away in accordance with type (+ or -) and strength of their charges (Buller 1909; Gregory 1957; Webster et al. 1988; Saar 2013; Saar and Salm 2014). Similarly, spores can move up when an electrostatic force comes from above (Lakkireddy and Kües 2017 - Chapter 5). In such manner, ballistospores ejected from their sterigmata and braked in their flights through the free air space are not fallen back by gravity into the open spaces in between hymenia of their mushrooms. Instead, when suitably charged they are attracted against gravity toward plastic lids of Petri dishes when the spore-ejecting mushrooms lie upside-down beneath the lids, thereby facing up their lamellas, ridges or pores. This observation has been made use of in a new method presented in this thesis (Lakkireddy and Kües 2017 - Chapter 5) which allows mostly sterile basidiospore collection from wild mushrooms for potential mycelial production after spore germination.

Whether monokaryotic from basidiospore germination, dikaryotic from mating between two compatible successfully isolated monokaryons or from isolation as such mycelium from mushroom tissues, isolated mycelial strains have good value for many biological studies in the laboratory. In this thesis, isolated mycelial cultures served in fungal growth tests on potential substrates (Chapter 3 – Part I and Lakkireddy et al. 2020 – Subchapter 4.2), for species determination by amplifying ITS-sequences from isolated DNA for sequencing (Lakkireddy et al. 2020 – Subchapter 4.2), for fruiting induction tests (Chapter 7), and for infection tests with mycopathogens (Lakkireddy et al. 2020 – Subchapter 4.2).

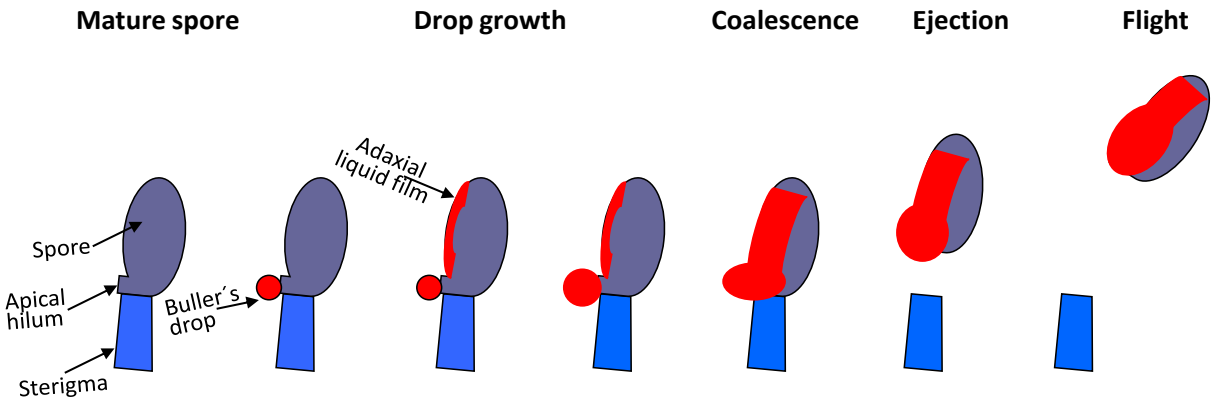

Fig. 22 Ballistospore ejection mechanism (after Buller 1922 and Fischer et al. 2010b).

There are however many more potential uses for isolated mycelia. Among applications to be named are genome-scale sequencing projects. Much can be learned from the acquired theoretical sequence data on ecology, fungal lifestyles and development (Floudas et al. 2012, 2014; Grigoriev et al. 2014; Kohler et al. 2015), including on fruiting body development of *Agaricomycetes* (Bao et al. 2013; Chen et al. 2016; Morin et al. 2012; Ohm et al. 2010; Stajich et al. 2010). Many genomes of *Agaricomycetes* are by now publically available on the Mycocosm website (Grigoriev et al. 2014) of the JGI (Joint Genome Institute at Walnut Creek, California; http://genome.jgi.doe.gov/programs/fungi/index.jsf) and in the GenBank database at NCBI (National Center for Biotechnology Information at Bethesda, Maryland; https://www.ncbi.nlm.nih.gov/). Use can be made of these data by searching the genome sequences for presence of interesting genes such as in this PhD study for searching of genes which are possibly linked to fungal fruiting body formation (Lakkireddy et al. 2011 – Chapter 6 as well as Chapters 7 and 8).

For most newly isolated species it is not straightforward to successfully induce fruiting body development in the laboratory, particularly if no (clear) data on environmental regulation are available from prior observations in nature. The number of species which have successfully been brought to fruiting in culture by empirical research is constantly rising with more and more experiences on different species and their cultural conditions for optimal growth and fruiting (Rühl and Kües 2007). For various Coprini for example, incubation at comfortable room temperature on artificial malt-extract or yeast- and malt-extract media or on dung in many instances can lead to good success (Navarro-González 2008; Badalyan et al. 2011; Lakkireddy et al. 2011 - Subchapter 8.1). However, another strategy is to deeply study a few well established model fungi in fruiting and use the knowledge which was generated on their genes related to fruiting for transfer onto other species. Two species mainly serve in international research on fruiting body development, i.e. the inkcap *C. cinerea* (Kües 2000; Kües and Navarro-González 2015; Stajich et al. 2010) and the split-gill fungus *S. commune* (Kües and Navarro-González 2015; Ohm et al. 2010). Other than the observation on fruiting bodies in nature of *S. commune* on wooden hosts (Lakkireddy et al. 2017 and Chapter 3 – Part II), a focus in this study on fruiting in laboratory work is on *C. cinerea* (Chapter 6 to 8).

The edible inkcap *C. cinerea* grows and fruits in nature on horse dung (Buller 1909, 1931; Kües 2000) but it is cultivated for human consumption in some Asian and African countries on agricultural wastes such as from sisal plantations and on the plant pest water hyacinth (*Eichhornia crassipes*) (Kües et al. 2007). Moreover, this model mushroom grows and completes its life cycle by fruiting body and basidiospore formation on artificial media (e.g. on YMG – 4 % yeast extract, 10 % malt extract and 4 % glucose or on the YMG/T –

version with extra 100 mg/l tryptophan added to allow also laboratory strains with defects in tryptophan biosynthesis good growth and development) under well defined environmental conditions – i.e. 25 °C in a 12 h light/12 h dark rhythm at high humidity on a substrate of good water activity. Vegetative growth in contrast can best occur at the higher temperature of 37 °C (Buller 1909, 1931; Kües 2000; Granado et al. 1997; Moore 1985; Kües et al. 2016).

Once placed onto a suitable substrate, the vegetative filamentous fungal mycelial growth of *C. cinerea* begins by formation of stronger first hyphae (the leading hyphae) in order to enter fresh substrate. Subsequently from the leading hyphae, generations of sub-terminal side branches arise and upon their confrontation they eventually will fuse via anastomoses into a larger hyphal network (Buller 1931; Kües and Navarro-Gonzaléz 2009; Lakkireddy et al. 2011 - Chapter 6). Upon receiving the right environmental signals for fruiting, the simple two-dimensional vegetative mycelial growth turns locally into an intense three-dimensional growth by massive formation of short side-branches of stunted growth and aggregations between these side-branches. This gives then a compact three-dimensional aggregated structure while tissue differentiation in these structures will finally shape the mushroom with all the specific cap and stipe tissues (Buller 1931; Kües 2000; Lakkireddy et al. 2011 - Chapter 6; Fig. 23).

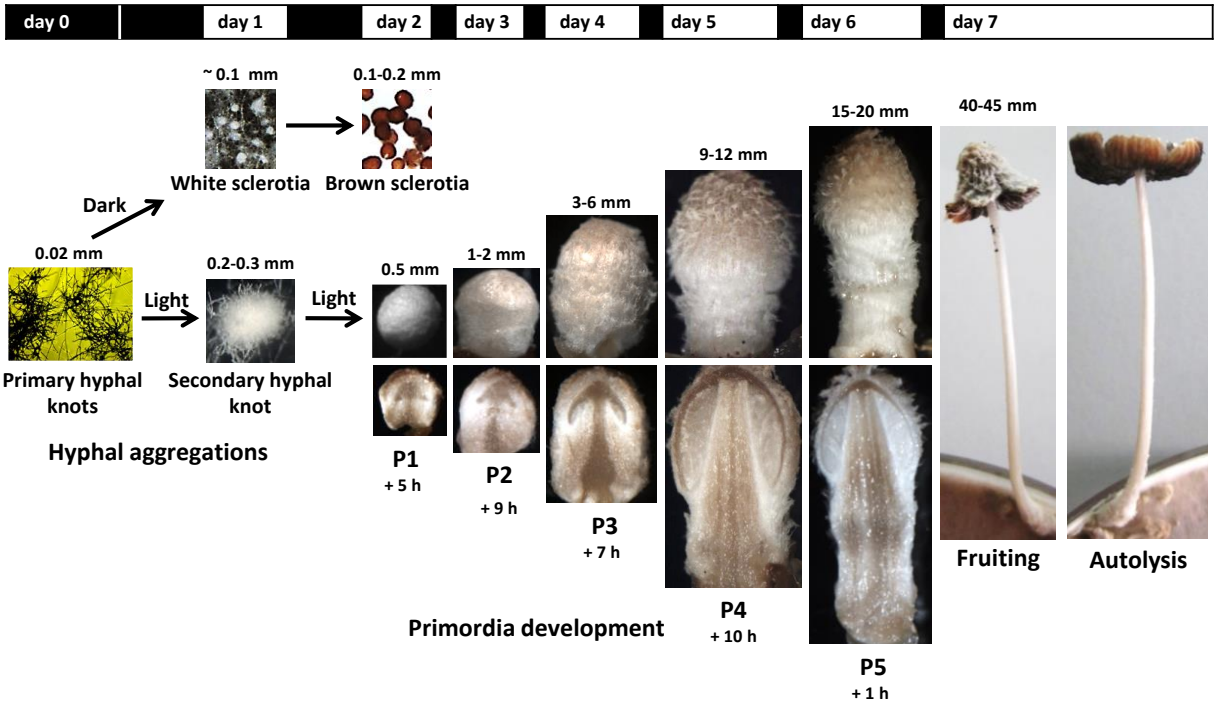

Fig. 23 Fruiting body pathway of a *Coprinopsis cinerea* dikaryon (strain PS1x2) under standard fruiting conditions.
(The figure was kindly provided by Shanta Subba, altered from Kües et al. 2016).

After mycelial growth, the developmental pathway initiates with primary and secondary hyphal knot development. Dark produced primary hyphal knots will transform into sclerotia when kept further in the dark and into secondary hyphal knots when a light signal is received. Development from secondary hyphal knots continues in a 12 h light/12 h dark rhythm with cap and stipe tissue differentiation in the growing primordia (P1 to P5 which are defined as the developmental stages reached at the morning of a new day when light switches on; the + h values indicate the actual age in extra hours after the light did switch on when the

structures shown in Fig. 23 were photographed). At the primordial stage P4 with the main tissues fully established, a light signal is required to induce karyogamy in the basidia within the hymenia which in turn is mostly completed at the stage P5. With karyogamy, fruiting body maturation starts. Subsequently over day 6, meiosis and basidiospore formation happens and occurs in parallel to stipe elongation and cap expansion. The mushrooms with mature spores are fully opened within the night phase of day 6. Fruiting bodies autolyse then their caps at the next morning on day 7 (Navarro-Gonzaléz 2008; Kües and Navarro-Gonzaléz 2015; Kües et al. 2016).

The first visible step in fruiting body development in basidiomycetes is thus the formation of small hyphal knots. In *C. cinerea*, primary hyphal knots as initial loose hyphal aggregates can be distinguished from secondary hyphal knots as compact undifferentiated hyphal aggregates. Primary hyphal knots form in the dark and have still the option open to enter into development of sclerotia (small, oval-round, dark-rinded resting structures) as an alternative pathway to fruiting. Dark and light conditions decide whether sclerotia develop (in the dark) or secondary hyphal knots (upon a light signal has been received) as a first fruiting - body-specific developmental structure. Genetical, the initiation of hyphal aggregation is controlled by the mating type genes in interconnection with light and in interaction with other environmental factors including nutrients and temperature (Boulianne et al. 2000; Kües 2000; Kües and Liu 2000; Kües et al. 1998, 2002; Matthews and Niederpruem 1972; Kües et al. 2016; Fig. 23). Once started, the development from a primary hyphal knot up to a mature open fruiting body releasing its spores first by the mechanism of catapulting off the mature ballistospores (Lakkireddy and Kües 2017 - Chapter 5) and later in liquid droplets by the inkcap-typical cap autolysis (Nagy et al. 2010, 2011, 2012a,b, 2013) takes for *C. cinerea* 7 days (Navarro-Gonzaléz 2008; Kües and Navarro-Gonzaléz 2015; Kües et al. 2016; Fig. 23).

Earlier work in *C. cinerea* and some other fungal species has suggested that lectins might contribute to fruiting body formation. Different types of sugar moieties-binding lectins may thereby mediate cellular aggregation of hyphae in development of mushrooms (Wang et al. 1998; Kües and Liu 2000; Walser et al. 2003). Galectins as β-galactoside binding proteins for example are lectins with a specific sugar-binding domain for β-galactoside sugars. By binding to such sugars in fungal cell walls, galectins of *C. cinerea* had been postulated to interlink fungal cells (Cooper 2002; Boulianne et al. 2000; Walser et al. 2003, 2004, 2005). Judged by their expression in *C. cinerea*, galectins and galectin-related lectin proteins apparently play certain roles during primary and secondary hyphal knot formation and during subsequent primordia differentiation. The two galectins CGL1 and CGL2 were reported to be expressed in parallel to the formation of hyphal knots and to be temperospatially regulated in an alike manner through the same environmental factors than the primary and the secondary hyphal knot formation (Boulianne et al. 2000; Bertossa et al. 2004). Moreover, expression of the later detected galectin-like protein CGL3 follows that of CGL2 during the developmental pathway (Wälti et al. 2006, 2008). The expression of the proteins is high in the outer cap and outer stipe tissues of the developing mushrooms. The *C. cinerea* galectins are secreted and localize to the cell walls and the extracellular matrix of the outer cap and stipe tissues (Walser et al. 2003, 2004, 2005). Recent studies employing molecular techniques such as gene silencing however showed that these proteins are not essential for hyphal aggregation and neither for proper fruiting body formation. Instead, the studies revealed a positive function in protection against grazing arthropods and nematodes (Wälti et al. 2006; Butschi et al. 2010; Bleuler-Martínez et al 2011; Sabotič et al. 2016). This goes along with findings on lectin genes in this PhD thesis from genome analysis of a larger selection of sequenced *Agaricomycetes*. The mushroom-forming species tend to have specific selections of lectin genes but the outfits of species with such genes overlap little between the different fungi. The

respective lectin genes analyzed in this PhD thesis are mostly only present in very few species. The patterns of sharing the rare genes appear rather random between species and do not fit with any closer evolutionary species relationships (Lakkireddy et al. 2011 – Chapter 6). Accordingly, while the findings on knocking down selected galectin genes (Wälti et al. 2006) do not rule out any additional supportive function of their encoded products in hyphal aggregation, the sparse distribution of these genes in only a few of the analyzed species with complete genomes (*C. cinerea* from the *Psathyrellaceae*, *Agaricales*; *L. bicolor* from the *Hydnangiaceae*, *Agaricales*; *H. irregulare*, *Bondarzewiaceae*, *Russuales*; Lakkireddy et al. 2011 – Chapter 6) makes a primary function in hyphal aggregation unlikely. Nevertheless, feeding studies of *A. cylindracea* galectins onto mycelium of the own species and onto mycelium of *Auricularia polytricha* induced formation of aggregates and primordia differentiation in both species (Sun et al. 2003; Luan et al. 2010). Application experiments of some other types of lectins to mycelium of some *Agaricomycetes* showed also fruiting-inducing effects (Lakkireddy et al. 2011 – Chapter 6). In terms of evolution in mushroom formation, it appears however too elaborate to have invented again and again proteins essential for mediation of hyphal aggregation. Other more widespread candidates for such plausible required functions must be sought for.

Some fruiting body-specific lectins contain domains that adopt a similar structure than bacterial pore-forming haemolytic actinoporins and other pore-forming haemolysins. Such pore-forming lectins and specific fungal haemolysins (comprising the specific fungal protein family of aegerolysins) have also been handled as candidate proteins for mediating hyphal aggregation in mushroom formation because some of them also induced fruiting in mycelial feeding experiments (Walser et al. 2003; Berne et al. 2009). However, rather than bridging in between different hyphae for hyphal aggregation, pore-forming lectins and more fungal haemolysins might play roles in membrane signalling and in interactions with lipid rafts in cellular membranes (Walser et al. 2003; Birck et al. 2004; Carrizo et al. 2005). Presence of one or more genes for types for pore-forming proteins is also not rare in mushrooms. However again, none of the genes for pore-forming proteins are present in all mushroom species, making also for these an essential core function in hyphal aggregation unlikely (Kurahashi et al. 2013; Nayak et al. 2013; Novak et al. 2014; Lakkireddy et al. 2011 – Chapter 6).

C. cinerea is especially suited to study fruiting body development on the genetic level (Kües 2000) including the mating-type control of sexual reproduction (Kües 2015b). *C. cinerea* is tetrapolar and has thus an *A* and a *B* mating type locus encoding homeodomain transcription factors and a pheromine-pheromone-recepter system, respectively (Kües 2000, 2015b). Mating type genes act not only as a master regulators of dikaryon development (Fig. 21) but they also control mushroom formation (Tymon et al. 1992; Kües et al. 1998, 2002), in addition to that fruiting body development is influenced by environmental factors such as light, temperature, nutrients and humidity (Kües et al. 1998, 2002; Kües 2000; Kües and Liu 2000). The self-compatible strain AmutBmut (*A43mut*, *B43mut*) is a very special homokaryon due to mutations in both mating type loci (Swamy et al. 1984). As the wildtype dikaryons (Fig. 23), homokaryon AmutBmut can form fruiting bodies. The strain can do this without mating to another strain due to the defects in the two mating type loci. The strain mimics in all aspects a fertile dikaryon, including that generation of uninucleate haploid single-celled asexual spores (oidia) on the AmutBmut homokaryon is light-induced (Swamy et al. 1984; Polak et al. 1997; Kertesz-Chaloupková et al. 1998; Boulianne et al. 2000). These uninucleate haploid spores are especially valuable for any kind of mutagenesis (Granado et al. 1997; Walser et al. 2001). The fruiting-deficient strain 6-031 for example is derived from homokaryon AmutBmut by UV mutagenesis. Mutant 6-031 has a defect at a very early step in fruiting body development. It does not give rise to secondary hyphal knots. Therefore, mutant

6-031 cannot reach the mature primordia and subsequent fruiting body stages for karyogamy, meiosis and basidiospore production. The defect in fruiting body initiation in this mutant had been complemented via transformation of a cosmid with integrated genomic *C. cinerea* DNA. Subcloning of active DNA fragments from the complementing cosmid revealed that a mutation in the gene *cfs1* (for a potential cyclopropane fatty acid synthase) was responsible for the loss of ability of the mutant to enter the fruiting pathway. The *cfs1* gene is thus essential in fruiting body development of *C. cinerea* (Liu et al. 2006). Accordingly, genome analyses of *Agaricomycetes* revealed that commonly one or more genes for cyclopropane fatty acid synthases are present in the fungi undergoing mushroom development (Subchapter 8.2). With the *cfs1* gene is likely one first genetic function detected that is central to the initiating processes of fruiting body development in the *Agaricomycetes*. This situation is this clearly different from those of lectin and haemolysin genes reported above (Lakkireddy et al. 2011 – Chapter 6).

C. cinerea cfs1 encodes a protein that is highly similar to bacterial Cfa (cyclopropane fatty acid synthase) enzymes (Liu et al. 2006). In *Escherichia coli*, enzyme Cfa is most of the time present in the cytoplasm and has been shown to transfer a methylene group from an S-adenosyl-L-methionine (SAM) to the double bond in the chains of unsaturated fatty acids (UFA) in phospolipids of cellular membranes. For conferring this biochemical reaction, the enzyme transiently attaches or integrates into a leaflet the cellular double membrane (Taylor and Cronan 1979; Grogan and Cronan 1997; Chang and Cronan 1999; Zhang and Rock 2008; Hari et al. 2018). Transfer of methylene leads to formation of a cyclopropane ring in the now cyclopropanated lipids (CFA) and this transformation alters membrane properties through changes in fluidity behaviour. In bacteria, CFA production is higher at stationary growth phases when nutrients become short and cells suffer nutritional stress. Increase on CFA production is also linked to several other stress conditions (e.g. acid, salt, osmotic, heat, cold). CFA production helps so the bacteria to survive under various stress situations, including acid stress conditions (Chang and Cronan 1999; Beil et al. 1991; Grogan and Cronan 1997; Alvarez-Ordonez et al. 2008, 2009; Shabala and Ross 2008; Zhang and Rock 2008; Charoenwong et al. 2011).

Functional expression of the *E. coli cfa* gene in the ascomycetous yeast *Saccharomyces cerevisiae* showed that the fungal membranes and unsaturated lipids are principly accessible by the bacterial enzyme for biochemical reactions. Similarly, plant Cfas can be functionally expressed in yeast (Grogan and Cronan 1997; Yu et al 2011; Liu et al. 2013). In fact, *S. cerevisiae* has no own *cfs1* gene it its genome (Liu et al. 2006) while *cfs1*-like fungal genes can be present in other ascomycetous yeasts and also filamentous ascomycetes (Subchapter 8.2). In the meantime, similar biochemical functions in CFA production have also been proven for an eukaryotic *cfa* gene from the pathogenic trypanosomatid *Leishmania major* (Oyola et al. 2012) and for a range of genes of plant origins (Bao et al. 2002, 2003; Yu et al. 2011, 2014). In view of the above mentioned results on induction of fruiting body development by addition of membrane integrating haemolysins (Walser et al. 2003; Berne et al. 2009; Lakkireddy et al. 2011 – Chapter 6), it is in any case interesting to note that likely a membrane-linked function in initiation of fruiting body development is blocked in the *cfs1* mutant of *C. cinerea* (Liu et al. 2006). Notably, addition of natural or synthetic surface-active substances such as saponins, 3-*O*-alkyl-D-glucose derivatives or polymeric 3-alkylpyridinium salts to nutrient media or onto established fungal mycelium were shown to stimulate fruiting body development in a range of *Agaricomycetes* (Magae 1999; Kües and Liu 2000; Magae et al. 2005; Magae and Ohara 2006; Berne et al. 2002, 2007, 2008). To better study and understand the biochemical function of *cfs1* of *C. cinerea*, the fungal gene and the *E. coli cfa* gene for comparison were cloned into an expression vector of *E. coli* (Lakkireddy and Kües 2014 - Subchapter 8.1). These constructs

were transformed into available isogenic *E. coli* wildtype and *cfa* mutant strains (Grogan and Cronan 1997; Chang and Cronan 1999). Results obtained from some acid incubation tests of the bacterial transformants support that the fungal gene can possibly replace the bacterial gene in conferring acid resistance to growing cells (Subchapter 8.2).

Concentrating on other potential genetic factors which will influence fruiting body development, nutrients and linked metabolic abilities have also to be considered in connection (Kües and Liu 2000). In axenic culture, commercial strains of *A. bisporus* were capable of producing primordia and mature sporophores on stimulating casing layers containing charcoal, anthracite coal, lignite and zeolite (Noble et al. 2003). In this case, fruiting inhibiting compounds need to be eliminated from the environment such as by absorption to the added materials. Alternatively, this is also possible when helper bacteria of the genus *Pseudomonas* are available which consume inhibiting organic compounds. Interestingly, such inhibiting compounds can also be of own fungal origin. Among, the bacteria consume volatile compounds like fungal-produced 1-octen-3-ol with which *A. bisporus* and other mushroom species negatively control the frequency of their own fruiting body formation (Park and Agnihotri 1969; Rainey 1991; Rainey et al. 1990; Cho et al. 2003; Noble et al. 2003; Zarenejad et al. 2012; Young et al. 2013; Colauto et al. 2016). Moreover, bacterial siderophore production for better supply of iron could also be an effect in enhancing fruiting (Rainey 1989; Ebadi et al. 2012) and possibly disease control by fungal (Singh et al. 2000, 2012) and bacterial mycopathogens (Henry et al. 1991). Presence of free-living nitrogen-fixing bacteria of the genus *Azotobacter* had positive influences on initiation of fruiting body development on *Hypsizygus ulmarius*. It remains still to be shown whether this is due to better nitrogen supply (Poonga and Kaviyarasan 2015). In connection, both the amounts as well as the types of nitrogen available for a fungus have their roles in initiation of fruiting (Morimoto et al. 1981, 1983; He and Suzuki 2003; Kües et al. 2004). As probably documented with the mushrooms shown in Figs. 11, 14 and 18, nitrogen-rich, respectively ammonium-rich biotopes in nature can be positive for fruiting of many of the Coprini. The ecological aspect of high nitrogen in an environment generally for the so-called ammonia fungi on fruiting body development was already mentioned above (Suzuki 2006, 2009).

Addition of other specific compounds to growth media can help in culture to stimulate mushroom production. For example, addition of calcium to cultures of *Cyathus stercoreus* promoted fruiting (Lu 1973). In *C. cinerea*, increasing the concentrations of magnesium sulphate supported fruiting body development (Casselton and Casselton 1966). Addition of copper can induce fruiting under unusual conditions such as in the dark or at 37°C where this typically does not happen (Navarro-Gonzaléz 2008) as can also addition of ammonia (Morimoto et al. 1981). Nutrient supplementation by specific vegetative oils helps fruiting of *A. bisporus* (Schisler and Sinden 1966; Wardle et al. 1969). Growth and fruit body formation of the edible mushrooms *Pleurotus pulmonarius* and *Stropharia rugosoannulata* were greatly influenced by addition of β-adenosine which was extracted from grass chaff (Domondon et al. 2004). Furthermore, addition of specific amino acids such as phenylalanine, methione and proline can positively influence fungal growth and development of different mushroom species (Fraser 1953; Fraser and Fujikawa 1958). In nature, socializing bacteria may feed such and other metabolites to a fungus. Bacteria might provide also missing vitamins to *Agaricomycetes* such as biotin and thiamin (Fig. 24) and growth factors such as IAA (indole acetic acid), (Poonga and Kaviyarasan 2015; Jiang et al. 2018). In conclusion, mushroom formation can be sparked by a variety of environmental chemical and biochemical nutritional factors.

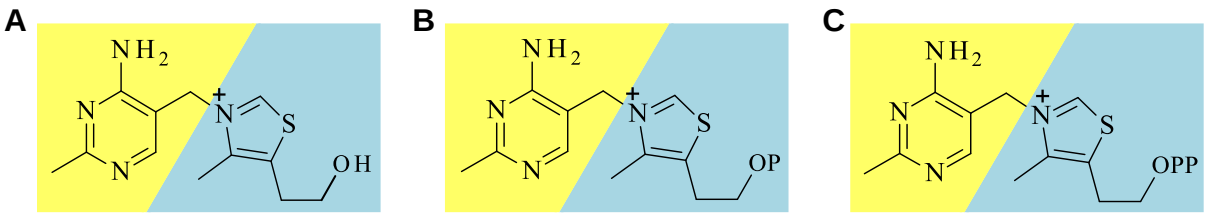

Fig. 24 Structure of A. thiamin, B. thiamin monophosphate (TMP) and C. thiamin diphosphate (TDP), also called thiamin pyrophosphate (TPP).
The pyrimidine moiety is yellow and the thiazole moiety blue underlaid.
The figure was constructed with the help of the program BIOVIA Draw Version 16.1 NET 2016 (Dassault Systèmes BIOVIA, San Diego, California).

Common in the *Agaricomycetes* is a deficit for thiamin production (Jennison et al. 1955; Ward 1962; Osborne and Thrower 1964; Impens 1972; Eul and Schwantes 1985L Gramss 1990a,b; Strzelczyk et al. 1991; Deveau et al. 2010; Jiang et al. 2018; Chapter 7). Nevertheless, fruiting bodies of *Agaricomycetes* contain measurable amounts of thiamin (Karosene et al. 1984; Furlani and Godoy 2008; Okwulehie et al. 2014; Afiukwa et al. 2013; Nile and Park 2014; Phan et al. 2014; Wang et al. 2014; Rahi and Malik 2016). Sufficient thiamin must be added to media for growth in culture and enzymatic substrate degradation and, in comparison, more for promotion of fruiting (Aschan 1954; Wessels 1965; Gold and Cheng 1979; Laetham 1983; Jonathan and Fasidi 2001; Joo et al. 2009; Levin et al. 2010; Fu et al. 2011; Atri and Guleria 2013; Lee et al. 2014; Chapter 7). Being unable to produce thiamin themselves, the auxotrophic fungal species must take it up in nature from the environment. Thiamin (vitamin B1, aneurin) is in nature produced by bacteria, plants, varied groups lower eukaryotes and a range of fungi but not by animals and humans (Guarro et al. 1999; Croft et al. 2006; Nosaka 2006; Begley et al. 2008, 2012; Judelson 2012; Bettencourt and Wins 2009; Jurgensen et al. 2009; Du et al. 2011; Lai et al. 2012; Fitzpatrik and Thore 2014; Manzetti et al. 2014; Kijima et al. 2016). Its major biologically active derivative (the actual co-factor form for enzymes) is thiamin diphosphate or thiamin pyrophosphate (abbreviated as TDP or TPP, respectively; Fig. 24 and 25), which serves as a cofactor for several enzymes involved in carbohydrate and amino acid metabolisms of cells (Hohmann and Meacock 1998; Kowalska and Kozik 2008; Begley et al. 2012; Jurgenson et al. 2009; Nosaka et al. 1993, 1994; Chatterjee et al. 2010; Ciquille et al. 2012; Manzetti et al. 2014; Liu et al. 2015). Furthermore, thiamin diphosphate has additional non-catalytic functions such as in regulation at promoters of genes for thiamin biosynthesis (Nosaka et al. 2005, 2012) and in binding to riboswitches (small non-coding regions of mRNA molecules) with regulatory purposes (Richter 2013; Moulin et al. 2013; Bocobza and Aharoni 2014; Moldovan et al. 2018). Thiamin compounds can confer resistance against reactive oxygen species (ROS) and serve as signaling molecules under stress conditions (Gigliobianco et al. 2010; Wolak et al. 2014, 2015).

Thiamin consists of a pyrimidine ring and a thiazole ring linked via a methylene bridge. The two distinct moieties are separately produced and then coupled to each other (Fig. 24 and 25). The major bacterial biosynthesis pathway is long understood and 16 different enzymes have been defined to comprise the whole biosynthetic pathway (Zurlinden and Schweingruber 1994; Jurgenson et al. 2009; Hazra 2010). Appointing chloroplasts in biosynthesis, plants produce thiamin in a pathway partially conserved with that of the bacteria. Synthesis of the pyrimidine moiety HMP-PP (4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate) is as in bacteria whereas the synthesis of the thiazole precursor HET-P (5-(2-hydroxyethyl)-4-methylthiazole phosphate) is like in baker´s yeast

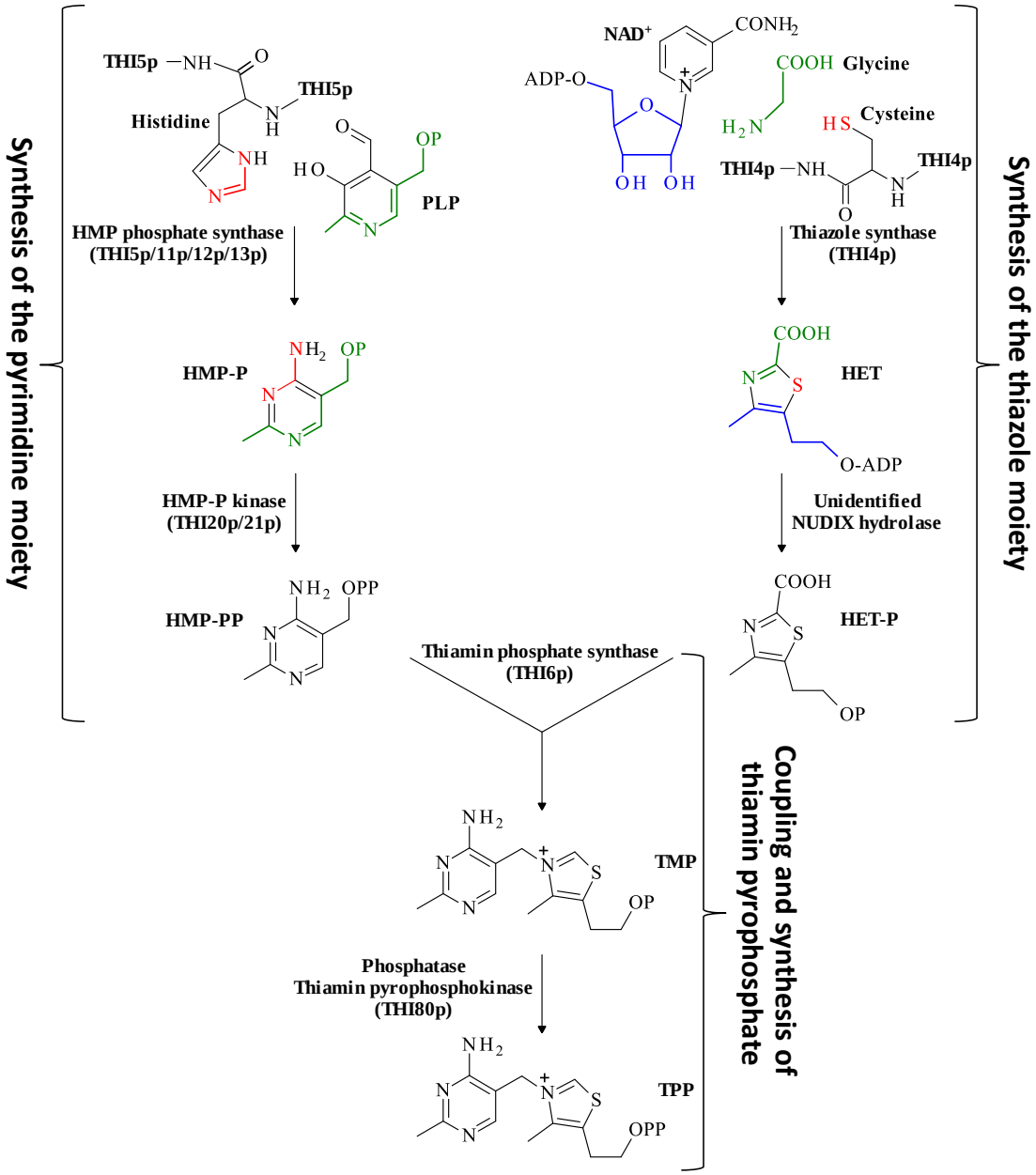

Fig. 25 The thiamin biosynthesis pathway in the ascomycetous yeast *Saccharomyces cerevisiae* (Jurgensen et al. 2009; Begley et al. 2012; Coquille et al. 2012; Fitzpatrick and Thore 2014; modified from Lai et al. 2012).

The precursor of the thiamin pyrimidine moiety is produced in two steps from PLP (pyridoxal phosphate) and a histidine originating from a single-turnover HMP phosphate synthase (either from THI5p, THI11p, THI12p, or THI13p) via HMP-P (hydroxymethyl pyrimidine phosphate; colors indicate origins of atoms) which is subsequently phosphorylated by a HMP-P kinase (THI20p or THI21p) to HMP-PP (hydroxymethylpyrimidine pyrophosphate). In the thiazole moiety branch, NAD^+ (nicotinamide adenine dinucleotide), glycine and an active-site cysteine of the suicidal THI4p (thiazole synthase) convert to HET (hydroxyethylthiamin; colors indicate origins of atoms) which is further transformed into HET-P (hydroxyethyl thiazole phosphate) by an unidentified Nudix hydrolase. HMP-PP and HET-P become coupled to TMP (thiamin monophosphate) by a thiamin phosphate synthase (THI6p). A phosphatase converts TMP to thiamin and thiamin pyrophosphokinase THI80p pyrophosphorylates the thiamin to give the active form TPP (thiamin pyrophosphate). The figure was constructed with the help of the program BIOVIA Draw Version 16.1 NET 2016 (Dassault Systèmes BIOVIA, San Diego, California).

(compare Fig. 25) employing NAD^+, glycine and a backbone cysteine of a sulfur donor protein to give HET (hydroxyethylthiamin) as a substrate for the HET-P synthase (named THI1 in plants) (Raschke et al. 2007; Jurgensen et al. 2009; Goyer et al. 2013; Pourcel et alal. 2013; Garcia et al. 2014; Dong et al. 2015; Mimura et al. 2016). Fungi undertake a fully different thiamin biosynthesis pathway with enzymes of other origins than in bacteria. In the best studied fungal pathway of thiamin de novo synthesis, i.e. in *Saccharomyces cerevisiae* (see complete pathway in Fig. 25), the biosynthesis of the two thiamin precursors HMP-PP and HET-P, differs in the starting molecules as well as in the active enzymes from those in the bacterial pathway (Nosaka 2006; Raschke et al. 2007; Jurgensen et al. 2009; Goyer et al. 2013; Pourcel et al. 2013; Mimura et al. 2016). The pyrimidine heterocycle HMP-PP is generated in *S. cereviseae* by HMP-P kinase THI20p (or THI21p whereas a third related protein THI22p is inactive) from HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) which originates from PLP (pyridoxal phosphate) and amino acid His66 of the single-turnover enzyme THI5p (THI11p, THI12p and THI13p are alternatives). The thiazole heterocycle HET-P is produced by the suicidal THI4p (using amino acid Cys205 as substrate) in combination with a glycine and NAD^+ (nicotinamide adenine dinucleotide) via formation of HET which is subsequently converted to HET-P by a yet unknown Nudix (for nucleoside diphosphatases linked to other moieties, X) hydrolase, an enzyme which catalyzes the hydrolysis of nucleoside diphosphates linked to another moieties (X), requires a divalent cation, such as Mg^{2+} or Mn^{2+}, for their activity and contains a highly conserved 23-residue Nudix motif (GX5EX7REUXEEXGU, where U = I, L or V), which functions as a metal binding and catalytic site; Srouji et al. 2017). HET-P and HMP-PP are then fused by THI6p to form thiamin monophosphate (TMP). A still to define phosphatase is thought to convert TMP into thiamin. Upon dephosphorylation of TMP, the thiamin pyrophosphokinase (diphosphokinase) THI80p pyrophosphorylates the free thiamin to the diphosphate ester (TTP) representing the main biologically active thiamin derivative (Nosaka et al. 1993, 1994; Llorente et al. 1999; Kawasaki et al. 2005; Kowalska and Kozik 2008; Jurgenson et al. 2009; Chatterjee et al. 2011; Begley et al. 2012; Coquille et al. 2012; Fitzpatrick and Thore 2014; Palmer et al. 2015; Zhang et al. 2016). PHO3p of *S. cerevisiae* as a periplasmic acid phosphatase has the ability to hydrolyse TMP to thiamin (Nosaka et al. 1989), while a selective intracellular phosphatase (Th2p) for specific TMP hydrolysis has now been detected in *Arabidopsis thaliana* giving evidence for the intracellular existence of the specific TMP hydrolysis step in plants (Mimura et al. 2016).

Using the key proteins of *S. cerevisiae* for thiamin biosynthesis, fungal genome tblastn searches on the JGI website (http://genome.jgi.doe.gov/programs/fungi/index.jsf) and in the GenBank database at NCBI (subbase whole-genome shotgun contigs¸ https://www.ncbi.nlm.nih.gov/genbank/wgs/) revealed that most *Agaricomycetes* have lost the HMP-P synthases and are therefore thiamin auxotrophs while variably, other thiamin biosynthesis genes may have been lost in addition (Chapter 7). Other fungi than the *Agaricomycetes* have also not always all genes present for the complete pathway. Different genes have been lost in different species or groups of fungi (Jiang et al. 2018; Chapter 7). For example, microsporidia seem to have kept none of the genes for the pathway whereas ascomycetes usually have retained some or all of the genes for thiamin production and may or may not need thiamin supplementation for growth and development (Robbins and Ma 1943; Barnett and Lilly 1948; Ward 1960; Esposito et al. 1962; Garraway 1973; Kawasaki et al. 1990; Schweingruber et al. 1991; Watanabe 1997; Ruiz-Roldan et al. 2008; Bi et al. 2010; Chhaya and Gupte 2010; Xu et al. 2012; Spanu 2012; Yoon et al. 2013; Cisse et al. 2014; Hoppenau et al. 2014; Liu et al. 2015; Iosue et al. 2016; Shimizu et al. 2016; Chapter 7). In consequence for fungal cultivation in case of incomplete thiamin biosynthesis pathways, resulting thiamin auxotrophy in fungal species might be overcome by addition of thiamin or by addition of the thiazole and pyrimidine precursors, depending on which of the genes of the

thiamin biosynthesis pathway are left. Uptake of thiamin and its precursors from the environment by such thiamin-dependent organisms implies presence of suitable transporters. Thiamin transporters have evolved several times in eukaryotes (Fankhauser et al. 1995; Enjo et al. 1997; Singleton 1997; Nosaka et al. 1989, 1998; Niederberger et al. 1996; Mojzita et al. 2006; Belenky et al. 2007; Vogl et al. 2008; Li et al. 2010; Manzetti et al. 2014; Wolak et al. 2015; Martinis et al. 2016; Qi et al. 2016). Uptake of thiamin can further positively be influenced by extracellular, periplasmic thiamin-binding proteins such as e.g. by PHO3p in *S. cerevisiae* which hydrolyses thiamin phosphates outside of the protoplast (Nosaka et al. 1989, 2005). An account on the distribution of genes for fungal thiamin transporters and *PHO3* homologs will also be presented in this study. All tested *Agaricomycetes* appear for example to have homologues to the *Schizophyllum pombe* thiamin transporter THI9p and many also carry a gene for a *S. cerevisiae* THI10p-like protein with or without another gene for a PHO3p-like phosphatase (Chapter 7).

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Chapter 2

Objectives of this Work and General Discussion

This chapter introduces into the specific objectives of this thesis and provides an overarching discussion of the diverse observations and data collected on mushroom production of *Agaricomycetes* and presented in this thesis work.

Contributions: Kiran Lakkireddy (KL) wrote a draft of the chapter, collected and read relevant literature, observed and photographed mushrooms in nature and selected from these the collection of photos shown in the chapter. The content, topics and structure of the chapter were refined through interactions with Ursula Kües (UK) and input of knowledge and further literature by UK. Weeradej Khonsuntia helped in collecting literature and supplied photos in Fig. 5. UK supplied photos in Fig. 5, Fig. 7 and Table 2 and took part in genome analyses for selected genes presented extra in the broader data discussion in this chapter. Bernd Kopka is thanked for providing raw climate data.
All colleagues are sincerely thanked for their kind support.

Chapter 2

Objectives of this Work and General Discussion

As summarized in the general introduction (Chapter 1), there are many reasons in science, both in basic mycological and ecological research and in medicinal and biotechnological application, and also just by personal interest as why to study fruiting bodies of *Agaricomycetes* and their development. Chapter 1 documents with many examples the richness in biodiversity of mushrooms in shapes, sizes, colours, substrate use, and other characters. While it is nice and enjoyable to photograph the great diversity of mushrooms, it is harder to actually identify them all. This takes a lot of knowledge combined with good observation skills and attention for small features and probably much patience to evaluate also any revealing microscopic features. Helpful in learning about fungi which occur and fruit under the climate conditions of Germany are good guide books and luckily those of Breitenbach and Kränzlin (1986, 1991, 1995), Bresinski and Besl (1990), Dähncke (2001), Flück (1995), Gerhardt (2010) and also others (Lange and Bayard Hora 1967; Jahn 1979; Reid 1980; Arora 1986; Kibby 1992; Jordan 1995; Læssøe and Lincoff 1998; Könemann Inc 1999) turned out to be very useful during this study to make oneself familiar with the wealth of basidiomycete mushrooms that were observed over the time of this PhD study (Chapters 1, 3, 4 and 5). For the Coprini (inkaps) as a frequently fruiting-body-autolysing dark-spored form group of *Agaricomycetes* of special ecology being in the main focus of this study, the guide books are however not sufficient to determine the multitude of similar looking species that occurred in observations in nature in this study. For the Coprini, the website of Kees Uljé on the former genus *Coprinus* with dichotonous species keys (http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm) further helped for the now valid four genera *Coprinus*, *Coprinellus*, *Corinopsis* and *Parasola* (Redhead et al. 2001) in morphologically differentiating various species.

The global question addressed in this PhD study is under which environmental conditions do mushrooms of *Agaricomycetes* form and can environmental and genetic factors with important roles in initiation of fruiting body development be defined.

It is clear that this is a very large question, far too large to be completely answered in a single PhD thesis. However, a PhD thesis like the current work can add some individual details to the growing knowledge on mushroom production of *Agaricomycetes* and help with work of others to come to a more global picture on what will determine the fruiting body production in nature (Andrew et al. 2017).

In the following, I will summarize the observations from nature and the laboratory on individual fungi and on specific aspects and details in the general process of fruiting body formation of *Agaricomycetes* from this PhD thesis and discuss their further significance for the fruiting process.

As already indicated in the introductory Chapter 1, different species will have different requirements for fruiting regarding environmental factors like substrate (and possibly hosts), temperature, light, and humidity. Many mushrooms appear in the autumn at lower temperature under higher humidity and reduced illumination in shorter days (see the guide books cited above). Some are winter mushrooms and form even under weather conditions with snow such as *Flammulina velutipes* (Fig. 1K in Chapter 1) and *Tubaria hiemalis* (Fig. 4K in Chapter 1; Fig. 1). Other species in contrast develop without difficulty in temperature ranges >20-25 °C and when matured may tolerate also higher values without directly dying (see as fleshy examples *Paneaolus olicaveus*, *Caloboletus colopus*, *Agaricus*

Fig. 1 Winter mushrooms found on the grounds of the North Campus of the University of Göttingen.
Colonies of *Tubaria hiemalis* mushrooms on wood chips beneath **A.-C.** *Prunus kurilensis* (Japanese alpine cherry) and **D.** *Lonicera korolkowii* (Blueleaf Honeysuckle) shrubs close to the Chemistry buildings of the University of Göttingen on the 02.12.2012 (1-2 °C night and day temperature, 94-97 % and 88-97 % humidity at night and day, respectively).
The weather conditions in the days and weeks before in November 2012 were relatively constantly cold and humid (compare Fig. A2 in Appendix III of Chapter 3). In particular, the temperature and humidity ranges in the eight nights and days before were
7-8/8-9 °C and 97-98/89-97 % (night/day; 24.11.2012),
8-11/9-14 °C and 73-88/53-69 % (night/day; 25.11.2012),
3-8/6-10 °C and 73-98/75-97 % (night/day; 26.11.2012-28.11.2012),
3-5/2-3 ºC and 91-97/82-94 % (night/day; 29.11.2012),
2-3/1-4 ºC and 83-86/62-84 % (night/day; 30.11.2012),
and 1-2/2-3 ºC and 78-91/82-89 % (night/day; 01.12.2012), respectively.
Note that mushrooms from both sites were taken for spore collection tests presented in Lakkireddy and Kües (2017) – Chapter 5.

xanthodermus, *Agaricus campestris*, *Limacella glioderma*, *Mycena inclinata* and *Suillus bovinus* in Fig. 2A, D-J and Table 1; Lakkireddy et al. 2020 – Subchapter 4.2), or mushrooms may appear at even higher temperatures such as ≥25°C or >30 °C and also survive at hotter temperatures (see the summer mushrooms *Russula rosea*, *Amanita strobiliformis* and *Amanita pantherina* as examples in Fig. 2B-E, Table 1). Some mushrooms develop on open meadows (Fig. 1C, E; Fig. 4A, B; Fig. 6C, E, F, J, K; Fig. 8D; Fig. 9A; Fig. 11; Fig. 12B-D, F-H, J, P; Fig. 14; Fig. 15B-D in chapter 1) and some actually appear also at warmer temperatures at places with even (some) direct sunshine (Fig. 2A; Fig. 3). However, many of the mushrooms prefer shadow and preferentially grow underneath shrubs and trees even if they are not mycorrhizal and not wood-decaying (e.g. the litter-degrading *Agaricus xanthodermus* which is presented in Fig. 17 in Chapter 1 and discussed in more detail in Lakkireddy et al. 2016, 2020 – Chapter 4). Different from most other wood-decay fungi, the weak shrub and tree pathogen *Schizophyllum commune* is contrariwise well adapted to light and preferentially forms its mushrooms on the sunny surface areas on fallen deadwood (typical situation at the

edges of forests and in forest glades) and also on the sunny side of twigs and branches of living wooden plants where these might be sun-burned (Jahn 1979; Breitenbach and Kränzlin

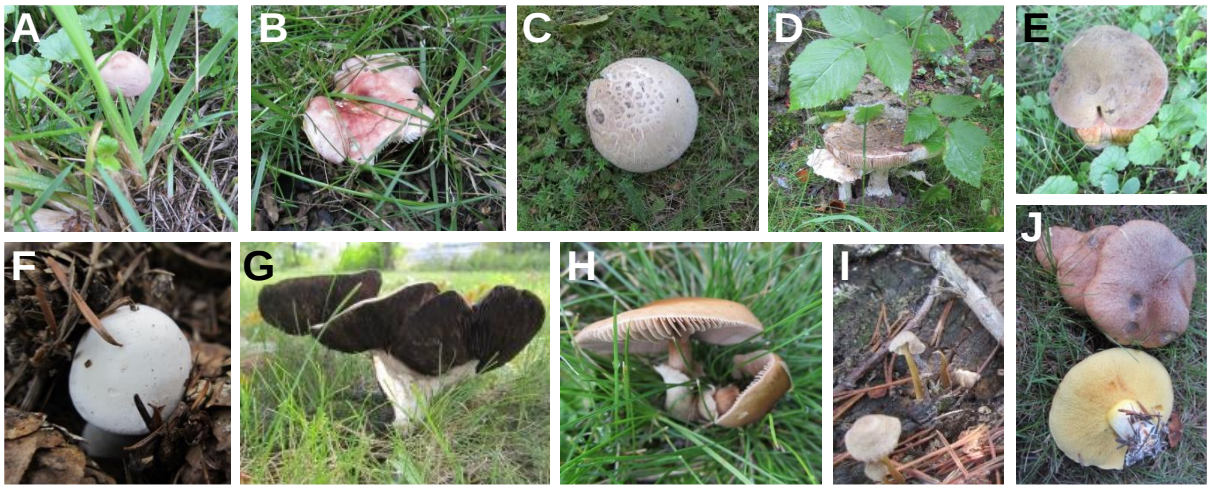

Fig. 2 Summer mushrooms found on the grounds of the North Campus of the University of Göttingen.
A. *Panaeolus olivaceus* at 27.06.2012 (night/day: 17-21/19-26 °C, 84-93/62-97 % humidity), **B.** *Russula rosea*, **C.** *Amanita strobiliformis* and **D.** *Amanita pantherina* at 27.07.2012 (17-22/26-34 °C, 71-90/41-83 % humidity), **E.** *Caloboletus calopus* at 30.08.2014 (8-15/15-26 °C, 89-95/49-82 % humidity), **F.** *Agaricus xanthodermus* at 05.09.2014 (15-17/16-25 °C, 85-96/54-91 % humidity), **G.** *Agaricus campestris* at 14.09.2014 (15-17/16-26 °C, 92-97/62-85 % humidity), **H.** *Limacella glioderma*, **I.** *Mycena inclinata*, and **J.** *Suillus bovinus* at 08.09.2014 (13-16/16-26 °C, 83-97/58-90 % humidity). For temperature and humidity ranges in the 5 days before photographing see Table 1 below and the climate diagrams in Fig. A2 in the Appendix III of Chapter 3.
Note that mushrooms from the sites shown in photos **A-C**, **E**, **H** and **J** were taken for spore collection tests presented in Lakkireddy and Kües (2017) - Chapter 5.

Table 1: Temperature (T) and humidity (H) values during fruiting of the fungi in the three days prior photographing as shown in Fig. 2*

Fungus (Fig.)	Parameter	5 d before (night/day)	4 d before (night/day)	3 d before (night/day)	2 d before (night/day)	1 d before (night/day)
Panaeolus olivaceus (2A)	T °C	15-18/18-23	11-18/18-20	12-14/12-23	12-15/13-19	12-14/12-19
	H %	79-98/45-73	71-94/56-75	60-88/63-89	83-91/64-92	77-87/62-86
Russula rosea (2B), *Amanita strobiliformis* (2C), *Amanita pantherina* (2D)	T °C	9-14/14-12	10-15/20-26	12-17/14-30	16-20/20-33	18-20/25-31
	H %	78-92/44-76	80-90/37-64	69-87/36-76	77-88/45-77	83-89/45-64
Caloboletus calopus (2E)	T °C	7-12/12-19	12-13/13-17	16-17/17-25	16-18/15-19	13-15/16-23
	H %	83-97/61-90	95-98/80-97	92-97/51-84	71-75/67-97	95-98/49-87
Agaricus xanthodermus (2F)	T °C	13-17/16-19	10-14/15-18	9-11/13-24	12-14/19-23	11-14/18-25
	H %	82-97/70-91	92-98/78-94	89-93/50-81	87-93/53-78	91-94/47-86
Agaricus campestris (2G)	T °C	10-12/14-19	13-15/15-18	17-21/17-22	11-14/09-15	6-9/14-19
	H %	91-98/58-85	85-92/59-87	84-100/50-81	98-100/72-95	93-96/49-93
Limacella glioderma (2H), *Suillus bovinus* (2J), *Mycena inclinata* (2I)	T °C	13-17/15-22	10-14717-25	14-16/16-20	11-14/15-20	9-13/15-23
	H %	90-97/56-82	80-93/59-83	88-92/57-85	69-79/48-72	70-88/48-70

*Grey shaded are days with temperature values ≥20 °C.

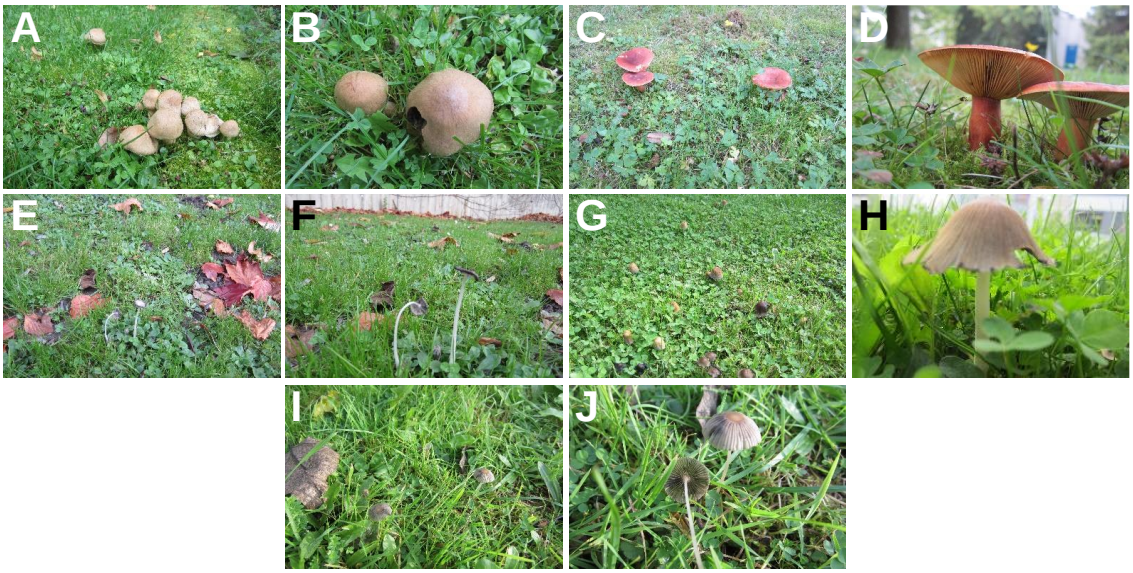

Fig. 3 Mushrooms found on days with mild to warm temperature conditions in lawns on the grounds of the North Campus of the University of Göttingen.
A., **B.** *Lacrymaria lacrymabunda* at 30.08.2014 (night/day: 14-16/17-20 °C, 91-97/70-88 % humidity), **C.**, **D.** the mycorrhizal *Lactarius rufus* in the neighborhood of a *Pseudotsuga menzii* (Douglas fir) tree and **E.**, **F.** *Coprinopsis semitalis* at 06.09.2014 (night/day: 16-18/17-26 °C, 91-95/65-92 % humidity), **G.**, **H.** *Coprinellus domesticus* at 08.09.2014 (night/day: 13-15/15-24 °C, 95-97/58-89 % humidity), and **I.**, **J.** *Parasola plicatilis* at 13.10.2014 (night/day 14-14/15-19°C, 90-95/79-97% humidity).

1991; Læssøe and Lincoff 1998; Conedera et al. 2007; Peddireddi 2008; Brazee et al. 2014; Fig. 16 in Chapter 1; see further information in Lakkireddy et al. 2017 and Chapter 3 – Part II). Likewise, several of the Coprini tolerate or prefer higher temperatures in nature for fruiting (Chapter 3 – Part I). However, to deduce solid conclusions from outside observations on properties and behaviour of fungi, sufficient independent observations on global mushroom communities and also per individual fungal species over the time are essential in combination with recording environmental parameters of potential influence (Kauserud et al. 2010; Büntgen et al. 2012, 2013, 2015; Boddy et al. 2014; Ágreda et al. 2015, 2016; Primicia et al. 2016; Schenk-Jäger et al. 2016; Alday et al. 2017a,b; Andrew et al. 2017; Chapter 3 and Lakkireddy et al. 2016, 2020 – Chapter 4).

The pleasure of documenting the beauty of fungal organisms by photography in this PhD thesis (Chapter 1) went along with recording places and local conditions of findings. This allowed also to interconnect them with weather data available for the North Campus of the University of Göttingen by a weather recorder station (Hygro-thermo transmitter - Adolf Thies GmbH & Co. KG, Thies Clima, Göttingen, Germany) at the Faculty of Forest Sciences and Forest Ecology (positioned in the open greenhouse at the building Büsgenweg 2). In this way, it was possible to deduce e.g. for the species *Coprinellus disseminatus*, *Coprinellus domesticus*, and *Coprinellus micaceus* from the form group of Coprini (Chapter 3 – Part I) and also for the agaric *A. xanthodermus* (Lakkireddy et al. 2020 – Subchapter 4.2) favored temperature and humidity conditions. Sufficient rainfall increasing the humidity and for most species decreasing the temperature is seen favorable to induce fruiting of these species at appropriate temperature ranges (about 15-25 °C in case of several closer observed Coprini, Chapter 3- Part I; about 15-20 °C in case of *A. xanthodermus*; Lakkireddy et al. 2020 – Subchapter 4.2). Fruiting of these saprotrophic species is apparently not necessarily very

much confined to (a) special month(s) of the year, particularly also not in years with higher average temperatures in the winter months and with sufficient rainfall in summer and autumn months, but it is positively affected by the proper combinations of humidity and temperature values. Under proper amounts of rainfall at favorable temperatures, mushrooms may be generated in several flushes such as observed for an *A. xanthodermus* colony underneath a Douglas fir (*Pseudotsuga menziesii*) in a meadow on the North Campus of the University of Göttingen (next to Büsgenweg 5; further information in Lakkireddy et al. 2020 – Subchapter 4.2) or repeatedly also at distinct places especially for the Coprini *C. disseminatus*, *C. domesticus*, and *C. micaceus* (more reading in Chapter 3 – Part I). A strong linkage of fruiting to rainfall has recently also been deduced by other researchers for other mushrooms species from observations in nature in other countries and ecosystems, such as in the temperate oceanic Britain (Kauserud et al. 2010; Boddy et al. 2014) and Norway (Kauserud et al. 2010, 2012; Boddy et al. 2014), in alpine Switzerland (Kauserud et al. 2012; Büntgen et al. 2013; Boddy et al. 2014; Andrew et al. 2016) and Austria (Kauserud et al. 2012; Boddy et al. 2014), in Mediterranean-type ecosystems (Büntgen et al. 2015; Agreda et al. 2015, 2016; Andrew et al. 2016; Primicia et al. 2016; Alday et al. 2017a,b), in Brazilian ecosystems (Braga-Neto et al. 2008; Paz et al. 2015), and in tropical Pacific Panama (Piepenbring et al. 2015). We can now add fruiting body production under the continental climate of Mid-Germany (South of Lower Saxony) as another climatic condition under which a connection between rainfall and temperature regimes on the one hand and fruiting body development on the other hand has been deduced from observations outside in nature. Moreover, the species range of saprotrophic *Agaricomycetes* at which we looked at was so far mostly neglected in the literature. The work performed and presented in this PhD thesis therefore offers new knowledge on several species of the Coprini (Chapter 3 – Part I) and also on the agaric *A. xanthodermus* (Lakkireddy et al. 2016, 2020 – Chapter 4).

From our observations it can be further noticed that rainfall tends to cause a drop in temperature and that fruiting happens then after the rainfall usually at reduced temperature when the air humidity (measured values of air humidity are used in this study) is still high (Fig. 2 and Table 1; Chapter 3; Lakkireddy et al. 2020 – Subchapter 4.2). This situation of a reduction in temperature very much resembles reports from artificial conditions required for many species for fruiting in culture in the lab or for commercial production of fruiting bodies in mushroom farms. For many cultivable species, mycelial growth is better at higher temperature values while fruiting occurs only when after growth the temperature is suitably decreased (Kües and Liu 2000; Rühl and Kües 2007). Moreover, the humidity in the air needs to be high but also the water potential within the growth substrate of a fungus is decisive. Fully grown wood blocks or chip bags of *Lentinula edodes* for example are thus soaked for several hours in water prior to transfer into suitable cultivation conditions with appropriate lower temperatures for induction of fruiting body development (e.g. Tokimoto et al. 1998; Ohga and Royse 2001; Royse and Sanchez-Vazquez 2001; Philippoussis et al. 2007; Rühl and Kües 2007). One should therefore have in mind that a (strong) rainfall will not only be responsible for reduction of temperature and for keeping the air humidity high by afterwards evaporation from rain-soaked soil and other wet material but it will also give the substrate the favorable water potential to mediate fungal fruiting body development. In congruence with this, fruiting in nature is reported to be sensitive to soil moisture (Tsujuno et al. 2009) and water resource availability pulses (Alday et al. 2017b). Following rainfall, fruiting body development can therefore initiate and continue over the few or more days that a species needs to complete the whole fruiting process, even if there might be no further rainfalls during this time. However, in the case that the air dries too much out, too early and too fast, mushroom development will not be completed, probably because of drying out of the structure when no further water can possibly be supplied from the substrate as replacement of evaporated water.

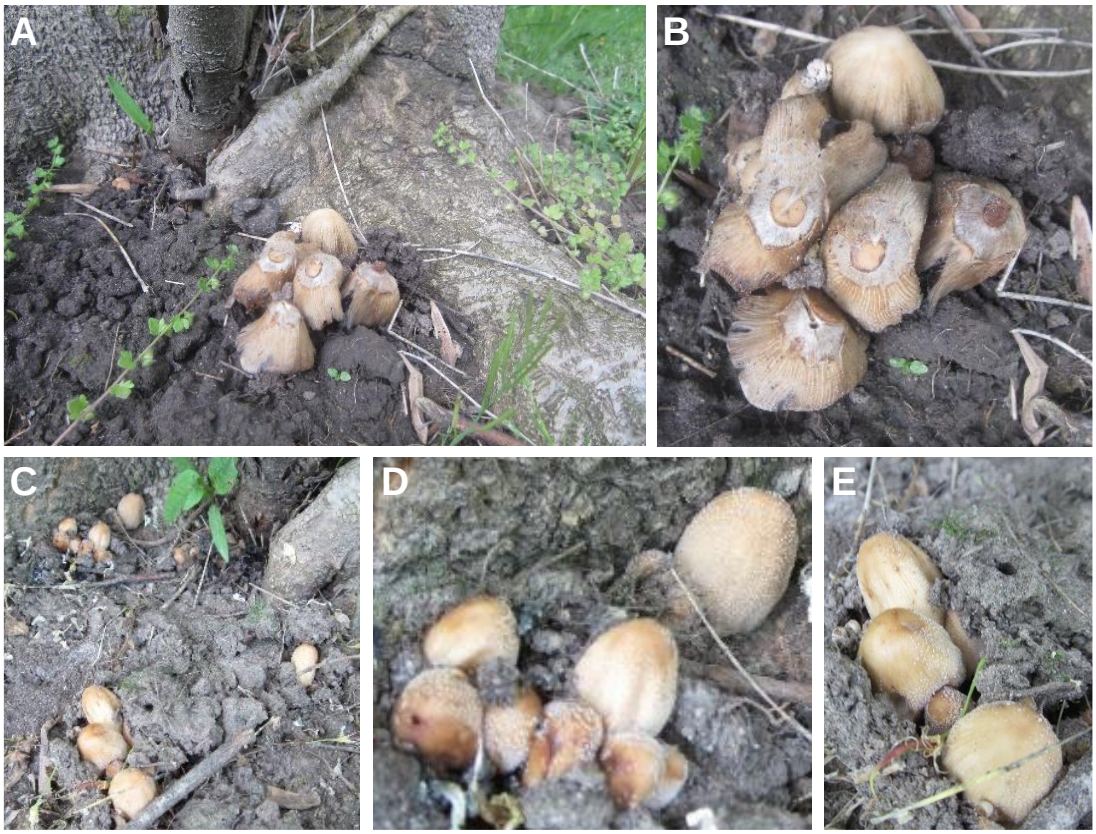

Fig. 4 Aborted developmental structures of *Coprinellus truncorum* underneath a *Cladrastis lutea* (Kentucky yellowwood) tree on the grounds of the North Campus of the University of Göttingen, photographed on 03.05.2013 (A, B) and 01.07.2013 (C-D).
Temperature and humidity values for the case shown in **A.** and **B.** were
prior to fruiting 12-19/18-26 °C and 67-79/43-67 % (night/day; 25.04.2013),
13-15/15-25 °C and 65-74/43-93 % (night/day; 26.04.2013),
and 5-7/5-7 °C and 100/98-100 % (night/day; 27.04.2013),
during development 4-6/8-15 °C and 85-98/49-77 % (night/day; 28.04.2013)
and 9-10/11-17°C and 71-80/34-75% (night/day; 29.04.2013),
and during drying 7-11/11-16 °C and 65-82/37-70 % (night/day; 29.04.2013-01.05.2013)
and 9-11/10-15 °C and 88-93/58-94 % (night/day; 02.05.2013 to 03.05.2013), respectively.
Temperature and humidity values for the case shown in **C.** and **D.** were
prior to fruiting 13-16/14-19 °C and 71-83/64-94 % (night/day; 24.06.2013)
14/14-16 °C and 83-89/68-94 % (night/day; 25.06.2013),
and 9-13/12-17 °C and 79-93/59-80 % (night/day; 26.06.2013-27.06.2013),
during development 11-15/14-19 °C and 71-92/62-94 % (night/day; 28.06.2013-29.06.2013),
and further at reduced humidity 6-13/14-18 °C and 81-91/58-72 % (night/day; 30.06.2013),
and at increased temperature 13-19/21-27 °C and 71-84/48-89 % (night/day; 01.07.2013), and
then 13-16/16-25 °C and 91-96/46-85 % (night/day; 02.07.2013), respectively,
which lead to abortion of the structures.
For further interest, compare the climate curves in Fig. A2 in Appendix III of Chapter 3.

Examples of fungal developing structures which dried out prior to that they could have undergone fruiting body maturation and basidiospore production are shown in Fig. 4 for *Coprinellus truncorum*, together with measured temperature and humidity values prior to fruiting, at initiation (likely one or two days after a rainfall/an increase in humidity) and during early steps of fruiting body development up to the differentiation of stipe and cap

tissues (the next days when the humidity was still reasonable high), and subsequent days during the phase of development when at a stage of early cap opening the respective young fruiting structures dried out by a drop in humidity (Fig. 4A and B) and, in another case of some still closed young mushrooms, in addition by an increase in temperature (Fig. 4C and D). Drop in humidity and possibly also increase in temperature are thus likely the reasons for the abortion of the closed or just starting to open young mushrooms. Other examples for drying out of primordia and young closed or just opening young mushrooms are documented for *C. micaceus* in Fig. 5I and J in this chapter, and for young fruiting bodies of *C. disseminatus* prior to sporulation, as shown in the Chapter 3 in the Fig. 7 and the corresponding environmental conditions-summarizing Table 3, with temperature and

Fig. 5. Decaying elm tree (*Tilia cordata*) stump in a lawn close to the Chemistry Buildings on the North Campus of the University of Göttingen

View from top on the partially decayed stump with brittle cubicle wood and flushes of *Coprinellus micaceus* young mushrooms with still closed caps **A.** at 30.09.2013 and **B.** at 02.11.2013. **C.** Much further deteriorated stump with young mushrooms nearby at 07.04.2014. **D.** Brittle wood residues at the place of the decayed stump (16.06.2014) and **E.** wood chips (ca. 2 to 4 cm long) taken to the lab for analysis (11.04.2014; see Chapter 3). **F.** View on the lawn with mushrooms and the open area presenting the place of the decayed stump at the 14.04.2017. **G.** A tuft of *C. micaceus* fruiting bodies above the root zone. **H.** Fruiting bodies of *Flammulina velutipes* at the stump zone. **I.** View on the lawn with mushrooms and the open area presenting the place of the decayed stump at the 21.05.2017. **J.** Enlarged view on a tuft of *C. micaceus* fruiting bodies formed at the edge of the stump zone. **K.** Enlarged view of brown stained rotted cubic residues of the stump. Photographs kindly supplied by W. Khonsuntia (**F**-**H**) and U. Kües (**L**-**K**).

humidity values that show that an increase to warmer temperatures (from about 15-20 °C up to highest temperatures of 25 to 27 °C at day times in the 1 to 6 days after the rainfall/increase in humidity) and a parallel drop in humidity (from >80-100 % down to at lowest 40-55 % humidity at day times in the 1 to 6 days after the rainfall/increase in humidity) was possibly negative for spore production in the caps which in turn remained thus white.

Weather conditions (humidity and temperature) were the most solid parameters which we got to connect to fruiting of *Agaricomycetes* in general and to fruiting of the species which in this PhD work were specifically better observed due to their relative often occurence (Chapter 3 and Lakkireddy et al. 2020 – Subchapter 4.2). When fruiting occurred on the grounds of the North Campus of the University of Göttingen, it usually happened in parallel for several closely and also distantly related species which is documented in own observations in the dated photos of multiple species of mushrooms in Fig. 1 to 20 of Chapter 1, in Fig. 1 to 6 of this chapter, and by photographing many more mushrooms from different *Agaricomycetes* over the time (not further shown and not evaluated in detail). However, in addition to the reported observations in this PhD thesis on climate conditions favourable for fruiting of selected fungi, some extra knowledge was collected regarding substrates on which certain species may fruit.

Noteworthy is the connection of a number of Coprini to fruit on likely nitrogen-enriched soils (Chapter 1 and 3). Many of the Coprini belong to the chemoecological, -physiological group of ammonia fungi, i.e. those fungi which can exist and (often) also fruit in presence of higher amounts of nitrogen of different organic or anorganic forms, among ammonium-nitrogen (Yamanaka 1999; Suzuki 2006; Imamura and Yumoto 2008; Sagara et al. 2008), and which influence the pH of their environments to switch to alkaline conditions (pH 8 to 10) by production of bases through ammonia release (Sagara 1975; Suzuki 2009a). *Coprinopsis cinerea* for example is a typical ammonia fungus which switches the pH during cultivation from slightly acidic to alkaline (Fukiharu and Hongo 1995; Fukiharu et al. 1997; Soponsathien 1998; Suzuki et al. 2003; Navarro-Gonzaléz 2008) and which fruits even under dark conditions on ammonia–rich media (Morimotro et al. 1981) while fruiting body development of the species is normally strictly under the control of light (Kües 2000; Kües and Navarro-Gonzaléz (2015).

Other Coprini reported to belong to the ammonia fungi are *Coprinopsis asiaticiphlyctidospora* (Fukiharu et al. 2014), *Coprinopsis austrophlyctidospora* (Raut et al. 011a; Fukihara et al. 2011), *Coprinopsis echinospora* (Lehmann 1976; Sagara 1975; Yamanaka 1995a,b; Soponsathien 1998; Sagara et al. 2008), *Coprinopsis laanii* (Sagara 1992), *Coprinopsis neocinerea* (Nguyen et al. 2019), *Coprinopsis neolagopus* (Sagara 1975; Soponsathien 1998; Imamura and Yumoto 2004), *Coprinopsis neophlyctidospora* (Raut et al. 2011b), *Coprinopsis novorugosobispora* (which was first published under the name *Coprinopsis rugosobispora* and might also be mistaken as a two-spore morphological variant of *Coprinopsis phlyctidospora*; Fukiharu et al. 2012; Gierczyk et al. 2014; Raut et al. 2015), *C. phlyctidospora* (Fukiharu and Hongo 1995; Suzuki et al. 2002; Raut et al. 2011; Gierczyk et al. 2014), *Coprinopsis tadians* (Soponsathien 1998), *Coprinopsis stercorea* (Sagara 1995; in other newer studies identified under the name *Coprinopsis tuberosa*, Sagara et al. 2008 and Barua et al. 2012), and likely also *Coprinopsis* aff. *radians* and *Coprinopsis strossmayeri* (Badalyan et al. 2011).

Effects of nitrogen sources on fruiting in culture were studied in Japan with *C. phlyctidospora*. Addition of ammonium salts and urea promotes fruiting in light and addition of urea promotes also the unusual fruiting in the dark (He and Suzuki 2003). Moreover, addition of urea to *C. stercorea* cultures supports fruiting in light (Morimoto et al.

Fig. 6 Fruiting bodies of *Flammulina velutipes* on or near to a decaying stump of an elm tree (*Tilia cordata*) close to the Chemistry buildings on the North Campus of the University of Göttingen.

Photographed **A.** on 18.09.2012 (night/day: 12-16/15-23 °C, 89-94 % humidity) growing in the vicinity of *Coprinellus xanthothrix* (larger mushrooms to the left; debris of smaller *F. velutipes* fruiting bodies at the right), **B.** on 28.10.2013 (night/day: 13-16/14-20 °C, 73-89/55-74 % humidity), **C.**, **D.** on 02.11.2013 (night/day: 8-10/10-14 °C, 90-97/88-100 % humidity) growing on the top surface of the decaying stump with multiple *Coprinellus micaeus* fruiting bodies growing on the outer surface of the stump and in the surrounding grass, **E.** on 09.01.2014 (night/day 7-10/9-12 °C, 85-95/75-86 % humidity), **F.** on 25.01.2014 (night/day: -3 to -4/-4 to -6 °C, 85-88/79-87 % humidity), **G.**, **H.** on 20.10.2014 (night/day: 13-16/13-17 °C, 84-101/64-83 % humidity), and **I., J.** on 20.11.2014 (night/day: 6-7/6-7 °C, 88-91/82-8 7% humidity).

1982). Application of urea in forests stimulated fruiting of ammonia fungi in field studies (Imamura and Yumoto 2004; Suzuki 2006). Urease activities in soil (mainly in the upper O layers) of hydrolysing urea into NH_3 and CO_2 have been implicated in the promotion of fruiting of ammonia fungi (Imamura et al. 2006). *Coprinopsis atramentaria*, *C. disseminatus* and *C. domesticus* from the genus *Coprinellus*, *Coprinus comatus*, and also a *Lacrymaria* species were observed during this study to fruit on nitrogen-rich substrate (Fig. 11 A-D; Fig. 14 A, B and Fig. 15C in Chapter 1; Fig. 5-11, Fig. 10 and Fig. 11 in Chapter 3), judging from the prevailing nitrogen-indicator plant communities (*Urtica* nettle species and ground ivy *Gleochoma hederacea*) and the fertilization of a meadow throughout a whole summer period by feces and urine of a sheep flock (year 2011). Barua et al. (2012) however list *C. atramentaria* and *C. comatus* as non-ammonia fungi. Most ammonia-fungi including *C. cinerea*, *C. echinosporus*, *C. neolagopus*, and *C. phlyctidospora* have good urease activities while the latter authors did not detect such activity for *C. atramentaria* and only weak urease activity for *C. comatus*. In accordance with measured urease activity for the species (Barua et al. 2012), *C. cinerea* has a urease gene (Navarathna et al. 2010; Strope et al. 2011). Moreover, nearly all other *Basidiomycetes* and so far all *Agaricomycetes* with sequenced genomes have also one or more urease genes as concluded from genome Blast searches with the *C. cinerea* urease sequence (EAU85273; unpublished observation).

Presence of a urease gene can thus not been taken as an indication that a species will belong to the chemoecological group of ammonia fungi.

When freshly chopped wood chips including bark from tree cuttings were provided as ground cover underneath trees on the grounds of the North Campus of the University of Göttingen, we frequently observed Coprini to occur in the running year as early phase fungi in the material, whereas fruiting was less often encountered in covers of wood chips of older ages (Navarro-Gonzaléz 2008; Fig. 10 in Chapter 1; see further information in the text and Table 1 of Chapter 3 – Part I and Table A1 in Appendix I of Chapter 3). It remains to be shown whether this effect of time relates to fast release and consumption of the easily accessible unconsumed nitrogen from the parenchymatic sapwood cells of fresh wood chips and from tissues of the bark. Fast release and consumption of the nitrogen source has been suggested to happen as prompt effects upon urea input into forest biotopes (Imamura et al. 2006). In the studies in Japan, ammonia fungi including *C. phlyctidospora* tended to fruit in summer and autumn, particularly at periods after addition of a nitrogen source (urea), but urea-stimulated fruiting happened also in winter and spring months at moderate temperatures and under rainfall while the vegetation types did not play a role in it (Imamura and Yumoto 2004; Fukiharu and Hongo 1995; Imamura et al. 2006). The less strict confined fruiting to a specific phase or months of the year resembles the observations on fruiting body formation of some of the Coprini species in this study (Chapter 3 – Part I).

Coprini are often dung and compost fungi (Redhead et al. 2001), thus in ecological sense coprophilous ammonia fungi (Suzuki 2009a,b) and their ecological environments tend to offer pulses of ample amounts of nitrogen sources (Lynch et al. 2006). While many ectomycorrhizal Agarics are in relation nitrophobic (Lilleskov et al. 2001, 2011), others which belong to the nitrophilic ectomycorrhizal genera *Hebeloma* and *Laccaria* are in chemoecological sense also ammonia fungi (Fukihara and Hongo 1995; Soponsathien 1998; Sagara et al. 2000; Suzuki et al. 2003; Imamura et al. 2006; Deng and Suzuki 2008; Imamura and Yumoto 2008; Maeno et al. 2014). Members of these ectomycorrhizal genera live species-dependent with narrow or broad host ranges in symbiosis with specific or several hardwood and sometimes softwood tree species (Marmeisse et al. 1999; Eberhardt et al. 2009) or as multi-host symbionts on many softwood and hardwood species (Kropp and Mueller 1999; Plett et al. 2015), respectively. Species of Coprini are early phase (EP) ammonia fungi and quickly sporulate, while the ectomycorrhizal species are late phase (LP) ammonia fungi, because they occur late in succession on nitrogen-rich substrates. Early-stage EP fungi are ruderal stress-tolerant strategists whereas late-stage EP fungi are combative ruderal strategists (Suzuki 2009a,b).

The ectomycorrhizal LP ammonia fungus *Hebeloma cylindrosporum* prefers inorganic over organic nitrogen sources, including nitrate (Marmeisse et al. 2004; Avolio et al. 2012). An ammonium-regulated nitrate assimilate gene cluster with a gene for a nitrate transporter Ntr2, a nitrate reductase Nar with nitrite as product, and a nitrite reductase Nir with ammonium as product has been studied in *H. cylindrosporium* (Jargeat et al. 2000; 2003). Similar gene clusters are also present in the ammonia fungi *C. cinerea* and *Laccaria bicolor* (Lucic et al. 2008; Navarro-Gonzaléz 2008; Kemppainen et al. 2010) and could contribute to the phenomenon of ammonia fungi. *nrt2* and *nar* genes have been amplified from more species of the genera *Hebeloma* and *Laccaria* as well as *nrt2* from *C. disseminatus*, *Coprinellus curtus*, *C. micaceus*, *Coprinellus radians*, *C. atramentaria*, and some *Psathyrella* species and gene *nar* also from *C. micaceus* (Slot and Hibbett 2007; Slot et al. 2007, 2010; Nygren et al. 2008) It is thought that the *nar-nrt2-nir* gene cluster in *Basidiomycetes* was acquired by a horizontal gene transfer early in evolution and that it has subsequently been lost in many *Basidiomycetes* lineages (Slot and Hibbett 2007; Slot et al. 2007). A nitrate

assimilate gene cluster is thus missing in many *Basidiomycetes* (Slot et al. 2007, 2010; Nygren et al. 2008), for example in the mushrooms *Agaricus bisporus* and *S. commune* (own observation from blasting the JGI Mycocosm page and the NCBI *Coprinopsis* (taxid:184431) whole genome database with Nar, NTr2 and Nir of *C. cinerea*, XP_001840500, XP_001840499, XP_001840498). *C. micaceus* (ID 1695059, 1670725, 1740823) and *Laccaria amethystina* (ID 679397, 679398, 132785; interrupted by two other genes for unknown proteins ID 616900 and 679402) have complete gene clusters but *Coprinellus pellucidus*, *Coprinopsis marcescibilis* (ID 672933, 646153, 758975) and *Coprinopsis sclerotiger* (ID 488624, 527127, 488624) not. A draft genome for *C. strossmayeri* was recently published (Banks et al. 2017) and contig FTPT01000151 was identified which carries DNA sequences for a complete *nar-nrt2-nir* gene cluster. *Lepista nuda*, another ammonia fungus (Sagara 1975; Soponsathien 1998), has three *nar* genes (ID 1277267, 1276612, 1276938) and an unlinked *nir* gene (ID 550928) but apparently no *nrt2* gene. There are however also possibilities to produce ammonium from organic compounds. The ectomycorrhizal *Hebeloma* spp. and *L. bicolor* are shown to have L-amino acid oxidase activities for release of organic-bound nitrogen as ammonium (Nuutinen and Timoneni 2008; Nuutinen et al. 2012). Again, *C. cinerea* (ID 2155), *C. micaceus* (ID 1662893, 1746453, 1819164), *C. marcescibilis* (ID 752962), *C. sclerotiger* (ID 529621, 529644), *C. strossmayeri* (on contigs FTPT01000146 and FTPT01000230), *L. amethystina* (ID 681274, 1732111) and *L. nuda* (ID 1305819, 1272826, 724903) have respective genes for related potential L-amino acid oxidases, but not *A. bisporus*, some strains of *S. commune* (strains Loenen and Tattone not whereas strain H4-8 has a gene, ID 2667289), *C. pellucidus* and many other *Agaricomycetes* (determined on the JGI Mycocosm page by blasting with *L. bicolor* L-amino acid oxidase 1, DAA34975). The mechanism(s) of ammonium production by ammonia fungi and the accompained environmental pH increase should be better evaluated on molecular level in the future.

Regarding possible substrates of further continuous debate among scientists is whether species of Coprini can grow on wood and decay it as a substrate and, moreover, whether there are tree pathogens among them (Gilbertson 1980; Redhead and Ginns 1985; Keirle et al. 2004; Ministry of Environment, Lands and Parks 1997; Redhead et al. 2001; Bagley and Richter 2002; Navarro-Gonzaléz 2008; Oliver et al. 2010; Badalyan et al. 2011). In this study, fruiting bodies of Coprini were repeatedly observed in beds with wood chips, on a stump of an elm tree (*Tilia cordata*) and on roots and stem bases of various living shrubs and trees (see Fig. 4 and Fig. 6 in this chapter; title page of this thesis with photo of *C. micaceus* on a decaying *T. cordata* tree stump photographed on 28.09.2013 on the grounds of the North Campus of the University of Göttingen; Fig. 10 and 11, Fig. 14 and 15, Fig. 18 and 19 in Chapter 1; Chapter 3 – Part I). The observations in this PhD thesis thus support the above cited former publications that various Coprini in nature can live and reproduce in close association with wood. This however does not answer whether they also attack wood and can degrade the wood lignocellulose. Some earlier publications (Ross 1976; Mitchel and Smith 1978; Gilbertson 1980; Redhead and Ginns 1985) reported before that *C. atramentarius* and *C. micaceus* fruit on brown-rotted aspen stumps. However, Redhead and Ginns (1985) deduced that the Coprini are late in the succession of wood decay fungi when the material is already softened in consistency and that the fungi may possibly be white-rotting. In this PhD study, repeated fruiting of *Coprinellus xanthothrix* on a decaying *T. cordata* tree stump (Fig. 6A; Fig. 19A-C in Chapter 1) was encountered at first in the years 2011 and again in 2012 and afterwards repeated fruiting of *C. micaceus* in the years 2011 to 2016 (see title page of this thesis; Fig. 4; Fig. 19D-G in Chapter 1; Fig. 12, 13 and 14 in Chapter 3 – Part I and see for both species the list of observation dates of fruiting events on the elm tree stump in Table A1 in Appendix I of Chapter 3). Fruiting of *C. micaceus* occurred again in 2017 while subsequently in the dry years 2018 and 2019 no further fruiting bodies appeared on the place

(Fig. 5F-K; W. Khonsuntia and U. Kües, personal communication). Before, fruiting bodies of *C. xanthothrix* were observed first in year 2010 upon felling the elm tree which suffered from decay in the stem base (M. Navarro-González, personal communication). The decaying stump fell part into brown brittle cubes as typical products on fungal brown-rot (Fig. 5D, E and K) and chemical analysis detected relative more lignin in the decayed material as compared to fresh wood (Chapter 3 – Part I).

Few reports in the literature state yet lignocellulolytic enzymatic activities of different Coprini, with *C. cinerea* being the best analyzed fungus with regards to the diversity of types of enzymes reported and also biochemically better described enzymes (see Table 2). Overall, there are some reports on cellulolytic and hemicellulolytic enzymes for potential degradation of wood polysaccharides, more on laccase activities and a few on some other types of auxiliary enzymes. All these enzymes might act in degradation of specific polymers from lignocellulosic plant biomass. However, secreted efficient heme peroxidases (lignin peroxidase, Mn-dependent peroxidase, versatile peroxidase) are commonly required as the phenoloxidases that effectively initiate wood decay by attack of the recalcitrant lignin in the case of white-rot fungi whereas extracellular reactive oxygen species produced through the chemical Fenton reaction with the help of Fe^{3+}-reducing fungal aromates are required in case of brown rots in order to break down linkages between lignin and polysaccharides in the wood (Martinez et al. 2009; Lundell et al. 2010; Wei et al. 2010; Eastwood et al. 2011; Fernandez-Fueyo et al. 2012; Floudas et al. 2012, 2015; Sigoillot et al. 2012; Hori et al. 2014; Levasseur et al. 2014; Riley et al. 2014; Rytioja et al. 2014; Floudas et al. 2015; Kües 2015; Manavalan et al. 2015; Nagy et al. 2016, 2017). *C. cinerea* has a generic fungal class II peroxidase of low-redox-potential (Kjalke et al. 1992; Baunsgaard et al. 1993; Cherry et al. 1999) which is ineffective with lignin (Petersen et al. 1994; Ruiz-Dueñas and Martínez 2009; Smith et al. 2009; Kersten and Cullen 2013). *C. disseminatus* (James et al. 2006), *C. micaeus* (ID 1668751, 1733396, 1749546), *C. pellucidus* (ID 592784), and *C. marcescibilis* (ID 217905, 649569, 705347, 754357, 760905) have genes for related generic peroxidases while the dung fungus *C. sclerotiger* has not. Furthermore, none of the *Coprinellus* and *Coprinopsis* species with completely sequenced genomes have genes for the peroxidases which are typical for lignin depolymerisation (Morgenstern et al. 2008; Ruiz-Dueñas et al. 2009). Peroxidase activities of some other Coprini have however been reported (Table 2), among Mn-dependent activity from *Paneolus papilionaceus* of the *Psathyrellaceae* (Heinzkill et al. 1998). In consistency, *P. papilionaceus* contains several genes for atypical Mn-depdendent peroxidases (ID 1392862, 1458747, 1458972, 1519804, 1561296, 1613629) of the kind described before in *Agrocybe aegerita* and *Agrocybe praecox* which have a conserved Mn-binding residue replaced (S40) as compared to the glutamate residue in the classical Mn-dependent peroxidases (Hofrichter and Ullrich 2006; Hildén et al. 2014). The Coprini of the genera *Coprinellus* and *Coprinopsis* might therefore be expected to be weak in lignocellulose degradation and the species of the genus *Panaeolus* to possibly be stronger (Heinzkill et al. 1998).

Presented in this PhD thesis in Chapter 3- Part I, wood decay tests in the lab with fresh elm tree wood failed to unravel any autonomous decay ability by isolated *C. xanthothrix* and *C. micaceus* dikaryons (Fig. 3E to L in Chapter 3), in support with the idea that the fungus is a later invader in succession of wood decay like other Coprini (Redhead and Ginns 1985; Peiris et al. 2008) and that the fungus colonizes the substrate when the wooden material has been partially decayed by other microbes including the lignin cover of the cellulose microfibrils in the plant cell walls. Oliver et al. (2010) showed for both hardwood and softwood that prior decay of wood by a brown-rot such as *Gloeophyllum trabeum* will accelerate wood decay by various *Coprinellus* strains (*C. micaceus*, *C. radians*) as compared to prior treatment by a white-rot such as *Trametes versicolor*. However, there were no such effects of enhanced wood

Table 2: Reports on lignocellulolytic enzymes of Coprini (kindly supplied by U. Kües)

Species	Enzymes	Reference(s)
Coprinellus congregatus	Laccase	Ross (1982); Kim et al. (2001)
Coprinellus disseminatus	Laccase, carboxymethyl cellulase, xylanases	Singh et al. 2009; Agnihotri et al. (2010); Lal et al. (2011, 2015)
Coprinellus micaeus	Laccase, peroxidase, cellulases, endo-β-1,4-glucanase, endo-β-1,4-xylanase	Guiraud et al. (1999); Sergentani et al. (2016)
Coprinellus radians	Laccase, peroxidases, aromatic peroxygenase (haloperoxidase Crp)	Ahn et al. (2007); Aranda et al. (2009); Liers et al. (2013); Reina et al. (2013)
Coprinopsis atramentaria	Laccase, peroxidase	Erden et al. (2009)
Coprinopsis cinerea	Laccases, generic peroxidase Cip, peroxygenase, cellulases, alkaline cellulase, carboxymethyl cellulase, cellobiohydrolases, GH131 glycoside hydrolase, β-glucosidase, xylanases, β-xylosidase, pyranose dehydrogenase, GH62 α-L-arabinofuranosidase, carbohydrate esterase, acetyl xylan esterase, pectinase	Long and Knapp (1991); Kjalke et al. (1992); Baunsgaard et al. (1993); Cherry et al. (1999); Guiraud et al. (1999); Schneider et al. (1999); Hoegger et al. (2004); Ikehata et al. (2005); Kilaru et al. (2006a); Hashimoto et al. (2010, 2011); Liu et al. (2009, 2010); Kaur et al. (2011); Tamura et al. (2012); Dutt et al. (2013); Juturu et al. (2013); Miyazaki et al. (2013); Rühl et al. (2013); Pan et al. (2014); Pathak et al. (2014); Babot et al. (2015); Raymond et al. (2015); Takeda et al. (2015, 2016); Karich et al. (2016); Lucas et al. (2016); Maan et al. (2016); Tonozuka et al. (2017)
Coprinopsis echinosporus	Laccase, peroxidase	Soponsathien (1998)¸ Ikehata et al. (2004)
Coprinopsis friesii	Laccase, peroxidases	Heinzkill et al. (1998)
Coprinopsis lagopus	Peroxidase	Ikehata and Buchanan (2002)
Coprinopsis macrocephalus	Peroxidase	Ikehata et al. (2004)
Coprinopsus phlyctidosporus	Laccase	Soponsathien (1998)
Coprinopsis scobicola	Cellulases, xylanases	Stephens et al. (1991)
Coprinopsis verticillata	Laccase, peroxidase	Ahn et al. 2007
Coprinopsis sp.	Laccase	Qasemian et al. (2012)
Coprinus sp.	Laccase	Soponsathien (1998)
Coprinus spp.	Peroxidase	Ikehata and Buchanan (2002); Ikehata et al. (2004, 2005)
Coprinus sp.	Cellulases	Guiraud et al. (1999)
Coprinus comatus	Laccase, carboxymethyl cellulase	Lu and Ding (2010); Li et al. (2010); Jiang et al. (2013); Li et al. (2014); Zhao et al. (2014)
Panaeolus sphinctrinus	Laccase, Mn-dependent peroxidase	Heinzkill et al. (1998)
Panaeolus papilionaceus	Laccase, Mn-dependent peroxidase	Heinzkill et al. (1998)
Parasola plicatilis	Laccase, peroxidase	Erden et al. (2009); Akdogan (2015); Akdogan and Topuz (2015)

decay when strains of *C. atramentaria* and *C. comatus* were tested as secondary invaders after *G. trabeum* and *T. versicolor*, respectively (Oliver et al. 2010). The results of Oliver et al. (2010), the former observations and the deductions from a literature review by Navarro-Gonzaléz (2008) and several of the observations in this study of Coprini on wood substrates (Fig. 4, Fig. 5 and the discussion above in this chapter; Fig. 10, Fig. 18 and 19 in Chapter 1; Chapter 3- Part I) may suggest that many species of the genus *Coprinellus* may be better adapted to grow and fruit on wooden substrates than species of other genera of Coprini, even if this might be (partially) mediated through the help of other microbes.

During our observations of fungal species on the stump of the *T. cordata* tree close to the Physic´s buildings on the North Campus of the University of Göttingen, fruiting bodies of *Flammulina velutipes* were also repeatedly noticed (Fig. 5F, H; Fig. 6), with and without simultaneous *C. xanthothrix* or *C. micaceus* fruiting (see list of dates of fruiting events on the elm tree stump in Table A1 in Appendix I of Chapter 3). This white–rot species might have helped in decay of the lignocellulose of the stump and may have helped *C. micaceus* to exist in the place and to fruit repeatedly over several years. However, it is possible that there were also other decay fungi contributing to the stump decay over the time which were overlooked by lack of fruiting bodies (Rinne et al. 2017) or being missed by not always controlling the elm tree stump for presence fruiting bodies. From observation of fruiting bodies, it is easily been done to conclude that the species which is visible by its fruiting bodies is responsible for the decay of the lignocellulosic substrate beneath. However, such ostensible conclusion from cursory observations in nature can also easily lead to misconcepts when unseen other species are indeed actibe in wood decay (Redhead and Ginns 1995; Peddireddi et al. 2005; Peddireddi 2008).

Occurrence of fruiting bodies on the decaying material in the other hand is no solid proof for an abilitity of a fungus to perform wood decay. The split gill fungus *S. commune* for example is often found as a first (pioneering) species to fruit on twigs, branches and stems (Fig. 16 in Chapter 1; Lakkireddy et al. 2017 and Chapter 3 – Part II; Peddireddy 2008) and the wood beneath the fungus can provide an impression of white-rot (Fig. 7). Fungal guide books thus do refer to the species as white-rot fungus (Jahn 1979; Arora 1986; Breitenbach and Kränzlin 1995) and scientific literature on the species does sometimes the same (Schmidt and Liese 1980; Ohm et al. 2010). However, while *S. commune* grows on and in wood vessels and sometimes is also seen in microscope preparations of wood samples to attack plant cell walls (Peddireddi et al. 2005; Erwin et al. 2008; Peddireddy 2008; Riley et al. 2014; Floudas et al. 2015; Koyani et al. 2016), wood decay tests in the laboratory usually fail to proof degradation abilities of *S. commune* through no and only little noticable weight losses (see the literature survey and the respective experiments by Peddireddi et al. 2005; Sexton et al. 1993; Ah Chee et al. 1998; Erwin et al. 2008; Peddireddi 2008; Wei et al. 2013; Schilling et al. 2015) but see the unique very contrasting report by Koyani et al. (2016) on high weight loss mediated by a *S. commune* strain on the most outermost sapwood of different tropical woods when grown on malt extract medium offering extra nutrients. However, even without significant weight loss physical properties such as bending strength can be affected by the fungus (Peddireddi 2008) and lignin properties of the wood might be altered (Schilling et al. 2015), even though the fungus lacks aggressive peroxidases and laccases and is neither a white rot nor a typical brown rot (Peddireddi et al. 2005; Riley et al. 2014; Floudas et al. 2015). Current discussions therefore target at possible alternatives as how *S. commune* can tap nutrients and may grow in wood to eventually form fruiting bodies on the host surface (Floudas et al. 2015). Various observations such as from *Pinus tabulaeformis* branches (Wang et al. 2005), twigs of *Eucalyptus* (Fischer et al. 1993), young *Aralia elata* (Chinese angelica-tree) plants (Wu et al. 2012) and, moreover, roots of Scots pine *Pinus sylvestris* (Smith et al. 2017), leaves of *Piper hispidum* (Jamaican pepper; Orlandelli et al. 2015) and banana (*Musa*

spp.; Assunção et al. 2010), leaves, rachis and petioles of the oil *palm Elaeis guineensis* (Rungjindamai et al. 2008) and pods of the Malvaceae tree *Theobroma gileri* (Thomas et al. 2008) suggest that *S. commune* may exist as an endophyte in the healthy living host without symptoms. In nature, the fungus will usually be noticed by fruiting body formation (Fig. 7; Fig. 16 in Chapter 1; Chapter 3 – Part II; Essig 1922; Peddireddi et al. 2005; Peddireddi 2008). Because the fungus occurs on twigs and branches of living trees and shrubs and freshly fallen dead twigs, branches and stems on and underneath the bark and because it can enter the functional sapwood (Jahn 1979; Peddireddi 2008; Fig. 7C-E; Fig. 16 in Chapter 1; Fig. 16 and 17 in Lakkireddy et al. 2017 and Chapter 3 – Part II), *S. commune* may most easily partake of the nutritional reserves present in the living (parenchymatic) cells of the tissues of the host (Floudas et al. 2015) with a certain potential to act as a sap-rot (= sapwood rot) following the death of bark, cambium and sapwood storage cells (Davidson et al. 1942; Luley and Kane 2009; Erwin et al. 2008; Takemoto et al. 2010). *S. commune* is often seen not alone on the wooden substrate but with fruiting bodies of other wood-rotting species (Lakkireddy et al.

Fig. 7 A., B., C. and E. *Schizophyllum commune* infested branches of a *Juglans ailantifolia* (Japanese Walnut) tree on the grounds of the North Campus of the University of Göttingen (15.03.2005) with mushrooms grown on the sunny upper side of the branches and C. a series of 1-cm-thick disks from the infested branch harvested from the cut shown in D. and E.

Starting with the disk D1 obtained directly at the cut of the branch (**D**, **E**), D with following numbers in subfigure **C.** refers to the consecutive cuts obtained from the >70 cm-long *S. commune* infection in the harvested branch. The blue-grey shades in the wood represent fungal pigments (Miles et al. 1956; Epstein and Miles 1966; Hosoe et al. 1999; Arun et al. 2015; Menezes et al. 2015) and mark the area of wood which has been occupied through growth by the fungus. Note also the white fruiting bodies on the outer surface of some of the discs (D1, D16, D27, D29). Note further that the tree was stressed through replanting in year 2003 due to construction works at its original growth place (Peddireddi 2008). Photos from the departmental collection supplied by U. Kües.

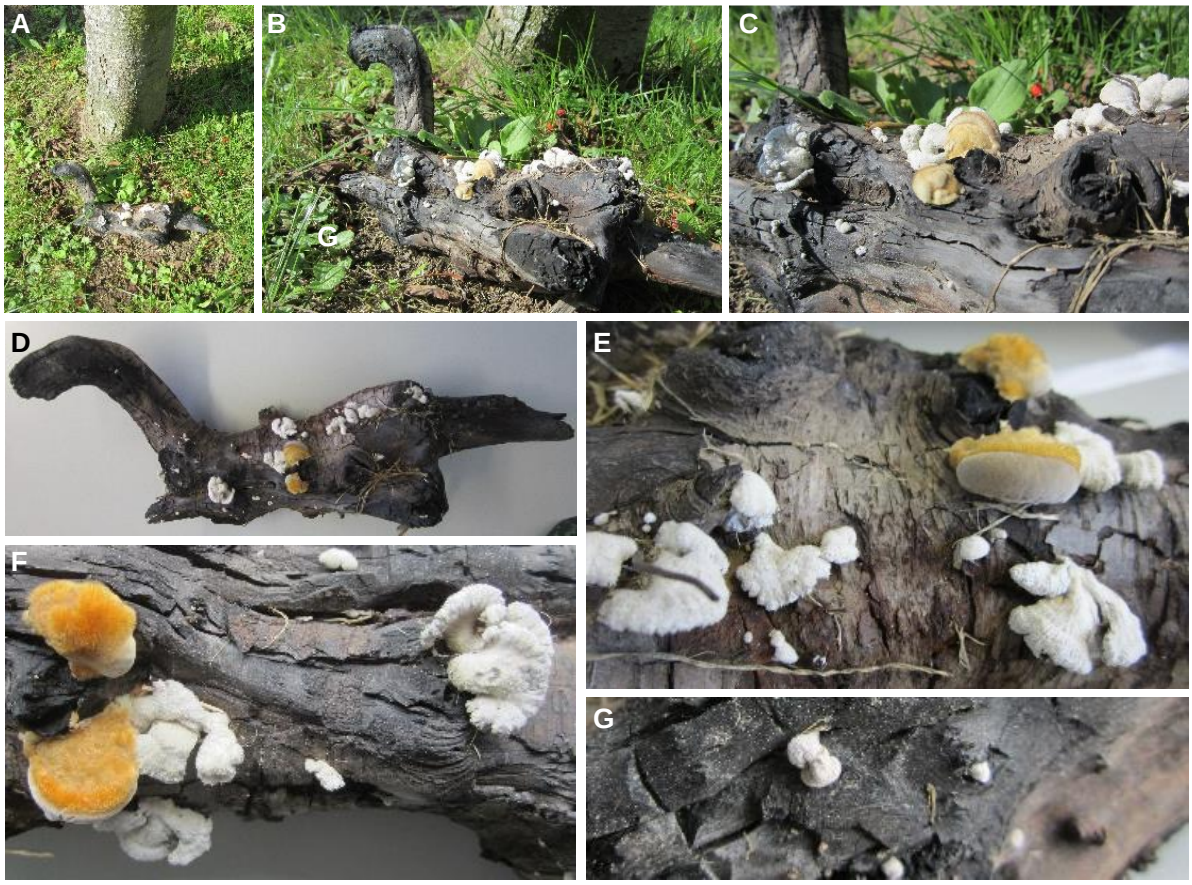

Fig. 8 Whitish *Schizophyllum commune* mushrooms with yellowish fruiting bodies of the polyporous basidiomycete *Inonotus hispidus* on a burnt branch of a cherry tree, found on 14.09.2014 at the Otto-Hahn-Strasse at the side of the Northern Campus of the University of Göttingen near the Police station, opposite to the place where the yearly Easterfire of the community of Weende is celebrated.
A.-C. Views at the place of discovery, **D.-G.** photos taken in the laboratory. Note the cracks in the unburned and in heat-darkened bark and the cracks in the charcoaled wood where mushrooms grew out.

2017 and Chapter 3 – Part II; Fig. A4 in Appendix V in Chapter 3; Essig 1922; Peddireddi 2008). The fungus may thus benefit from the foreign wood decay activities (Erwin et al. 2008; Floudas et al. 2015). Furthermore, *S. commune* can act as mycoparasite on other fungi which offers another potential for the requisition of nutrients (Takemoto et al. 2010). *S. commune* can grow at a reduced water potential as compared to other wood- and litter-decomposing species (Koske and Tessier 1986; Castillo and Demoulin 1997) which may give a competitious advantage to the species over other more aggressive wood-decaying fungi to confine to the sunny sides of wooden substrates (see discussion above and Fig. 6A and B) which easily dry out by sun and wind activities and can thus be harder to live in (Jahn 1979).

Moreover, *S. commune* produces also mushrooms on burnt barked wood (Fig. 8; Fig. 16 in Lakkireddy et al. 2017 and Chapter 3 – Part II). The fungus has some ability to grow on charcoal (tested in artificial system under addition of a nitrogen source; Wengel et al. 2006) and may occur and fruit in much increased frequencies on surface-burnt barked branches and stems upon forest fires (Toole 1957; Wylie and Shanahan 1976; Schmidt and Liese 1980; Conedera et al. 2010; Takemoto et Greeshma et al. 2016). While pyrolysis and burning heat alters the chemical composition of the material including replacing the natural

wood organic extractives by very different compounds and aromatic pyrolysis products (Kymäläonen et al. 2014), the effects and influences on fungal wood decay are little understood, whether e.g. some better to the situation adapated species by air-born spore fall will be primary invaders of the burned substrate or whether mycelia surviving a by fire protection through the host´s bark (Rosell 2016; Pausas 2017) will quickly colonize from inner wood tissues (Edman and Erikson 2016). Regarding spores of the worldwide distributed species *S. commune*, there is commonly no shortage of spores in the air (James et al. 1999; James and Vilgalys 2001; Nieuwenhuis et al. 2013) while the re-occuring observation that *S. commune* fruits rather on the still barked areas of burned branches and stems (Fig. 8) might support the second hypothesis.

C. xanthothrix (Fig. 6A; Fig. 19A to C in Chapter 1) and *C. micaceus* on the elm tree stump close to the Chemistry buildings at the North Campus of the University of Göttingen (Fig. 5A- J in this chapter; Fig. 19D-G in Chapter 1; Fig. 12 and Fig. 14 in Chapter 3 – Part I) and *C. disseminatus*, *C. micaceus*, *C. xanthothrix*, and *Psathyrella candolleana* of the Coprini on stem bases of living trees (Fig. 18A, C, F and Fig. 19H, I, L-W in Chapter 1) often fruited also from bark which covered (decaying) wood underneath. Similar observations by Buller (1924) were interpreted by the famous mycologist as living from easily accessible nutrients from the secondary phloem of the host. As for *S. commune* (see above), there are also increasing reports on Coprini to live as endophytic fungi within different organs of plants (roots, bark and wood, leaves, fruits), among *C. cinerea* and *C. micaceus* in sapwood of *Platanus* stems without and with cancer symptoms, respectively (Robles et al. 2015), *C. micaceus* in the wood of young stems of *Broussinetia papyrifera* and *Celtis occidentalis* (de Errasti et al. 2010), *C. domesticus* in bark and branches of *Taxus globosus* (Rivera-Orduña 2011), different *Coprinellus* species in sapwood of *Hevea* (Martin et al. 2015), *Theobroma cacao* (Crozier et al. 2006), and *Theobroma gileri* (Thomas et al. 2008), and in *Pinus tabulaeformis* branches (Wang et al. 2005). According to these reports, *Coprinellus* species appear particularly often to be already at place with direct access to nutrients when a tree weakens and wood decay could be initated. Possibly therefore, fruiting bodies of *Coprinellus* species were seen by Navarro-Gonzaléz (2008) and in this work comparably often to be associated with living shrubs and trees (Fig. 18 in Chapter 1; see text and e.g. Fig. 5 to 11 in Chapter 3 – Part I and also Table A1 in Appendix III of Chapter 3). However, other potential biological interactions for example such as with bacteria should also not be forgotten (Hoppe et al. 2014; Rinne et al. 2017). Wood-associated *Coprinopsis variegata*, *C. atramentaria* and *C. comatus* can attack lines of living bacteria, possibly for nitrogen and other nutrient supply (Barron 1988; Thorn and Tsuneda 1992; de Boer and van der Wal 2008) which lignocellulose-rich wood sources do not offer in excess (Merrill and Cowling 1966; Levi and Cowling 1969; Hoppe et al. 2014; Rinne et al. 2017) and which for efficient wood decomposition rates in particular also at early stages of decay must be transported to the wood from external sources (Rayner and Boddy 1988; Alban and Pastor 1993; Bebber et al. 2011; Philpott et al. 2014) including to large parts from bacteria (Rinne et al. 2017). However, microbivory could also serve to satisfy the need for more specific essential compounds, such as the vitamin thiamin which most *Agaricomycetes* from the phylum of *Basidiomycetes* including Coprini are unable to produce themselves and thus to need to be taken up from the environment (Chapter 7).

Accordingly, it is not surprising that feeding with thiamin can influence both, fungal growth and the efficiency of fruiting (Chapter 7). Genome analyses documented in Chapter 7 revealed that it is the *thi5* gene for a HMP phosphate synthase (compare Fig. 25 in Chapter 1) which is missing in many *Basidiomycetes* and, more specifically, in most *Agaricomycetes* and which makes these fungi in consequence thiamin auxotrophic. This contrasts the situation in the *Ascomycetes*, the sister phylum of the *Dikarya* whose species are often, while not always

thiamin prototrophic (see Chapter 7 and also Fig. 3 in Jiang et al. 2018). It is unclear whether loss of the thiamin biosynthesis gene *thi5* in many of the *Basidiomycetes* is just a random event in early evolution of the phylum without further consequences as long as thiamin will be supplied for by other organisms in the environment or whether there might be even an advantage for the fungi from the loss of the gene (Helliwell et al. 2013; Kraft and Angert 2017; further dicussed in Chapter 7). For example, feeding experiments showed for many species that thiamin is not only required for mycelial growth but also for fruiting body development (Aschan 1954; Wessels 1965; Gold and Cheng 1979; Leatham 1983; Jonathan and Fasidi 2001; Joo et al. 2009; Fu et al. 2011; Atri and Guleria 2013; Chapter 7). Thiamin could act for example as an environmental sensor for required biological nutritional sources for the fungi in nutritional limited and unbalanced environments and in the decision of inducing fruiting at appropriate times and help to overcome impeding stresses (Chapter 7).

While so far mostly biological interactions with possible beneficial effects for growth and on fruiting body production of selected *Agaricomycetes* were discussed, mushrooms are in contrast also endangered by various types of biological threats – they may for example be eaten by animals and humans (see Chapter 1) and they might be attacked by infections of microbial origin (Lakkireddy et al. 2016, 2020 – Chapter 4). In this study at the first time on the 1st of September 2015 (15-17/20-27 °C; 75-87/42-66 % humidity at night/day), a single young mushroom of *A. xanthodermus* was observed which was attacked by a mitosporic fungus (Fig. 17C in Chapter 1; Fig. 1F in Lakkireddy et al. 2016 - Subchapter 4.1 and Fig. 1 E-L and 2 in Lakkireddy et al. 2020 – Subchapter 4.2). While we caught interest in this observation and brought the diseased mushroom for mycoparasite identification (*Hypomyces odoratus* which causes cobweb desease on mushroom species; Lakkireddy et al. 2020 – Subchapter 4.2**)** and further study into the institute, in the next flush of *A. xanthodermus* fruiting two weeks later at the 14th of September 2015 (Fig. 20 in Chapter 1; Fig. 3 in Lakkireddy et al. 2020 – Subchapter 4.2) at suitable temperature and humidity conditions (15-16/17-23 °C and 92-98/56-89 % humidity night/day at the day) many patches of the pathogen *H. odoratus* were seen in the meadow, particularly also on places of former *A. xanthodermus* fruiting, and on several newly appeared primordia and young mushrooms. Whether patches of mycoparasites might have hindered already in certain instances the outgrowth of new fruiting body primordia, this is not possible to deduce from the observations of no initiation at these places. However, the mycopathogens attacked developmental structures of all ages of *A. xanthodermus* from the very early visible primordial stages onwards whereas mycelium in laboratory tests had certain degrees of resistance (Lakkireddy et al. 2020 – Subchapter 4.2), which makes it quite likely that the mycopathogen hinders also fruiting body initiation.

From the overarching discussion of own observations in nature explained in further details in the Chapters 3 and 4 of the PhD thesis and from resultant conclusions presented in the current chapter, it is obvious that new details on regulation of the fruiting behaviour of selected species and ecological groups of *Basidiomycetes* were realized from the observations presented. However, laboratory work on specific isolates can also help in better understanding of fruiting of *Agaricomycetes* because laboratory work allows application of distinctive conditions and targeted changes of selected parameters.

For laboratory work, it is essential to obtain suitable sterile cultures from unsterile mushrooms as they are collected in the wild. Two types of mycelia may be obtained from a typical Basidiomycete mushroom – either a fertile dikaryon obtained from attentive surgery of non-contaminated inner mushroom stipe or cap tissues (Badalyan et al. 2011) or sterile monokaryotic mycelia which germinated from basidiospores plated on an agar growth medium (Lakkireddy and Kües 2017 – Chapter 5). Both principles of strain isolation were

used in this study (Chapter 3 and Lakkireddy et al. 2016, 2020 – Chapter 4, respectively). Moreover, a new technique of basidiospore isolation for subsequent spore plating was presented which is less prone to contaminations by other fungal spores or bacterial cells as compared to classical basidiospore isolation methods. The method makes use of the special active basidiospore release mechanism which is inherent to most mushroom-forming *Agaricomycetes* and which bases on the hygroscopic Buller´s droplet for sudden spore catapulting from the basidospore-producing basidium as described first by Buller (1909, 1922) and more recently by Webster et al. (1988, 1989), (Money (1998), and Pringle et al. (2005), as well as in Chapter 1 and in Lakkireddy and Kües (2017) - Chapter 5.

The principle biological function of fruiting body formation is sexual reproduction via recombined meiotic spores (Kües 2000). The fruiting body thereby serves as an organ to position the masses of meiotic basidiospores produced most optimal for easy distribution (Kües and Navarro-Gonzaléz 2015). The typical mushroom shape offers that i. mature basidiospores are propelled off from the surface of the spore-producing hymenia via the fusion of the Buller´s droplet at the hilar apex of the spore with a hygroscopic liquid film on the surface of the spore (McLaughlin et al. 1985; Webster and Davey 1985; Webster et al. 1989, 1995; Ingold 1992; Money 1998; Pringle et al. 2005), that ii. the basidiospores enter the free air space between hymenia and fall down out of the cap once the catapulting energy has been used up (Turner and Webster 1991; Stolze-Rybczynski et al. 2009; Fischer et al. 2010a,b), and that iii. large clouds of spores released together at the same moment from a mushroom generate motions in the air which will help to bring the spore clouds from the wind-still region beneath a mushroom up into the air with more motion above for further long-distance carriage through the moving air (Buller 1934; Deering et al. 2001; Dressaire et al. 2015, 2016). This PhD thesis shows that electrostatic charging of the basidiospores which accumulated during the movement of the air through static friction (Saar and Salm 2014) is a further physical force which can act on spore release and distribution. Indeed, it is this electrostatic charging that can be used in the selective isolation of basidiospores (Lakkireddy and Kües 2017 – Chapter 5). By the new basidiospore isolation method, bulks of basidiospores can thus be harvested from up-side down positioned mushrooms with a much reduced risk of any contamination by unwanted microbes which are not propelled off from a surface into the air.

On the one hand, the electrostatic forces obtained from the fast flight of the catapulted basidiospores though the air play a key role to subsequently drive the charged spores up by attraction of plastic lids of sterile Petri-dishes positioned above reversed mushrooms and to then attach the charged spores to the plastic surfaces of the electrostatically active lids. On the other hand, the further trick is that spores of any other fungi or bacteria are usually not moved up toward and stick to the lids, likely because of a missing activating starting pulse and possibly also by an insufficient cellular charging. Attached basidiospores can next be harvested from the plastic lids in sterile solution for further use without much fear of disturbing contaminations by other microbes. If there were any contaminations encountered in the experiments performed, they were usually of bacterial origin which subsequently can be suppressed in growth by addition of suitable antibiotics to a fungal medium (Lakkireddy and Kües 2017 – Chapter 5).

The new basidiospore isolation method which makes use of spore charging is broadly applicable in the *Agaricomycetes*. In this PhD study, a total of 66 different *Agaricomycetes* species with an active ballistospore propulsion mechanism were shown to release basidiospores against gravity into the air by attracting forces of plastic lids of Petri dishes positioned above subside-down laid mushrooms. Moreover, for two gasteroid species (*Geastrum rufescens* and *Geastrum striatum*) which lost in evolution the catapulting

mechanism for basidiospore release (Hibbett et al. 1997; Wilson et al. 2011), bulks of basidiospores could be forced into the air by mechanical pushing the mushroom spore sacs. They were then sufficiently charged in order to be attracted and strongly attached to the plastic surface of a Petri dish lid placed above (Lakkireddy and Kües 2017 – Chapter 5). Charging of ballistospores is thus widely distributed in the *Agaricomycetes*, and evidence for spore charging is now available in the literature for over 100 different species (Saar and Parmasto 2014; Lakkireddy and Kües 2017 – Chapter 5). Among the positively tested cases of electrostatic spore attraction to plastic lids positioned above fruiting bodies was also the laboratory fruiting-competent strain AmutBmut of the model *Agaricomycete C. cinerea* (Swamy et al. 1984; Kertesz-Chaloupková et al. 1998; Boulianne et al. 2000), as principle of proof that also the ephemeral fruiting bodies of Coprini can be used in the new technique for contamination-safe basidiospore isolation (Lakkireddy and Kües 2017 – Chapter 5).

Species which fruit and sporulate in culture, thereby allowing also Mendelian genetic studies, and which moreover can be transformed by isolated DNA are particularly useful for laboratory studies. *C. cinerea* is such an important model fungus for the *Agaricomycetes*, in particular also for studies of fruiting body development, because the fungus can easily and in short time fruit in culture and is thus amendable to Mendelian genetics (Kües 2000; Walser et al. 2001; Stajich et al. 2010; Kües and Navarro-Gonzaléz 2015; Kües et al. 2016; Fig. 23 in Chapter 1). The fungus can also easily be transformed by an outstanding effective DNA transfer technique using protoplasts of uninuclear asexual spores (oidia) under application of different selection markers (Binninger et al. 1986; Granado et al. 1997; Cummings et al. 1999; Kilaru et al. 2009; Collins et al. 2010; Dörnte and Kües 2012, 2016, 2017) and efficient constitutive or also a few differentially regulated promoters (Bertossa et al. 2004; Kilaru et al. 2006b; Cheng et al. 2009; Heneghan et al. 2009; Muraguchi et al. 2011; Sugano et al. 2017). Moreover, gene silencing (Namekawa et al. 2005; Wälti et al. 2006; Heneghan et al. 2007; Costa et al. 2008), knocking-out genes (Nakazawa et al. 2011; Dörnte and Kües 2017), marker recycling (Nakazawa et al. 2015), and most recently the CRISPR/Cas9-technique for genome editing (Sugano et al. 2017) have all also become possible for *C. cinerea*.

In the past, various genes implicated in fruiting body development have been cloned and studied on molecular level by transformation in suitable *C. cinerea* wildtype and mutant strains (Boulianne et al. 2000; Kües 2000; Muraguchi and Kamada 1998, 2000; Inada et al. 2001; Arima et al. 2004; Bottoli 2001; Terashima et al. 2005; Liu et al. 2006; Muraguchi et al. 2008; Kamada et al. 2010; Kuratani et al. 2010; Nakazawa et al. 2010, 2016; Ando et al. 2013; de Sena-Tomas et al. 2013; Shioya et al. 2013; Masuda et al. 2016). For studies in this PhD, in particular the fruiting-body-specific galactin genes of *C. cinerea* for small secreted β-galactoside-binding proteins (Boulianne et al. 2000) and the esssential fruiting body development gene *cfs1* for a potential cyclopropane fatty acid synthase (Liu et al. 2006) have been considered (Lakkiredy et al. 2011 - Chapter 6; Lakkireddy and Kües 2014 - Subchapter 8.1; Subchapter 8.2).

Expression of the *C. cinerea* galectin genes *cgl1* and *cgl2* correlates spatiotemporally with initiation of fruiting body development (Boulianne et al. 2000; Bertossa et al. 2004; Walser et al. 2003; Wälti et al. 2006, 2008) and the gene products are specifically found in outer cap and stipe tissues (Walser et al. 2005). The originally anticipated biological function in strengthening hyphal aggregation for mushroom formation (Boulianne et al. 2000) has however later been dismissed when Wälti et al. (2006), Tietz et al. (2009), Butschi et al. (2010) and Bleuler-Martínez et al. (2011) found that the galectins are not essential for fruiting and that the galectins are toxic to small mushroom-grazing small animals and therefore suggested rather a protection role for these secreted proteins (see Chapter 1 for more background information and also the review by Sabotič et al. 2016). Since then, protection

roles against predators have be postulated and proven for several more secreted small fruiting-body-specific proteins of *Agaricomycetes* (Novak et al. 2015; Sabotič et al. 2016). However, early in this PhD work as one of the first scientific work done, I looked at the genomes of *Agaricomycetes* which were at the time available in order to find orthologous galectin genes in other species than *C. cinerea*. Of in total 27 different analyzed genomes, only *C. cinerea*, *Heterobasidion irregulare* and *L. bicolor* had such galectin genes (Lakkireddy et al. 2011 - Chapter 6). Similar for genes of other fungal lectins (5 different ones were analyzed), for genes of membrane pore-forming haemolytic proteins (3 different ones were analyzed) and for genes of chimeric lectin-membrane pore-forming proteins (3 different ones were analyzed) which also somehow relate to the initial aggregation phase in the process of fruiting body production in other *Agaricomycetes* it was found that variably some species share the presence of genes for a certain type of protein with each other while other species do not have any related genes (Lakkireddy et al. 2011 - Chapter 6). Other authors in later studies came to similar conclusions (Novak et al. 2015; Sabotič et al. 2016; Kües and Badalyan 2017). With time, further lectins and haemolysins of other protein subfamilies were described from more mushrooms (Novak et al. 2015; Sabotič et al. 2016). It could therefore be possible that in the end all *Agaricomycetes* may have genes for one or the other small lectin type and possibly also genes for some membrane-pore forming proteins. In my genome analyses with the 27 different fungal species at the early stage in my PhD work, I did not detect copies of genes for any of the proteins tested in the jelly fungus brown-rot *Dacryopinax* sp. (now listed by the JGI as *Dacryopinax primogenitus*; McLaughlin et al. 2016) from the *Dacrymycetes* and in the brown-rot *Boletales* species *Coniophora puteana* and the polyporous white-rot *Ganoderma* sp. from the *Agaricomycetes* (Lakkireddy et al. 2011 - Chapter 6). A further check of the genomes these three species with others lectins described at newer time from mushroom-forming *Basidiomycetes* (see Table 3; Sabotič et al. 2016) variably detected related gene copies for some while not for all lectins in the two *Agaricomycetes* but none in the morphologically less developed jelly fungus from the evolutionary older *Dacrymycetes* (Hibbett et al. 2014). At least in species of the *Agaricomycetes* it is thus quite common to have some type(s) of lectin molecules (Lakkireddy et al. 2011 - Chapter 6; Sabotič et al. 2016).

It is furthermore eye-catching from the comparative genome analyses presented in Chapter 6 (Lakkireddy et al. 2011) and further in Table 3 that no two species are identical in terms of all the respective genes for lectins and membrane-pore-forming haemolytic proteins they have and respectively also in the types of genes they do not have. The broader analyses

Table 3 Putative fungal lectins found by tBLASTn searches with selected known lectins on the JGI Mycocosm website in genomes of three distinct species of *Agaricomycotina*

Lectin used in tBLASTn searches	Accession number	*Dacryopinax primogenitus*	*Coniophora puteana*	*Ganoderma* sp.
Coprinopsis cinerea CCl2	ACD88750	-	ID 119225	-
Macrolepiota procera MpL	AEE98238	-	ID 85956	ID 11969, 55641, 92636, 122237, 122239, 122267, 139346, 146072
Boletus edulis BEL	F2Z266	-	-	-
Laccaria bicolor LbTec2	EDR12168	-	-	-
Marasmius oreades MOA	3EF2_A	-	-	-
Grifola frondosa GFL	BAE43874	-	-	-
Hygrophorus russula HRL	BAL02996	-	-	ID 16441, 108191

with genomes of further species of *Agaricomycetes* published by Sabotič et al. (2016) confer similar impressions of very high variabilities between different fungal species in their irregular sparse equipment with such types of genes. There is generally also no clear closer and uninterrupted evolutionary relationship between species which share same types of genes (Lakkireddy et al. 2011 - Chapter 6; Novak et al. 2015; Sabotič et al. 2016). Horizontal transfer of genes for lectins and membrane-pore-forming haemolytic proteins such as from bacteria is discussed as one possibility as how the irregular sparse distribution patterns of such genes might be explained (Moran et al. 2012; Wohlschlager et al. 2014; Künzler 2015; Sabotič et al. 2016). Horizontal gene transfers (HGTs) into some but not all species would support the argument that the gene products do not have a general function required for fruiting body development. However, evolutionary HGT events are difficult to proof, even through application of solid computer methods. Supported by a deeper analyzed example of an apparently false claim of an HGT event from bacteria into a fungus, strong warnings are expressed to not too airily claim events of HGTs in fungal evolution just by an observed scattered distribution of genes over different taxa (Dupont and Murray 2017).

Wherever the origins of the genes for the various lectins and membrane-pore-forming proteins detected in *Agaricomycetes* may reside, the variable distribution of the genes in the genomes of *Agaricomycetes* makes it unlikely that any of the encoded proteins are essential hyphal aggregation factors for fruiting body formation (Lakkireddy et al. 2011 - Chapter 6; Novak et al. 2015; Sabotič et al. 2016). On the other hand, a supporting function in hyphal aggregation is not per se excluded even if another task such as in fungal defense has been experimentally assigned to a specific protein (Lakkireddy et al. 2011 - Chapter 6). For a potential role in hyphal aggregation, lectins would be required to recognize specific sugar residues and sugar chains present on the hyphal surfaces. However, of the characterized mushroom lectins only some overlap in their sugar-binding specificities. Mushroom lectins of different types tend to differ in the distinct sugars they recognize and interact with (Sabotič et al. 2016). Again the very distinct sugar-specificities of distinct proteins argue against a general function of the proteins in hyphal aggregation for fruiting body development because with so different types of sugar-binding proteins one would need to postulate that the diffferent the fungal species in parallel co-evolution had variably developed also the species-specific projection of different sugars on hyphal surfaces. While the cell wall structures with their sugar-components and the surrounding fungal glucan sheaths of *Agaricomycetes* are generally still understudied (Wessels 1993, 1994; Ruthes et al. 2015, 2016), little evidence for the existence of such situations of lectin-specific ligands in fungal cell walls are so far provided (Walser et al. 2005). In conclusion, despite of proven effects of specific lectins and haemolytic membrane-pore-forming proteins on induction of fruiting body formation and a correlation of gene expression with fruiting body initiation and further development (Lakkireddy et al. 2011 - Chapter 6; see also Chapter 1 for further information), because the genes for specific lectins and haemolytic membrane-pore-formning proteins were found to be clearly not of general nature to all *Agaricomycetes* (Lakkireddy et al. 2011 - Chapter 6; Novak et al. 2015; Kües and Badalyan 2017) they were then therefore not considered for further experimental work in this PhD study.

The situation for gene *cfs1* shown by Liu et al. (2006) to be essential for fruiting body initiation in *C. cinerea* is clearly different. A tblastn crosscheck with the *Agaricomycetina* genomes (in total 223 genomes at 03.06.2017) present on the Mycocosm website (Grigoriev et al. 2014) of the JGI (Joint Genome Institute at Walnut Creek, California; http://genome.jgi.doe.gov/programs/fungi/index.jsf) in June 2017 revealed that most of *Agaricomycetina* with sequenced genomes have one or more copies for *cfs* genes. A *cfs1* gene is missing i. in the yeast-like *Basidioascus undulatus* as a species from the basic *Genimibasidiomycetes* (*Geminibasidiales*, *Geminibasidiaceae*) which does not form fruiting

bodies (Nguyen et al. 2013), ii. in the yeast *Cryptococcus neoformans* var. *grubii* H99 from the *Tremellomycetes* (*Tremellales*, *Tremellaceae*) unlike in the close relative *Cryptococcus neoformans* var. *neoformans* JE21 which both also do not form fruiting bodies (Loftus et al. 2005; Janbon et al. 2014), and iii. as in the only species of the *Agaricomycetes* in the orchid mycorrhizal *Tulasnella calospora* (Kohler et al. 2015) from the *Tulasnellaceae* (*Cantharellales*) which forms primitive resupinate sparse basidiocarps of generative hyphae which intermingle into other resupinate fungi (Roberts 1994; Wells 1994; https://www.aphyllo.net/spec.php?id=1204800). In summary, the broad distribution of *cfs1*-homologs in nearly all *Agaricomycetes* suggests that the *cfs1* gene function will likely be of higher importance for the fungi.

By homology to bacterial enzymes, the fruiting essential *C. cinerea cfs1* gene has been postulated to encode a cyclopropane fatty acid synthase (Liu et al. 2006; Lakkireddy and Kües 2014 – Subchapter 8.1). Cfa from *Escherichia coli* has a stress-related function (Grogan and Cronan 1997; Chen and Ganzle 2016) and has been shown to catalyse the cyclopropanation of unsaturated phospholipids in the bacterial membrane by transfer of a methyl group from *S*-adenosyl-L-methionine to the double bond of an unsaturated fatty acid chain (Taylor and Cronan 1979; Grogan and Cronan 1997; Chang and Cronan 1999; Courtois et al. 2004; Zhang and Rock 2008; Chapter 1). In the meanwhile, such enzymatic function has also been proven for some eukaryotic enzymes from plants (Bao et al. 2002, 2003; Yu et al. 2011, 2014) and of the trypanosomatid *Leishmania infantum* (Oyola et al. 2012).

The biochemical function of the enzyme encoded by gene *cfs1* of *C. cinerea* was addressed in experimental work in this PhD (Chapter 8). In order to proof the enzymatic Cfs1 function, it was decided to heterologously express the *C. cinerea cfs1* gene in *E. coli* (Lakkireddy and Kües 2014 – Subchapter 8.1). *cfs1* cDNA (Liu 2004; Liu et al. 2006) was subcloned by Loos (2002) into the 5.7 kb *E. coli* expression vector pET-16b from the pET expression system of the company Novagen (Darmstadt, Germany) in the functional 5´ to 3´ reading direction behind the T7*lac* promoter to give construct pET-16b*cfs1*. In this study, the negative control pET-16b-derived vector pET-16*cfs1*-inv with the *cfs1* cDNA cloned in the non-functional inverted direction relative to the T7*lac* promoter was generated (Lakireddy and Kües 2014 – Subchapter 8.1). Both constructs with the *C. cinerea* DNA as well as derivatives pET-16b*cfa* as pET-16b*cfa*-inv as positive and negative controls containing the *E. coli cfa* gene in sense and in antisense direction to the T7*lac* promoter subcloned also in this study (Lakkireddy and Kües 2014 – Subchapter 8.1) were expressed in *E. coli* strain BL21(DE3), following the pET system manual of Novagen (2006). Heterologously expressed *C. cinerea* Cfs1 and recombinantly expressed *E. coli* Cfa were both found expressed in *E. coli* strain BL21(DE3) as judged from intensely stained bands of ca 55 kDa and 45 kDa, respectively in size in 2-D gels of separated total proteins (Fig. 2; Subchapter 8.2) and then further from proteomics analysis of peptides from excised bands from IPTG- (isopropyl β-D-1-thiogalactopyranoside) induced and also from non-induced cells (Table 2; Subchapter 8.2). Because the strain BL21(DE3) is a wildtype for *E. coli* Cfa production, the proof for a functional eukaryotic enzyme Cfs1 in the bacterial host required a change of strains. *E. coli* YYC1272 is a mutant with a defect in the *E. coli cfa* gene and ZK126 is the corresponding wildtype strain (Chang and Cronan 1999). Both strains were transformed with all four constructs and transformants were submitted to an acid resistance test as described in Chang and Cronan (1999). However, within the limitations of the sensitivity of the method, proteomics analysis of gel-exised bands of expected protein sizes (ca. 45 and 55 kDa) did not detect any peptide of neither Cfa nor Cfs1 (Subchapter 8.2). While this could indicate the the proteins were not expressed based on lack of a T7 RNA polymerase for activity at the T7*lac* promoter of the plasmids, Loos (2002) performed a western blot with total proteins from IPTG-induced and non-induced pET-16b*cfs1*-transformed mutant YYC1272 cells and found

evidences of degradation products of the recombinantly produced protein. The results indicated some cryptic background expression from the T7*lac* promoter when LacI-repression on the *lacO* operator cloned in pET-16b downstream to the T7*lac* promoter was lifted in presence of IPTG. Results then in this study from acids tests then much varied. In some tests, it appeared that mutant clones with the sense constructs were better resistant than clones with other constructs and these experiments could suggest that the two proteins were indeed both expressed from the plasmids and mediated acid stress resistance. In other series of experiments, however, experiments gave erratic results, possibly by technical difficulties to perform all steps in the protocols with several clones at the same time perfectly controlled to equal cell numbers and incubation times (Subchapter 8.2). A clear positive conclusion in this thesis on the function of the *C. cinerea* Cfs1 protein is therefore not possible. To substantiate such in the future, it would appear best to delete the cfa^+ gene from the strain BL21(DE3) in which we know that both proteins are easily overexpressed and use such cells then in acid tests. Alternatively, the enzymes mighte be purified and used in *in-vitro* tests for enzymatic functions as described before for overexpressed recombinant Cfa obtained from the cfa^+ gene cloned in vector pET-24(+) (Courtois et al. 2004).

Liu et al. (2006) reported that the gene *cfs1* is differentially regulated, with light-induced expression in vegetative mycelium in parallel to fruiting body induction and with a further increase in gene expression during primordia development at 25°C under a 12 h dark/12 h light regime. Similarly, Plaza et al. (2014) came to the same conclusions in transcriptomic studies where the authors compared expression in early-stage primordia with that in vegetative mycelium grown at 37°C in dark. The authors found a 64-fold increase in expression of *cfs1* in the young primordia produced at 25°C under a 12 h dark/12 h light regime. Gene expression studies (Burns et al. 2010; Stajich et al. 2010; Cheng et al. 2013; Plaza et al. 2014; Cheng et al. 2015; Muraguchi et al. 2015) identified many more differentially expressed genes during different developmental stages in *C. cinerea*. Generally, there are multitudes of genes in *Agaricomycetes* which might act in fruiting. The typical genomes of *Agaricomycetes* harbor more than 10000 potential genes, often about 12000 to 15000 and sometimes coming in number even close to 20000 (for examples see Martin et al. 2008; Ohm et al. 2010; Stajich et al. 2010; Eastwood et al. 2011; Floudas et al. 2012, 2015; Morin et al. 2012; Wawrzyn et al. 2012; Bao et al. 2013; Collins et al. 2013; Lu et al. 2014; Park et al. 2014; Riley et al. 2014; Kohler et al. 2015; Kües et al. 2015; Chen et al. 2016; Nagy et al. 2016; Pulman et al. 2016; Yang et al. 2016). The expression data from *C. cinerea* (see citations above) and other mushrooms (see cited genome papers for e.g. *Agaricus bisporus*, *L. bicolor*, and *S. commune* and other original papers e.g. by Chum et al. 2008, 2011; Lu et al. 2014; Zhou et al. 2014; Zhang et al. 2015; de Freitas Pereira et al. 2017) suggests that large parts of the genes of a mushroom species potentially act directly or indirectly in fruiting body development. How to find out more about them? As presented in Chapter 6 and Chapter 8, interesting candidate genes for tasks in fruiting body development might be selected from the bulk of genes available and then individually be studied. While such procedure can lead to a dismissal of some genes for further research on fruiting body development by findings of minor importance for this complex process of reproduction (see as examples the galectin genes in Chapter 6; Wälti et al. 2006; Tietz et al. 2009; Butschi et al. 2010; Bleuler-Martínez et al 2011; Sabotič et al. 2016), other genes in contrast continue to be generally interesting for the process of fruiting body development in the *Agaricomycetes* and such may experience more research in the future (see as example the *cfs1* gene in Chapter 8).

Instead of starting from a gene which is present in a genome, Chapter 7 then followed another strategy in genome research in which missing genes for the biosynthetic pathway of thiamin production were defined. Since thiamin has clearly positive effects on growth and fruiting body formation in *Agaricomycetes* (Aschan 1954; Wessels 1965; Gold and Cheng

1979; Leatham 1983; Jonathan and Fasidi 2001; Joo et al. 2009; Fu et al. 2011; Atri and Guleria 2013; Chapter 7), the findings of this study open up the possibility by genetic engineering of e.g. the model mushroom *C. cinerea* to reinstall the complete biosynthetic pathway in the future and to test whether fruiting will be enhanced by internal thiamin production. Experiments in this direction are already ongoing (U. Kües, personal communication).

As presented especially in Chapters 1 to 4 in this thesis and is more discussed above in this chapter, fruiting body development of species of the *Agaricomycetes* in nature is much influenced by climate conditions and available growth substrates for the fungi. Species distinguish in their needs. In laboratory research, effects of environmental factors on genomes, including light (Tang et al. 2013, 2016; Plaza et al. 2014; Zhang et al. 2015), changes in temperature (Morin et al. 2012; Eastwood et al. 2013; Foulongne-Oriol et al. 2014; Fu et al. 2016; Liu et al. 2017), aeration (Eastwood et al. 2013), and nutrients (Zhang et al. 2017), might be followed up in transcriptomic and also proteomic approaches to unravel and understand the complex environmental regulation of fruiting body development of individual species on the genetic level and through comparisons what might be common between species. Improved DNA and RNA sequencing techniques by now also allow to use biological material directly from nature, such as dikaryotic non-sterile mushrooms as found in the forests (Kohler et al. 2015). Techniques thus become independent from culturing species under established fruiting conditions in the laboratory. Thus, observations in nature on correlations of fruiting of a mushroom species with environmental conditions might in the future be accompanied by genomic and transcriptomic molecular studies to indentify genes required for the production of fruiting bodies and the right combination of environmental factors that will activate these genes for fruiting. This is particularly interesting and important for species which can not be cultured and induced to fruiting in the laboratory. On the long run, it will thus be possible to interlink controlled studies on fruiting body development from the laboratory with data accumulating from observing and analysing fruiting bodies of non-model species together with relevant environmental parameters in nature.

Studying fruiting body development in *Agaricomycetes* at best will thus recurrently go the experimental path 'From nature to the laboratory and back to nature'. While the individual observations and contributions on aspects in fungal fruiting in this PhD thesis on the one hand may all rather appear very specific, this PhD study with its individual foci of study is an example as how to collect jigsaw pieces in nature and in the laboratory and contribute them to the major questions that need to be solved by science in the processes of fruiting in the *Agaricomycetes*.

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Chapter 3

Observations on Fruiting Body Development of Selected *Agaricomycetes* in Nature

This chapter represents observations on fruiting body development of selected *Agaricomycetes* in nature. Focus is given to the form group of Coprini and further dung fungi of the family of *Psathyrellaceae* on the one hand and on the weak tree pathogen and supposed wood decay fungus *Schizophyllum commune* on the other hand. Fruiting body development in nature is described for selected species, connections of fruiting to climate conditions and, where possible, links to nutrients are given.

Contributions: Kiran Lakkireddy (KL), Mónica Navarro-Gonzaléz (MNG), Ursula Kües (UK), Weeradej Khonsuntia (WK) and Shanta Subba observed and photographed mushrooms in nature, KL and MNG compiled data on mushroom growth and recorded basic steps in fruiting body development of selected species. KL and UK analyzed data. KL together with MNG isolated mycelial strains, KL and Zuleima Quiñones sequenced ITS regions of selected fungi. Fabian Herz (FH) and KL analyzed chemical compositions of wood samples. FH performed the wood decay test after standard EN113, Karin Lange and UK performed fruiting experiments on wood in the laboratory. KL drafted and wrote a first version of the chapter. UK supplied material for the introduction and discussion and helped to improve structure and writing of the chapter. WK and UK provided Fig. A4. Volker Meng kindly provided information on identities on various tree species and Bernd Kopka raw climate data. All colleagues are sincerely thanked for their kind support.

Data of this thesis on occurrence of *S. commune* as pathogen on shrubs and trees on the grounds of the North Campus of the University of Göttingen have been included in a conference proceedings shown as an appendix of this chapter and indicated in the text: Lakkireddy K, Zia A, Khonsuntia W, Kües U (2017) *Schizophyllum commune* as an early sapwood colonizing fungus. In: Proceeding Book. The 3rd Asia Pacific APRC 2017 Rubber Conference. 16th-17th November 2017. Prince of Songla University, Surat Thani Campus, Surat Thani, Thailand, pp 171-176

Observations on Fruiting Body Development of Selected *Agaricomycetes* in Nature

Coprini (inkcaps) are a frequently fruiting-body-autolysing dark-spored form group (former and now rejected family *Coprinaceae*) of *Agaricomycetes* with four defined genera belonging variably either to the *Agaricaceae* or to the *Psathyrellaceae*. With other dung fungi from the *Psathyrellaceae*, many of them appear on grasslands while a range of species also associates with lignicolous wastes, wood chips and debris and in some instances also with living trees. In this work, observations on the occurrence of mushrooms of species of the genera *Coprinellus*, *Coprinopsis*, *Lacrymaria*, *Panaeolus*, *Parasola*, and *Psathyrella* of the *Psathyrellacacae* and the genus *Coprinus* from the *Agaricaceae*, in the years from 2011 to 2016 on such biotopes are reported, in total individual data on 56 different species, mostly from observations on the North Campus of the University of Göttingen. Depending on the genus and species, fruiting bodies preferentially occurred in two main periods of time in a year in spring and in autum (e.g. *Coprinellus domesticus* and *Coprinellus micaceus*), or from April or May in spring over the summer months June-July-August and in the autumn months September till November (e.g. *Coprinellus disseminatus* and *Coprinellus truncorum*), mainly in summer months (*Psathyrella candolleana*), or fungi fruited preferentially only in autumn months (e.g. *Coprinellus tardus*, *Coprinellus xanthothrix*, *Coprinus comatus*, *Coprinospsis atramentaria* and many species of other genera of the *Psathyrellaceae*). Most of the observed species (in total 46) were recorded as fruiting on meadows of which 21 were only observed on meadows suggesting that ecologically the majority of these fungi are grassland fungi. Among were several *Coprinopsis* species and *Psathyrella* species, many of which also occured in wood chip beds. Various *Coprinopsis* and *Lacrymaria* are considered as ammonia fungi by their ability to withstand high nitrogen content in substrates such as by input into meadows through dung of grazing animals.

In total, fourteen species were detected to occasionally fruit within plant litter, ten species were observed to fruit on dead wood and five in connection to living trees. More often, fruiting bodies were associated with wood chip beds (26 species) from chopping cut branches and stems as a modern gardening practice. *Coprinopsis* species more likely link to wood chip beds, *Coprinellus* species more likely to wood of living trees and compact deadwood. Of the Coprini, *C. comatus* from the *Agaricaceae* and *C. disseminatus*, *C. domesticus*, *C. micaceus*, *C. truncorum*, *C. xanthothrix*, and *C. atramentaria* from the *Psathyrellaceae* were species most often observed. Particularly many observations were obtained on fruiting body development of the species *C. disseminatus*, *C. domesticus* and *C. micaceus* on the grounds of the North Campus of the University of Göttingen. Progress in fruiting of individual structures was photographed and followed up over the time for these three species as well as once for *C. truncorum*. Fruiting body development of the species occurred in most months of the year, but usually after a few days of adequate rainfalls which lead to substrate moisturing, increased humidity levels over 2 or more days (>80 % to mostly >90 % at nights and days), and drops in temperature. Sufficient rainfall apparently helped to induce fruiting body development. Humidity levels also played roles in further development since fast reduction in humidity caused drying and stop of development. During the progress in the individual completed fruiting processes, humidity values of >80 to >90 % at nights and of >75 to >90 % at days were recorded but in some instances there were also periods of 80-90 % humidity at nights with 70-80 % and even lower values with 80-90 % of humidity at days.

The complete process from first appearance of primordia over growth and development to the young mushroom, stretching stipes and opening of caps, and final

decaying of the mushrooms differed in apparent length between times of appearance, in total between 8 and 23 days for *C. disseminatus*, between 12 and 20 days for *C. domesticus*, and between 9 and 14 days in *C. micaceus*. Mainly temperatures appear to be a cause for these varieties. Broadly variable temperature ranges of minimum-maximum 8-20/12-24 ºC/, 5-14/10-19 ºC, and 7-16/10-24 ºC (night/day) prior to rainfall and fruiting of *C. disseminatus*, *C. domesticus* and *C. micaceus*, respectively were recorded. Induction of fruiting occurred for the three species at temperature ranges of variably 5-6 to 14-16/8-11 to 15-20 ºC (night/day), around 5-13/8-17 °C (night/day) and 7-15/7-16 °C (night/day), respectively, for all species upon drops in temperature by up to 8/9 °C (night/day). Afterwards temperatures stayed in the same lower temperature ranges or in some instances increased again somewhat by up to 5/6 °C for *C. disseminatus* and up to 5/7 °C for *C. micaceus*. Particularly of *C. disseminatus*, higher temperatures (11-15/17-23 °C night/day) after induction seem to speed up the whole fruiting process as compared to lower temperature values (2-10/3-13 °C night/day). The species *C. disseminatus* was thus less dependent in fruiting on the time of the year than any of the other species observed in this study.

Further observations on the North Campus of the University of Göttingen presented in this chapter related to the weak tree pathogen and poor wood decay fungus *Schizophyllum commune* from the family of *Schizophyllaceae*. For this species, an account is given on tree and shrub species on which this fungus appeared on branches and twigs of living specismens. *S. commune* preferentially forms mushrooms on the sunny side of barked wood. Release of the bark and callus formation were observed as defence reactions of a *Juglans ailantifolia* tree as a measure to overcome damages on a partially killed and partially debarked branch caused by a *S. commune* infection.

Introduction

Agaricomycetes display a fascinating diversity in life history schemes (e.g. saprotrophic on easy or more difficult to degrade plant wastes using different decay schemes, plant pathogenic, animal pathogenic, mycotrophic, mycorrhizal, endophytic) as well as in morphology of their mushrooms (size, colour and shape of the fruit bodies) while the life styles as well as the shape and features of mushrooms do not necessarily reflect direct phylogenetic relationships (Ohm et al. 2010; Stajich et al. 2010; Kües and Navarro-González 2015; Halbwachs et al. 2016; Chapter 1). Mushroom formation is so far generally only poorly understood, even for the two well studied model species *Coprinopsis cinerea* (Kües 2000; Stajich et al. 2010) and *Schizophyllum commune* (Ohm et al. 2010; Pelkmans et al. 2016).

Factors which are affecting fruiting body production are examined mainly for the model species and a restricted assortment of species which are commercially cultivated for mushroom production and which can be grown and brought to fruiting on artificial media (Kües and Liu 2000; Rühl and Kües 2007). Different species have their individual needs on the conditions which allow them to fruit. For every novel species, the specific conditions for fruiting have therefore to be newly established by empirical methods. A combination of environmental factors including the correct temperature range, light conditions and sufficient humidity is important for fruiting body initiation and maturation, as is good aeration to avoid any CO_2 accumulation and the nutritional status of a fungus for conditioning both, appropriate metabolic resources and sufficient energy (Kües and Liu 2000; Rühl and Kües 2007; Moore et al. 2008). Failure in correct conditions of any of these factors can alter or suppress basidiome production and the implementation of projected morphologies (Moore et al. 2008; Kües and Navarro-Gonzaléz 2015).

Also in nature, a wide variety of environmental factors will influence development of fruiting bodies. In a constantly changing natural environment, the timing and the efficiency of fruiting body production differs dramatically from year to year (Moore et al. 2008; Boddy et al. 2014). Global climate warming however triggered for a number of species to have two fruiting seasons in year, in spring and autumn, and to begin fruiting earlier and to continue longer in the year (Gange et al. 2007; Rutishauser et al. 2007; Kauserud et al. 2010, 2012). Other studies show an increase in fruiting body numbers and in species richness linked to global warming (Büntgen et al. 2013). Since active mycelial growth is needed for accumulation of enough energy and biochemical resources for fruiting, the earlier and longer sporophore production seen now in many historical studies on fruiting body production over the time could be an evidence for that the mycelium of the respective species must be active in late winter and early spring and also later in summer and during the autumn (Gange et al. 2007, 2013; Ágreda et al. 2015).

Differences in fruiting behavior between fungi of different life styles were not obvious in a study on moist mid-latitude Swiss forests (Büntgen et al. 2013). Possibly, in this study the environmental conditions in the moist forests were ideal for fruiting? Remarkable differences in fruiting phenologies are more frequently apparent in a same forest between mycorrhizal species and saprotrophs and between litter degraders and wood decay fungi. These differences might reflect seasonability of carbohydrate availability. Mycorrhizal species get their carbohydrates from their hosts and are thus bound in their actions to the hosts´ photosynthetic activities (Martin et al. 2008; Kohler et al. 2015). Litter degraders and wood-decomposing fungi obtain their carbohydrates from own degrading activities (Floudas et al. 2012; Morin et al. 2012). However, litter degraders are thereby more variably affected in their fruiting activities by interlinked plant phenologies such as by the seasonal litter entry though leaf shedding while substrate quality can overwhelm climate effects for wood-decayers. Mycelial growth and spatial prevalence are prerequisites for fruiting. Required for the enhanced fruiting activities, the nowadays by climate change likely increased mycelial activity potentially affects the forest ecosystems by higher decay rates and faster biomass turnover (Gange et al. 2007; Högberg et al. 2010; Sato et al. 2012; Büntgen et al. 2013; Boddy et al. 2014; Ágreda et al. 2015; Piepenbring et al. 2015; Heegaard et al. 2016; Venugopal et al. 2016). Intra- and inter-annual effects of climatic variability are of consequence for the different fungal activities, for both the spatial fungal prevalence and the mushroom yields. Individual species might thereby react differentially (Ágreda et al. 2016; Heegaard et al. 2016; Primica et al. 2016).

Type of forests – deciduous or coniferous – (Gange et al. 2007), forest stand age (Martínez de Aragón et al. 2007; Taye et al. 2016), geographical regions, amplitudes as well as large and fine scales (Kauserud et al. 2008, 2010, 2013; Andrew et al. 2016; Heegard et al. 2017), and land-use practices (Azul et al. 2009; Griffith et al. 2012; Newbound et al. 2012) can all have an impact on the overall fruiting behaviour over the time. Soil type and chemical properties (Newbound et al. 2012; Taye et al. 2016), soil moisture and water content – which must be above certain required water activity values but also not too high – determine success in general and in taxa-specific fruiting body formation and development (Querejeta et al. 2003; Lilleskov et al. 2009; Pinna et al. 2010; Büntgen et al. 2013; Piepenbring et al. 2015). Sporocarp production fails when minimum climatic conditions are not fulfilled (Gange et al. 2007; Martínez de Aragón et al. 2007). Precipitation in right amounts and at right times is a main factor (Krebs et al. 2008; Baptista et al. 2009; Büntgen et al. 2012, 2015; Yang et al. 2012; Ágreda et al. 2015; Piepenbring et al. 2015; Primicia et al. 2016; Taye et al. 2016) together with evaporation (Newbound et al. 2010; Ágreda et al. 2016) and the interrelations with temperature (Newbound et al. 2010; Pinna et al. 2010; Büntgen et al. 2013; Diez et al. 2013, 2014; Narimatsu et al. 2015; Venugopal et al. 2016).

The type of food substrate as well as its amount plays another key role in fruiting body formation. With better supply of resources, fruiting bodies of symbiotic and saprotrophic species can increase in overall size which can go along with concomitant reduction in absolute numbers of fruiting bodies per area (Bässler et al. 2016). As already stated, mycorrhizal fungi obtain carbon from their host plants. They have generally larger fruit bodies than saprotrophic fungi but there are no general differences in spore size and spore shape between species of different life style (Halbwachs and Bässler 2012; Bässler et al. 2015, 2016). The production yield of mycorrhizal fruiting bodies depends on the transferred amount of current photosynthates from a tree to an agaricomycetous fungus (Högberg et al. 2008; Smith and Read 2008). Mycorrhizal fungi further receive water from plants through hydraulic lift for transfer to the sporocarps (Querejeta et al. 2003; Lilleskov et al. 2009). The hosts are therefore decisive in the fruiting activities of the ectomycorrhizal species (see above). Independent on any host activities, dung fungi living on restricted substrates fruit much faster than litter decomposers and the wood decay fungi are slower than the latter (Cooke and Rayner 1984; Rayner and Boddy 1988).

As indicated from the citations in the text above, most studies on fruiting behavior of *Agaricomycetes* in nature focus on species within forests. Grassland studies in contrast are thus much more sparse than studies in forests (Griffith and Roderick 2008; Jordal et al. 2016). This might not be as surprising under the consideration that in Europe 80% of species of macrofungi are present in woodlands whereas only an estimated 10% are specialized to grasslands (Arnold 1992). Grassland biotopes differ greatly from forests in that they are typically low in lignin and scant in any fungal successions, among influenced by animal grazing and input of dung. Fungal life styles in grasslands include litter decomposers, dung fungi, terricolous species and root endophytes (Griffith and Roderick 2008). Grasslands can contain over 70 different species of macrofungi which are considered to be specifically adapted to the biotope (Jordal et al. 2016). Among are mainly waxcaps, often more than 30 different *Hygrocybe* spp., and, often, they occur in close association with living or dead plant fine roots (Halbwachs et al. 2013a,b; Griffith et al. 2013). It is estimated that about 200 to 400 species in Northern Europe are mainly found in grassland. Other than from the genus *Hygrocybe* dominating in grasslands, macrofungi of grassland biotopes are frequently species belonging to the families of *Clavariaceae*, *Entolomataceae* and *Gloeglossaceae* (comprising together with *Hygrocybe* the CHEG fungi, a name coined from the initials of the four fungal group names) and the genera of *Camarophyllopsis* from the *Clavariaceae*, *Dermoloma* (when included in the ecological group name of grassland mushrooms: CHEGD fungi) and *Porpoloma*, both from the *Tricholomataceae* (Arnold 1992; Griffith et al. 2013). However, there can be many more species, 200-300 different ones from other genera, that appear in grasslands but these may have other ecological preferences. Most of these are dung or dung-associated species such as *Panaeolus* spp. (see Fig. 10 O,P, 12F,G, and 13 F,G in Chapter 1 and Fig. 2A in Chapter 2), litter saprotrophs like *Mycena* spp. (see Fig. 12 J,K in Chapter 1) or terricolous species e.g. of the genus *Agaricus* (see Fig. 6 A,B, 8F, 9F, 17, and 20 in Chapter 1 and Fig. 2F,G in Chapter 2), (Griffith et al. 2004, 2013; Jordal et al. 2016). Less disturbed unfertilised grasslands, 'seminatural grasslands' need to be distinguished from other grasslands, e.g. lawns, parks and road verges, defined by different management measures and intensities (Jordal et al. 2016). Animal grazing, sward management and particularly fertilizer input (nitrogen but also phosphate) can threaten the fungal communities. Among the CHEG fungi, especially *Hygrocybe* spp. react on disturbances (Griffith et al. 2004, 2012). *Hygrocybe virginea* and *Hygrocybe conica* are the most resistant (Griffith et al. 2004) which were also observed in this PhD study (Fig. 1B,E and 8D in Chapter 1).

The grounds of the North Campus of the University of Göttingen can be defined as grasslands of different management intensities which are intermingled with shrub and tree

vegetations. Our group focusses already for some time on the often fruiting-body-autolysing dark-spored form group of Coprini (inkaps) as target of research, including the model fungus of basidiomycete research *Coprinopsis cinerea* (Navarro-Gonzaléz et al. 2004; Naumann et al. 2007; Navarro-Gonzaléz 2008; Badalyan et al. 2011a,b; Lakkireddy et al. 2011 – Chapter 6). This chapter reports on occurrence of Coprini on the grounds of the North Campus of the University of Göttingen in the years 2011 to 2016. By ecology, the Coprini are mainly dung fungi. Many of these taxa are well known to be deliquescent and to have autodigestive chitinases and glucanases (Kües 2000; Redhead et al. 2001; Nagy et al. 2009, 2010, 2012a; Yoo and Choi 2013; Liu et al. 2015; Niu et al. 2016; Zhou et al. 2016) in order to liquify their caps for basidiospore release. Based on molecular taxonomy, the classical Coprini (former large genus *Coprinus* and former family of *Coprinaceae*) have been subdivided into four distinct genera, *Coprinellus*, *Coprinopsis* and *Parasola* which were included into the *Agaricales* family of *Psathyrellaceae* and separated from the now very small genus *Coprinus* which belongs to the *Agaricales* family of *Agaricaceae* (Redhead et al. 2001). The family of *Psathyrellaceae* comprises dark-spored agarics which have soft, delicate fruiting bodies. Their basidiospores are dark, brown or reddish, and they are difficult to assign to species. The *Psathyrellaceae* contain among others the six genera *Coprinellus*, *Coprinopsis, Coprinus, Panaeolus*, *Parasola*, and *Psathyrella* (Redhead et al. 2001; Keirle et al. 2004; Nagy 2005; Nagy et al. 2009, 2012a,b, 2013a,b; Örstadius et al. 2015). In addition to species of the mostly autodigesting form group Coprini, species of the non-autolysing *Panaeolus* and *Psathyrella* genera were also recorded in this study as additional dung fungi.

Another attention was here given to the split gill fungus *Schizophyllum commune* from the family of *Schizophyllaceae* (*Agaricales*). This species is a second model fungus for the *Agaricomycetes* and completes its life cycle in about 10 days on defined media (Ohm et al. 2010; Kües and Navarro-Gonzaléz 2015). The split gill fungus is a widespread fungus all over the world and occurs in temperate and tropical climate zones. In temperate zones, the fruiting bodies of the species are frequently observed on recently fallen twigs, branches and stems at sunny places (Jahn 1979; Læssøe and Lincoff 1998; Breitenbach and Kränzlin 1995; Takemoto et al. 2010). Moreover, over 200 plant species have been listed on which the fungus forms fruiting bodies (Cooke 1962). *S. commune* is a weak tree and shrub pathogen and may also infect living wooden species, such as through wounds in the bark (Takemoto et al. 2010). The fungus grows on wood but has only a limited ability for lignocellulose degradation (Ohm et al. 2014; Floudas et al. 2015). It seems to have a less aggressive wood decomposition mechanism in between white and brown rot (Riley et al. 2014; Floudas et al. 2015).

Important for the correct understanding of this study, observations and recording in this study did not follow a particulate strong design and may therefore not be free of subjective bias (Heilmann-Clausen and Læssøe 2012; Griffith et al. 2013). The former first observations on Coprini as well as on *S. commune* on the grounds of the North Campus of the University of Göttingen were rather randomly by accidental notice (Navarro-Gonzaléz et al. 2006; Peddireddi et al. 2006; Navarro-Gonzaléz 2008; Peddireddi 2008). Giving more attention to these groups of fungi in this work however resulted in more regular rounds of observations throughout the year and in recognition of patterns where and when some specific dung fungi will fruit and it provided further information on plant host ranges of the pathogenic *S. commune*.

Material and Methods

***Mushroom inventories*.** Wild mushrooms of the family of *Psathyrellaceae* were recorded on the North Campus of the University of Göttingen and neighbouring areas of the village of

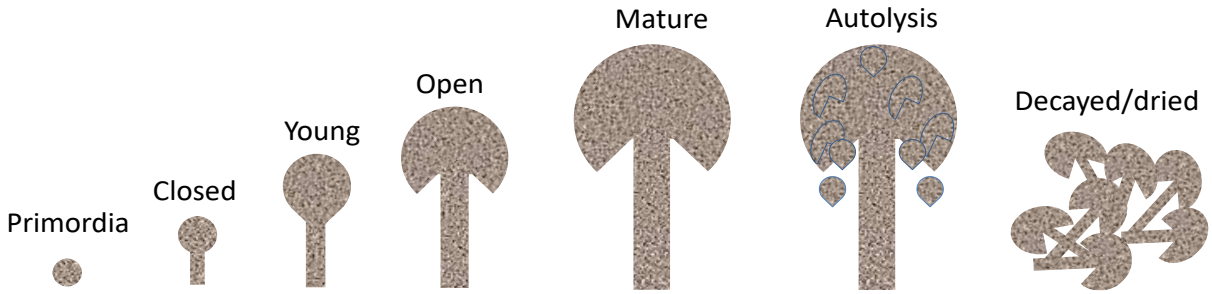

Fig. 1 Scheme of mushroom stages.

Göttingen-Weende from 05.2011 to 11.2016 (for raw data, see Table A1 in Appendix I). Mushrooms were photographed using a Cannon IXUS 115 H5 digital camera (12.1 megapixels; Canon, Krefeld, Germany). Developmental stages and ages (C = closed; Y = young, O = open; M = mature) were noted as indicated in Fig. 1. Ecological parameters of mushrooms growth (biotope, substrate, host trees) and morphological characters of the mushrooms were recorded for species determination. Inventories over larger areas were done throughout the year in roughly weekly basis but not necessarily regularly every day throughout the year in a type of systematic design, unless mushroom seasons were expected by time of year in combination by a suitable temperature range linked with rainfall or when mushroom production was expected triggered by rainfall at suitable temperature ranges. In addition, certain places of mushroom appearance were regularly passed throughout the year by crossing the whole ground in daily walks during the week during lunch breaks. Any unexpected mushroom appearance caused direct cross-checking of all potential mushroom places known on the ground.

Inspections for the long-lasting fruiting bodies of *S. commune* on twigs and branches of living trees were done predominantly in autumn-winter months on days after rainfall when after shedding the leaves in fall a clear sight on all branches was possible and mushrooms were well expanded by moisture and therefore well visible by their whitish colour on dark grounds of the barks. A *Juglans ailantifolia* tree at the West side of the Ernst-Caspari-House of the Göttingen Center for Molecular Biosciences (GZMB) at the split of the Burckhardtweg from the Justus-von-Liebig-Weg observed already longer before this work by former members of the group (Peddireddi et al. 2006; Peddireddi 2008) was frequently visited throughout the year.

***Mushroom identification*.** The field guide of Breitenbach and Kränzlin (1995) and the *Coprinus* website of Kees Uljé (http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm) were used in species identifications and the MycoBank database (http://www.mycobank.org/) was considered for current species names and higher classification. In some cases, species identification was confirmed by isolating DNA from mycelial cultures (Zolan and Pukkila 1986) and by amplifying and sequencing the ITS sequences with basidiomycete-specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') following methods as described earlier (Naumann et al. 2007). Specific sequence results are given in Fig. A1 in Appendix II of this chapter.

Isolation of mycelia. Dikaryotic mycelial cultures (referred to as D for dikaryon with consecutive numbers if more than one strain was isolated per site) were obtained by sterile surgery from inner tissues of stipes of freshly collected mushrooms (Badalyan et al. 2011b). Small dissected pieces of inner stipe tissue were laid onto YMG/T medium (Granado et al.

1997) with antibiotics (100 µg/ml ampicillin, 50 µg/ml kanamycin). Plates were cultured for 2-6 days at 25 °C. Outgrowing basidiomycete mycelia were transferred to fresh YMG/T plates for further growth as pure cultures. Monokaryotic cultures were obtained by incubation of freshly collected mushrooms in upside-down position in plastic Petri-dishes for 18 h for basidiospore collection by electrostatic attraction in the plastic lids above as described in Lakkireddy and Kües (2017) – Chapter 5. Spores were harvested from the lids in 200 µl sterile water and suitable dilutions of spores were plated onto YMG/T for spore germination during 2 to 4 days incubation at 25 °C. Germlings (referred to as s for spore or M for monokaryon with consecutive numbers if more than one strain was isolated per site) were then transferred for further growth onto fresh YMG/T. An overview of monokaryotic and dikaryotic strains isolated from collected mushrooms during this work and from earlier more random collections at same sites are given with strain codes in Table A2 in the Appendix II to this chapter. Mycelia were stored for future work at -80°C by transferring 10 to 20 small agar plugs (1-2 mm in Ø) of freshly grown mycelium into 1 ml sterile 15 % glycerol in 2.5 ml Nunc^{Tm} cryotubes (Roskilde, Denmark) and shock-freezing by direct transfer into a -80°C freezer (Walser et al. 2001).

Climate data collection. Climate data (temperature and humidity measured in 1-hour steps) are routinely collected on the grounds recorded on the North Campus of the University of Göttingen through a hygro-thermo transmitter (Adolf Thies GmbH & Co. KG, Thies Clima, Göttingen, Germany) installed at the greenhouses at Büsgenweg 2. Raw data were introduced into the program Excel (Microsoft, Redmond, WA) for creating monthly climate graphs.

Determination of lignin, cellulose and hemicellulose. The composition of fresh wood broadleaf trees (acer, *Acer platanoides*; beech, *Fagus sylvatica*; elm, *Tilia cordata*, and poplar, *Populus tremulus*) and of decaying wood collected from an infested elm (*T. cordata*) tree stump (at the Justus-von-Liebig-Weg at the north side of Physic´s building Friedrich-Hund-Platz 1) was determined.

The TAPPI method (TAPPI 1985) was used for lignin determination. In brief, crushed wood samples (particle size ≤ 8mm) were ground into a powder in a Retsch Mixer Mill MM 400 (Haan, Germany) to a particle size of 0.4 mm in diameter. Ofen-dried samples adjusted to 1 g were gently mixed in glass beakers (on ice) with 15 ml 72% sulphuric acid (10-15°C in temperature) and the glass beakers covered with a lid (to prevent evaporation) were placed into a water bath at 20°C for 2 hours under occasional gently shaking. Then, the material was transferred into a 1 l-sized round bottom glass flask filled with 250 ml water. Any remainders in the glass beaker were washed out with water (total volume 95 ml) and added also into the round bottom glass flasks with the samples to obtain a final volume of liquid of 360 ml with a 3% sulphuric acid concentration. Then, the samples were cooked for 4 hours using a reflux condenser to conserve the volume of total liquid. After cooling to room temperature over night, the samples were filtered using filter paper of known weight and washed with water. Stored filter papers with the lignin material were dried at 105°C for 24 hours in aluminium cups of known weights. Total acid-unsoluble lignin (Klason-lignin) content in a sample was determined by weighing the aluminium cup, filter paper and the lignin with subsequent subtraction of the weight of the aluminium cup and the filter paper.

For determination of the cellulose content of wood samples, 2x 10 ml of the cooked supernatant from the lignin determination described above (cleared by centrifugation from any unsoluble debris) were taken and diluted to need (10- or 20-fold). The glucose content of solutions as measures for the cellulose was enzymatically determined with the help of glucose oxidase/horseradish peroxidase and *o*-dianisidine reagent from a SIGMA-Glucose Assay Kit GA60-20 (Merck, Darmstadt, Germany) through spectrophotometric measurements at 540 nm

in comparison to a standard curve with values obtained from glucose standard solutions (20, 40, 60 and 80 µg glucose/ml), following all instructions of the supplier.

Determination of the hemicellulose content of wood samples was done via pentose determination of centrifuged supernatant from the lignin determination described above, after 10-fold dilution using orinol (5-methylresin) reagent (40 mg solved in 20 ml $FeCl_3$ solution prepared as follows: 0.15 g $FeCl_3$ x 6 H_2O was solved in 10 ml water to which 30 % HCL was then added to give a final volume of 200 ml) and xylose standard solutions (7.813, 15.625, 31.25, 62.5, ans 125 µg xylose/ml) after Hernell and King (1952). Samples were mixed 1:3 in volume with the orinol reagent, the mixtures were 20 min incubated in a water bath at 100°C, quickly cooled down to room temperature (using ice), after a rest period of 30 min measured in a specterophotometer at 660 nm, and the pentose content in samples calculated in comparison from a standard curve obtained from the samples with known xylose content.

Wood decay tests after standard EN113 (European standard 1997). Ofen-dried sterile wood blocks (50 mm x 25 mm x 15 mm, longitudinal x tangential x radial (L x T x R; six to eight samples for each wood species and fungus) of acer (*A. platanoides*), beech (*F. sylvatica*), elm (*T. cordata*), and poplar (*P. tremulus*) were incubated for 16 weeks at 22 ± 1 °C in the dark in glass jars (lid closed with Parafilm to block aeration) on a metal grid laid just above the aerial mycelium of fungi prior grown at 25 °C on malt extract agar medium (4 % malt extract, 2 % agar). Controls of wood blocks in same numbers were performed on jars with inoculated malt extract agar medium. Wood blocks were weighted prior and after incubation. *Coprinellus disseminatus* isolates G3 (D1), G3 (D2), and G15 (s1), *Coprinellus micaceus* isolates G2-1 (D1), G2-2 (D2), 1-OH (Badalyan et al. 2011b), and MN-7 (Naumann et al. 2007), *Coprinellus xanthothrix* G1 (D1) and G18 (D1), *Coprinopsis atramentaria* G33(s1) and G34 (s1), and *Coprinus comatus* isolates G30 (s1) and MN-1 (1-2004) were used [*C. micaceus* isolates G2-1, G2-2, 1-OH, MN-7, and MN-1 (1-2004) were kindly supplied by M. Navarro-Gonzaléz; according to a personal communication by S. Badalyan strain G2-1 is possibly *C. xanthothrix*; for origins of all other strains see Table A2]. All tested strains were dikaryons.

Results and Discussion

Part I: Coprini and related *Psathyrellaceae* species in nature

Fruiting of* Psathyrellaceae *over the year. Appendix I (Table A1) and III (Fig. A2) give an overview on the months over the main period of observation (from 09.2011 to 11.2016) when fruiting bodies of any species of the family of *Psathyrellaceae* were noticed on the North Campus of the University of Göttingen and in its closer neighbourhood of the village of Weende (a single observation from 05/2011 on the North Campus of the University of Göttingen and observations at selected times from two other locations are also added for completeness of documentation of all collected data). In summary from all observations, there appear to be two main seasons in the year when fruiting bodies are formed – variably in June, July and August and more consistent in September-October-November –, while fruiting in the autumn period was more abundant than fruiting in the summer months (Fig. A2 in Appendix III). Furthermore, there are the occasional observations of fruiting in other months of the year: Year-wise, mushrooms of *Psathyrellaceae* appeared

- in year 2011 in May (an extra single random observation mentioned already above), **September**, October, **November**, and December,
- in year 2012 in January, April, May, **June**, **July**, August, September, **October**, and November,
- in year 2013 in April, May, June, July, **September**, **October**, and **November**,

- in year 2014 in April, May, July, **August**, **September**, **October**, and **November**,
- in year 2015 in April, May, **July**, **August**, **September**, **October**, and **November**, and
- in year 2016 in January, March, April, **June**, July, August, **September**, **October**, and **November**.

Months given in bold were those with multiple observations of fruiting of same and of different species.

Species range of* Psathyrellaceae *and of* Coprinus comatus *on different substrates. In total, 56 different species were observed over the time, 12 species from the genus *Coprinellus*, 10 species from the genus *Coprinopsis*, 1 species from the genus *Coprinus*, 1 species from the genus *Lacrymaria*, 12 species from the genus *Panaeolus*, 2 species from the genus *Parasola*, and 18 species from the genus *Psathyrella* (Table 1). Many of the saprotrophic species occurred on grassland and/or on wood chips (Table 1), supporting that frequently, the species of the monitored genera of the *Psathyrellacecae* are familiarized with grasslands (Fig. 11, 14, and 15 in Chapter 1; Fig. 3A-B and 3E-J in Chapter 2) and that they can enter also new man-made biotopes such as wood chip beds (Fig. 10 in Chapter 1), even when the different habitats might have been over-fertilized in terms of nitrogen (Fig. 11, Fig. 14 and Fig. 18 in Chapter 1), (Van de Bogart 1979; Uljé and Noordeloos 1999; Schafer 2001; Keirle et al. 2004; Shaw et al. 2004; Nagy 2005; Navarro-Gonzaléz 2008; Gierczyk et al. 2011; Nagy et al. 2012b; 2013b).

Twenty of the observed species occurred only in meadows and lawns (Table 1). However, many of these were only one time seen. A bias of underrepresentation through too few observations per species can thus not be excluded. However, species observed only in grassland were frequently from the genera *Panaeolus* (8 of in total 12 species found) and *Psathyrella* (6 of in total 18 species):

- *Coprinellus heptemerus* (1 case), *Coprinellus plagioporus* (1 case),
- ***C. atramentaria*** (22 cases), *Coprinopsis narcotica* (2 cases), *Coprinopsis semitalis* (1 case),
- *Panaeolus acuminatus* (1 case), *Panaeolus ater* (1 case), *Panaeolus campanulatus* (2 cases), *Panaeolus cinctulus* (3 cases), *Panaeolus foenisecii* (1 case), *Panaeolus olivaceus* (2 cases), *Panaeolus rickenii* (2 cases), *Panaeolus semiovatus* (1 case),
- ***Parasola plicatilis*** (20 cases),
- ***Psathyrella atrolaminata*** (9 cases), *Psathyrella bifrons* (3 cases), *Psathyrella pennata* (1 case), *Psathyrella phegophila* (1 case), *Psathyrella piluliformis* (1 case), and *Psathyrella prona* (2 cases).

In particular the genus *Panaeolus* might thus be adapted to grassland situations, in accordance with conclusions from earlier reports (Griffith et al. 2004, 2013; Jordal et al. 2016). Moreover, occurrence of species of the genus *Psathyrella* is also not uncommon in grasslands (van Wabern 1977; Griffith and Roderick 2008). Of other genera, *C. atramentaria* and *P. plicatilis* (indicated in the text above in bold) were very often found in grasslands suggesting that meadows are a preferred natural biotope for the two species. However, the extensive literature survey by Navarro-Gonzaléz (2008) compiled particular many reports on *C. atramentaria* fruiting bodies as having been observed with logs, roots, stumps, and standing trees of broadleaf tree species such as *Fagus*, *Fraxinus*, *Populus*, *Salix*, *Ulmus*, and also *Quercus*. There is also an exceptional report of fruiting of *P. plicatilis* on a *Tilia* stump at the end phase of decay (Navarro-Gonzaléz 2008).

In this study, only 3 of in total 12 *Coprinellus* species and 3 of in total 10 *Coprinopsis* species were found only on grassland, and 8 species of *Panaeolus*, 1 species of *Parasola* and 6 species of *Psathyrella* with only observations of fruiting on grassland were counted. Species of these first two genera *Coprinellus* and *Coprinopsis* with often short life cycles and

Table 1 Fruiting bodies of *Psathyrellaceae* observed on different substrates on the grounds of the North Campus of the University of Göttingen in between 09/2011 and 12/2016 (a single observation from 05/2011 is included).

Species	Biotope*	Number of colonies/places	Times of occurrence
Genus *Coprinellus*			
Coprinellus disseminatus	On stem or roots of a living tree	7	38
	Dead wood	2	2
	Wood chips	1	3
	Plant litter	6	21
	Meadow	6	14
	Total	**22**	**78**
Coprinellus domesticus	On stem or roots of a living tree	1	3
	Dead wood	2	6
	Plant litter	1	5
	Meadow	11	29
	Total	**15**	43
Coprinellus flocculosus	Dead wood	1	1
	Meadow	1	1
	Total	**2**	**2**
Coprinellus heptemerus	Meadow	1	2
	Total	**1**	**2**
Coprinellus impatiens	Wood chips	1	1
	Meadow	1	2
	Total	**2**	**3**
Coprinellus marculentus	Wood chips	1	2
	Meadow	1	1
	Total	**2**	**3**
Coprinellus micaceus	Dead wood	3	20
	Plant litter	2	8
	Meadow	6	11
	Total	**11**	**39**
Coprinellus plagioporus	Meadow	1	1
	Total	**1**	**1**
Coprinellus subimpatiens	Meadow	1	1
	Total	**1**	**1**
Coprinellus tardus	Plant litter	1	1
	Meadow	1	7
	Total	**2**	**8**
Coprinellus truncorum	On stem or roots of a living tree	3	16
	Dead wood	1	1
	Meadow	1	1
	Total	**5**	**18**
Coprinellus xanthothrix	On stem or roots of a living tree	1	3
	Dead wood	4	9
	Total	**5**	**12**

* Data were summarized from Table A2 (Appendix II). Five classes of biotopes/substrates were considered: On stem or roots of living tree, dead wood, wood chips (beneath trees and shrubs), plant litter (leaves, needles, maybe some wood debris) and meadow. For every species, only those substrates are mentioned on which mushrooms were observed.

Table 1 continued

Species	Biotope*	Number of colonies/places	Times of occurrence
Genus *Coprinopsis*			
Coprinopsis acuminata	Dead wood	1	1
	Total	**1**	**1**
Coprinopsis atramentaria	Meadow	6	22
	Total	**6**	**22**
Coprinopsis cothurnata	Wood chips	1	1
	Total	**1**	**1**
Coprinopsis ephemeroides	Wood chips	1	1
	Total	**1**	**1**
Coprinopsis narcotica	Meadow	1	2
	Total	**1**	**2**
Coprinopsis lagopus	Wood chips	1	1
	Total	**1**	**1**
Coprinopsis macrocephala	Wood chips	2	3
	Meadow	1	1
	Total	**3**	**4**
Coprinopsis picacea	Wood chips	1	1
	Plant litter	1	1
	Total	**2**	**2**
Coprinopsis semitalis	Meadow	1	1
	Total	**1**	**1**
Coprinopsis stercorea	Wood chips	1	1
	Total	**1**	**1**
Genus *Coprinus*			
Coprinus comatus	Wood chips	1	1
	Plant litter	3	3
	Meadow	10	16
	Total	**14**	**20**
Genus *Lacrymaria*			
Lacrymaria lacrymabunda	Dead wood	1	1
	Wood chips	1	1
	Plant litter	1	1
	Meadow	7	14
	Total	**10**	**17**
Genus *Panaeolus*			
Panaeolus acuminatus	Meadow	1	1
	Total	**1**	**1**
Panaeolus antillarum	Wood chips	1	1
	Meadow	1	1
	Total	**2**	**2**
Panaeolus ater	Meadow	1	1
	Total	**1**	**1**
Panaeolus campanulatus	Meadow	1	2
	Total	**1**	**2**

Table 1 continued

Species	Biotope*	Number of colonies/places	Times of occurrence
Panaeolus cinctulus	Meadow	1	3
	Total	**1**	**3**
Panaeolus dunensis	Wood chips	1	1
	Total	**1**	**1**
Panaeolus foenisecii	Meadow	1	1
	Total	**1**	**1**
Panaeolus olivaceus	Meadow	2	2
	Total	**2**	**2**
Panaeolus papilionaceus	Wood chips	1	1
	Meadow	5	7
	Total	**6**	**8**
Panaeolus rickenii	Meadow	1	2
	Total	**1**	**2**
Panaeolus semiovatus	Meadow	1	1
	Total	**1**	**1**
Panaeolus solidipes	Plant litter	1	1
	Total	**1**	**1**
Genus *Parasola*			
Parasola auricoma	Wood chips	3	7
	Total	**3**	**7**
Parasola plicatilis	Meadow	11	20
	Total	**11**	**20**
Genus *Psathyrella*			
Psathyrella atrolaminata	Meadow	7	9
	Total	**7**	**9**
Psathyrella bifrons	Meadow	2	3
	Total	**2**	**3**
Psathyrella candolleana	On stem or roots of a living tree	4	9
	Dead wood	1	1
	Wood chips	1	1
	Meadow	2	2
	Total	**8**	**13**
Psathyrella conopilus	Dead wood	1	1
	Wood chips	6	8
	Plant litter	2	2
	Meadow	5	6
	Total	**14**	**17**
Psathyrella corrugis	Wood chips	1	1
	Total	**1**	**1**
Psathyrella gracilis	Wood chips	1	1
	Plant litter	1	1
	Meadow	4	4
	Total	**6**	**6**
Psathyrella leucotephra	Wood chips	1	3
	Total	**1**	**3**

Table 1 continued

Species	Biotope*	Number of colonies/places	Times of occurrence
Psathyrella marcescibilis	Wood chips	1	1
	Meadow	4	4
	Total	**5**	**5**
Psathyrella microrhiza	Wood chips	3	4
	Plant litter	1	1
	Meadow	3	4
	Total	**7**	**9**
Psathyrella multipedata	Plant litter	1	2
	Meadow	2	2
	Total	**3**	**4**
Psathyrella ochracea	Wood chips	1	1
	Total	**1**	**1**
Psathyrella pennata	Meadow	1	1
	Total	**1**	**1**
Psathyrella phegophila	Meadow	1	1
	Total	**1**	**1**
Psathyrella piluliformis	Meadow	1	1
	Total	**1**	**1**
Psathyrella prona	Meadow	2	2
	Total	**2**	**2**
Psathyrella pseudogracilis	Wood chips	1	1
	Meadow	1	1
	Total	**2**	**2**
Psathyrella spadiceogrisea	Wood chips	2	2
	Plant litter	2	2
	Meadow	2	6
	Total	**6**	**10**
Psathyrella tephrophylla	Wood chips	1	1
	Plant litter	2	2
	Meadow	1	1
	Total	**4**	**4**

ephemeral mushrooms might thus be able to adapt broader to variable substrates offered to a fungus upon occasion. A subgroup of species (8 of in total 12 *Coprinellus* species; 7 of total 10 *Coprinopsis* species; the single *Coprinus* species; the single *Lacrymaria* species; 3 of in total 12 *Panaeolus* species; 1 of in total 2 *Parasola* species; 11 of in total 18 *Psathyrella* species) was observed in connection with wood, mostly in fragmented state (Table 1), further supporting former observations (Navarro-Gonzaléz 2008) that Coprini and relatives can grow on wood chips and wood litter. Species listed before by Navarro-Gonzaléz (2008) to fruit in connection with wood are shown in the following in simple italic fond, species not previuosly listed by Navarro-Gonzaléz (2008) to fruit in connection with wood are given in italic and bold:

- *C. disseminatus*, *Coprinellus domesticus*, *Coprinellus flocculosus*, *Coprinellus impatiens*, *Coprinellus marculentus*, *C. micaceus*, *Coprinellus truncorum*, and *C. xanthrotrix*,

- *Coprinopsis acuminata*, ***Coprinopsis cothurnata***, ***Coprinopsis ephemeroides***, *Coprinopsis lagopus*, *Coprinopsis macrocephala*, ***Coprinopsis picacea***, and ***Coprinopsis stercorea***,
- *C. comatus*,
- ***Lacrymaria lacrymabunda***,
- ***Paneolus antillarum***, ***Paneolus dunensis***, and ***Paneolus papilionaceus***,
- *Parasola auricoma*,
- ***Psathyrella candolleana***, ***Psathyrella conopilus***, ***Psathyrella corrugis***, ***Psathyrella gracilis***, ***Psathyrella leucotephra***, ***Psathyrella marcescibilis***, ***Psathyrella microrhiza***, ***Psathyrella multipedata***, ***Psathyrella ochracea***, ***Psathyrella pseudogracilis***, ***Psathyrella spadiceogrisea***, and ***Psathyrella tephropylla***.

Navarro-Gonzaléz (2008) collected literature with a focus on the classical form group of Coprini (i.e., the new genera *Coprinellus*, *Coprinopsis*, *Parasola*, and *Coprinus*) while the genera *Panaeolus* and *Psathyrella* was not included in her literature survey. No new *Coprinellus* species but four new *Coprinopsis* species can now be added to the list of Coprini which may grow and fruit in connection with a wooden substrate.

In addition, at least some species of the Coprini occur also with living woody plants (Fig. 18 and Fig. 19, Chapter 1; Fig. 4, Fig. 5 and Fig. 6A-D in Chapter 2; Table 1), in the case of species of *Coprinellus* also in accordance with earlier reports (Navarro-Gonzaléz 2008): *C. disseminatus* was observed on *Acer pseudoplatanus* (11.09.2015; Fig. 19J-K in Chapter 1), *C. truncorum* on *Cladrastis lutea* (01.07.2013; Fig. 19P-Q in Chapter 1), *C. xanthothrix* on *A. pseudoplatanus* (29.08.2012; Fig. 19H-I in Chapter 1), and *P. candelloleana* on *Phellodendron amurense* (05.11.2011; Fig. 19R-S in Chapter 1).

In summary of all observations, 17 species were monitored on five or more different spots and often with repeated occurrence (Table 1 and Table 2). Of these, observations on

Table 2 Summary of observations (total individual ≥ 5) on repeated fruiting on individual sites of species of the *Psathyrellaceae* on the grounds of the North Campus of the University of Göttingen in between 09/2011 and 12/2016.

Species	Number of different sites (with repeated fruiting)	Times of re-occurrence per site	Total individual observations
Coprinellus disseminatus	22 (8)	Up to 32	78
Coprinellus domesticus	15 (6)	Up to 12	43
Coprinellus micaceus	11 (5)	Up to 17	39
Coprinellus tardus	2 (2)	Up to 3	8
Coprinellus truncorum	5 (1)	Up to 14	18
Coprinellus xanthothrix	5 (2)	Up to 6	12
Coprinopsis atramentaria	6 (4)	Up to 9	22
Coprinus comatus	14 (5)	Up to 3	20
Lacrymaria lacrymabunda	10 (4)	Up to 6	17
Panaeolus papilionaceus	6 (2)	2	8
Parasola auricoma	3 (3)	Up to 3	7
Parasola plicatilis	11 (4)	Up to 4	20
Psathyrella atrolaminata	7 (3)	2	9
Psathyrella candolleana	8 (2)	Up to 5	13
Psathyrella conopilus	14 (3)	Up to 5	17
Psathyrella gracilis	6 (1)	2	6
Psathyrella microrhiza	7 (2)	2	9
Psathyrella spadiceogrisea	6 (1)	5	10

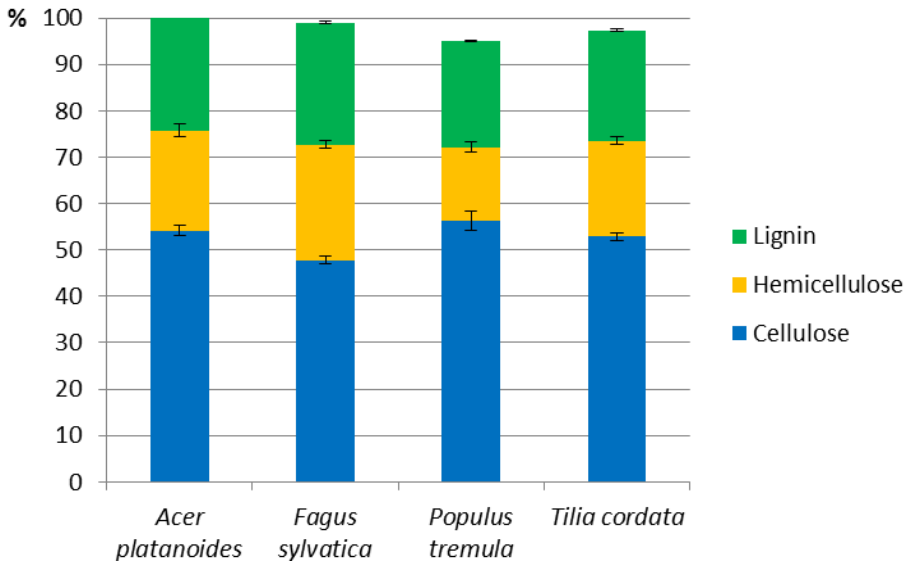

Fig. 2 Relative content of lignin, hemicellulose, and cellulose in wood blocks of different broadleaf trees used in wood decay tests after standard EN113 with isolates of Coprini. Average values and standard deviations were calculated from four different measurements per sample. Data kindly provided by F. Herz.

C. atramentaria, *P. plicatilis*, and *P. atrolaminata* were grassland-specific and *C. xanthothrix* wood-specific, while *C. disseminatus*, *C. domesticus*, *C. micaceus*, *C. truncorum*, *C. comatus*, *L. lacrymabunda*, *P. papilionaceus*, *P. candolleana*, *P. conopilus*, *P. gracilis*, *P. marcescibilis*, *P. microrhiza*, and *P. spadiceogrisea* were all observed in connection with grasslands and in connection with wooden substrates and sometimes also in combination with other plant litter such as leaves, needles and probably some smaller wood debris (species marked above in bold). In conclusion, most of the saprotrophic species found several times in this study occurred on diverse types of microhabitats (meadow, plant litter, wood chips and other wooden substrate, some also on living plants; Table 1). Mostly, the plant materials on which mushrooms were seen were rather loose in package which allowed good aeration. The different habitats with types of loose plant material, among the new man-made habitat of wood chip beds, imply that the organic materials used for growth may not be as strict as possibly anticipated for described grassland and coprophilus species of *Psathyrellaceae* with use of decaying grass and straw. The observations suggest that an airy environment could likely be of importance for the development of the species on certain organic materials.

Coprini and wood as substrate. To evaluate the possible use of wood by Coprini as a nutrient resource better, several wood decay tests after standard EN113 (1997) were performed by F. Herz with selected *C. disseminatus*, *C. micaceus*, *C. xanthothrix*, *C. atramentaria*, and *C. comatus* isolates obtained in this study or from M. Navarro-Gonzaléz (see Material and Methods) and with wood blocks of broadleaf trees *A. platanoides*, *F. sylvaticus*, *Populus tremulus*, and *T. cordata*, respectively (see Fig. 2 for wood polymer compositions). All tested strains grew onto samples of the different wood sources (see examples in Fig. 3). However, significant weight loss was in no case encountered after 16 weeks incubation. The results indicate that if these species have a wood decaying character, they are likely not primary degraders of sound wood.

Previously, the ammonia fungi *C. cinerea*, *Coprinopsis echinospora*, *Coprinopsis neolagopus*, and *Coprinopsis phlyctidospora* were all reported to grew on an agar medium with fresh beech wood sawdust added as sole nutrient source (Soponsathien 1998). Very

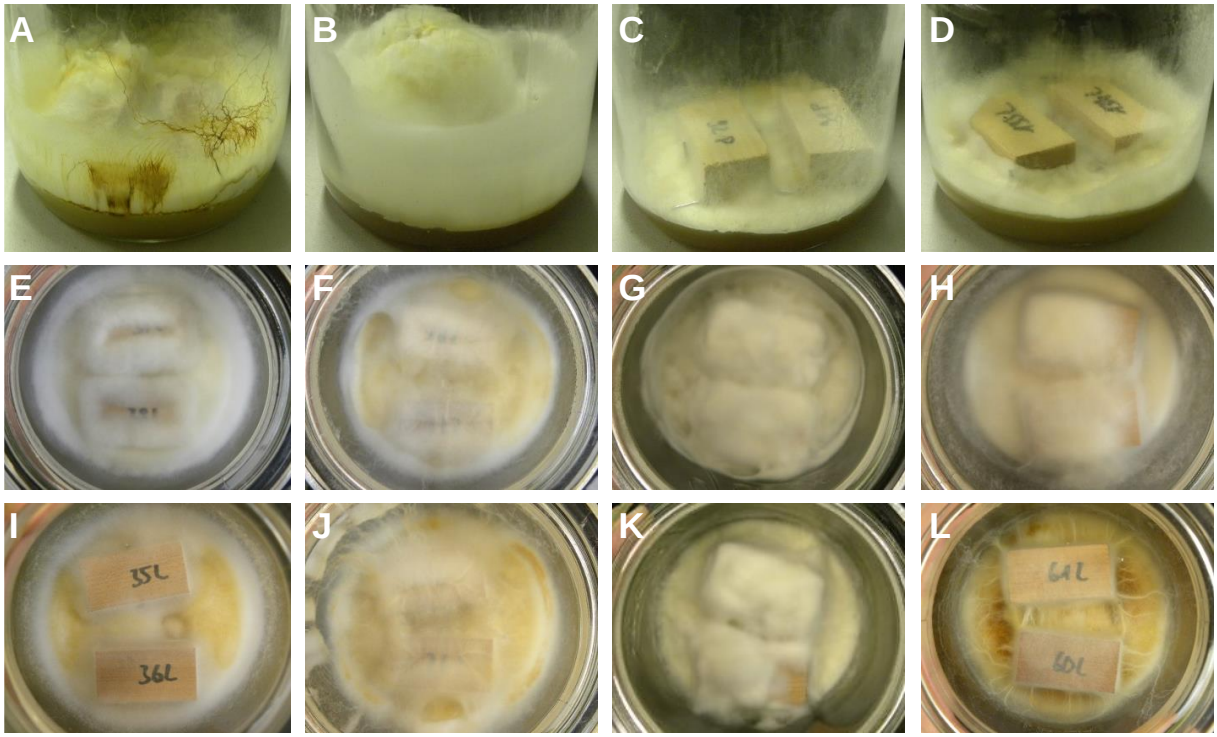

Fig. 3 Selected views on *Tilia cordata* wood block decay tests following standard EN113 with various Coprini isolates after 12 weeks (A to D) and 16 weeks incubation (E to L). **A.** *Coprinellus disseminatus* G15 (s1), **B.** *C. disseminatus* G3 (D2), **C.** *Coprinopsis atramentaria* G34 (s1), **D.** *Coprinus comatus* G30 (s1), **E.** and **I.** *Coprinellus micaceus* 1-OH, **F.** and **J.** *C. micaceus* MN7, **G.** and **K.** *Coprinellus xanthothrix* G18 (D1), **H.** and **L.** *C. xanthothrix* G1 (D1). Note the development of yellow-stained mycelium (a specialized mycelium known under the name ozonium; Badalyan et al. 2011a) in several of the 16-week-old *Coprinellus* cultures.
Wood test was performed and photos were taken by F. Herz.

likely therefore, even if several Coprini may be unable to significantly decay lignocellulose of intact wood, these fungi may take advantage of easily accessible nutrients which are present in fresh wood other than the compact lignocellulose. Other data from literature suggests that *C. atramentaria* may function as a secondary wood rot while *C. comatus* may use non-wood plant debris as resource for growth. Both species utilize cellulose from pre-white-rotted *A. pseudoplatanus* wood (Mohamed and Dix 1988). In another study with field-degraded maple sapwood blocks and with in the laboratory white-rot-predegraded poplar wood blocks, *C. atramentaria, C. cinerea* and *C. comatus* strains did not significantly further decay the wood unlike most strains of *C. micaceus* and *Coprinellus radians*. Weight loss of brown-rot predegraded poplar wood by *C. atramentaria, C. cinerea* and some *C. comatus* strains in contrast was significant and for some *C. micaceus* and *C. radians* strains the weight loss was higher as compared to on white-rot predegraded wood (Oliver et al. 2010). Species of Coprini may thus exist in nature as late degraders of broadleaf wood and such Coprini may thus take the opportunities provided by other wood-decaying fungi. Because Coprini were repeatedly observed in nature to fruit on brown-rotted wood of broadleaf trees (Ross 1976; Mitchel and Smith 1978; Gilbertson 1980; Redhead and Ginns 1985) while at least some species do produce enzymes typical for white rot (Heinzkill et al. 1998) and while Coprini phylogenetically do not belong into classical fungal clades with typical brown-rot species (Hibbett and Donoghue 2001), conflicting reports exist in the literature in whether Coprini

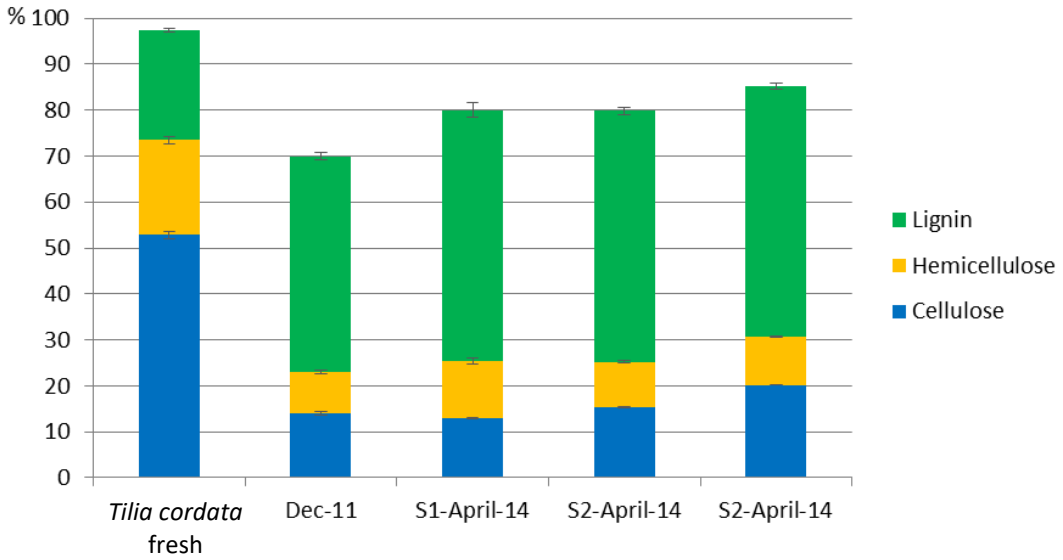

Fig. 4 Relative content of lignin, hemicellulose, and cellulose in brown-stained decaying wood samples taken at different dates from a *Tilia cordata* tree stump on the grounds of the North Campus of the University of Göttingen as compared to *T. cordata* fresh wood as control.

Average values and standard deviations were calculated from two or different measurements per decaying sample. Fresh wood data are from Fig. 2. Data kindly provided by F. Herz.

may be white or brown rots (Gilbertson 1980; Redhead and Ginns 1985). While this question is still not finally solved, the more common believe is that any wood-decaying Coprini should rather be white rots (Redhead and Ginns 1985; Navarro-Gonzaléz 2008).

Previously by Navarro-Gonzaléz (2008) and further in this study, fruiting bodies of two different *Coprinellus* species, i.e. first *C. xanthothrix* and then *C. micaceus*, were successively observed over the time on the same *T. cordata* tree stump on the grounds of the North Campus of the University of Göttingen. The tree stump appeared to decay in form a brittle cubicle brown rot manner (Fig. 19A-G in Chapter 1; Fig. 5 in Chapter 2; Table A1 in the Appendix I), in accordance to other reports from the literature on occurrence of Coprini species on apparently brown-rotted wood of broadleaf trees (Ross 1976; Mitchel and Smith 1978; Gilbertson 1980; Redhead and Ginns 1985) and in accordance to the findings in the report by Oliver et al. (2005) that various Coprini species can make use of the available cellulose in such brown-rotted wood. Further in this study, brown-stained partially decayed *T. cordata* wood samples (Fig. 5E in Chapter 2) were taken from the stump in 12/2011 and in 04/2014 and the Klason lignin content in the samples was determined as the values of solid weight of residues left after 3% sulphuric acid treatment for 4 h at 60°C, following TAPPI (1985). A value of 47.0 ± 0.84 % (n = 4) was obtained from the sample 12/2011 (Fig. 4) and values of 52.7 ± 4.0 % (n = 4), 55.3 ± 0.6% (n = 3) and 51.7 ± 4.5% (n = 3) for three different samples (S1 to S2) taken from different parts of the stump in 04/2014. The Klason lignin content in the latter samples were analyzed a second time with similar results (Fig. 4). Compared to the Klason lignin content in fresh *T. cordata* wood (23.9 ± 0.3%; Fig. 2 and Fig. 4), there was apparently an increase in lignin content. This further suggests that the stump had undergone a brown-rot-like decay or, also possible, decay by a soft-rot mechanism in which preferentially the carbohydrate polymers of plant cell walls are degraded (Martinez et al. 2005). As compared to in fresh wood, the measured cellulose and hemicellulose content in all decaying samples was accordingly much reduced from about 52.9 % cellulose in fresh elm wood to values between 12.96 and 15.3 % and from about 20.6 % hemicellulose in fresh elm

wood to values between 9.0 and 12.43 % (Fig. 4). Note that in the decaying samples from the elm-tree-stump, the calculated values for Klason lignin, cellulose and hemicellulose did not add up to total values approaching 100 % (Fig. 4). While the nature of other material was not further determined, it can be concluded that the relative amount of Klason lignin in comparison to the amounts of cellulose and to hemicellulose in relation is even higher. Consumption of polysaccharides by *C. xanthothrix* and *C. micaceus* might have contributed to this observed increase in lignin content. *C. micaceus* has previsously been observed to fruit on wood in combination with the large fruiting bodies of *Peziza* species from the phylum of *Ascomycota* (Pady 1941; Singh 1998). *Peziza* species can be early invaders of wood with soft-rot properties and they may prepare thereby the wood for usage by other fungi (Duncan and Eslyn 1966; Käärik 1974). Many other microscopic ascomycetous species are candidates for such early activities on fresh wood (Lumley et al. 2000, 2001; Fukusawa et al. 2009) and, moreover, also for activities in intermediately and well decayed wood (Fukusawa et al. 2009, 2017). Presence of such species would remain unnoticed in visual observations in nature auch as performed in this study when checking on fruiting bodies which are recognisable by the naked eye. Molecular strategies such as ITS sequencing from bulk DNA isolated from wood are best applied to reveal all microscopic species which are potentially involved in wood decay. Notably, outcomes from such molecular studies on presence of fungal communities and the individual species distribution within wood and over the consecutive decay stages can differ from deductions made from pure inventory studies of easily visual fruiting bodies (Kubartová et al. 2012).

Repeatedly in this study observing fruiting bodies of *Basidiomycetes* on the decaying *T. cordata* stump on the grounds of the North Campus of the University of Göttingen, also fruiting bodies of *Flammulina velutipes* were observed on the stump either alone or together with *Coprinellus* mushrooms (Fig. 6 in Chapter 2). *F. velutipes* is a white-rot fungus with abilities of lignin degradation [Pal et al. 1995; Park et al. 2014; Xie et al. 2017; see however also a contradictory report by Boyle et al. (1992) of lack of lignin degradation by *F. velutipes*]. *F. velutipes* will thus likely not have been responsible for the observed increase in lignin content. It is however possible that white-rot activities by *F. velutipes* paved the way to the cellulose and hemicellulose for the two *Coprinellus* species. *F. velutipes* (known also under a common name as winter mushroom) is a species with fruiting activities preferentially in colder months at preferred temperatures of 15 °C and below (Ingold 1981; Fultz 1988; Fig. 6 in Chapter 2; Table A3 in the Appendix II) which grows and colonizes wood in a temperature range between 4 and 22 °C (Miłkowski and Łakomy 2007). This is different to *C. xanthothrix* and *C. micaceus* which prefer temperatures around 25-28°C for growth (Guiraud et al. 1999; Badalyan et al. 2008; Navarro-Gonzaléz 2008) and which fruit at room temperature in the laboratory (Badalyan et al. 2001a). In this study with the observations of fruiting on an elm stump in nature, fruiting bodies of *C. xanthothrix* and *C. micaceus* occur more likely in warmer months as compared to *F. velutipes* (Table A3 in the Appendix IV). In conclusion from this, while the fungi shared with the elm tree stump the same place for their development, their temperature preferences are very different. A possibility therefore is that the white rot *F. velutipes* being physiologically active in colder months of the year helps via production of lignocellulolytic enzymes the *Coprinellus* species being physiologically active in warmer months of the year in enzymatic consumption of polysaccharides in the warmer seasons. Cellulase and hemicellulose activities have been reported for *C. micaceus* (Sergentani et al. 2016).

Mushroom development of Coprini in nature. Observations on the grounds of the North Campus of the University of Göttingen revealed that fruiting body formation of various Coprini reoccured over the months and years at the same distinct places (Table A1 in Appendix I; Table 2). This was particularly true for species of the genus *Coprinellus* (see

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage
		Night	Day	Night	Day	
-	08.11.15	16 (13-17)	15 (13-17)	76 (75-86)	65 (62-78)	?
-	09.11.15	15 (14-16)	15 (15-17)	85 (66-95)	67 (62-95)	?
-	10.11.15	14 (13-14)	15 (14-15)	75 (68-98)	84 (75-98)	?
-	11.11.15	13 (11-14)	15 (11-16)	98 (72-98)	75 (65-98)	?
-	12.11.15	12 (11-13)	14 (12-15)	78 (70-81)	75 (74-82)	?
A	13.11.15	9 (7-12)	13 (12-14)	91 (80-94)	80 (75-82)	Primordia
B	14.11.15	6 (6-7)	7 (5-9)	95 (80-98)	90 (72-97)	Primordia/Closed
C	15.11.15	8 (7-12)	12 (9-13)	99 (97-100)	98 (94-100)	Young
D	16.11.15	11 (10-12)	11 (10-12)	94 (88-99)	85 (77-88)	Young/Open
E	17.11.15	11 (10-14)	12 (10-15)	90 (80-92)	95 (74-99)	Open/Mature
F	18.11.15	11 (11-14)	12 (12-15)	90 (74-95)	68 (63-71)	Mature
G	19.11.15	10 (07-12)	12 (09-13)	84 (71-98)	75 (65-100)	Mature/Decaying
H	20.11.15	6 (5-8)	7 (7-8)	84 (82-97)	93 (85-95)	Decaying
I	21.11.15	3 (2-5)	6 (4-7)	84 (80-91)	75 (65-90)	Decayed

Fig. 5 Development of *Coprinellus disseminatus* mushrooms over the time.
A. to **I.** Period of mushroom observation from 13.11.2015 to 21.11.2015. Mushrooms appeared underneath a *Prunus sargentii* (Sargent´s cherry) tree on the grounds of the North Campus of the University of Göttingen (larger cherry tree between Microbiology Building and Göttingen Center for Molecular Biosciences, numbered here as Tree 1; same tree as in Fig. 6, code KL95A in Table A1 in Appendix I). The table beneath gives main temperature and humidity values at night (12 h period from 9 PM to 9 AM) and at day (12 h period from 9 AM to 9 PM) and in brackets the temperature range encountered over a respective 12 h period to enable easy comparisons with developmental stages in mushroom development in other observations on the species – see Fig. 6 to Fig. 11. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting phases noticed first three days later on still wet soil. ?: not observed.

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage
		Night	Day	Night	Day	
-	17.10.16	10 (8-14)	17 (12-18)	95 (88-97)	70 (70-88)	?
-	18.10.16	12 (11-13)	16 (13-18)	96 (78-97)	69 (60-75)	?
-	19.10.16	7 (6-11)	8 (7-10)	97 (95-100)	91 (82-99)	?
-	20.10.16	7 (6-8)	8 (7-9)	95 (90-97)	95 (85-95)	?
-	21.10.16	7 (6-7)	7 (6-7)	93 (92-99)	97 (96-99)	?
-	22.10.16	5 (3-6)	7 (6-9)	98 (92-98)	98 (85-99)	?
-	23.10.16	4 (2-7)	9 (7-11)	95 (92-97)	80 (70-91)	?
-	24.10.16	6 (6-9)	9 (7-9)	96 (94-98)	88 (87-96)	?
A	25.10.16	8 (8-9)	10 (9-11)	98 (95-99)	95 (94-98)	Primordia/Closed
B	26.10.16	8 (8-10)	11 (9-12)	95 (95-99)	83 (74-90)	Primordia/Closed
C	27.10.16	9 (8-12)	13 (10-14)	89 (87-92)	82 (78-88)	Closed
D	28.10.16	11 (10-11)	13 (12-13)	88 (87-91)	83 (82-87)	Young
E	29.10.16	8 (7-13)	12 (8-13)	94 (85-97)	70 (62-96)	Young/Open
F	30.10.16	7 (6-11)	12 (8-12)	95 (85-97)	78 (77-91)	Open, mature
G	31.10.16	10 (8-11)	13 (11-13)	91 (90-94)	73 (72-87)	Mature
H	01.11.16	6 (5-12)	13 (10-14)	95 (82-96)	74 (69-83)	Mature
I	02.11.16	6 (6-7)	8 (7-9)	95 (90-97)	85 (76-93)	Mature/Decaying
J	03.11.16	5 (4-7)	8 (7-8)	95 (94-96)	92 (90-94)	Decaying
K	04.11.16	6 (5-8)	8 (6-9)	92 (80-94)	74 (73-80)	Decayed
L	05.11.16	6 (6-7)	9 (7-9)	92 (80-94)	84 (80-90)	Decayed
M	06.11.16	6 (4-7)	8 (7-9)	90 (81-92)	74 (70-82)	Decayed
N	07.11.16	5 (5-6)	6 (5-6)	88 (80-90)	84 (84-87)	Decayed
O	08.11.16	2 (1-5)	3 (2-4)	90 (83-91)	82 (82-90)	Decayed

Fig. 6 Development of *Coprinellus disseminatus* mushrooms over the time.
A. to **O.** Period of observation from 25.10.2016 to 08.11.2016. Mushrooms appeared underneath a *Prunus sargentii* (Sargent´s cherry) tree on the grounds of the North Campus of the University of Göttingen (same site as in Fig. 5; larger Tree 1, code KL95A in Table A1 in Appendix I). Note that the soil appeared to be wet throughout the whole observation time. The table beneath gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first four days later. ?: not observed.

examples of fruiting of same colonies at different time points as documented in Fig. 5 to Fig. 14) which appear to have a stronger affinity to biotopes connected to living trees and also to dead wood of trees that takes its time to decay (Table 1; Table A1 in Appendix 1), as compared to species of the genera *Coprinopsis* and of *C. comatus* which more generally showed preferences for grassland biotopes which may have different nutrient inputs over the years (e.g. by faster decay of the herbaceous plant material and by nitrogen inputs as through human actions and animal grazing periods). However, more species of the genus *Coprinopsis* also occured as early invaders and fruiters in ephemeral manmnade wood-chip beds (Table 1

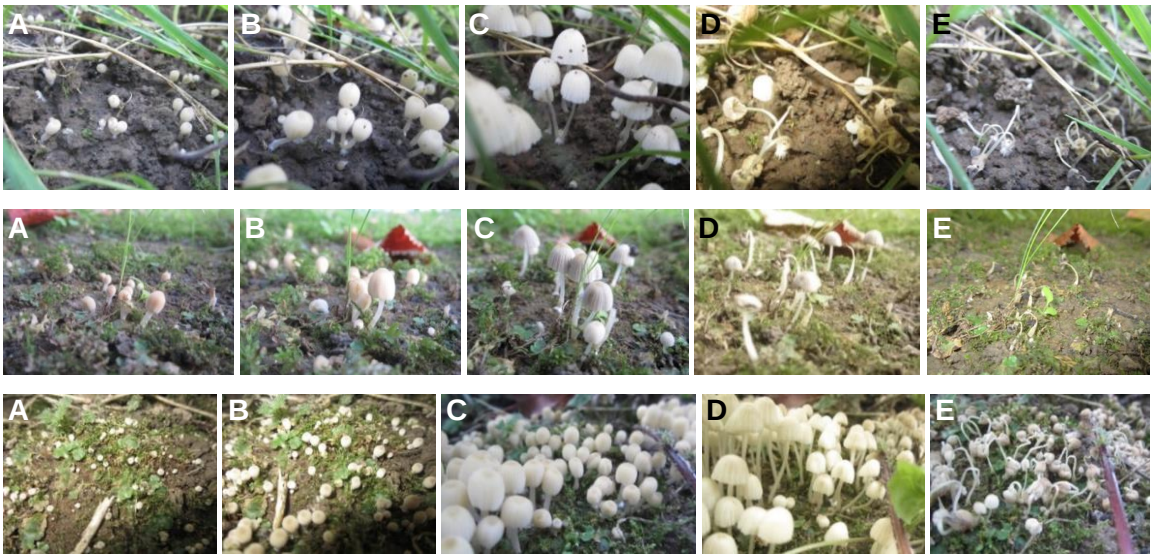

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage
		Night	Day	Night	Day	
-	16.09.16	17 (16-20)	23 (18-24)	95 (58-98)	58 (58-89)	?
-	17.09.16	16 (15-18)	17 (16-17)	94 (85-97)	96 (91-97)	?
-	18.09.16	15 (16-17)	20 (16-21)	95 (89-96)	72 (55-85)	?
-	19.09.16	14 (13-16)	15 (13-20)	85 (75-87)	80 (58-100)	?
-	20.09.16	9 (8-17)	20 (16-25)	95 (73-96)	45 (42-73)	?
-	21.09.16	15 (14-21)	20 (17-25)	95 (71-97)	62 (55-88)	?
-	22.09.16	11 (10-16)	23 (10-24)	80 (80-95)	50 (50-86)	?
A	23.09.16	11 (10-15)	20 (15-24)	85 (80-90)	65 (45-70)	Young/Primordia
B	24.09.16	11 (10-15)	20 (18-25)	87 (88-95)	64 (42-72)	Open/Young
C	25.09.16	11 (9-16)	20 (18-27)	87 (80-94)	60 (40-70)	Mature/Open
D	26.09.16	12 (11-15)	17 (14-21)	86 (75-91)	70 (60-75)	Dried/Mature
E	27.09.16	14 (13-17)	20 (18-24)	86 (84-91)	60 (55-76)	Dried

Fig. 7 Development of *Coprinellus disseminatus* mushrooms over the time as documented by three different groups of mushrooms within the same larger colony of fruiting bodies. **A.** to **E.** Period of observation from 23.09.2016 to 27.09.2016. Mushrooms appeared underneath a *Prunus incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen (smaller cherry tree between Microbiology Building and Göttingen Center for Molecular Biosciences, numbered here as Tree 2; same site as in Fig. 5 and Fig. 6, same tree as in Fig. 8 to Fig. 11, code KL95A in Table A1 in Appendix I). The table beneath gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting phases noticed first four days later on still wet soil. ?: not observed.

Fig. 8 Development of *Coprinellus disseminatus* mushrooms over the time (two distinct concurrent clusters of mushrooms in same larger colony are shown). **A.** to **M.** Period of observation from 08.10.2016 to 20.10.2016. Mushrooms appeared underneath a *Prunus incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen (same site as in Fig. 5 and Fig. 6, smaller Tree 2 as in Fig. 7 and Fig. 9 to Fig. 11; code KL95A in Table A1 in Appendix I). Note that the soil appeared to be wet throughout the whole observation time. The corresponding table (Fig. 8 continued, next page) gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first four and two days later. ?: not observed.

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage	
		Night	Day	Night	Day	Cluster 1	Cluster 2
-	02.10.16	13 (12-14)	17 (13-19)	95 (93-98)	72 (53-95)	?	?
-	03.10.16	12 (11-12)	13 (11-15)	96 (83-97)	94 (75-98)	?	?
-	04.10.16	11 (10-12)	17 (12-18)	62 (51-74)	98 (88-99)	?	?
-	05.10.16	9 (5-9)	12 (6-14)	80 (74-86)	64 (59-89)	?	?
-	06.10.16	8 (8-11)	11 (7-12)	84 (81-92)	93 (83-92)	?	?
-	07.10.16	11 (9-11)	14 (10-15)	90 (85-92)	80 (65-90)	?	?
A	08.10.16	9 (7-10)	11 (8-12)	91 (76-95)	80 (73-90)	Young/ Primordia	Primordia
B	09.10.16	8 (6-10)	10 (8-11)	93 (82-96)	80 (75-85)	Young/ Primordia	Primordia
C	10.10.16	7 (7-11)	12 (11-13)	92 (80-93)	65 (63-84)	Closed/ Primordia	Closed
D	11.10.16	6 (4-9)	11 (9-11)	94 (91-96)	88 (80-90)	Open/ Young	Closed
E	12.10.16	8 (7-9)	9 (7-10)	94 (88-95)	85 (81-90)	Mature/ Young	Young
F	13.10.16	7 (7-8)	9 (8-9)	90 (85-91)	87 (80-90)	Decaying/ Open	Young
G	14.10.16	7 (6-10)	11 (9-12)	87 (83-90)	70 (75-86)	Decayed/ Open	Open
H	15.10.16	9 (8-13)	13 (13-15)	90 (86-92)	77 (76-87)	Mature	Mature
I	16.10.16	10 (7-13)	18 (11-21)	96 (88-98)	66 (53-80)	Mature	Mature
J	17.10.16	10 (8-14)	17 (12-18)	95 (88-97)	70 (70-88)	Decaying	Mature
K	18.10.16	12 (11-13)	16 (13-18)	96 (78-97)	69 (60-75)	Decayed	Decaying
L	19.10.16	7 (6-11)	8 (7-10)	97 (95-100)	91 (82-99)	Decayed	Decayed
M	20.10.16	7 (6-8)	8 (7-9)	95 (90-97)	95 (85-95)	-	Decayed

Fig. 8 continued.

and Table A1 in Appendix I; see above). In most instances initially, we had observations only on older structures in fruiting body development (e.g. mature mushrooms fully developed in size which caught the eyes) but with time and better experience, there were also increasing observations on earlier structures and the course of fruiting body development over the time (see Fig. 5 to Fig. 15).

Studying the consecutive processes of mushroom development in nature is generally quite difficult, due to various factors. In the beginning in any outside studies, it is hard to monitor environmental effects in the field on development of fruiting structures on fungal mycelium. It can be impossible to detect in nature the very early structures on development as known from laboratory work, particularly if they are microscopic small primary and secondary hyphal knots, which present the first undifferentiated hyphal aggregates with which *Basidiomycetes* might initiate the fruiting process (Kües and Navarro-Gonzaléz 2015). Upon initiation of specific tissue formation by the secondary hyphal knots, the structures grow in size and with increase in size they become eventually (better) detectable by the naked eye in form of recognisable fruiting body primordia (Kües and Navarro-Gonzaléz 2015). Subsequent development can then be visually followed up with growth of the structures over their maturation process till finally also their deterioration.

Observations over a whole developmental process can be done in nature upon fortuitous detection of early stages in fruiting body development, preferentially as young as possible (usually primordia). Better for targeted inventories is when one already knows the

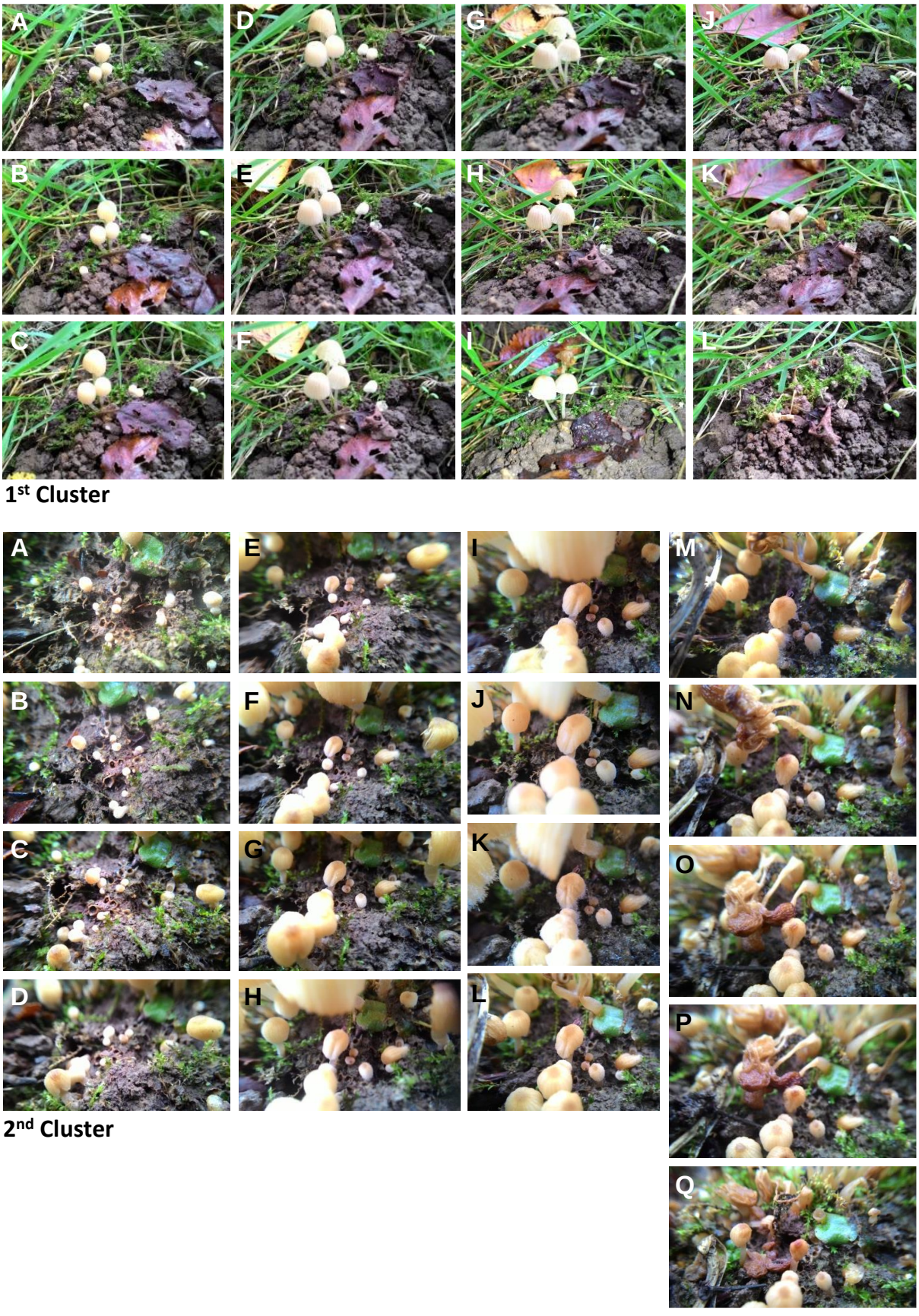

Fig. 9 Development of *Coprinellus disseminatus* mushrooms over the time (two distinct concurrent small clusters of mushrooms within the same larger colony are shown). **A.** to **Q.** Period of observation from 02.11.2016 to 18.11.2016. Further explanations are given on the next page (Fig. 9 continued).

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage	
		Night	Day	Night	Day	Cluster 1	Cluster 2
-	16.10.16	10 (7-13)	18 (11-21)	96 (88-98)	66 (53-80)	?	?
-	17.10.16	10 (8-14)	17 (12-18)	95 (88-97)	70 (70-88)	?	?
-	18.10.16	12 (11-13)	16 (13-18)	96 (78-97)	69 (60-75)	?	?
-	19.10.16	7 (6-11)	8 (7-10)	97 (95-100)	91 (82-99)	?	?
-	20.10.16	7 (6-8)	8 (7-9)	94 (91-99)	95 (85-97)	?	?
-	21.10.16	7 (6-7)	7 (6-7)	93 (92-99)	97 (96-99)	?	?
-	22.10.16	5 (3-6)	7 (6-9)	98 (92-98)	98 (85-99)	?	?
-	23.10.16	4 (2-7)	9 (7-11)	95 (92-97)	80 (70-91)	?	?
-	24.10.16	6 (6-9)	9 (7-9)	96 (94-98)	88 (87-96)	?	?
-	25.10.16	8 (8-9)	10 (9-11)	98 (95-99)	95 (94-98)	?	?
-	26.10.16	8 (8-10)	11 (9-12)	95 (95-99)	83 (74-90)	?	?
-	27.10.16	9 (8-12)	13 (10-14)	89 (87-92)	82 (78-88)	?	?
-	28.10.16	11 (10-11)	13 (12-13)	88 (87-91)	83 (82-87)	?	?
-	29.10.16	8 (7-13)	12 (8-13)	94 (85-97)	70 (62-96)	?	?
-	30.10.16	7 (6-11)	12 (8-12)	95 (85-97)	78 (77-91)	?	?
-	31.10.16	10 (8-11)	13 (11-13)	91 (90-94)	73 (72-87)	?	?
-	01.11.16	6 (5-12)	13 (10-14)	95 (82-96)	74 (69-83)	?	?
A	02.11.16	6 (6-7)	8 (7-9)	95 (90-97)	85 (76-93)	Young	Closed
B	03.11.16	5 (4-7)	8 (7-8)	95 (94-96)	92 (90-94)	Young	Closed
C	04.11.16	6 (5-8)	8 (6-9)	92 (80-94)	74 (73-80)	Open	Closed
D	05.11.16	6 (6-7)	9 (7-9)	92 (80-94)	84 (80-90)	Open	Closed
E	06.11.16	6 (4-7)	8 (7-9)	90 (81-92)	74 (70-82)	Mature	Closed
F	07.11.16	5 (5-6)	6 (5-6)	88 (80-90)	84 (84-87)	Mature	Young
G	08.11.16	2 (1-5)	3 (2-4)	90 (83-91)	82 (82-90)	Mature	Young
H	09.11.16	2 (0-3)	4 (3-4)	92 (85-95)	82 (79-86)	Mature	Young
I	10.11.16	2 (2-3)	4 (3-4)	96 (94-97)	94 (91-96)	Mature	Young
J	11.11.16	1 (-1-3)	4 (3-4)	94 (88-96)	85 (83-96)	Dried	Young
K	12.11.16	-3 (-4-2)	2 (-1-2)	92 (73-93)	74 (70-90)	Dried	Dried
L	13.11.16	1 (-1-2)	3 (1-4)	74 (73-83)	64 (63-69)	Decayed	Dried
M	14.11.16	-3 (-4-3)	5 (3-7)	74 (73-83)	64 (63-69)	Decayed	Dried
N	15.11.16	4 (-2-5)	4 (3-4)	96 (67-97)	94 (95-97)	Decayed	Dried
O	16.11.16	7 (6-10)	9 (8-10)	97 (63-97)	98 (93-99)	Decayed	Dried
P	17.11.16	10 (9-10)	9 (9-10)	90 (86-95)	94 (88-98)	Decayed	Dried
Q	18.11.16	12 (10-12)	8 (5-9)	79 (77-88)	98 (91-100)	Decayed	Dried

Fig. 9 continued

A. to **Q.** Mushrooms appeared underneath a *Prunus incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen (same site as in Fig. 5 and Fig. 6, smaller Tree 2 as in Fig. 7, Fig. 8, Fig. 10 and Fig. 11; code KL95A in Table A1 in Appendix I; see corresponding photos in Fig. 9 on the previous page). Note that the soil appeared to be wet throughout the whole observation time. The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first 10 and six days later. ?: not observed.

places where mycelia of species of interest reside in soil or in plant substrates. Even better for the earliest detection as possible is when environmental climate conditions for induction are identified for fruiting at known places and the likelihood of appearance of a flush of fruiting body development can thus reliably be predicted for a time period in connection with climate conditions. As described below, in this study such behaviour could be learned with time in order to then recognize and predict with a certain likelihood of accuracy for selected places on

Fig. 10 Concurrent development of different clusters of multiple smaller-sized *Coprinellus disseminatus* and larger *Coprinellus domesticus* fruiting bodies at a same site. **A.** to **L.** Period of observation from 08.10.2016 to 19.10.2016, the same time of observation as in Fig. 8. Further explanations are given on the next page (Fig. 10 continued).

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage in cluster			
						Cluster 1		Cluster 2	
		Night	Day	Night	Day	*C. disseminatus*	*C. domesticus*	*C. disseminatus*	*C. domesticus*
-	02.10.16	13 (12-4)	17 (13-19)	95 (93-98)	72 (53-95)	?	?	?	?
-	03.10.16	12 (11-12)	13 (11-15)	96 (83-97)	94 (75-98)	?	?	?	?
-	04.10.16	11 (10-12)	17 (12-18)	62 (51-74)	98 (88-99)	?	?	?	?
-	05.10.16	9 (5-9)	12 (6-14)	80 (74-86)	64 (59-89)	?	?	?	?
-	06.10.16	8 (8-11)	11 (7-12)	84 (81-92)	93 (83-92)	?	?	?	?
-	07.10.16	11 (9-11)	14 (10-15)	90 (85-92)	80 (65-90)	?	?	?	?
A	08.10.16	9 (7-10)	11 (8-12)	91 (76-95)	80 (73-90)	Primordia	Primordia	Primordia	Primordia
B	09.10.16	8 (6-10)	10 (8-11)	93 (82-96)	80 (75-85)	Primordia	Primordia	Primordia	Primordia
C	10.10.16	7 (7-11)	12 (11-13)	92 (80-93)	65 (63-84)	Primordia	Closed	Closed	Primordia
D	11.10.16	6 (4-9)	11 (9-11)	94 (91-96)	88 (80-90)	Closed	Closed	Closed	Primordia
E	12.10.16	8 (7-9)	9 (7-10)	94 (88-95)	85 (81-90)	Closed	Young	Closed	Closed
F	13.10.16	7 (7-8)	9 (8-9)	90 (85-91)	87 (80-90)	Closed	Young/Primordia	Young	Young/Closed
G	14.10.16	7 (6-10)	11 (9-12)	87 (83-90)	70 (75-86)	Young	Open/Closed	Young	Open/Young
H	15.10.16	9 (8-13)	13 (13-15)	90 (86-92)	77 (76-87)	Young	Mature/Open	Open	Mature/Open
I	16.10.16	10 (7-13)	18 (11-21)	96 (88-98)	66 (53-80)	Open	Decaying/Young	Mature	Mature
J	17.10.16	10 (8-14)	17 (12-18)	95 (88-97)	70 (70-88)	Mature	Decaying/Mature	Mature	Decayed/Decaying
K	18.10.16	12 (11-13)	16 (13-18)	96 (78-97)	69 (60-75)	Mature	Decayed/Decaying	Mature/Decayed	Decayed
L	19.10.16	7 (6-11)	8 (7-10)	97 (95-100)	91 (82-99)	Decaying/Decayed	Decayed	Decayed	Decayed

Fig. 10 continued.

A. to **L.** Mushrooms appeared underneath a *Prunus incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen (same site as in Fig. 5 and Fig. 6, smaller Tree 2 as in Fig. 7 to Fig. 9 and Fig. 11; code KL95A and KL97D in Table A1 in Appendix I; see corresponding photos in Fig. 10 on the previous page). The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting phases noticed first four days and respectively one day later. Note for case **F.** of the observation series of Cluster 1 the delayed development of a single *C. domesticus* fruiting body seen in the lower right corner at the edge of the photo shown in Fig. 10F. ?: not observed.

Fig. 11 Concurrent development of different clusters of multiple smaller-sized *Coprinellus disseminatus* and larger *Coprinellus domesticus* fruiting bodies at a same site. **A.** to **M.** Period of observation from 09.11.2016 to 21.11.2016, overlapping in time of observation with Fig. 9. Further explanations are given on the next page (Fig. 11 continued).

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage in cluster			
						Cluster 1		Cluster 2	
		Night	Day	Night	Day	*C. disseminatus*	*C. domesticus*	*C. disseminatus*	*C. domesticus*
-	01.11.16	6 (5-12)	13 (10-14)	95 (82-96)	74 (69-83)	?	?	?	?
-	02.11.16	6 (6-7)	8 (7-9)	95 (90-97)	85 (76-93)	?	?	?	?
-	03.11.16	5 (4-7)	8 (7-8)	95 (94-96)	92 (90-94)	?	?	?	?
-	04.11.16	6 (5-8)	8 (6-9)	92 (80-94)	74 (73-80)	?	?	?	?
-	05.11.16	6 (6-7)	9 (7-9)	92 (80-94)	84 (80-90)	?	?	?	?
-	06.11.16	6 (4-7)	8 (7-9)	90 (81-92)	74 (70-82)	?	?	?	?
-	07.11.16	5 (5-6)	6 (5-6)	88 (80-90)	84 (84-87)	?	?	?	?
-	08.11.16	2 (1-5)	3 (2-4)	90 (83-91)	82 (82-90)	?	?	?	?
A	09.11.16	2 (0-3)	4 (3-4)	92 (85-95)	82 (79-86)	Open/Mature	Decaying	Mature	Primordia
B	10.11.16	2 (2-3)	4 (3-4)	96 (94-97)	94 (91-96)	Open/Mature	Young	Mature	Primordia
C	11.11.16	1 (-1-3)	4 (3-4)	94 (88-96)	85 (83-96)	Open/Mature	Young/Open	Mature	Primordia
D	12.11.16	-3 (-4-2)	2 (-1-2)	92 (73-93)	74 (70-90)	Open/Mature	Open	Mature	Primordia
E	13.11.16	1 (-1-2)	3 (1-4)	74 (73-83)	64 (63-69)	Decayed	Open	Mature/Decaying	Primordia
F	14.11.16	-3 (-4-3)	5 (3-7)	74 (73-83)	64 (63-69)	Decayed	Open	Decayed	Open
G	15.11.16	4 (-2-5)	4 (3-4)	96 (67-97)	94 (95-97)	Decayed	Open	Decayed	Open
H	16.11.16	7 (6-10)	9 (8-10)	97 (63-97)	98 (93-99)	-	Open	Decayed	Open
I	17.11.16	10 (9-10)	9 (9-10)	90 (86-95)	94 (88-98)	-	Open	Decayed	Open
J	18.11.16	12 (10-12)	8 (5-9)	79 (77-88)	98 (91-100)	-	Decaying	Decayed	Open
K	19.11.16	5 (5-6)	7 (6-8)	86 (82-88)	78 (75-81)	-	Decaying	Decayed	Open/Aging
L	20.11.16	5 (5)	12 (7-14)	82 (78-84)	60 (59-65)	-	Decaying	-	Open/Aging
M	21.11.16	12 (11-13)	13 (12-15)	58 (55-67)	53 (51-58)	-	Decaying	-	Open/Aging

Fig. 11 continued.

A. to **L.** Mushrooms appeared underneath a *Prunus incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen (same site as in Fig. 5 and Fig. 6, smaller Tree 2 as in Fig. 7 to Fig. 11; codes KL95A and KL97D in Table A1 in Appendix I; see corresponding photos in Fig. 11 on the previous page). The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting phases noticed first six days later. Note for case **I.** of the developmental series of Cluster 1 the delayed development of a *C. domesticus* fruiting body seen at the lower edge slightly to the middle of the photo. ?: not observed.

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage	
		Night	Day	Night	Day	1st flush	2nd flush
-	02.10.12	8 (8-10)	18 (12-21)	90 (84-91)	61 (56-78)	?	?
-	03.10.12	14 (13-16)	17 (16-18)	79 (72-83)	65 (64-70)	?	?
-	04.10.12	14 (13-16)	12 (10-15)	92 (85-94)	92 (86-95)	?	?
-	05.10.12	9 (9-13)	16 (10-18)	90 (81-94)	88 (78-96)	?	?
-	06.10.12	10 (8-15)	15 (09-17)	92 (87-98)	85 (79-93)	?	?
-	07.10.12	7 (6-11)	13 (10-14)	94 (67-95)	60 (62-96)	?	?
A	08.10.12	6 (4-8)	14 (4-14)	92 (82-95)	60 (50-94)	Closed	Primordia
B	09.10.12	6 (5-8)	12 (5-14)	92 (67-93)	55 (50-94)	Young	Primordia
C	10.10.12	5 (4-10)	10 (6-12)	90 (85-92)	78 (65-80)	Young	Primordia
D	11.10.12	4 (2-9)	10 (8-14)	92 (90-96)	72 (55-85)	Open	Closed
E	12.10.12	7 (6-9)	10 (9-12)	94 (90-97)	78 (73-85)	Open	Closed
F	13.10.12	8 (6-11)	12 (10-15)	90 (80-94)	70 (60-85)	Mature	Young
G	14.10.12	8 (7-11)	11 (10-12)	92 (80-95)	70 (60-82)	Autolysis	Young

Fig. 12 Successive development of two flushes of *Coprinellus micaceus* mushrooms at a same site over the time.

A. to **G.** Periods of observation of the two partially overlapping flushes from 08.10.2012 to 14.10.2012. Mushrooms appeared on and next to a stump of a *Tilia cordata* (elm) tree in a lawn on the grounds of the North Campus of the University of Göttingen, north to the Physics buildings (same site as in Fig. 13 and Fig. 14; code KL112 in Table A1 in Appendix I). Further observations were precluded by maintenance activities of university gardeners cutting the lawn. The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first two days later.?: not observed.

the grounds of the North Campus of the University of Göttingen for some of the observed Coprini species.

Establishing time courses of fruiting body development in nature can help in cases of observation of only older fruting structures to calculate back the likely time of induction and initiation of their development. These data can then be linked to environmental climate conditions which existed at that time in nature. Accordingly, with good knowledge on the overall progress and the typical length of developmental process, later stages in development and mature fruiting bodies detected as first observation of a fruiting event at a given place becomes then also useful surrogates to study inducing effects of fruiting by previous environmental climate conditions that were recorded for a region. This will be further exemplified in the following section where observations on climate conditions in induction of fruiting body development of Coprini are considered.

***Temperature and humidity effects on fruiting of* Psathyrellaceae *and* Coprinus comatus *in nature*.** For the 17 species with observations on at least 5 different places of appearance and with places of repeated occurrence of fruiting (see the summary in Table 2), it appeared possible by the higher numbers of individual observations per species and the availability of various recorded time courses of fruiting body development (Fig. 5 to Fig. 15) to confer more details on climate conditions under which their fruiting bodies preferentially appear.

In accordance with the literature (Orton and Watling 1979; Breitenbach and Kränzlin 1995; Keirle et al. 2004; Noordeloos et al. 2005; Navarro-Gonzaléz 2008; http://www.grzyby.pl/coprinus-site-Kees Uljee/species/Coprinus.htm), the species *C. disseminatus* (14 different places with up to 32 times observed re-occurrence per single site; in total 79 individual observations), *C. domesticus* (10 different places with up to 1 times observed re-occurrence per single site; in total 44 individual observations), and *C. micaceus* (10 different places with up to 17 times observed re-occurrence per single site; in total 39 individual observations) from the genus *Coprinellus* were especially often appearing and reappearing with successive fruiting periods at the same locations (Table 2). Because particularly many observations were available for these three species including the several fully recorded time courses (Fig. 5 to Fig. 14), we used these first to analyse connections between temperatures and humiditiy values and the appearance of fruiting bodies, respectively. However, as explained already above, the actual developmental stages already reached at the first time point of a new mushroom detection had to be considered in the analysis to link climate conditions with the respective time of mushroom induction (Fig. 5 to Fig. 14; Table 3).

To describe the course of fruiting body development of distinguished species over the time better in detail, noticed appearance of mushrooms of the observed Coprini on the day of detection were categorized into five possible distinct developmental stages (primordia, closed, young, open, and matured, see scheme in Fig. 1), depending on what was found on the day of first observation (Appendix I, Table A1). Subsequently, progresses in the distinct developmental stages over the time were also recorded where possible. To carefully document the respective observed stages was of eminent importance because the whole fruiting process takes several days from the first obvious visible sign of fruiting (such as that primordia appear) over closed and open mushroom stages to finally mushroom degradation (see theoretical scheme in Fig. 1 and for impressions the actual serial observations on colonies of *C. disseminatus* in Fig. 5 to Fig. 11, of *C. domesticus* in Fig. 10 and Fig. 11, of *C. micaceus* in Fig. 12 to Fig. 14, and further of *C. truncorum* in Fig. 15.

In several instances of mushroom observations, we recorded humidity and temperature on daily basis prior to and while fruit body development of the species started and continued up to mushroom decay when fruiting body development was fully completed (Fig. 5, Fig. 6, Fig. 8 to Fig. 12, Fig. 14, Fig. 15) or, when weather conditions became unfavourable for

fruiting body maturation, up to the stage when the structures were drying out prior to maturation (Fig. 7, Fig. 9, Fig. 13). Moreover, sometimes we were not able to record the full process when e.g. gardening work on the grounds of the Northern Campus of the University of Göttingen interfered with completing our observations such as by mowing the lawn (Fig. 12). The humidity and temperature ranges before and during the observed successful fruiting events for *C. disseminatus*, *C. domesticus*, *C. micaceus* and *C. truncorum* (data listed in series) comprised

i. temperature ranges variably from about 5-12 to 16-20 °C (night) and 10-14 to 18-24 ºC (day), 5-14 °C (night) and 12-18 °C (day), 7-16 °C (night) and 10-25 ºC (day), and 6-11 °C (night) and 7-10 °C (day), respectively prior to rainfall and fruiting,

ii. temperature ranges variably from about 2-10 to 13-18 °C (night) and 5-12 to 12-21 ºC (day), 4-12 °C (night) and 5-15 °C (day), 6-15 °C (night) and 6-19 ºC (day), and 2-9 °C (night) and 7-11 °C (day), respectively upon rainfall, moisturing of the growth substrate and an increase in humidity to highest values (>80 % to mostly >90 % both

Fig. 13 Successive development of six different clusters of *Coprinellus micaceus* mushrooms on the remnants of a former stump of a *Tilia cordata* (elm) tree in a lawn on the grounds of the North Campus of the University of Göttingen, north to the Physics buildings (same site as in Fig. 12 and Fig. 14; code KL112 in Table A1 in appendix I). **A.** to **J.** Periods of observation from 25.10.2014 to 04.11.2014. Further explanations are given on the next page (Fig. 13 continued).

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage in cluster					
		Night	Day	Night	Day	1	2	3	4	5	6
-	17.10.14	13 (13)	16 (14-17)	93 (93-96)	87 (84-97)	?	?	?	?	?	?
-	18.10.14	11 (10-13)	19 (15-21)	97 (94-98)	70 (66-92)	?	?	?	?	?	?
-	19.10.14	13 (12-14)	22 (15-24)	91 (89-95)	73 (61-85)	?	?	?	?	?	?
-	20.10.14	14 (13-16)	16 (13-17)	95 (82-100)	72 (64-84)	?	?	?	?	?	?
-	21.10.14	12 (9-13)	13 (10-13)	87 (82-95)	79 (77-99)	?	?	?	?	?	?
-	22.10.14	7 (6-9)	7 (7-9)	90 (86-98)	97 (89-98)	?	?	?	?	?	?
-	23.10.14	9 (8-11)	14 (9-14)	83 (78-94)	75 (61-87)	?	?	?	?	?	?
-	24.10.14	11 (8-11)	16 (11-17)	92 (78-95)	62 (57-85)	?	?	?	?	?	?
A	25.10.14	10 (10-12)	13 (12-15)	80 (78-85)	75 (68-75)	Primordia	Primordia	?	?	?	?
B	26.10.14	10 (10-12)	13 (12-15)	83 (82-96)	75 (62-78)	Closed	Primordia	?	Primordia	?	?
C	27.10.14	10 (8-12)	14 (12-18)	88 (90-97)	73 (62-80)	Young	Primordia	Primordia	Primordia	Primordia	?
D	28.10.14	8 (7-9)	10 (10-11)	87 (82-95)	72 (63-80)	Young	Closed	Primordia	Closed	Primordia	?
E	29.10.14	6 (5-9)	11 (9-13)	88 (82-97)	72 (63-80)	Mature	Young	Primordia	Young	Closed	Primordia
-	30.10.14	9 (9-10)	11 (10-13)	95 (93-97)	91 (88-92)	?	?	?	?	?	?
F	31.10.14	13 (12-16)	17 (16-19)	91 (82-94)	78 (70-80)	Autolysis	Mature	Closed	Mature	Young	Primordia
G	01.11.14	10 (9-13)	17 (15-21)	93 (85-97)	78 (70-80)	Decayed	Mature	Young	Autolysis	Mature	Closed
H	02.11.14	11 (10-13)	17 (14-19)	92 (85-94)	78 (66-82)	Decayed	Autolysis	Mature	Autolysis	Autolysis	Closed
I	03.11.14	13 (13-14)	15 (15-16)	83 (82-85)	76 (60-80)	Dried	Dried	Autolysis	Dried	Autolysis	Young
J	04.11.14	14 (13-16)	16 (15-19)	88 (72-88)	70 (60-73)	Dried	Dried	Dried	Dried	Dried	Open

Fig. 13 continued.

A. to **J.** The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first three days later – note first in **E.** closer to the center of the area of the now much decayed elm tree stump the delayed development of another *C. micaceus* fruiting body. ?: not observed.

Fig. 14 Development of *Coprinellus micaceus* mushrooms at a same site over the time. **A.** to **J.** Period of observation from 22.10.2016 to 31.10.2016. Further explanations are given on the next page (Fig. 13 continued).

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage	
		Night	Day	Night	Day	Cluster 1	Cluster 2
-	16.10.16	10 (7-13)	18 (11-21)	96 (88-98)	66 (53-80)	?	?
-	17.10.16	10 (8-14)	17 (12-18)	95 (88-97)	70 (70-88)	?	?
-	18.10.16	12 (11-13)	16 (13-18)	96 (78-97)	69 (60-75)	?	?
-	19.10.16	7 (6-11)	8 (7-10)	97 (95-100)	91 (82-99)	?	?
-	20.10.16	7 (6-8)	8 (7-9)	94 (91-99)	95 (85-97)	?	?
-	21.10.16	7 (6-7)	7 (6-7)	93 (92-99)	97 (96-99)	?	?
A	22.10.16	5 (3-6)	7 (6-9)	98 (92-98)	98 (85-99)	Primordia	Primordia
B	23.10.16	4 (2-7)	9 (7-11)	95 (92-97)	80 (70-91)	Primordia	Primordia
C	24.10.16	6 (6-9)	9 (7-9)	96 (94-98)	88 (87-96)	Closed, Young	Closed
D	25.10.16	8 (8-9)	10 (9-11)	98 (95-99)	95 (94-98)	Young, Open	Young
E	26.10.16	8 (8-10)	11 (9-12)	95 (95-99)	83 (74-90)	Open, Mature	Open
F	27.10.16	9 (8-12)	13 (10-14)	89 (87-92)	82 (78-88)	Mature	Mature
G	28.10.16	11 (10-13)	13 (11-13)	89 (87-93)	84 (82-86)	Autolysis	Autolysis
H	29.10.16	10 (7-12)	13 (11-13)	91 (90-97)	66 (62-68)	Decayed	Autolysis
I	30.10.16	9 (6-11)	12 (10-12)	90 (85-95)	84 (77-90)	Decayed	Decayed
J	31.10.16	10 (8-10)	13 (10-13)	91 (84-94)	80 (72-87)	Decayed	Decayed

Fig. 14 continued.

A. to **J.** Mushrooms appeared on and next to a decayed stump of a *Tilia cordata* (elm) tree (note the brown remnants of the former stump around and between fruiting bodies of Cluster 1) in a lawn on the grounds of the North Campus of the University of Göttingen, north to the Physics buildings (same site as in Fig. 12 and Fig. 13; code KL112 in Table A1 in Appendix I). The table gives main values and ranges of temperatures and humidities at night and day phases as explained in the legend of Fig. 5. Grey underlaid are dates with a remarkable increase in humidity prior to the observed fruiting noticed first 10 and six days later. ?: not observed.

at nights and at days) over 2 or more days with often simultaneous drops in temperature by in the highest cases falls of up to 8/9 °C (night/day), and

iii. temperature ranges variably from about 0-14 to 8-21 °C (night) and 3-14 to 10-27 °C (day), 0-14 °C (night) and 3-21 °C (day), 2-13 °C (night) and 4-19 °C (day), and 8-13 °C (night) and 10-14 °C (day), respectively (Fig. 5 to Fig. 15; Table 3).

With more experience on correlations of fruiting with weather conditions, especially rainfall, predictive sampling became thus possible for the three species *C. disseminatus*, *C. domesticus*, and *C. micaceus*. Basically, a high increase in humidity was required for at least 2 or 3 days and achieved through sufficient rainfall, along with a reduction in temperature (at warmer climates) by about 3 to 4 °C and even more (5 to 7 °C) both at nights and at days prior to that primordia for the species would become recognizable. Because fruiting of *C. disseminatus*, *C. domesticus*, and *C. micaceus* was often paralleled by fruiting of other *Psathyrellaceae* (Appendix II, Table A2), including of *C. truncorum* (Fig. 15; Table 3), other species of the family appear to follow similar humidity and temperature schemes.

From the various data collected, we then compiled in Table 3 the likely temperature and humidity values at the time of mushroom induction. Table 4 then summarizes the fruiting events in the different months for those species that were several different times observed to fruit during the years 2011 to 2016. In the following text, a catalog of short overviews on

environmental conditions for fruiting of selected repeatedly fruiting species of the *Psathyrellaceae* and also *C. comatus* from the *Agariaceae* is given, with a main focus on wood-related species of the genus *Coprinellus* summarizing the observations for future usage:

1. **Coprinellus disseminatus.** The relatively small fruiting bodies of the appear always in masses (looking as if seeded = *disseminatus*) and in almost all months in a year (Table 4; Table A1 in Appendix I), except at extended periods of very low temperature (less than 4 °C as e.g. occurring in many December months) and in periods of lack of rain (thus throughout most of the very dry years 2018 and 2019; observations kindly provided by W. Khonsuntia and U. Kües). *C. disseminatus* is commonly rather considered a non-autolysing species (Kühner 1929; Lange 1939; Orton and Watling 1979; Hoppele et al. 1999; Redhead et al. 2001; Keirle et al. 2004; Padamsee et al. 2008). Own observations show rather drying out of matured mushrooms or full collapsing of aged mushrooms with no specific cap autolysis (see Fig. 5 to Fig. 11), similarly as reported in Padamsee et al. (2008) who also observed the mushrooms 'collapsing' while not considering this feature as deliquescence. However, differently from Padamsee et al. (2008), Nagy et al. (2011, 2013b) marked the species as

Photo	Date	Temperature (°C)		Humidity (%)		Mushroom stage
		Night	Day	Night	Day	
-	19.10.16	7 (6-11)	8 (7-10)	97 (95-101)	91 (82-99)	?
-	20.10.16	7 (6-8)	8 (7-9)	94 (91-99)	95 (85-97)	?
-	21.10.16	7 (6-7)	7 (6-7)	93 (92-99)	97 (96-99)	?
-	22.10.16	5 (3-6)	7 (6-9)	98 (92-98)	98 (85-99)	?
-	23.10.16	4 (2-7)	9 (7-11)	95 (92-97)	80 (70-91)	?
-	24.10.16	6 (6-9)	9 (7-9)	96 (94-98)	88 (87-96)	?
A	25.10.16	8 (8-9)	10 (9-11)	98 (95-99)	95 (94-98)	Young
B	26.10.16	8 (8-10)	11 (9-12)	95 (95-99)	83 (74-90)	Open
C	27.10.16	9 (8-12)	13 (10-14)	89 (87-92)	82 (78-88)	Mature
D	28.10.16	11 (10-13)	13 (11-13)	89 (87-93)	84 (82-86)	Decayed
E	29.10.16	10 (7-12)	13 (11-13)	91 (90-97)	66 (62-68)	Dried

Fig. 15 Development of *Coprinellus truncorum* mushrooms over the time.
A. to **M.** Period of observation from 24.10.2016 to 28.10.2016. Mushrooms appeared underneath a felled stem of a pine tree placed on logs from poplar (Büsgenweg 5, E - side of bldg.; code L161 in Table A1 in Appendix I and Fig. A3 in Appendix IV) on the grounds of the North Campus of the University of Göttingen.

Table 3 Summary on climate data linked to fruiting activities of selected *Coprinellus* species as deduced from data presented in Fig. 5 to Fig. 15.

Species	Fig.	First stage observed	Days (d) prior observations*	Temperature (°C) at induction		Decrease in temperature (- °C)		Humidity range (%) at induction		Days from induction to death*
				Night	Day	Night	Day	Night	Day	
Coprinellus disseminatus	5	Primordia	2-4	13-15	15	3-7	2	75-98	75-84	11-13
	6	Primordia/ Closed	3-6	7-12	7-16	5-7	8-9	93-98	91-98	14-17
	7	Young/ Primordia	4-7	14-16	15-20	5-7	3-8	85-95	72-96	8-12
	8	Cluster 1 Young/ Primordia	2-5	8-12	11-17	3-4	1-6	62-96	93-98	10-16
		Cluster 2 Primordia	2-5	8-11	11-17	2-3	1-5	80-84	64-93	13-15
	9	Cluster 1 Young	7-14	4-8	8-11	0-8	5-9	95-97	83-98	19-23
		Cluster 2 Closed								Aborted
	10	Cluster 1 Primordia	1-6	8-13	11-17	2-4	2-6	62-96	72-98	13-18
		Cluster 2 Primordia								
	11	Cluster 1 Open/ Mature	6-7	5-6	8-13	0-1	5	95	85-92	>11-12?
		Cluster 2 Mature								
Coprinellus domesticus	10	Cluster 1 Primordia	1-6	8-13	11-17	2-4	2-6	62-96	72-98	12-18
		Cluster 2 Primordia								
	11	Cluster 1 Young	7-8	5-6	8-13	0-1	5	95	85-92	16-17
		Cluster 2 Primordia	6-7							19-20
Coprinellus micaceus	12	1st flush Closed	2-4	12-15	12-16	0-5	1-5	90-92	85-92	9-11
		2nd flush Primordia								>9-11 Razed
	13	Cluster 1 Primordia	3	7	7	5	6	90	97	11
		Cluster 2 Primordia	3							12
		Cluster 3 Primordia	5							14
		Cluster 4 Primordia	4							12
		Cluster 5 Primordia	5							14
		Cluster 6 Primordia	7							>14
	14	Cluster 1 Primordia	1-3	7	7-8	5	8-9	94-97	91-97	10-12
		Cluster 2 Primordia								
Coprinellus truncorum	15	Young	1-6	4-7	7-9	1-3	0-1	93-98	80-97	6-11

* From estimated induction/start of development

Table 4 Preferred months of fruiting of selected species of Coprini and related *Psathyrellaceae* through the years*

Species	Number of times of fruiting observed in a month in the years/over the years 2011-2016												
	01	02	03	04	05	06	07	08	09	10	11	12	Total
Coprinellus disseminatus	2	-	-	3	1	5	5	7	20	24	11	1	79
Coprinellus domesticus	-	-	-	1	1	-	-	-	11	13	17	-	44
Coprinellus micaceus	-	-	1	5	1	1	-	-	5	16	10	-	39
Coprinellus tardus	-	-	-	-	-	-	1	-	1	2	4	-	8
Coprinellus truncorum	-	-	-	-	2	3	4	2	4	2	1	-	18
Coprinellus xanthothrix	-	-	-	-	1	-	4	1	5	1	-	-	12
Coprinopsis atramentaria	-	-	-	-	1	-	-	-	7	8	6	-	22
Coprinopsis macrocephala	-	-	-	-	-	1	-	-	3	-	-	-	4
Coprinus comatus	-	-	-	-	-	-	-	-	5	11	4	-	20
Lacrymaria lacrymabunda	-	-	-	-	-	1	-	1	6	7	3	-	18
Panaeolus papolionaceus	-	-	-	-	-	-	-	-	4	-	4	-	8
Parasola auricoma	-	-	-	-	-	-	-	-	-	4	3	-	7
Parasola plicatilis	-	-	-	-	-	-	-	-	7	9	4	-	20
Psathyrella artolaminata	-	-	-	-	-	-	-	-	5	2	2	-	9
Psathyrella candolleana	-	-	-	-	-	2	2	2	6	-	1	-	13
Psathyrella conopilus	-	-	-	-	-	-	1	-	5	7	4	-	17
Psathyrella gracilis	-	-	-	-	-	-	-	-	1	5	-	-	6
Psathyrella marcescibilis	-	-	-	-	-	-	-	-	-	5	-	-	5
Psathyrella microrhiza	-	-	-	-	-	-	-	2	1	2	2	2	9
Psathyrella multipedata	-	-	-	-	-	-	-	-	1	3	-	-	4
Psathyrella spadiceogrisea	-	-	-	-	-	1	-	-	3	2	3	1	10
Psathyrella tephrophylla	-	-	-	-	-	-	-	-	-	2	1	1	4

* Data were compiled from all observations listed in Table A2 in Appendix I

deliquescent because these latter authors see the processes of ‘drying out’ and ‘collapsing’ resulting in loss of moisture before that autodigestion could have started. With this classification, they stepped forward from an earlier classification of ‘collapsing’ being an intermediate feature between deliquescent and non- deliquescent (Nagy et al. 2009).

In this study, in years with sufficient rain, this species fruited readily from late spring to late autumn (months April to November) and also on occasions in December and January (Table A1 in Appendix I). We deduce from the data presented here in Table 3 that *C. disseminatus* can start fruiting at a somewhat broader temperature range with main values from as low as 4-7/5-9 °C (night/day; Fig. 9) to as high as 10-16/20-23 °C (night/day; Fig. 7) at humidity values above 80 % (humidity ranges were mostly around up to 98 % at nights and around 70 to 90 % at days). Humidity came with rain that fell usually over a period of several days one to four days prior to any apparent primordia detection (Table 3). Adequate rainfall over two or more days usually caused drops in temperatures of several °C as compared to temperatures at the days before [such as calculated temperature differences in between prior or at and after by 1-8/2-9 °C (night/day) in dependence of the actual temperatures that were prevailing previous to a rainfall; see Table 3 and compare data in Fig. 5 to Fig. 11]. Both parameters, sufficient humidity in the air and also in the substrate are apparently important for fruiting. Occasional outliers of less humidity in the air might be tolerated for some hours of a night (such as 45 to 74 %; Fig. 8 and Fig. 10) or a day period (such as 42 to 73 %; Fig. 7,

Fig. 8 and Fig. 10) without directly aborting the induction of the fruiting process (Table 3; see as examples data for individual days and nights in Fig. 5, Fig. 8, and Fig. 10). The soil soaked with water by rainfall might have protected the fungi from drying. On the other hand, a single strong rainfall on very dry soil might not be sufficient to water the ground well enough to induce fruiting or to complete fruiting of induced primordia, such as was for example observed in the fall of the very dry year 2019 by S. Subba and U. Kües (personal communications) on the known fruiting places of *C. disseminatus* underneath the *P. sargentii* tree (Sargent´s cherry; referred to as Tree 1 in this work; Fig. 5; Table A1 in Appendix I) and underneath the *P. incisa* tree (Fuji cherry; Tree 2 in this work; Fig. 8 and Fig. 9; Table A1 in Appendix I) growing between the Microbiology Building and the GZMB on the grounds of the North Campus of the University of Göttingen.

While all recorded time courses of fruiting body development of *C. disseminatus* (Fig. 5 to Fig. 11) were from months September to November with temperature ranges during the fruiting process of mainly about 4 to 15 °C (nights) to about 7 to 20 °C (days) and even below (see data in Fig. 5 to Fig. 11), the species is less restricted in fruiting by the temperature range. Fruiting bodies of the species were observed at higher temperature ranges of about: 19-21/20-27 °C (night/day) in September 2011; 5-20/20-27 °C (night/day) in August 2014; 17-22/22-35 °C, 9-18/19-28 °C, and 9-18/20-29 °C (night/day) in July and August of 2015; 12-19/20-28 °C and 12-21/2-33 °C (night/day) in June and September 2016, respectively (compare data in Table A1 in Appendix I and Fig. A2 in Appendix III).

Deduced further from the observations on *C. disseminatus* in this study, the temperature ranges present at the time of initation of fruiting and during fruiting body development influenced the speed of the developmental processes. At lower temperature ranges (below 10 °C), the whole processes took an estimated time of 15 and more days whereas at higher temperature ranges (>15 °C) the process can be as fast as an estimated 8 to 12 days (Table 3). Importantly, all steps of fruiting body development can be affected in length by temperatures. At lower temperature ranges such as 10-15 °C or below at night/day, development from firstly recognized primordia to mature and decaying fruiting bodies took variably 7 to >12 days (Fig. 5, Fig. 6, Fig. 8 to 11). In comparison, the fastest series of events that we recorded was at a temperature range of around 14 to 16/15 to 25 °C (night/day) shown in Fig. 7 took 4 to 5 days only.

In summary, *C. disseminatus* can thus initiate and complete formation of fruiting bodies over a broad range of day temperatures at 5-10 °C to 25 °C. At lower temperature, the fruiting process from likely induction by rainfall up to mushroom decay lasted between 10 to 13 days and even up to 17 to 18 days and more (Fig. 5 to Fig. 6; Fig. 8 to Fig. 11; note sometimes after maturation with drying the cap out rather than decaying it), while at higher temperatures, the whole process from induction of fruiting by rainfall to mushroom maturation and then decay may be as fast as only 5 to 7 or 8 days (Fig. 7; own observations not further documented; personal communication by W. Khonsuntia, S. Subba and U. Kües from further observations in years 2017 and 2019). Overall, decisive for fruiting of *C. disseminatus* is thus apparently the increase in humidity for induction of fruiting, with a possible decrease in temperature, but then also sufficient water availability is required during the further fruiting process. Repeatedly, developing structures of *C. disseminatus* dried out prior to completion of development at times when the air became very dry and, with it, also the substrate (Fig. 9 and Fig. 11). Periods of lower humidity in the air but with still sufficient water reserves in the substrate can however be tolerated, probably in combination with a lower temperature less stressing on the water availability offering by the substrates (Fig. 8).

2. **Coprinellus domesticus.** Fruiting bodies of this (partially) deliquescent species (Badalyan et al. 2011; Nagy 2011) were three times encountered in groups of two, three and more

successively developing mushrooms and some also as individuals underneath a *P. incisa* (Fuji cherry) tree on the grounds of the North Campus of the University of Göttingen, together with *C. disseminatus* mushrooms (Fig. 10 and Fig. 11; Table A1 in Appendix I). The length of fruiting body development from induction by rainfall till mushroom decay was with 12 to 20 days, similar to the length of *C. disseminatus* fruiting occurring at the same time periods in parallel (Fig. 10 and Fig. 11). However, flushes of *C. disseminatus* fruiting bodies were not always accompanied by mushroom production by *C. domesticus*, but only in November 2014 and in October and November 2016. Similarly at all other places (14 in number) where *C. domesticus* fruiting were observed in the years 2011 to 2016 (Table 2; Table A1 in Appendix I), with only three exceptions in April and May 2013 and June 2012, all other incidences of fruiting of *C. domesticus* (40 in toal; compare Table 2 and Table 3) occured in autumn in the months September, October and November, suggesting a more restricted regulation of fruiting by the season of the year. Fruiting bodies connected in nine instances clearly to trees or to dead wood. When fruiting on meadow biotopes (Table A1 in Appendix I), at least in some instances, it was at known places of former growth of recently felled trees (sites Grisebachstr. 10; Julia-Lermintowa-Weg 3) or on grassland with shrubs and trees (sites Büsgenweg 2 and 4), suggesting that the wood-related fungus probably interacted with wooden roots burried in the underground.

The preferential fruiting in autum months implies that *C. domesticus* reacts much more sensitive on lower temperature conditions. Fruiting body development was observed after one to three days rainfalls with humidity increase at nights and days to >90 % at temperature ranges of 8-13/11-17 °C (night/day; see Table 3 and compare Table A1 in Appendix I with entries in Fig. A2 in Appendix III). We noticed in November 2016 also initiation of fruiting body development even at very low temperature (in the range of 2 °C to 5 °C at nights and 4 ° to 8 °C at days sometimes; Fig. 11, Table 3) and high humidity levels of 90-97/82-96 % (night/day) and certain proceedings of fruiting body development then at even lower temperatures in the zero °C temperature range (-4 to 2/-1 to 4 °C night/day; Fig. 11, Table 3) but the mushrooms could not fully complete development. Due to cold shock effects, development was apparently blocked at the young open stage while finally the not fully matured structures dried out.

3. **Coprinellus micaceus.** This readily deliquescent species releasing at least part of its basidiospores by autolysis (Fig. 12 to 14; Buller 1910; Nehemiah 1973; Keirle et al. 2004) showed two phases of fruiting in the year, in spring in the months March to June and in autumn with the months September to November (Table 4). In both periods of the year, initiation of fruiting linked to rainfall two to three days prior to when primordia were seen (Fig. 12 to Fig. 14 and see entries in Table A1 in Appendix I and Fig. A2 in Appendix III).

Accompained therefore by high humidity values prior and often also during the fruiting process with mostly high humidity peaks ≥85-95 % at nights and humidity levels reduced to ≥45-85 % over the days (see humidity curves for April and June 2012, May 2013, April 2014, and March and April 2016 in Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I; as exceptions, humidity levels in April 2012 and April 2015 were lower in the range of 50-70/30-50 % night/day, see humidity curves in Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I), temperature ranges in spring (at days of mushroom detection) were:

- 7-10/12-19 °C and 16-18/18-21 °C (night/day) in April and June 2012,
- 13-16/16-20 °C (night/day) in May 2013,
- 15-18/16-21 °C and 8-11/12-18 °C (night/day) in April 2014,
- 10-14/15-20 °C (night/day) in April 2015, and

- 5-8/7-12 °C and 2-8/10-18 °C (night/day) in March and April 2016 (compare Table A1 in Appendix I with entries in Fig. A2 in Appendix III).

Accompained throughout by highest humidity levels of ≥75-100 % over the whole night periods combined with humidity ranges ≥45-80 % over the day periods (see humidity curves for October 2012, September 2013, October 2014, and September, October and November 2015 in Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I) or with even higher humidity ranges ≥65-95 % over the day periods (see humidity curves for September 2011, October and November 2013, September, October and November 2014, October and November 2015, and October and November 2016 in Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I), similar to spring temperature ranges in autumn (at days of mushroom detection) were:

- 9-14/11-19 °C (night/day) in September 2011,
- 3-8/8-12 °C (night/day) in October 2012,
- 4-10/11-19 °C, 10-14/14-21 °C, and 8-10/8-12 °C (night/day) in September, October and November 2013,
- 10-15/16-20 °C, 9-14/12-19 °C, 10-12/12-15 °C, 12-15/13-20 °C, 9-15/12-20 °C, and 13-14/14-15 °C (night/day) in September, October and November 2014,
- 10-14/14-19 °C, 15-16/16-20 °C, 6-8/8-11 °C, and 12-13/13-14 °C (night/day) in September, October and November 2015; 6-8/8-10 °C, and
- 5-8/6-10 °C (night/day) in October and November 2016, respectively (compare Table A1 in Appendix I with entries in Fig. A2 in Appendix III).

Periods of fruiting as observed over the time lasted between 9 and >14 days (Table 3).

4. **Coprinellus tardus.** Regarding preferences of times of fruiting of this autolysing species (Lange 1939) during the year and related environmental conditions, this species behaved very similar than *C. domesticus* with a preference for fruiting in the autumn months September to November (Table 4). When fruiting bodies of *C. tardus* were detected, usually *C. domesticus* and various other species of Coprini fruited also (see Table A1 in Appendix I and Fig. A2 in Appendix III).

5. **Coprinellus truncorum.** Fruiting bodies of this (partially) deliquescent fungus (Fig. 15; Nagy et al. 2010) grow as typically in larger clusters at repeated times on the base of a weakened hardwood tree (*C. lutea*) and the underground tree roots (Fig. 19P-Q in Chapter 1 showing an event on 01.07.2013). Clusters of the species were found also on other trees (Table A1 in Appendix I) or connected to a dead poplar wood log as first seen in 2016 (Appendix IV, Fig. A3) and then again in 2017 and also in May 2019 in several clusters at the log and in the surrounding meadow (W. Khonsuntia, S. Subba and U. Kües, pers. communication).

Clusters were observed from an early summer month (May) until the autumn months October and November (Table 4). *C. truncorum* initiated formation of fruiting bodies also after rainfall, with first developments noticed up to 3 to 4 days later, when temperatures were mostly tentatively at around 6 to 15 °C (compare Table A1 in Appendix I with entries Fig. A2 in Appendix III). Accompained by highest humidity levels of ≥80-100 % over the night periods and ≥70-90 % over the day periods (see humidity curves for November 2011, July 2012, July 2013, October 2014, 2015 and 2016 Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I), of ≥60-80 % over the night periods and ≥50-70 % over the day periods (see humidity curves for May and June 2013, August 2015, September 2016 Fig. A2 in Appendix III combined with the entries in Table A1 in Appendix I) and variably in ranges of about ≥60-90 % over the night periods and 40-80 % over the day periods (see humidity curves for in May 2015, August 2015 and June 2016 Fig. A2 in Appendix III

combined with the entries in Table A1 in Appendix I), temperatures ranges at individual fruiting periods (at days of mushroom detection) were:

- 8-10/11-18 °C (night/day) in November 2011,
- 14-20/20-27 °C (night/day) in July 2012,
- 8-11/11-16 °C, 10-12/12-22 °C, and 8-12/12-28 °C (night/day) in May, June and July 2013,
- 12-15/13-18 °C (night/day) in October 2014,
- 7-12/13-21 °C, 14-19/19-22 °C, 15-18/18-21 °C and 10-15/15-20 °C (night/day) in May, July, August and September 2015, and
- 16-18/18-22 °C, 16-18/18-22 °C, 16-22/22-32 °C, and 8-10/10-14 °C (night/day) in June, August, September and October 2016, respectively (compare Table A1 in Appendix I with entries in Fig. A2 in Appendix III).

6. **Coprinellus xanthothrix.** This better known wood-related species (Table 1 and Table A1 in Appendix I) with (partially) deliquescent fruiting bodies (Badalyan et al. 2011; Nagy et al. 2010, 2013b) favored to our observations for fruiting the summer period of July to September (Table 4) with, after 2 to 4 days of rainfall, higher temperature ranges of 17-22/21-29 °C, 15-18/19-21 °C, 16-20/21-28 °C, 11-18/18-22 °C and 10-14/15-21 °C (night/day) as measured in the months September 2011, July, August and September 2012, and September 2015, respectively (compare Table A1 in Appendix I with entries in Fig. A2 in Appendix III). Humidity values were in these fruiting periods ≥70-95 % over the night hours and ≥50-86 % over the day hours (compare Table A1 in Appendix I with the respective humidity curves in Fig. A2 in Appendix III). Temperatures in the fruiting periods in Mai 2012 with temperature values of 9-11/12-14°C (night/day) [with one day before also relatively high temperatures of 10-18/19-27°C (night/day)] and with 9-13/14-19 °C (night/day) in October 2016 were also relatively warm. Humidity values in these two fruiting periods were 73-94/53-72 % (night/day) and 90-99/76-89 % (night/day), respectively (compare Table A1 in Appendix I and Fig. A2 in Appendix III).

7. Other species. From Table 4 and the descriptions of the six species above it emerges that the various wood-related species within the *Coprinellus* genus prefer for fruiting variable periods (spring, summer and/or autumn) on the year, with some tolerating higher temperatures (*C. disseminatus*, *C. truncorum* and *C. xanthothrix*), whereas others fruit mainly in one (autumn: *C. domesticus*, *C. tardus*) or two fruiting periods (spring and autumn: *C. micaceus*) of the year at moderate or low temperature. Most other species from other genera observed in this studied more frequently in fruiting were restricted to autumn months (*C. atramentarius*, *C. comatus*, *P. plicatilis*, *P. conopilus*, *P. gracilis*, *P. microrhiza*, *P. multipedia*, *P. spadiceogrisea*, and *P. tephrophylla*; Table 4; Fig. A1 in Appendix III).

The wood-related *P. candolleana* (Table 1) was the only other species that had also a preference for fruiting in summer months (June to September) at temperature ranges of 14-16/18-21°C, 14-19/20-25°C, 11-18/19-22°C, 12-14/15-19°C, 16-19/20-27°C, 10-15/16-19°C and 17-23/24-36°C (night/day) in the months of September 2011, July and September 2012, September 2013, August 2014, September 2015 and June 2016, respectively, observed after 2 to 4 days of rainfall and with humidity levels of ≥60-90 % over night periods and over might periods and ≥50-80 % over day periods (compare Table A1 in Appendix I and entries and curves in Fig. A2 in Appendix III).

In summary from the observations, apart from the genus *Coprinellus*, in the *Psathyrellaceae* family regardless of the genera, most of the species appear to be ready to fruit in the early autumn months in September and October and possibly also in November in the year. There is thus no fixed temperature and humidity to all mushrooms of the family for

fruiting in the fields. Climate data of temperature and humidity graphs (Fig. A1 in Appendix III) showed as tendencies after an inducing rainfall when the humidity raised up that the temperature went down, and when afterwards the temperature raised up that the humidity went often down. Fruiting initation is known from species cultivated in the laboratory to be inducable by reducing the temperature while some subsequent increase in temperature favors good progress in fruiting body development (Kües and Liu 2000; Rühl and Kües 2007). It is well known that in nature, fungal growth and developmental process on the mycelium are highly influenced by environmental factors. The data here support further that fruiting phenologies of observed fungal species are highly sensitive to low or high temperature and to low and high humidity. However, the optimum comvination of both is decisive for any developmental processes to happen.

Initation of mushroom growth and continuation of fruiting body development and, where present such as typically in *Coprinopsis* species, in specific *Coprinellus* species and also in *C. comatus* (Buller 1910, 1911; Bush 1974; Redhead et al. 2001; Nagy et al. 2012a, b, 2013a, b), also cap autolysis processes for basidiospore release take place within limits of tolerance at optimal temperatures and humidities. Temperature and humidity in combination have vital roles in mushroom development. If temperature falls below the optimum or rises above the optimum, any initiated developmental processes will delay or be aborted. However, without sufficient humidity in the air and a too low substrate moisture capacity, fruiting body development will not happen even when temperature ranges are optimal. The two years 2018 and 2019 with two dry hot summers in series are examples for such lack in mushroom production when unlike in the previous years with good rainfall fruiting of any Coprini and other *Psathyrellaceae* was rarely or not observed on the ground of the North Campus of the University of Göttingen (W. Khonsuntia, S. Subba and U. Kües, personal communications).

Part II: Fruiting body development of *S. commune* on twigs and branches in nature

In nature, fruiting bodies of the wood-inhabiting *S. commune* are observed on twigs and branches of various tree species. Fruiting bodies are longer lasting for one, two or more years. Over 260 different plant host species are known for the fungus (Cooke 1961).

In this study, *S. commune* was often observed on the grounds of the North Campus of the University of Göttingen, on living hardwoods [see Fig. 16 A-D on various living hardwoods, Fig. 17A-D and also Fig. 7A-E in Chapter 2 with infested branches of a *Juglans ailantifolia* (Japanese Walnut) tree], as early colonizers on recently fallen (see Fig. 3G and also Fig. 13 B in Chapter 1) or *S. commune* was found on felled while still barked deadwood (Fig. 16E-G and Fig. A4 in Appendix V) and once also on burned wood (Fig. 16H and also Fig. 8A-G in Chapter 2). Fruiting bodies usually appeared on the sides of wooden substrates best exposed to the sun, most often in S or W directions. When formed on other sides of the substrates, shadows of buildings, other trees or other stems of a shrub blocked sunshine from S and/or W (Fig. 16 to Fig. 18; Table 5). Fruiting bodies were often seen born in cracks of the bark or in lenticells s natural bark openings (Fig. 16A-G; see also cracks in the bark of a felled *Acer* tree in Fig. A4 in Appendix V and also in Fig. 2 in the conference proceedings by Lakkireddy et al. 2017 as shown in Appendix V of this chapter). Mushrooms of strong wood-decaying fungal species were often also found on the same or on directly connected branches and stems, below and above the fruiting bodies of *S. commune* (Fig. A4 in Appendix V; Table 5; see also Fig. 8 in Chapter 2; Peddireddi 2008).

S. commune itself is only a weak degrader of wood (Floudas et al. 2015). The fungus may live from the easily accessible nutrients provided by the parenchym in the sapwood, the

Fig. 16 *S. commune* fruiting bodies on living hardwoods (A. *Gleditsia trecanthos* f. *inermis*, B. *Prunus incisa*, C. *Juglans ailantifolia*, D. *Syringa tigerstedtii*) and deadwood (branches of E. *Fagus sylvatica* and F. *Acer pseudoplatanus*, G. cut *Quercus robur* stem, H. burned wood of unknown species).
Infested trees were found on the grounds of the North Campus of the University of Göttingen, **A.** on a meadow at the East side to the Forestry building on Büsgenweg 5, **B.** between the Microbiology Building and the Göttingen Center for Molecular Biosciences (smaller cherry tree numbered here as Tree 3), **C.** at the West side of the Ernst-Caspari-House of the Göttingen Center for Molecular Biosciences (GZMB) at the split of the Burckhardtweg from the Justus-von-Liebig-Weg, and **D.** on the North side of Physic´s building Friedrich-Hund-Platz 1. This figure has been published as Fig. 1 in a Conference Proceedings by Lakkireddy et al. (2017) as shown in Appendix V.

cambium and the bast but it may also profit from the presence of strong white-rotting species which more easily degrade the wood (Floudas et al. 2015; Almási et al. 2019). On the longer run however, the perennial fruiting bodies of *S. commune* will be replaced by mushrooms of other fungi (see Fig. A4B in Appendix V as an example). That the bark- and sapwood-fungus *S. commune* is not only colonizing bark or the cambium zone in between bark and wood but also enters the sapwodd zone becomes clear from blue-greyish stained fungal occupied wood in harvested branches (Peddireddi 2008; Fig. 7C-E) and also from mushroom production tests performed by K. Lange and U. Kües with freshly harvested infected twigs and branches of attacked broadleaf species (Fig. 19). Within short incubation periods of a few days in humid conditions in aquaria at room temperature in the laboratory und illumination by sunshine, hyphal aggreagates appeared on the sapwood surfaces of cuts of branches and developed further over stalk and apical pit stages, under peripheral cap expansion and split gill (pseudolamellae) formation into fully developed mushrooms (Fig. 19).

Where living trees and shrubs were attacked in nature as seen in this PhD study on the grounds of the grounds of the North Campus of the University of Göttingen by fruiting body production on twigs, branches or also younger stems, this correlated quite often with either replanting or with previous road and ground digging works in close neighborhood with likely root stress for the plants (root damage, disturbances in water supply; Lakkireddy et al. 2017 in Attachment V of this chapter). Among other living hosts (Table 5), we have observed fruiting

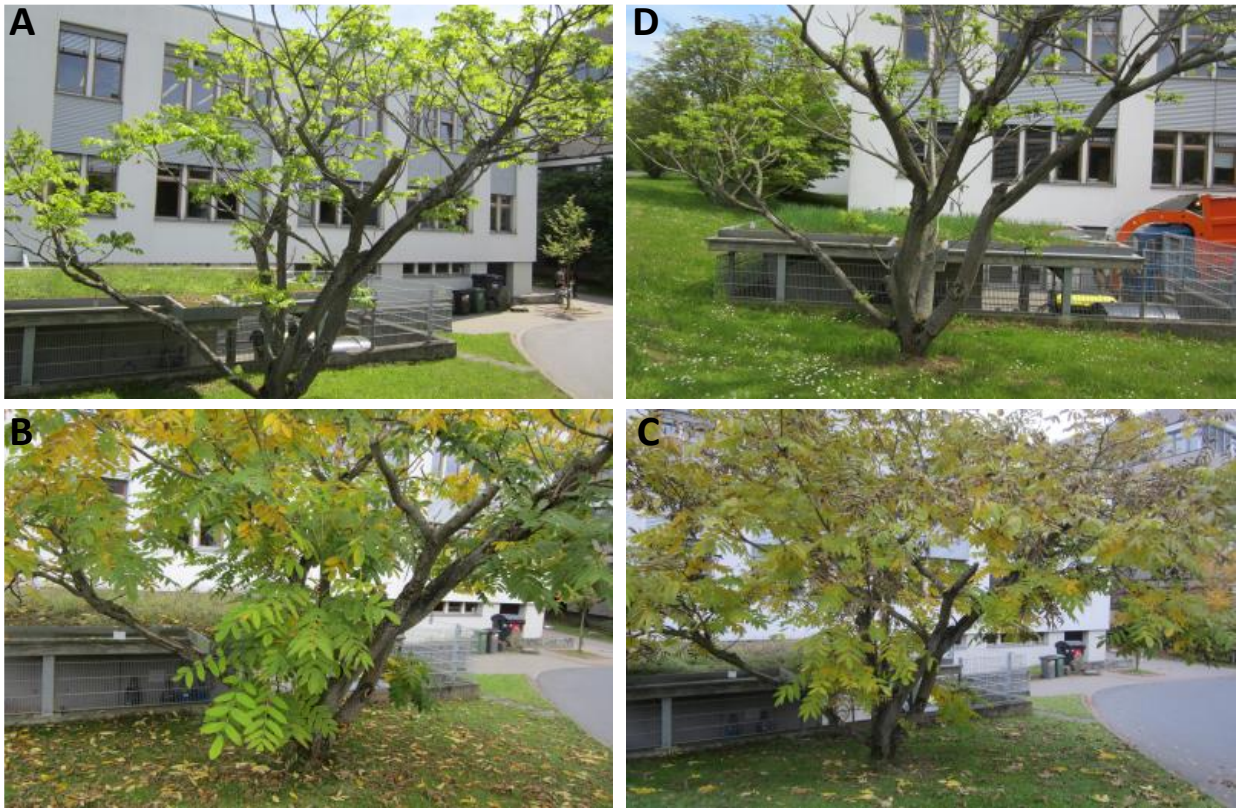

Fig. 17 *Juglans ailantifolia* tree on the grounds of the North Campus of the University of Göttingen at the West side of the Ernst-Caspari-House of the Göttingen Center for Molecular Biosciences (GZMB) with a main still alive branch (in the photos the foliated outer left branch) previously wounded by a *Schizophyllum commune* infection (Peddireddi et al. 2006; Peddireddi 2008).
Photos were taken in spring on **A.** 13.05.2012 and **B.** 15.05.2013 and in autumn on **C.** 26.10.2014 and **D.** 25.10.2015

bodies on branches and stems of walnuts (Fig. 16 and Fig. 17 this chapter; Fig. 16G in Chapter 1; Fig. 7 in Chapter 2), cherry (Fig. 19), and lilac (Fig. 16K in Chapter 1). All this wooden plants experienced in previous years digging and building work nearby (observations by U. Kües) which could have stressed their roots. For example, the Japanese Walnut tree (*Juglans ailantifolia*) shown in Fig. 17 and Fig. 18 in this chapter and in Fig. 7 in Chapter 2 was replanted in course of the construction of the GZMB building shown in the back of the photos (Peddireddi 2008; Peddiredi et al. 2016). The three cherry trees on the other hand between the Microbiology Building and the GZMB – i.e. the larger *P. sargentii* (Sargent´s cherry) tree = Tree 1 in this thesis with a large *C. disseminatus* colony underneath (Fig. 5 and Fig. 6; Fig. 19L to M in Chapter 1) and the smaller *P. incisa* (Fuji cherry) trees = Tree 2 in this study with a large *C. disseminatus* colony underneath (Fig. 7 to 11; Fig. 18 A to B in Chapter 1) and, respectively, = Tree 3 in this thesis infested by *S. commune* (Fig. 16B; Fig. 19A) as well as at the base with *Hypholoma fasciculare* (Fig. 1I in Chapter 1) and *Mycena* sp. (Table 5) and *C. disseminatus* appearing in close neighborhood to the stem base of the tree (Table 5) – experienced root damages by digging activities when an electrical bar was to be installed over the path to regulate entry to parking spaces. The *Syringa tigerstedtii* bush with infested branches at the North side of the Physic´s building Friedrich-Hund-Platz 1 (Fig. 16D) was replanted in course of road and pavement work and it was possibly negatively

affected again with later road diggings for underground pipe works (UK, personal communication).

The observations thus suggest that the weak pathogen *S. commune* may attack more easily weakened shrubs and trees. When found on branches and twigs of living trees, branches may (partially) die. Stretches of the branches with fruiting bodies increased often with time while the sapwood of the better shadowed opposite sides of branches were still fully functional (Fig. 16A.-D, Fig. 17 and Fig. 18; Fig. 17 in Chapter 2), which was best documented by the example of the J. *ailantifolia* tree next to the GZMB building (Fig. 18). With time, a tree however might successfully fight the fungal pathogen by drying out the surfaces of the resulting wound after loosing the protecting bark and further by callus formation for successive closing of the wounds (Fig. 18).

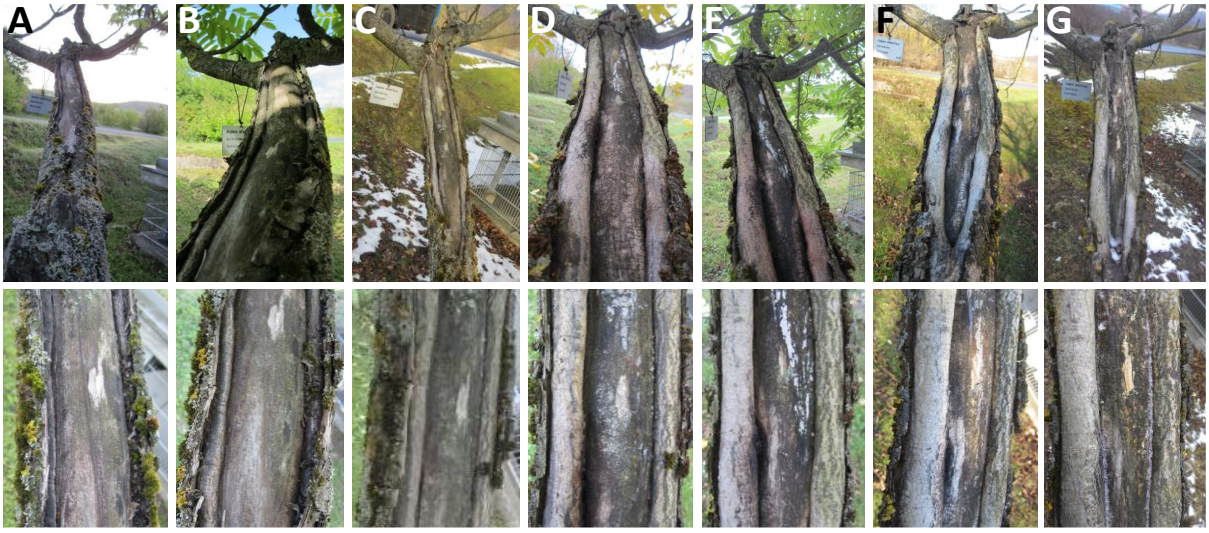

Fig. 18 Callus formation over the time on a branch of the *Juglans ailantofolia* tree growing on the grounds of the North Campus of the University of Göttingen at the West side of the GZMB, wounded on the top sun-directed side by an infestation with *Schizophyllum commune* which resulted over the time in bark loss (Peddireddi et al. 2006; Peddireddi 2008).

Upper rows show top views of the affected branch and lower rows enlarged sections. Photographs were taken on **A.** 13.05.2012, **B.** 29.08.2012, **C.** 03.02.2014 (top), 22.09.2013 (photo), **D.** 26.10.2014 (top), 04.08.2014 (bottom), **E.** 05.09.2015 (top), 25.10.2015 (bottom), **F.** 27.02.2016, and **G.** 25.01. 2017.

Part III: Concluding remarks and summarizing general discussion on observed fungi and their fruiting behaviour

In terrestrial ecosystems, organisms of the *Psathyrellaceae* family species may have different life styles and may grow on different substrates, as shown in this thesis by documentations in Chapters 1 and 2 and further here in Chapter 3, variably in meadows, among plant litter, under bushes and shrubbery, in wood chip beds, on stumps and other wood blocks, under trees and at tree-bottoms). *C. disseminatus*, *C. domesticus* and *C. micaceus* were repeatedly been observed to grow on areas together with e.g. nitrophilous *Urtica* sp. [under a *P. incisa* cherry tree (Fig. 18 A-D in Chapter 1) and in plant litter under a shrubbery with elderberry *Sambucus nigra* (elder; Fig. 18 E-G in Chapter 1) and other bushes (code G3

Table 5 Mushrooms of *S. commune* and other *Agaricomycetes* on living shrubs and trees (data were published in a conference proceedings by Lakkireddy et al. 2017 as shown in Appendix V; see also Fig. 16 in Chapter 1 and Fig. 7 in Chapter 2)

Plant Name	Branch condition	Other fruiting bodies on the same substrate	Direction*
Acer caudatum	Living stem	*Flammulina velutipes* + *Radulomyces molaris* (below)	W (N,S)
Acer ginnala (multi-stem free standing)	Living stem	*Auricularia auricula-judae* (above)	E, W (S)
Alnus glutinosa	Living stem	*Ganoderma lucidum* (same level, NE)	SW
Corylus tibetica	Dead branch	*Exidia glandulosa* + *Trametes pubescens* (connected dead branch)	W, S (N)
Cotoneaster moupinensis	Living branch	-	E
Crataegus monogyna	Living stem	-	W
Euonymus sanguineus	Many living branches and twigs	*Trametes versicolor* (2 branches, above)	E, S, W
Gleditsia triacanthos f. *inermis*	Living stem	-	W
Juglans ailantifolia (free standing)	Living branches	*Antrodiella fragrans* (above) *Trametes* sp. (above) *Coniophora arida* (below)	S, SW
Juglans mandschurica	Living branch	*F. velutipes* (below) *Trametes multicolor* (connected branch)	W
Lonicera korolkowii	Living branch	-	W, S
Lonicera maximowiczii	Living branch	*Skeletocutis nivea* (below)	W, NW
Malus angustifolia	Living stem	-	W
Malus spec. cf. *coronaria*	Living stem	*Bjerkandera adusta* (above)	W
Malus x *zumi*	Living stem	-	SE
Prunus incisa	Living stem	*Hypholoma fasciculare* + *Coprinellus disseminatus* + *Mycena* sp. (below at stem base)	W
Salix discolor	Living branch	-	SW (N)
Syringa tigerstedtii	Dead branch	*E. glandulosa* + *Byssomerulius corium* (connected branch)	W, E
Syringa wolfii	Living branch	-	E, NW
Viburnum burejaeticum	Living branch	*Skeletocutis subincarnata* (below)	NE, SE

* Main geographic directions of mushroom formation on the substrate are given without brackets. Further directions with only a few mushrooms are shown in brackets.

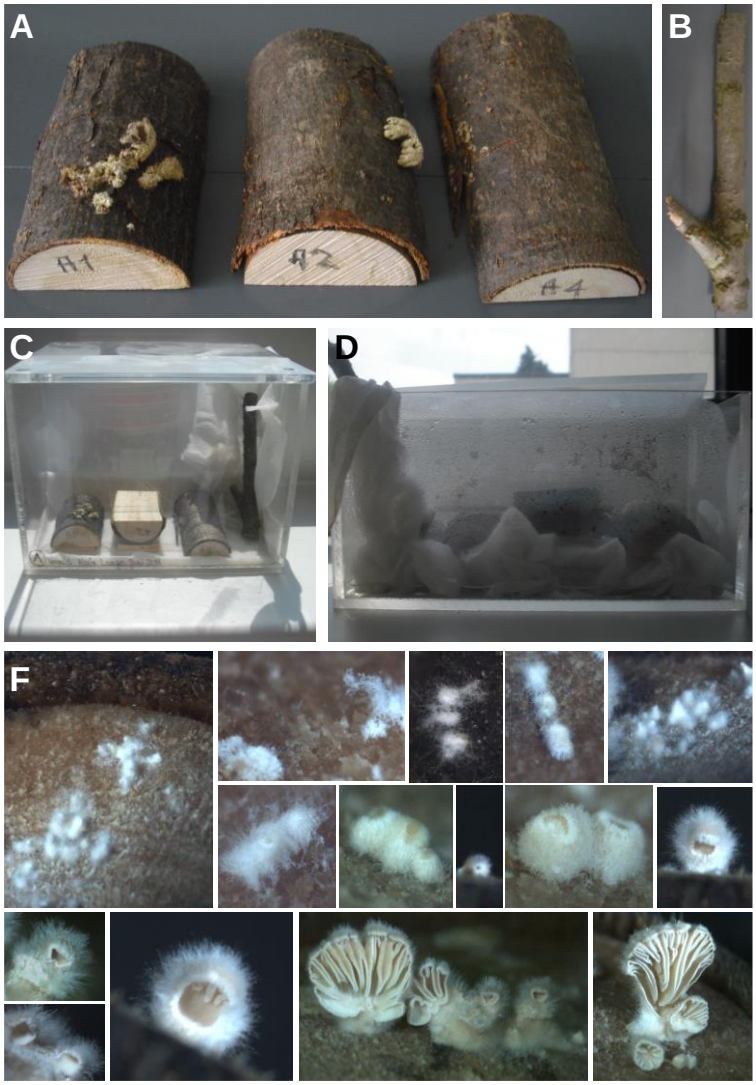

Fig. 19 Cultivation of *Schizophyllum commune* for fruiting body production on infested wood in the laboratory.

A. Freshly harvested *Prunus incisa* branches (from the smaller Tree 3 on the grounds of the North Campus of the University of Göttingen, between Microbiology Building and Göttingen Center for Molecular Biosciences) and **B.** *Syringa tigerstedtii* twig (from the North side of Physic´s building Friedrich-Hund-Platz 1) infested with *Schizophyllum commune*. **C.** and **D.** Experimental set-up: Infested twigs and branches were laid onto wet paper in aquaria for creating a constant humid atmosphere. The aquaria were covered by slightly shifted glass plates to leave vents for aeration and then incubated at room temperature on a bench at a laboratory window. Water was added daily to need in order to keep the paper sufficiently wet. Spacers were required between paper and wood in order to avoid water logging of the samples. **C.** When placed at an East window aquaria were exposed through the mornings to strong sunshine which resulted in effective fruiting body development on the surfaces of wood and bark. **D.** Less sunshine was achieved by partial shading through hanging wet paper tissues over the light-exposed glass surfaces of the inner aquarium walls and by partial closing of Venetian blends. Shadowing the aquaria by wet paper or by partially closed blends delayed and reduced frequencies of fruiting. However, when positioned at South windows, blends needed to be fully closed during the noon hours to avoid overheating the system. **F.** All stages in fruiting body development from hyphal aggregate over stalk and apical pit formation, peripheral cap expansion, split gill (pseudolamellae) formation and fully developed mushrooms were observed to grow out from sapwood. The experiment was performed and photos were provided by K. Lange and U. Kües.

in Table A1 in Appendix I)] as nettle plants which are favouring soils with high nitrogen and phosphate content and report nitrogen-rich soil conditions (Mullerova et al. 2014). Fungi that tolerate, grow and develop well on nitrogen-rich grounds are chemoecologically considered as ammonia fungi (He and Suzuki 2003; Suzuki et al. 2003; Suzuki 2006, 2009a,b). They may invade soils quickly after a large input of ammonium-nitrogen (Suzuki 2009b) or decompose urea to ammonia (Imamura et al. 2006). A range of Coprini from the genus *Coprinopsis* has been described as ammonia fungi (Suzuki 2009b; see also species compilation in Chapter 2 of this thesis). Many of the *Coprinopsis* species relate to dung (Redhead et al. 2001) and their basidiospores rapidly germinate in presence of ammonium-nitrogen under neutral to weak alkaline conditions and fruiting body development can be promoted (He and Suzuki 2003; Suzuki 2009b). *Coprinellus* species were so far not much considered in the literature as "ammonia fungi" with exception of one publication that suggested a *Coprinellus* aff. *radians* strain to possibly belong to the chemoecological group of ammonia fungi by a best growth at neutral pH 7.0 and good growth from weak acidic to alkaline pHs between pH 6.0 to pH 9.0 (Badalyan et al. 2011a). Future work may concentrate more on the aspect on nitrogen effects on occurence of species of the genus *Coprinellus*.

Occurrences of Coprini in nature in this study have been deduced by appearance of the macroscopic fruiting bodies. Mushroom formation is not a spontaneous process but requires sufficient nutrients and a range of favorable environmental factors. Initially, the fungal mycelium needs water and nutrients to grow to sufficient colony sizes in order to subsequently develop fruiting bodies under optimal environmental conditions (Moore et al. 2008; Kües and Navarro-González 2015). The fruiting phenology of fungal species is highly sensitive to environmental factors like temperature and humidity (Pinna et al. 2010). Mushrooms may emerge within hours or days from the ground or their fungal substrates while the speed of fruiting body development is not constant over the whole process. The early phases of fruiting, such as the formation of a primordium up to the basic tissue maturation, may take in relation much longer time than the usually very fast phases of stipe elongation and cap opening of short-lived fleshy mushrooms (Halbwachs et al. 2016). In nature, high humidity (such as >90 %) and optimal temperatures will support initiation of fruiting body production while the favorable climatic conditions may vary between mushroom species and the fruiting-permissive conditions within tolerable limits can also fluctuate for a species (Peay et al. 2010). In this study with time, it became obvous that sufficient rainfall to well moisture the soil favoured production of fruiting bodies of Coprini a few days later. Induction of fruiting by rainfall was accompanied by an increase in humidity and a drop in temperature (Fig. 5 to Fig. 15; Table 3). As seen by data of fungal species repeatedly observed at same colony places (Fig. 5 to Fig. 14), fruiting was allowed over some temperature ranges but with apparent effects on speed of developments. Lowering temperatures to sub-optimal does not necessarily block fruiting but will alter fungal growth, delay the speed of primordia development and fruiting body maturation, enhance the duration of the mature fruiting bodies for spore shedding and, in the autodigestible Coprini, possibly also the autolysis process. Evidence for such environmental influences on the general time course of fruiting body development for some of the observed Coprini in this study is presented in this Chapter in the summarizing Table 3.

Fruiting of *C. disseminatus* and *C. domesticus* was repeatedly been observed in the soil close to stems of living cherry trees (Fig. 5 to Fig. 11; Fig. 18A-D and Fig. 19 L-M in Chapter 1; Table A1 in Appendix I). *C. disseminatus* occurred also in the vicinity of other living shrubs such as *S. nigra*, often together with *C. micaceus* (compare Fig. 18 E-G and Fig. 19 N-O in Chapter 1 showing the same *S. nigra* bush; codes G3 and G117B in Table A1 in Appendix I), or also on *Acer pseudoplatanus* (sycamore) together with *C. xanthothrix* (Fig. 19 H-K in Chapter I; code G1B in Table A1 in Appendix I). *C. xanthothrix* (Fig. 19 A-C

in Chapter 1; Fig. 6A in Chapter 2) and *C. micaceus* (Fig. 12 to Fig. 14; Fig. 5 in Chapter 2) were further observed on a dying to dead stump of *Tilia cordata* (elm tree) which initially after felling up to year 2014 was still able to produce new living shoots (M. Navarro-Gonzaléz und U. Kües, personal communcations). Biotopes with upgrown living trees supply some degree of shading and likely better stability of environmental conditions. For example, the shade to certain degrees will block negative effects on fruiting by too much light, through reducing the dose of light on the one hand and mitigating opposing effects on temperature and humidity on the other hand. Air temperature may impact soil temperature and also soil moisture, as a microclimate with seasonal differences (in summer stronger and in winter marginal) in grassland more than under coppice with reduced solar input while soil microclimates underneath high forest covers are much more stable over the year until air temperatures of freezing (Morecroft et al. 1998). Over the time in this study, fruiting was observed of in total 56 different species of Coprini. Species of the genus *Coprinopsis* as well as of the genus *Psathyrella* correlated more likely with grassland biotopes and/or fresh artificial wood beds (Table 1). Nitrogen input in these biotopes can be high such as by grazing animals in meadows (Fig. 11 in Chapter 1) and by chopping of fresh barked branches with living phloem into wood chips (Fig. 10 in Chapter 1) and will thus favour species which ecologically are ammonia fungi.

On the other hand,9 distinct species of the genus *Coprinellus* of a total of 12 observed were found in instances where they were associated with natural situations linked to (buried) dead wood or growing on or near living trees and wooden shrubs (Table 1), indicating wood to be a habitat for these species. Wood decay tests with single species or in combination with other wood degraders should be done to mre deeply unravelled the extend which which species of the *Coprinellus* can make use of wood for their nutrition. Wood decay abilities may generally be more pronounced in the genus *Coprinellus* than in other species of the Coprini. A literature compilation of enzymes with possible functions in lignocellulose degradation from *Coprinellus* species is presented in Table 2 of Chapter 2 of this thesis. However so far, also wood decay abilities of *Coprinellus* species are much understudied (Redhead and Ginns 1985; Redhead et al. 2001; Oliver et al. 2010; Badalyan et al. 2011; Lee et al. 2018). Oliver et al. (2010) studied *Coprinellus radians* isolates on in-field-predigested wood blocks and observed wood weight loss induced by *C. radians* in dependence of the advances in progress of the former wood decay in nature. The authors therefore suggested that *Coprinellus* species are characterized as late state decay fungi which decompose wood that had been chemically and physically affected before by other degrative processes. During observations in this study, we however have detected fruiting bodies variably on wood which appeared still to be more intact (e.g. see Fig. A3 in Appendix III) and on wood that showed (over the time) signs of advanced decay such as brown rot on an elm tree stump (Fig. 13 and Fig. 14). Most interesting was a change in *Coprinellus* species from first the repeatedly fruiting of *C. xanthothrix* (Fig. 19A-C in Chapter 1; Fig. 6A in Chapter 2; Table A1 in Appendix I) on the elm tree stump north to the Physic´s buildings on the North Campus of the University of Göttingen that initially had still abilities to shoot with fresh young branches to later with proceeding wood destruction to *C. micaceus* Fig. 12 to Fig. 14; Fig. 19 D-G in Chapter 1; Fig. 5 and Fig. 6 C-D in Chapter 2; Table A1 in Appendix I) on the same elm tree stump. Mushrooms of the white rot *F. velutipes* were also observed at times on the stump (Fig. 1K in Chapter 1; Fig. 5H in Chapter 2) suggesting that possibly several different organisms contributed to the decay of the stump. Reports in the literature indicate that *Coprinellus* species in high frequency were encountered as endophytes in wood and pods of *Theobroma cacao* (Crozier et al. 2006) and in wood and leaves rubber trees of the genus *Hevea* (Martin et al. 2015). *C. micaceus* was further detected as endophyte in trees from Argentinia (de Errasti et al. 2010). Fungal endophytes may serve as priority colonizers for later initiation of wood

decay (Song et al. 2017). Under such a premise, *Coprinellus* species might in contrast contribute already early to the decomposition of the wood of their hosts.

Observations in this work were additionally collected on *S. commune* as an Agaricomycete distantly related to any of the Coprini. *S. commune* is traditionally considered as an early white rot wood decay fungus on fallen and still barked branches and stems with weak pathogen actions on mostly broadleaf trees. Following analysis of the genome together with microscope analysis of the degradation behaviour of *S. commune* in wood decay tests, more recently the fungus is considred a 'grey rot' with limited wood decay activities in between white and brown rot (Floudas et al. 2015; Kirker 2019). *S. commune* is also a possible endophyte in leaves, rachis and petioles of oil palms (*Elaeis guineensis*), leaves of tea plant (*Camellia sineasis*), coffee (*Coffea arabica*), teak (*Tectona grandis*), rain tree (*Samanea saman*), and the medical shrub *Piper hispidum*, and needles of pines (*Pinus tabulaeformis*), (Wand et al. 2005; Chareprasart et al. 2006; Agusta et al. 2006; Rungjindamai et al. 2008; Pinruan et al. 2010; Orlandelli et al. 2012; Bongiorno et al. 2016). Life as an endophyte may better enable weak tree pathogens and weak wood decay fungi to use the resource wood as the first prior to replacement by more aggressive competitors (Song et al. 2017). In the case of the poor wood degrader *S. commune*, a conditional life as an endophyte may help the fungus to access easily accessible nutrients both in bark and the sapwood of weakened or dying trees prior to that the organism is replaced by other more aggressive wood decay fungi (Peddireddi et al. 2006; Floudas et al. 2015; Almási et al. 2019).

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Chapter 3 - Appendix I

Table A1 Occurrence of fruiting bodies of species of the family of *Psathyrellaceae* on the grounds of the North Campus of the University of Göttingen and neighbouring grounds.
(Fruiting bodies observed on some extra places, i.e. in the inner town of Göttingen, in the Schloßgarten of Karlsruhe and in the town of Hann.Münden are also listed but in brackets; * same code = same site; C = closed, O = open, Y = young, and M = mature mushroom).

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Coprinellus disseminatus	Büsgenweg 2, W - side of building (bldg)	21.09.15	G3B	C,Y,O	On tree[1]
	Büsgenweg 3, N - side of bldg	20.09.15	G3A	C,Y,O	Meadow
	Büsgenweg 4, N - side of bldg. (plant litter under and in the neighbourhood of a fully shredded and then regrown shrubbery with *Sambucus nigra* as the main shrub and some other non-identified bushes)	29.09.14	G3	C	Plant litter
		16.10.14	G3	C,Y,O,M	Plant litter[2]
		20.10.14	G3	M	Plant litter
		08.09.15	G3	M	Plant litter
		10.09.15	G3	M	Plant litter
		21.09.15	G3	C,Y,O	Plant litter
		25.09.15	G3	M	Dead wood
		28.10.15	G3	C,Y,O	Plant litter
		30.10.15	G3	C,Y,O,M	Plant litter
		05.11.15	G3	M	Plant litter
		16.11.15	G3	C	Under tree[3]
		19.11.15	G3	M	Under tree[3]
		28.12.15	G3	C,Y,O,M	Plant litter
		02.06.16	G3	M	Plant litter
		20.06.16	G3	C,Y,O	Plant litter
		01.10.14	G3	C	Plant litter
		05.11.16	L120A	M	Plant litter
	Büsgenweg 4, W-N - side of bldg	27.06.12	G3F	M	Meadow
		22.09.13	G3F	M	Meadow
		16.10.14	G3F	M	Meadow
	Christophorusweg 12, S - side of bldg	18.09.15	L20C	M	Plant litter
		24.10.15	L20C	C,Y,O	On tree[2]
	Friedrich-Hund-Platz 1, N - side of bldg	27.04.14	L20B	C	Plant litter
	Grisebachstraße 10, E-S - side of bldg	29.09.14	KL95B	M	Plant litter
		05.08.14	KL95B	Y	Dead wood
		04.09.14	KL95B	M	Plant litter
		04.10.14	KL95B	Y	Meadow
		22.09.15	KL95B	M	Plant litter
	Julia-Lermontowa-Weg 3, E - side of bldg	11.09.11	G15	M	Meadow
		05.05.11	G15	Y	Meadow
		02.01.12	G15	M	Meadow
		31.08.12	G15	Y	Meadow
		04.09.14	G15	M	Meadow
		16.10.14	G15	M	Meadow
		02.10.15	G15	Y	Meadow
	Julia-Lermontowa-Weg 3, N - side of bldg	07.10.16	L144	M	Wood chips
		22.10.16	L144	C,Y,O,M	Wood chips
		05.11.16	L144	C	Wood chips
	Justus-von-Liebig-Weg 11, S-W - side of bldg	04.08.14	KL95A	C,Y,O,M	Under tree[4]
		15.10.14	KL95A	C,O	Under tree[4]
		20.10.14	KL95A	C,Y,O,M	Under tree[4,5]
		29.10.14	KL95A	C	Under tree[4,5]
		31.10.14	KL95A	C,Y,O	Under tree[4,5]
		01.11.14	KL95A	C,Y,O,M	Under tree[4,5]
		09.11.14	KL95A	M	Under tree[4]
		18.07.15	KL95A	C	Under tree[4]
		20.07.15	KL95A	C,Y,O	Under tree[4]
		25.07.15	KL95A	C	Under tree[4]
		27.07.15	KL95A	C	Under tree[4]
		01.08.15	KL95A	C	Under tree[4]

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Coprinellus disseminatus (continued)	Justus-von-Liebig-Weg 11, S-W - side of bldg (continued)	16.08.15	KL95A	C	Under tree[4]
		22.08.15	KL95A	C	Under tree[4,5]
		01.09.15	KL95A	C	Under tree[4]
		21.09.15	KL95A	C	Under tree[4]
		07.10.15	KL95A	C,O	Under tree[4]
		20.10.15	KL95A	C	Under tree[4,5]
		05.11.15	KL95A	C	Under tree[4]
		09.11.15	KL95A	C	Under tree[4,5]
		01.01.16	KL95A	C,Y,O	Under tree[4]
		11.04.16	KL95A	C	Under tree[4]
		14.04.16	KL95A	C,Y,O	Under tree[4,5]
		03.06.16	KL95A	M	Under tree[4]
		21.06.16	KL95A	C,Y,O,M	Under tree[4,5]
		02.07.16	KL95A	M	Under tree[4,5]
		04.09.16	KL95A	C,Y,O,M	Under tree[4,5]
		12.09.16	KL95A	M	Under tree[4,5]
		23.09.16	KL95A	C	Under tree[4,5]
		08.10.16	KL95A	C	Under tree[4]
		29.10.16	KL95A	C	Under tree[4]
		08.11.16	KL95A	C,M	Under tree[4]
	Nohlstraße 99, E - side of bldg	21.11.15	L20D	M	Plant litter
	(Platz der Göttinger Sieben 4, N - side of bldg	24.09.15	G3D	M	Plant litter)
	Rosenbachweg 6, W - side of bldg	14.10.14	L20E	O	Meadow
	Rosenbachweg 8, N - side of bldg	26.10.16	L165	C,Y,O,M	Meadow
	Tammannstraße 6, E - side of bldg	29.10.14	G3E	C	Close to tree base[3]
		31.10.14	G3E	M	Close to tree base[3]
		03.08.14	G3E	M	Close to tree base[3]
Coprinellus domesticus	Am Faßberg 11, S - side of bldg	06.11.11	G63	M	Meadow
	Büsgenweg 2, N - side of bldg	12.11.14	L64D	C,Y,O	Meadow
		04.09.14	L99A	M	Meadow
		05.09.14	L99A	M	Meadow
		30.09.15	L99A	M	Dead wood
		01.10.15	L99A	M	Meadow
	Büsgenweg 4, N - side of bldg	05.11.15	L64D	C	Meadow
		09.11.12	L70	Y,M	Plant litter
		05.11.13	L70	M	Plant litter
		09.11.13	L70	M	Plant litter
		09.11.14	KL97B	M	Plant litter
		13.11.14	L64D	C	Plant litter
	Christophorusweg 12, S - side of bldg	24.10.15	L99B	M	Meadow
	Grisebachstraße 10, E-S - side of bldg	07.11.11	KL55	Y,M	Dead wood
		29.04.13	KL97E	M	Dead wood
		27.09.13	L64C	M	Dead wood
		22.10.14	L64B	C	Meadow
		24.10.14	L64C	M	Dead wood
		26.10.14	L64B	M	Meadow
		01.11.14	KL97E	M	Meadow
		28.10.15	L64B	M	Meadow
		02.11.15	L120C	C	Meadow
		17.11.15	L64B	M	Meadow
		20.11.14	L64C	M	Dead wood
		21.10.16	KL97E	C,Y,O,M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	15.09.11	G27	C,O	Meadow
		11.09.11	G2	C,O	Meadow
		05.11.11	G49	M	Meadow
		16.06.12	L3C	M	Meadow
		28.05.13	KL13	C	Meadow
		08.09.14	KL55	M	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Coprinellus domesticus (continued)	Julia-Lermontowa-Weg 3, E - side of bldg (continued)	16.10.14	KL97A	Y	Meadow
		09.11.14	KL97A	C,Y,O	Meadow
		21.09.15	G2	C,Y,O,M	Meadow
		23.10.15	G2	M	Meadow
		26.10.15	G2	M	Meadow
	Julia-Lermontowa-Weg 3, W - side of bldg	28.10.16	L169	Y,M	Meadow
	Justus-von-Liebig-Weg 11, S-W - side of bldg	09.11.14	KL97D	C	On tree[4]
		30.10.16	KL97D	Y,M	On tree[4]
		09.11.16	KL97D	C	On tree[4]
	(Karlsruhe University Campus	29.09.13	KL97C	M	Meadow)
	Stumpfe Eiche 15A, W-N - side of bldg	26.10.14	L64A	M	Meadow
		19.09.15	L99C	M	Meadow
		22.09.13	L99C	M	Meadow
Coprinellus flocculosus	Büsgenweg 5, N - side of bldg	23.10.13	KL114	M	Meadow
	(Hann. Münden	08.07.14	L83	M	Dead wood)
Coprinellus heptemerus	Büsgenweg 4, N - side of bldg	18.11.14	KL203	M	Meadow
		05.11.11	G48	M	Meadow
Coprinellus impatiens	Julia-Lermontowa-Weg 3, E - side of bldg	21.10.16	L121B	M	Meadow
		31.10.16	L121C	M	Meadow
	Robert-Koch-Straße 33, W - side of bldg	22.08.15	L121A	C,Y,O,M	Wood chips
Coprinellus marculentus	Am Faßberg 11, S - side of bldg	06.11.11	G69	M	Wood chips
	Tammannstraße 6, N - side of bldg	09.10.16	L143	M	Meadow
	Büsgenweg 3, W-N - side of bldg	04.09.14	L95	M	Wood chips
Coprinellus micaceus	Burckhardtweg 2, N - side of bldg	23.09.11	G31	C,O	Meadow
	Büsgenweg 2, N - side of bldg	31.10.14	KL109B	M	Dead wood
	Büsgenweg 4, N - side of bldg	04.11.11	G117B	M	Plant litter
		15.06.12	L3B	Y,M	Plant litter
		03.11.13	L3A	M	Plant litter
		25.09.15	G117B	M	Plant litter
		07.10.15	G117B	M	Plant litter
		12.11.15	G117B	M	Plant litter
		05.11.16	G117B	M	Plant litter
	Friedrich-Hund-Platz 1, N - side of bldg	30.03.16	KL112	M	Dead wood
		09.04.16	KL112	C,Y,O,M	Dead wood
		10.05.13	KL206	M	Dead wood
		28.09.13	KL206	C	Dead wood
		28.10.13	KL206	C	Dead wood
		31.10.13	KL206	C	Dead wood
		07.04.14	KL206	C	Dead wood
		11.04.14	KL206	C,Y,O,M	Dead wood
		27.10.14	KL112	C,Y	Dead wood
		01.11.14	KL112	C,Y,O,M	Dead wood
		20.11.14	KL112	C,Y,O,M	Dead wood
		25.04.15	KL112	M	Dead wood
		17.10.15	L117	C,Y,O,M	Dead wood
		21.10.15	L117	C,Y,O,M	Dead wood
		10.10.12	KL112	C	Dead wood
		25.10.14	KL112	C	Dead wood
		22.10.16	L117	C	Dead wood
	Friedrich-Hund-Platz 1, N-W - side of bldg	05.11.14	L115	M	Meadow
		09.11.14	KL112	M	Meadow
		20.10.16	L115	M	Meadow
		29.10.16	L115	C,Y,O,M	Meadow
	Grisebachstraße 10, E-S - side of bldg	20.04.12	G122	M	Dead wood
		25.09.15	G122	M	Dead wood
		06.11.15	G122	C	Meadow
		09.11.15	G122	M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	04.09.14	KL94A	C,Y,O,M	Meadow
		26.10.14	KL109	C,Y,O,M	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Coprinellus micaceus (continued)	Stumpfe Eiche 20, S - side of bldg	11.10.14	KL94B	C,Y	Plant litter
	Tammannstraße 6, W - side of bldg	25.10.16	L162	M	Meadow
	Theodor-Heuss-Str.11, N - side of bldg	13.10.15	L120B	C,Y,O,M	Meadow
Coprinellus plagioporus	Julia-Lermontowa-Weg 3, E - side of bldg	16.10.14	KL98	M	Meadow
Coprinellus subimpatiens	Julia-Lermontowa-Weg 3, E - side of bldg	15.09.11	G26	M	Meadow
Coprinellus tardus	Am Faßberg 11, W - side of bldg	06.11.11	G60	M	Meadow
		06.11.11	G119	M	Plant litter
	Grisebachstraße 10, E-S - side of bldg	22.09.13	KL116	C	Meadow
	Grisebachstraße 2, S-E - side of bldg	03.11.15	L120D	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	24.07.12	L53	M	Meadow
		13.11.14	L64E	C,Y,O,M	Meadow
		17.10.16	L53	C,Y,M	Meadow
	Rosenbachweg 2, E - side of bldg	22.10.16	L158	M	Meadow
Coprinellus truncorum	Am Faßberg 11, S - side of bldg	03.11.11	G117A	Y,M	Meadow
	Büsgenweg 1, S - side of bldg	05.07.12	L56	C,Y,O,M	On tree[6]
		13.05.13	L56	Y,M	On tree[6]
		05.06.13	L56	C	On tree[6]
		01.07.13	L56	C	On tree[6]
		20.10.14	L56	Y,O	On tree[6]
		13.05.15	L56	C,Y,O,M	On tree[6]
		14.07.15	L56	M	On tree[6]
		26.07.15	L56	C	On tree[6]
		25.08.15	L56	M	On tree[6]
		09.09.15	L56	C	On tree[6]
		03.06.16	L56	C	On tree[6]
		13.06.16	L56	M	On tree[6]
		09.08.16	L56	C	On tree[6]
		13.09.16	L56	M	On tree[6]
	Büsgenweg 5, E - side of bldg	24.10.16	L161	C,Y,O,M	Dead wood
	Goßlerstraße 68, S - side of bldg	11.09.15	L120A	M	On tree[3]
	Hermann-Rein-Straße 2, N - side of bldg	28.09.16	L56	M	On tree[3]
Coprinellus xanthothrix	Büsgenweg 2, N - side of bldg	22.09.15	G1A	M	Dead wood
	Büsgenweg 4, W-N - side of bldg	29.08.12	G1B	C	On tree[7]
		18.09.15	G1B	C	On tree[7]
		04.10.16	G1B	C	On tree[7]
	Friedrich-Hund-Platz 1, N - side of bldg	12.05.12	G1	C,M	Dead wood
		17.07.12	G1	C,Y,O,M	Dead wood
		24.07.12	G1	C	Dead wood
		18.09.12	G1	Y	Dead wood
		10.09.11	G1	C	Dead wood
		31.07.12	G1	C	Dead wood
	Grisebachstraße 10, E-S - side of bldg	19.07.12	G1	C	Dead wood
	Julia-Lermontowa-Weg 3, E - side of bldg	13.09.11	G18	C	Dead wood
Coprinopsis acuminata	(Karlsruhe University Campus	29.09.13	KL124	C,M	Dead wood)
Coprinopsis atramentaria	Am Faßberg 7B, N-E - side of bldg	18.10.16	L142B	M	Meadow
	An der Lutter 22, E - side of bldg	29.09.11	G109	M	Meadow
		28.09.11	G34	M	Meadow
		28.09.11	G35	M	Meadow
		28.09.11	G36	M	Meadow
	Burckhardtweg 2, N-E - side of bldg	27.09.11	G33	M	Meadow
		05.11.11	G53	C,M	Meadow
		11.11.11	G78	C	Meadow
	Grisebachstraße 10, E-S - side of bldg	06.11.13	KL81A	M	Meadow
		19.05.14	KL81A	M	Meadow
		14.10.14	KL81A	M	Meadow
		15.10.14	KL96	M	Meadow
		06.11.14	KL96	C	Meadow
		07.10.15	KL81A	C	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Coprinopsis atramentaria (continued)	Grisebachstraße 10, E-S - side of bldg (continued)	23.10.15	KL81A	C	Meadow
		09.11.15	KL81A	M	Meadow
		21.10.16	KL96	C	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	22.09.13	KL81B	C,M	Meadow
		28.09.14	KL81B	C,M	Meadow
		26.10.14	KL111	M	Meadow
		09.11.14	KL96	M	Meadow
	Tammannstraße 6, N - side of bldg	15.10.16	L142A	C,Y,O,M	Meadow
Coprinopsis cothurnata	Büsgenweg 3, N-W - side of bldg	17.10.16	L145	C,Y,O,M	Wood chips
Coprinopsis ephemeroides	An der Lutter 26, N - side of bldg	28.10.15	L137	M	Wood chips
Coprinopsis lagopus	Tammannstraße 6, N - side of bldg	22.10.16	L155	C,Y,O,M	Wood chips
Coprinopsis macrocephala	Büsgenweg 1, S-W - side of bldg	23.09.11	G29	M	Wood chips
	Büsgenweg 1, S-W - side of bldg	13.06.12	L1	M	Wood chips
	Büsgenweg 3, N - side of bldg	19.09.15	L119	M	Meadow
	(Karlsruhe University Campus	29.09.13	KL122	C,M	Wood chips)
Coprinopsis narcotica	Julia-Lermontowa-Weg 3, E - side of bldg	01.09.14	L93	M	Meadow
		28.08.14	L90	M	Meadow
Coprinopsis picaea	Burckhardtweg 2, N-E - side of bldg	23.10.14	KL61	M	Meadow
	(Karlsruhe University Campus	29.09.13	KL123	M	Wood chips
Coprinopsis semitalis	Büsgenweg 4, W-N - side of bldg	23.10.14	KL61	M	Meadow
Coprinopsis stercorea	(Karlsruhe University Campus	29.09.13	KL123	M	Wood chips)
Coprinus comatus	Am Faßberg 11, W - side of bldg	23.09.11	G30	M	Plant litter
	An der Lutter 22, N - side of bldg	28.09.11	G37	M	Wood chips
	An der Lutter 26, N - side of bldg	04.10.15	KL9D	M	Meadow
	Burckhardtweg 2, N-E - side of bldg	05.11.11	G54	M	Meadow
		09.11.11	G121	M	Meadow
	Büsgenweg 5, N - side of bldg	26.10.15	KL9E	M	Meadow
		22.10.16	KL9E	M	Meadow
		02.11.16	KL9E	M	Meadow
	Friedrich-Hund-Platz 1, N-W - side of bldg	11.10.12	KL9F	C,M	Meadow
		29.10.16	L160B	M	Meadow
	Grisebachstraße 10, N - side of bldg	15.10.13	KL9A	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	31.10.16	L166C	M	Meadow
	(Hiroshimaplatz 3, N - side of bldg	17.09.15	KL9B	C,M	Plant litter)
	(Karlsruhe University Campus	29.09.13	KL125	M	Meadow)
	(Platz der Göttinger Sieben 2, N - side of bldg	24.10.16	L160A	C,Y,O,M	Meadow)
	Stumpfe Eiche 15A, W-N - side of bldg	19.09.15	KL9C	M	Meadow
		15.10.16	KL9C	M	Meadow
	Tammannstraße 6, N-W - side of bldg	26.10.16	L166A	Y	Meadow
		01.11.16	L166A	Y	Meadow
	Tammannstraße 8, N - side of bldg	26.10.16	L166B	Y	Plant litter
Lacrymaria lacrymabunda	Büsgenweg 4, N - side of bldg	04.09.14	KL104	C	Meadow
	Büsgenweg 5, N - side of bldg	05.11.11	G51	M	Plant litter
	Friedrich-Hund-Platz 1, N - side of bldg	10.09.11	G4	C	Plant litter
		25.06.12	L4A	M	Dead wood
	Friedrich-Hund-Platz 1, N-W - side of bldg	29.10.16	L164B	Y,M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G7	M	Meadow
		18.09.12	KL33	M	Wood chips
	Rosenbachweg 4, N - side of bldg	16.10.16	L147	C,Y,O,M	Meadow
	Rosenbachweg 8, N - side of bldg	26.10.16	L164A	M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	22.09.13	KL104	C	Meadow
		30.08.14	KL28	M	Meadow
		28.09.14	L4B	M	Meadow
		26.10.14	KL110	M	Meadow
		09.11.14	KL104	M	Meadow
		09.11.14	L4C	C,Y,O,M	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Lacrymaria lacrymabunda (continued)	Stumpfe Eiche 15A, W-N - side of bldg. (continued)	15.10.16	KL104	M	Meadow
	Tammannstraße 6, N - side of bldg	22.10.16	L156A	Y,M	Meadow
		25.10.16	L156B	M	Meadow
Panaeolus acuminatus	Tammannstraße 6, N - side of bldg	27.10.16	L168	Y,M	Meadow
Panaeolus antillarum	Büsgenweg 2, N - side of bldg	18.09.15	L125	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	30.10.15	L139	M	Wood chips
Panaeolus ater	Julia-Lermontowa-Weg 3, E - side of bldg	14.09.11	G23	M	Meadow
Panaeolus campanulatus	Büsgenweg 4, W-N - side of bldg	27.06.12	KL119	M	Meadow
		22.09.13	KL118	M	Meadow
Panaeolus cinctulus	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G10	M	Meadow
		27.06.12	L14A	M	Meadow
		12.09.11	G13A	M	Meadow
Panaeolus dunensis	Goldschmidtstraße 5, S-E - side of bldg	15.10.13	KL94	M	Wood chips
Panaeolus foenisecii	Büsgenweg 4, N - side of bldg	13.10.14	KL87	M	Meadow
Panaeolus olivaceus	Am Faßberg 11, S - side of bldg	06.11.11	G68	M	Meadow
	Büsgenweg 5, N - side of bldg	02.07.13	L58	M	Meadow
Panaeolus papilionaceus	Am Faßberg 11, N - side of bldg	06.11.11	G65	M	Meadow
	Büsgenweg 2, N - side of bldg	05.09.14	L103	Y	Meadow
		24.09.14	L109	M	Meadow
	Büsgenweg 3, N - side of bldg	26.11.14	L116	M	Wood chips
	Büsgenweg 4, N - side of bldg	05.11.11	G56	M	Meadow
	Büsgenweg 4, W-N - side of bldg	12.11.14	L14B	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	21.09.15	L127	M	Meadow
		29.09.15	L127	M	Meadow
Panaeolus rickenii	Büsgenweg 5, N - side of bldg	22.09.15	L128	M	Meadow
		05.11.15	L140	M	Meadow
Panaeolus semiovatus	Büsgenweg 2, N - side of bldg	22.09.13	KL117	M	Meadow
Panaeolus solidipes	Stumpfe Eiche 20, S - side of bldg	08.09.15	L124	M	Plant litter
Parasola auricoma	Büsgenweg 3, N - side of bldg	28.10.16	L170A	Y,M	Wood chips
		01.11.16	L170A	Y	Wood chips
	Büsgenweg 5, E - side of bldg	28.10.16	L170B	Y,M	Wood chips
		30.10.16	L170B	Y,M	Wood chips
		02.11.16	L170B	Y,M	Wood chips
	Hermann-Rein-Straße 8A, E - side of bldg	29.10.16	L170C	M	Wood chips
		05.11.16	L170C	Y,M	Wood chips
Parasola plicatilis	Am Faßberg 11, S - side of bldg	05.11.11	G39	M	Meadow
	Am Faßberg 7B, E - side of bldg	19.09.15	L118C	M	Meadow
	Büsgenweg 3, N-E - side of bldg	18.10.16	L152A	M	Meadow
	Büsgenweg 4, N - side of bldg	06.11.11	G57	M	Meadow
		13.10.14	KL73	M	Meadow
		21.11.14	KL99	M	Meadow
		07.09.15	L118A	M	Meadow
	Büsgenweg 5, N - side of bldg	11.11.11	G76	M	Meadow
		18.09.15	L118B	M	Meadow
	Friedrich-Hund-Platz 1, N-W - side of bldg	29.10.16	L152C	M	Meadow
	Grisebachstraße 10, E-S - side of bldg	16.10.12	L57	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	01.09.14	L92	M	Meadow
		16.10.14	KL99	M	Meadow
		19.09.16	L141	M	Meadow
		28.10.16	L152B	M	Meadow
	Rosenbachweg 6, W - side of bldg	14.10.14	KL74	M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	22.09.13	KL29	M	Meadow
		22.09.13	KL73	M	Meadow
		26.10.14	L142	M	Meadow
	Tammannstraße 6, N-W - side of bldg	31.10.16	L152D	M	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Psathyrella atrolaminata	Burckhardtweg 2B, S-E - side of bldg	05.11.11	G71	M	Meadow
		18.11.11	G85	M	Meadow
	Büsgenweg 5, N - side of bldg	12.09.11	G9	M	Meadow
		13.09.11	G17	M	Meadow
	Büsgenweg 5, E - side of bldg	19.10.16	L153A	Y,M	Meadow
	Grisebachstraße 10, E-S - side of bldg	27.10.16	L153B	M	Meadow
	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G12	M	Meadow
		12.09.11	G14	M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	24.09.13	L63	M	Meadow
Psathyrella bifrons	Büsgenweg 2, N - side of bldg	27.10.15	L135	M	Meadow
	Tammannstraße 6, N - side of bldg	15.10.16	L149	C,Y,O,M	Meadow
	Tammannstraße 6, W - side of bldg	25.10.16	L163	Y,M	Meadow
Psathyrella candolleana	Büsgenweg 2, N - side of bldg	24.09.13	L60	M	Meadow
	Friedrich-Hund-Platz 1, N - side of bldg	06.09.12	L11	M	Dead wood
	Goldschmidtstraße 5, S-E - side of bldg	22.06.16	L113	Y,M	On tree[8]
	Grisebachstraße 10, E-S - side of bldg	08.09.15	L114	M	On tree[9]
	Grisebachstraße 10, W - side of bldg	23.06.16	L114	Y,M	On tree[9]
	Julia-Lermontowa-Weg 3, E - side of bldg	14.09.11	G19	M	Meadow
		18.09.12	KL34	M	Wood chips
	Tammannstraße 6, W-N - side of bldg	09.07.12	L33	M	On tree 1[10]
		03.08.14	L33	M	On tree 1[10]
		09.07.12	L34	C,M	On tree 2[10]
		06.09.12	L34	M	On tree 2[10]
		04.08.14	L34	M	On tree 2[10]
	Tammannstraße 8, N - side of bldg	01.11.16	L172	M	On tree[11]
Psathyrella conopilus	Am Faßberg 11, N - side of bldg	06.11.11	G70	C	Wood chips
	Burckhardtweg 2, S-E - side of bldg	19.10.11	G112A	M	Dead wood
		03.11.11	G116	M	Meadow
	Büsgenweg 1, S-W - side of bldg	18.07.12	L50	C	Wood chips
	Büsgenweg 2, N - side of bldg	05.09.14	KL103	M	Meadow
		24.09.14	L108	M	Meadow
		25.09.15	L129	M	Wood chips
		12.10.15	L129	M	Wood chips
		26.10.15	L129	M	Wood chips
	Büsgenweg 4, N - side of bldg	04.09.14	L98	M	Meadow
		14.10.16	L150A	M	Wood chips
	Büsgenweg 5, N - side of bldg	06.11.11	G58	M	Meadow
	Grisebachstraße 10, W - side of bldg	02.11.16	L150C	M	Plant litter
	Grisebachstraße 10, E-S - side of bldg	11.09.11	G5	M	Meadow
	Hermann-Rein-Straße 2, N - side of bldg	29.10.16	L150B	M	Plant litter
	Robert-Koch-Straße 33, W - side of bldg	11.10.14	KL83	M	Wood chips
	Stumpfe Eiche 15A, W-N - side of bldg	26.10.14	KL103	M	Wood chips
Psathyrella corrugis	Büsgenweg 3, N-W - side of bldg	20.10.16	L154	Y,M	Wood chips
Psathyrella gracilis	Hermann-Rein-Straße 8A, E - side of bldg	29.10.16	L148D	Y,M	Plant litter
	Julia-Lermontowa-Weg 3, E - side of bldg	27.10.16	L148B	M	Meadow
	Justus-von-Liebig-Weg 8, W - side of bldg	27.10.16	L148C	Y,M	Meadow
	Justus-von-Liebig-Weg 11, S-W - side of bldg	16.10.16	L148A	C,Y,O,M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	22.09.13	KL108A	M	Meadow
		26.10.14	KL108B	M	Wood chips
Psathyrella leucotephra	Büsgenweg 2, N - side of bldg	30.09.15	L130	M	Wood chips
		09.10.15	L130	C,Y,O,M	Wood chips
		27.10.15	L130	M	Wood chips
Psathyrella marcescibilis	Büsgenweg 3, N-E - side of bldg	18.10.16	L151	M	Meadow
	Büsgenweg 5, N - side of bldg	07.10.15	L131	M	Meadow
	Christophorusweg 12, S - side of bldg	24.10.15	L133	M	Wood chips
	Julia-Lermontowa-Weg 3, E - side of bldg	26.10.15	L134	M	Meadow
	Justus-von-Liebig-Weg 3, E - side of bldg	22.10.15	L132	M	Meadow

Table A1 continued

Mushroom	Place and Direction	Date	Code*	Stage*	Substrate
Psathyrella microrhiza	Am Faßberg 11, N - side of bldg	28.08.12	G66	M	Meadow
	Büsgenweg 1, S-W - side of bldg	27.12.11	G98	M	Wood chips
		27.12.11	G104	M	Wood chips
	Büsgenweg 1A, E - side of bldg	29.09.13	L87	M	Meadow
		12.08.14	L87	M	Meadow
	Büsgenweg 2, W - side of bldg	29.10.16	L171A	Y,M	Wood chips
	Büsgenweg 3, N - side of bldg	01.11.16	L171C	Y,M	Wood chips
	Hermann-Rein-Straße 8A, E - side of bldg	29.10.16	L171B	M	Plant litter
	Julia-Lermontowa-Weg 3, E - side of bldg	12.11.14	KL113	M	Meadow
Psathyrella multipedata	Friedrich-Hund-Platz 1, N-E - side of bldg	27.10.16	L167	M	Meadow
	Stumpfe Eiche 15A, W-N - side of bldg	22.09.13	KL107	M	Meadow
		26.10.14	KL107	Y,M	Plant litter
		15.10.16	KL107	Y,M	Plant litter
Psathyrella ochracea	An der Lutter 26, N - side of bldg	28.10.15	L138	M	Wood chips
Psathyrella pennata	Julia-Lermontowa-Weg 3, E - side of bldg	18.09.15	L126	M	Meadow
Psathyrella phegophila	Julia-Lermontowa-Weg 3, E - side of bldg	01.09.14	KL105	M	Meadow
Psathyrella piluliformis	Tammannstraße 6, N - side of bldg	15.10.16	L146	C,Y,O,M	Meadow
Psathyrella prona	Büsgenweg 2, N - side of bldg	28.10.15	L136	M	Meadow
	Büsgenweg 4, N - side of bldg	05.09.15	L123	M	Meadow
Psathyrella pseudogracilis	Julia-Lermontowa-Weg 3, E - side of bldg	17.10.11	G111	M	Meadow
	Robert-Koch-Straße 33, W - side of bldg	22.08.15	L122	Y,M	Wood chips
Psathyrella spadiceogrisea	Burckhardtweg 2, N - side of bldg	27.06.12	L19A	M	Meadow
	Büsgenweg 1, S-W - side of bldg	27.12.11	G102	M	Wood chips
	Büsgenweg 3, W - side of bldg	05.11.16	L157C	Y,M	Plant litter
	Hermann-Rein-Straße 8A, E - side of bldg	05.11.16	L157B	Y,M	Plant litter
	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G11	M	Meadow
		04.09.14	L97	M	Meadow
		19.09.14	L106	M	Meadow
		23.10.14	KL101	M	Meadow
		18.11.14	L97	M	Meadow
	Tammannstraße 6, N - side of bldg	22.10.16	L157A	Y,M	Wood chips
Psathyrella tephrophylla	Am Faßberg 11, S - side of bldg	06.11.11	G59	M	Plant litter
	Büsgenweg 1, S-W - side of bldg	27.12.11	G97	M	Wood chips
	Büsgenweg 2, W - side of bldg	09.10.16	G97B	M	Plant litter
	Julia-Lermontowa-Weg 3, E - side of bldg	22.10.16	L159	M	Meadow

[1] *Cotinus americanus*
[2] *Sambucus nigra*
[3] Unknown tree species
[4] *Prunus sargentii* (Sargent´s cherry) tree (Tree 1 in Fig. 5 and Fig. 6)
[5] *Prunus incisa* (Fuji cherry) tree (Tree 2 in Fig. 7 to Fig. 9)
[6] *Cladrastis lutea* (Kentucky yellowwood) tree
[7] *Acer pseudolatanus* (sycamore) tree
[8] *Platanus x hispanica*
[9] *Ailanthus altissima*
[10] *Phellodendron amurense* (Amur cork tree) tree
[11] *Crataegus wattiana*

Chapter 3 - Appendix II

Table A2 Monokaryotic and dikaryotic strains isolated from mushrooms of *Psathyrellaceae* found on the grounds of the North Campus of the University of Göttingen and in the closer neighborhood

Species	Place and geographic direction	Collection date	Isolate	
			Code*	Type*
Coprinellus disseminatus	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G15	M
	Busgenweg 4, N - side of bldg	11.09.11	G3	D,M
Coprinellus domesticus	Julia-Lermontowa-Weg 3, E - side of bldg	11.09.11	G2	D,M
		14.09.11	G27	D
		11.05.11	G49	D
	Am Faßberg 11, S - side of bldg	11.06.11	G63	D
	Büsgenweg 2, N - side of bldg	04.09.14	L99A	D
Coprinellus heptemerus	Büsgenweg 4, N - side of bldg	11.05.11	G48	D
Coprinellus marculentus	Büsgenweg 3, W-N - side of bldg	04.09.14	L95	D
Coprinellus micaceus	Büsgenweg 4, N - side of bldg	03.11.11	G117B	D
	Burckhardtweg 2, N - side of bldg	23.09.11	G31	D
Coprinellus subimpatiens	Julia-Lermontowa-Weg 3, E - side of bldg	14.09.11	G26	D,M
Coprinellus tardus	Am Faßberg 11, W - side of bldg	06.11.11	G119	D
		06.11.11	G60	D
Coprinellus truncorum	Büsgenweg 1, S - side of bldg	05.07.12	L56	D,M
Coprinellus xanthothrix	Julia-Lermontowa-Weg 3, E - side of bldg	13.09.11	G18	D,M
	Büsgenweg 2, N - side of bldg	10.09.11	G1A	D,M
Coprinopsis atramentaria	Burckhardtweg 2, N-E - side of bldg	27.09.11	G33	D
	An der Lutter 22, E - side of bldg	28.09.11	G34	D
		28.09.11	G35	D
		28.09.11	G36	D
	Burckhardtweg 2, N-E - side of bldg	05.11.11	G53	D
Coprinopsis cothurnata	Büsgenweg 3, N-W - side of bldg	17.10.16	L145	D
Coprinopsis lagopus	Tammannstraße 6, N - side of bldg	22.10.16	L155	D
Coprinopsis narcotica	Julia-Lermontowa-Weg 3, E - side of bldg	28.08.14	L90	D
		01.09.14	L93	D
Coprinus comatus	Am Faßberg 11, W - side of bldg	23.09.11	G30	D
	An der Lutter 22, N - side of bldg	28.09.11	G37	D
	Burckhardtweg 2, N-E - side of bldg	11.10.11	G54	D
Coprinus picacea	Burckhardtweg 2, N-E - side of bldg	11.01.11	G41	D
Lacrymaria lacrymabunda	Friedrich-Hund-Platz 1, N - side of bldg	10.09.11	G4	M
	Büsgenweg 5, N - side of bldg	05.11.11	G51	D
	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G7	D,M
Panaeolus cinctulus	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G13A	M
Panaeolus olivaceus	Am Faßberg 11, S - side of bldg	11.06.11	G68	D
Panaeolus papilionaceus	Am Faßberg 11, N - side of bldg	11.06.11	G65	D
	Busgenweg 2, N - side of bldg	05.09.14	L103	D
		24.09.14	L109	D

* Code + date relate to sites in Table A1; M = monokaryotic and D = dikaryotic mycelium

Table A2 continued

Species	Place and Direction	Collection date	Isolate	
			Code*	Type*
Parasola plicatilis	Büsgenweg 5, N - side of bldg	11.11.11	G76	D
	Julia-Lermontowa-Weg 3, E - side of bldg	01.09.14	L92	D
Psathyrella atrolaminata	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G12	M
	Julia-Lermontowa-Weg 3, E - side of bldg	12.09.11	G14	M
	Büsgenweg 5, N - side of bldg	13.09.11	G17	M
		09.12.11	G9	M
Psathyrella candolleana	Julia-Lermontowa-Weg 3, E - side of bldg	14.09.11	G19	D
	Goldschmidtstraße 5, S-E - side of bldg	22.06.16	L113	D
	Grisebachstraße 10, E-S - side of bldg	08.09.15	L114	D
	Tammannstraße 6, W-N - side of bldg	09.07.12	L33	D
		09.07.12	L34	D
Psathyrella conopilus	Burckhardtweg 2, S-E - side of bldg	19.10.11	G112A	D,M
	Büsgenweg 2, N - side of bldg	24.09.14	L108	D
	Büsgenweg 4, N - side of bldg	04.09.14	L98	D
	Grisebachstraße 10, E-S - side of bldg	09.11.11	G5	M
Psathyrella microrhiza	Büsgenweg 1, S-W - side of bldg	27.12.11	G104	D
	Büsgenweg 1A, E - side of bldg	12.08.14	L87	D
Psathyrella spadiceogrisea	Büsgenweg 1, S-W - side of bldg	27.12.11	G102	D
	Julia-Lermontowa-Weg 3, E - side of bldg	09.12.11	G11	D,M
		19.09.14	L106	D
		04.09.14	L97	D
Psathyrella tephrophylla	Am Faßberg 11, S - side of bldg	06.11.11	G59	D

A **ITS sequence of L33 identifies the fungus as *Psathyrella condolleana* (99 % identity to type strain FN386114 and isolate KX022943)**

TGGCGTTGGTNGTAGCTGGCTCCTAGGAGCATTGTGCACGCCCGTCATTCATATCTTTCCACCTGTGAACCATCTGTAGGCCTGG
ATACCCCTCGCTTTGGCAACAAAGCGGATGCAAGGATTGCTGCGTCGACAAGGCCGGCTCTCTTTGAATTTCCAGGTTCTATGTC
TTTTACACACCCCATTCGAATGATTTAGAATGTAGTCAATGGGCTTTCATGCCTATAAAAAACTATACAACTTTCAGCAACGGAT
CTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACCTCACCAGTTTTGTAACGAG
ACAGGTGAAGGCTTGGATGTGGGGGTTTTGCAGGCTGCCTCAGTGCTGGTCTGCTCCCCTGAAATGCATTAGCGAGCTCATACTG
AGCTTCCGTCTATTGGTGTGATAATTATCTACGCCGTGGATTGGACTCATGCTTGCTTCTAACCGTCCGCAAGGACACTTTACTT
GACCAATTTGACCTCAAATCANGTAGGACTACCCGCTGAACTTAAGCATATCAATAA

B **ITS sequence of L56 identifies the fungus as *Coprinellus truncorum* (99 % identity to type strain JN159562 and 98 % identity to FM87007 and FM87 with 1 % gap)**

GACCTGCGGAAGGATCATTAACGAATAACTNTGGTGTCTTGGTTGTAGCTGGCTCCTCGGAGCATTGTGCACGCCCGCCATTTTT
ATCTATCCACCTGTGCACCGATTGTAGGTCTGGATGACTCTCGCGCTCTCTGAGTGCGGATGCGAGGATTGCCCTCTTAACGGAG
GTGTCTCTCCTCGAATTTCCAGGCTCTACGTCTTTTTACACACCCCAAACGCATGATATAGAATGCAGTCAATGGGCTTGATCGC
CTATAAAACACTATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCA
TTAAATTCTCAACCTCACCCGTTTTCTGAACGGTTCTCCGAGGCTTGGATGTGGGGGTTTGTGCAGGCTGCCTCAGCGCGGTCTG
CTCCCCTGAAATGCATTAGCGAGTTCGTACTGAGCTCCGTCTATTGGTGTGATAATTATCTACGCCGTGGACAGGGTTTAGACTC
GCTTCTAACCGTCCGCAAGGACAATACCTTTGACAATTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCATA
A

Fig. A1 Sequences of ITS regions of (A) isolate L33 (D) and (B) isolate L56 (D).
Note that the sequence of isolate L34 (D) is identical to that of isolate L33 (D) found under two different (neighbouring) *Phellodendron amurense* (Amur cork tree) trees.

Chapter 3 - Appendix III

Fig. A2 Temperature and humidity graphs of the Göttingen North Campus of months with occurrence of mushrooms of the family *of Psathyerellaceae* as indicated (red spots; see following pages 166 to 176).

Abbreviations used for species names are generated from the first letter of a genus name followed by the first three letters of the species affix – please see Table A1 in Appendix I for full names.

Y = young mushroom; c = cloded mushroom; o = open mushroom; m = mature mushroom

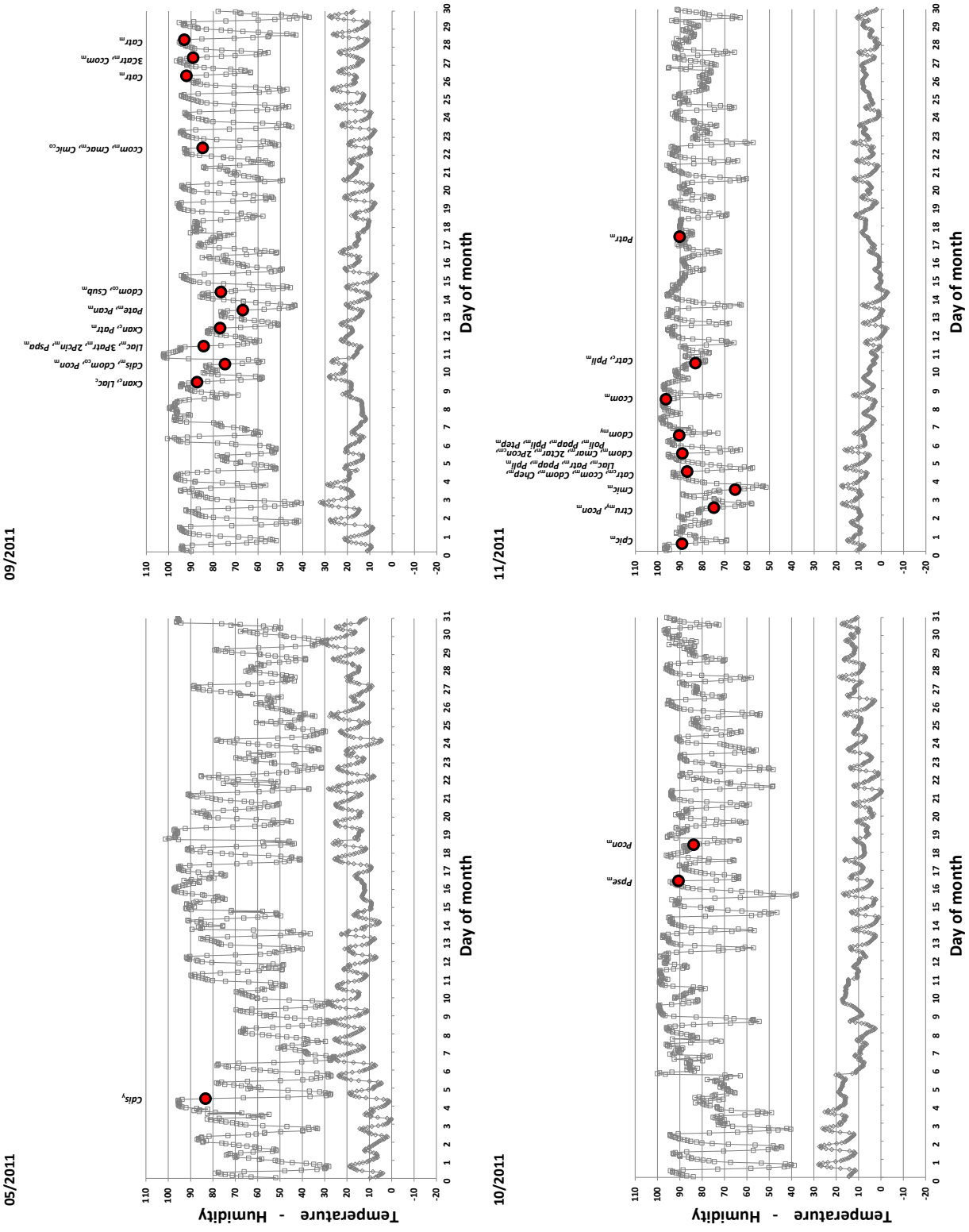

09/2011
Day of month
Cxon_C Llac_C
Cdis_Y Cdom_Co Pcon_E
Llac_E 3Potr_E 2Pclin_E Pspa_E
Cxon_C Potr_E
Pote_E Pcon_E
Cdom_Co Csub_E
Ccom_E Cmac_E Cmic_Co
Catr_E
3Catr_E Ccom_E
Catr_E
11/2011
Day of month
Potr_E
Catr_C Ppil_E
Ccom_E
Cdom_W
Poil_E Ppop_E Ppil_E Ptep_E
Catr_W Ccom_E Cdom_E Chep_E
Llac_E Potr_E Ppop_E Ppil_E
Cdom_E Cmar_E 2Catr_E 2Pcon_E
Cmic_E
Ctru_W Pcon_E
Cpic_E
05/2011
Temperature - Humidity
Day of month
Cdis_Y
10/2011
Temperature - Humidity
Day of month
Ppse_E
Pcon_E

Fig. A2

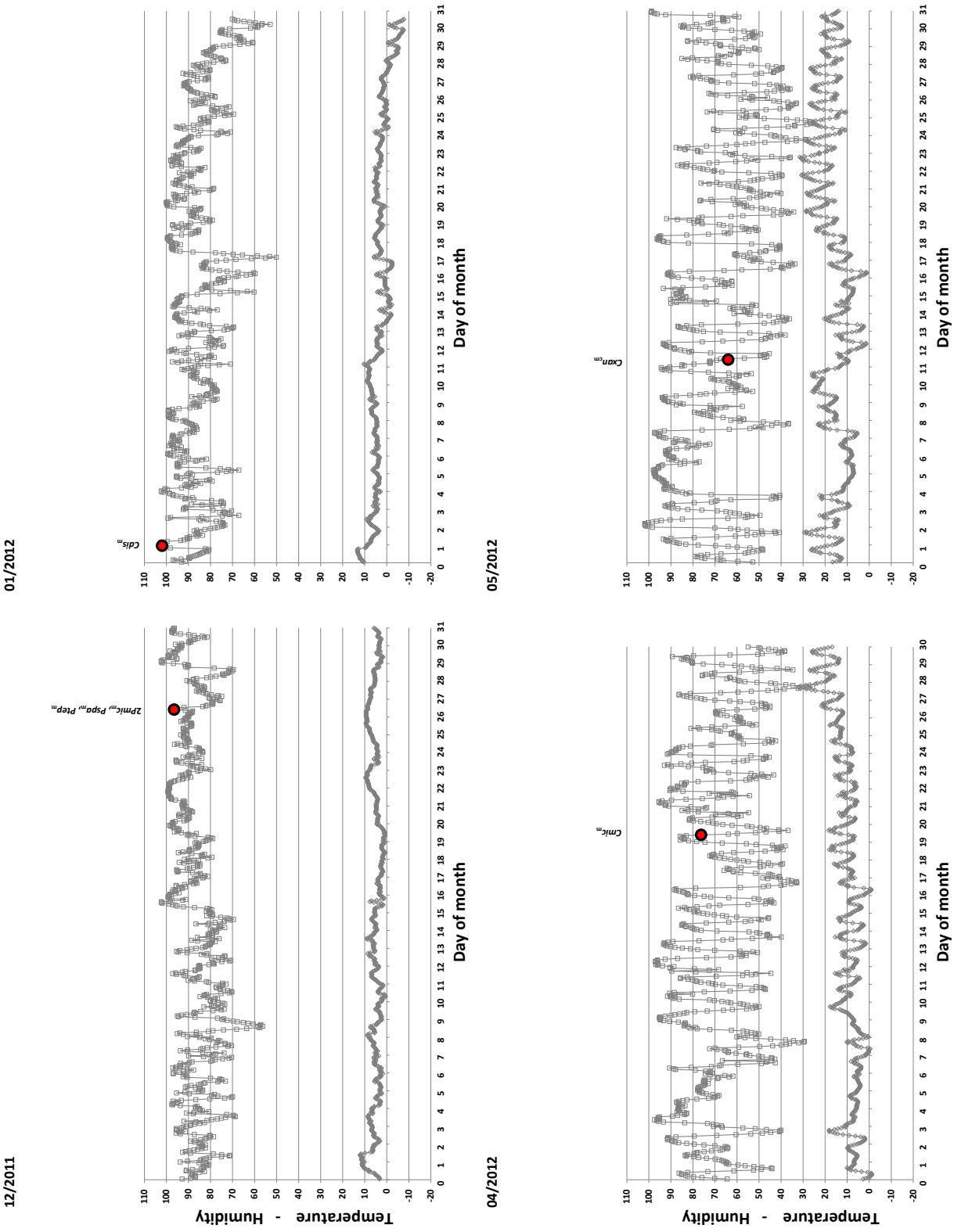


Fig. A2 continued

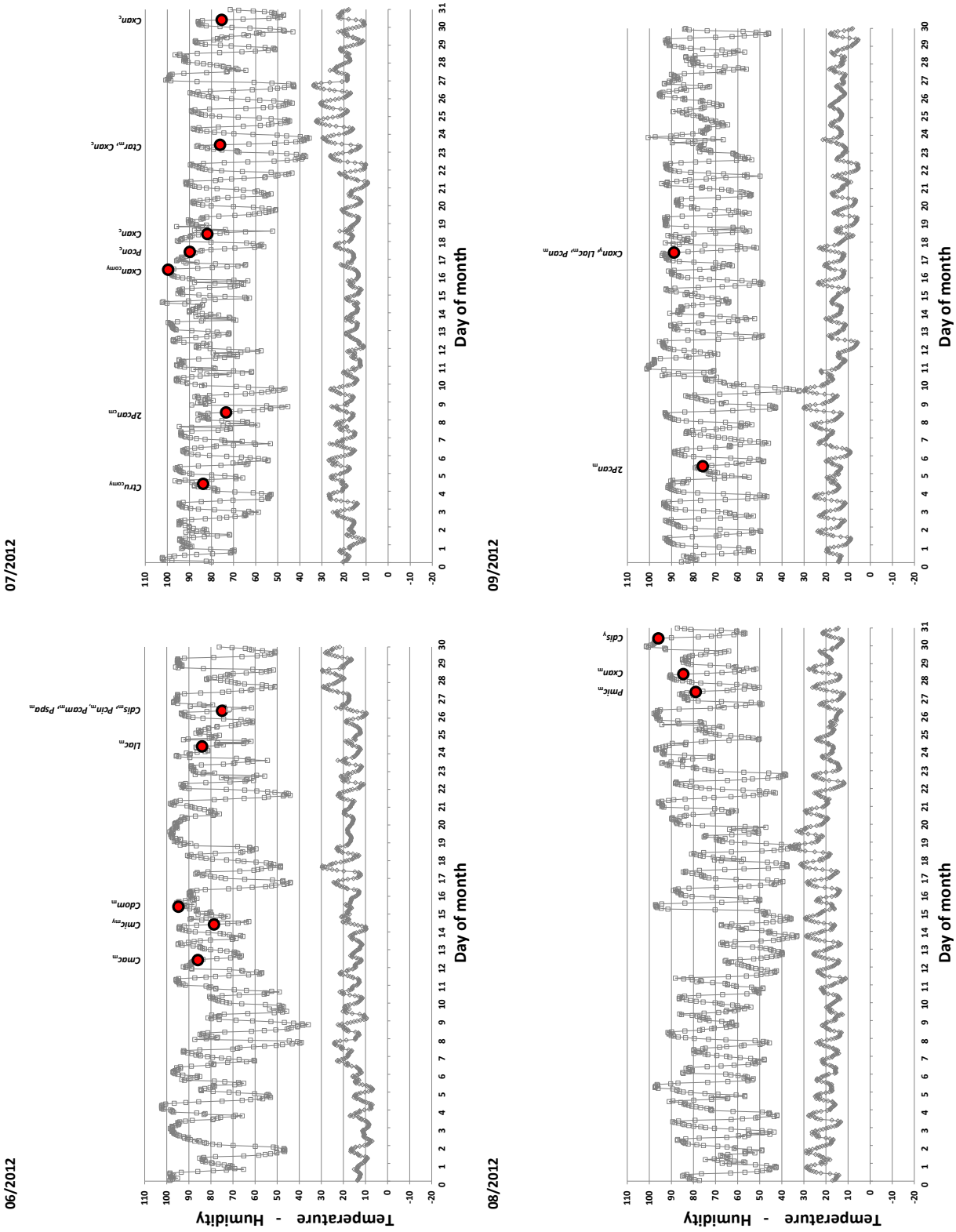


Fig. A2 continued

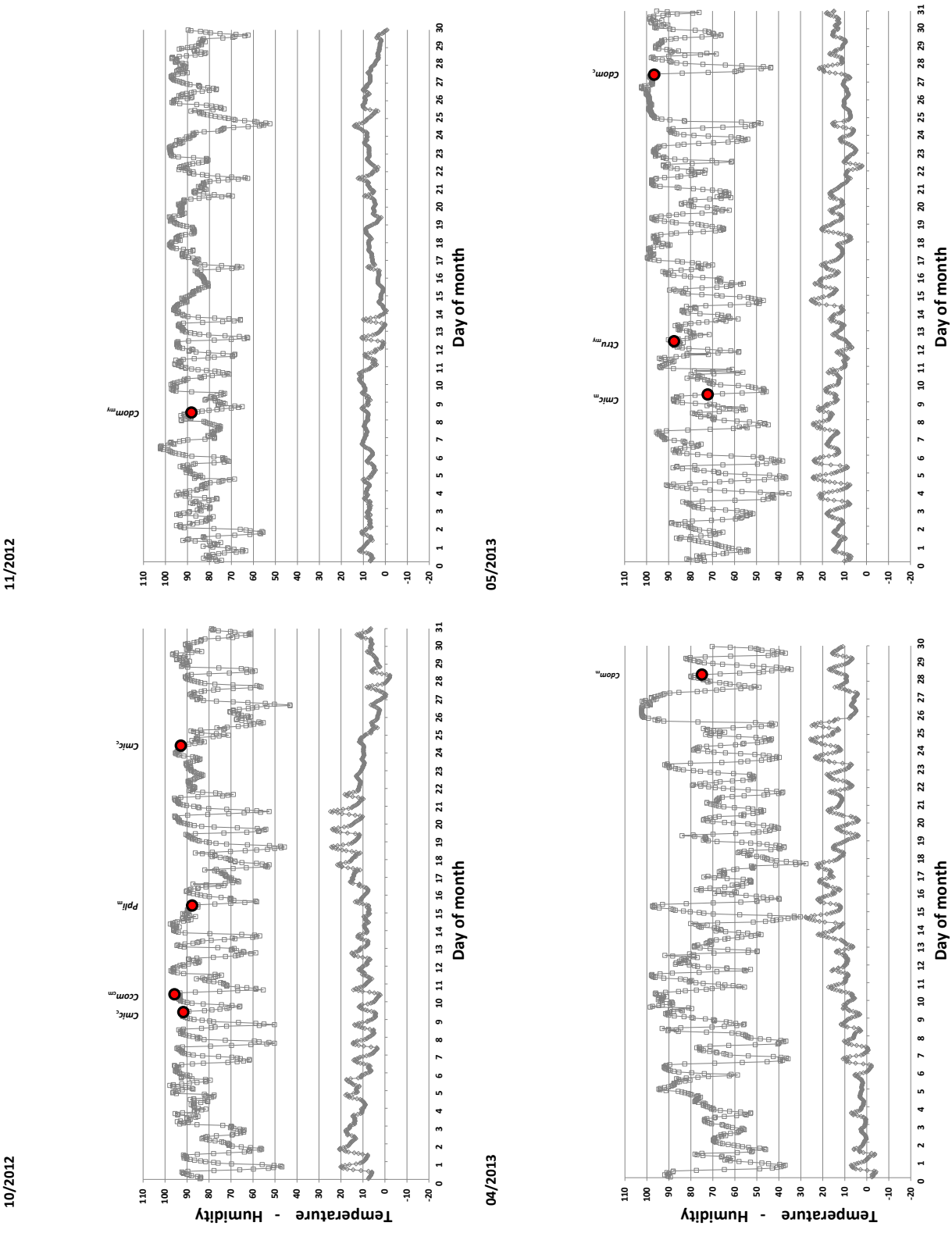


Fig. A2 continued

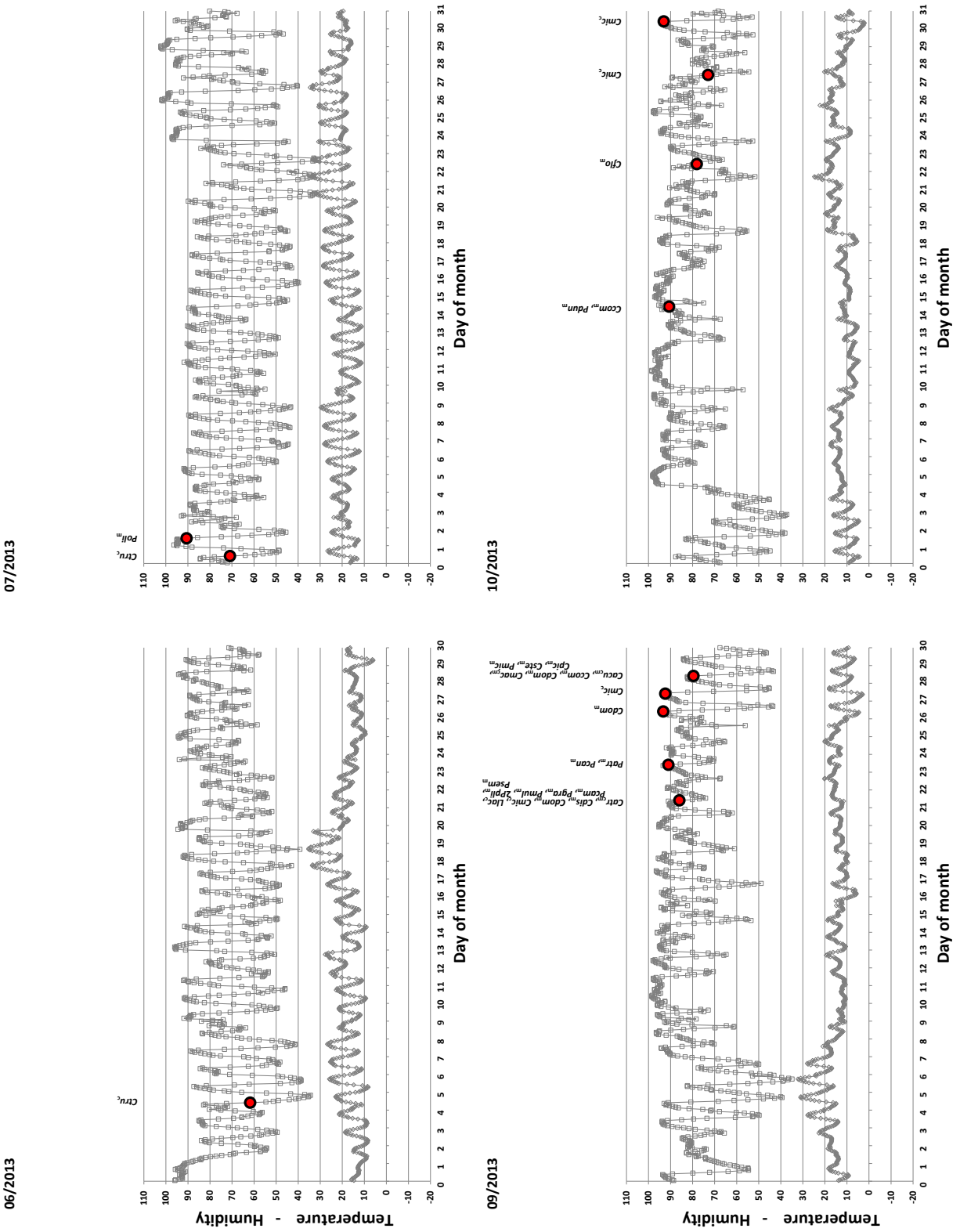
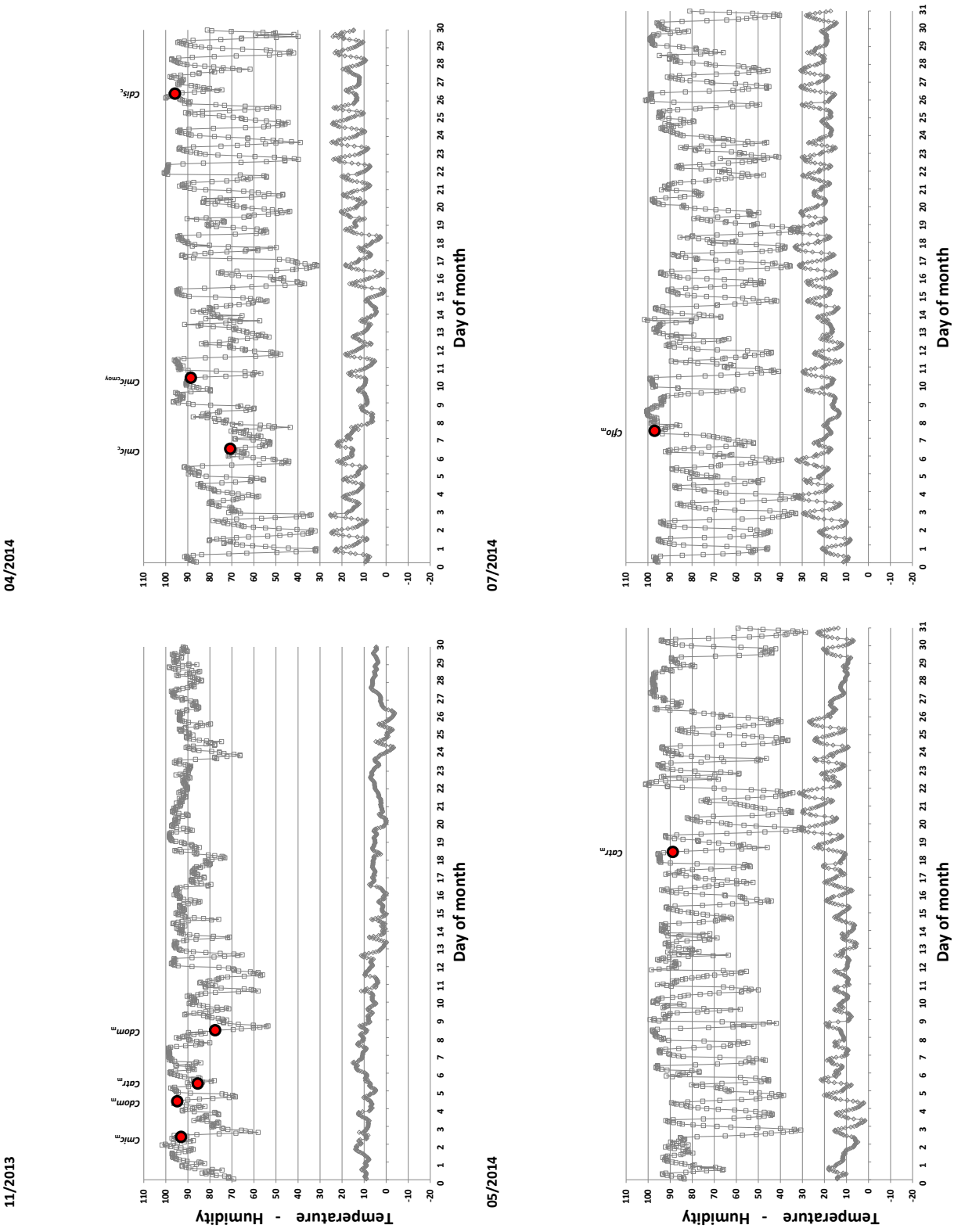


Fig. A2 continued



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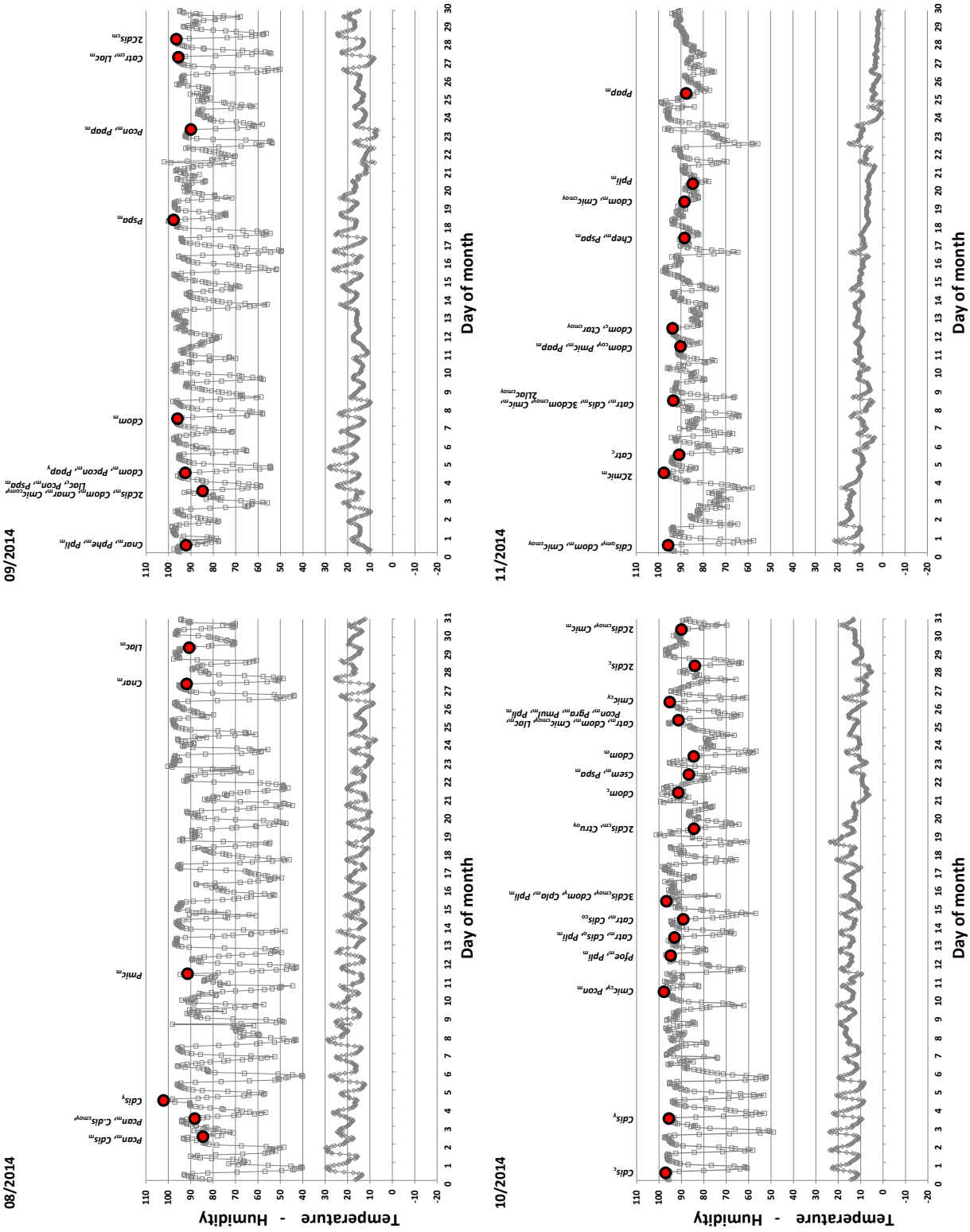
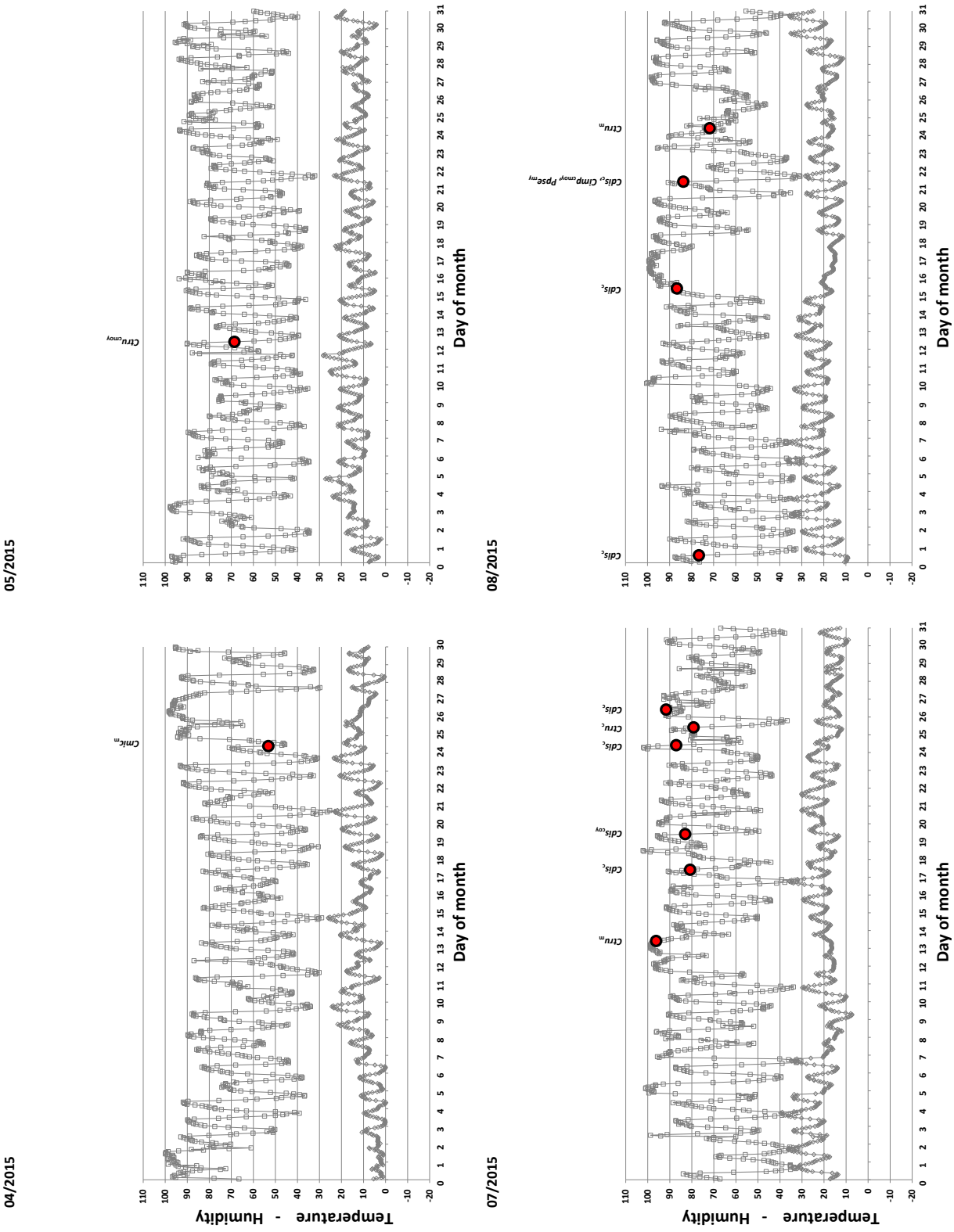


Fig. A2 continued



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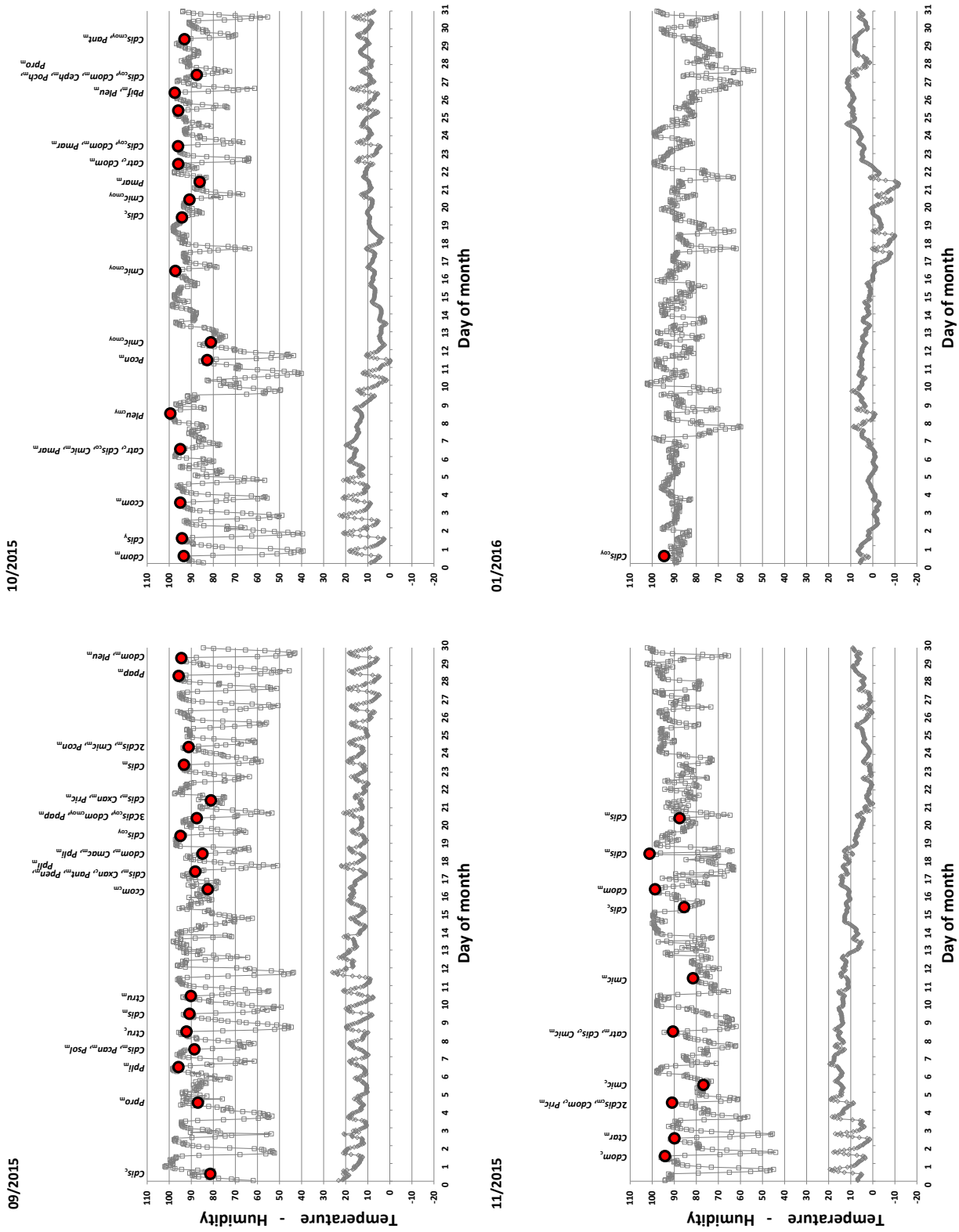

Fig. A2 continued

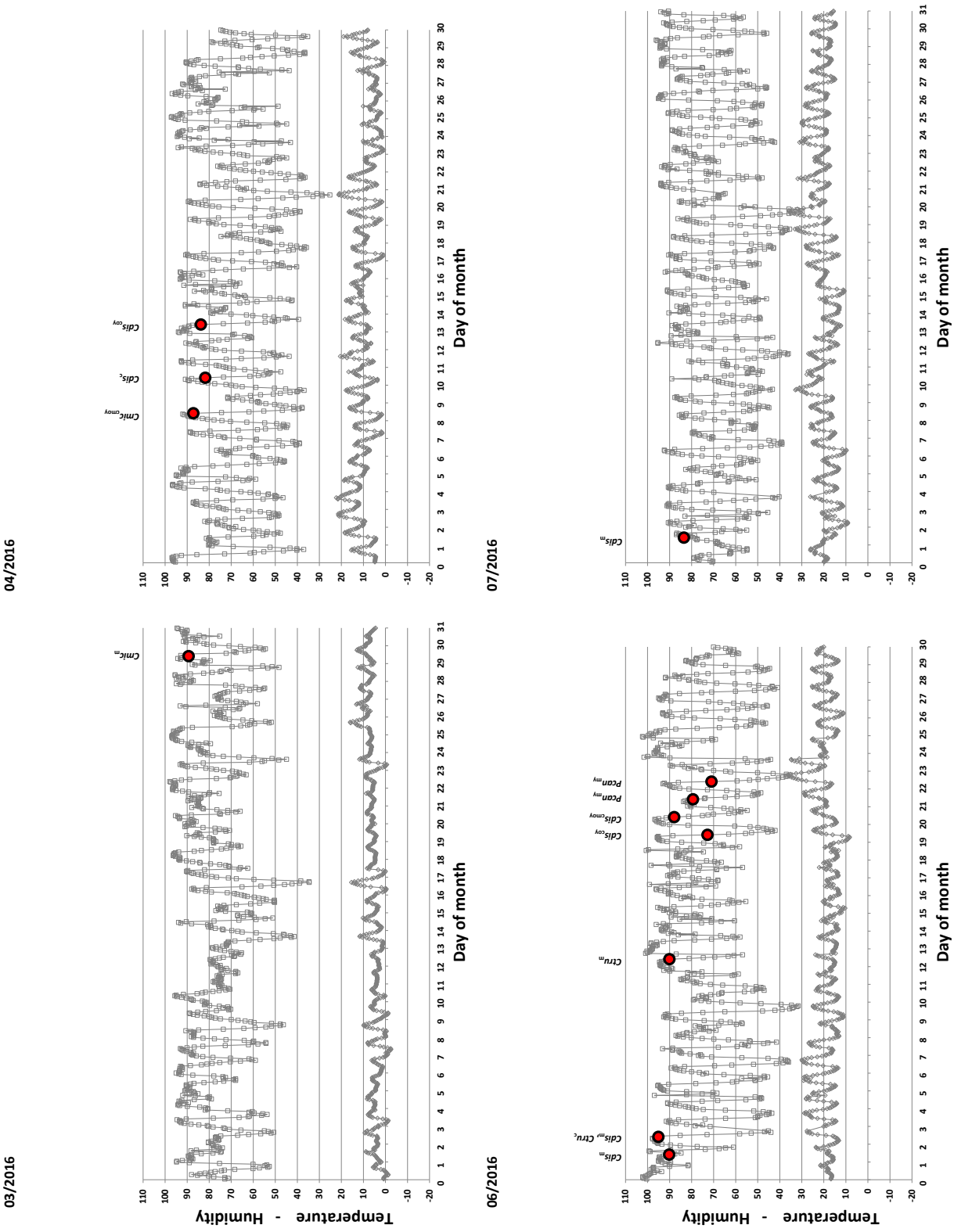

Fig. A2 continued

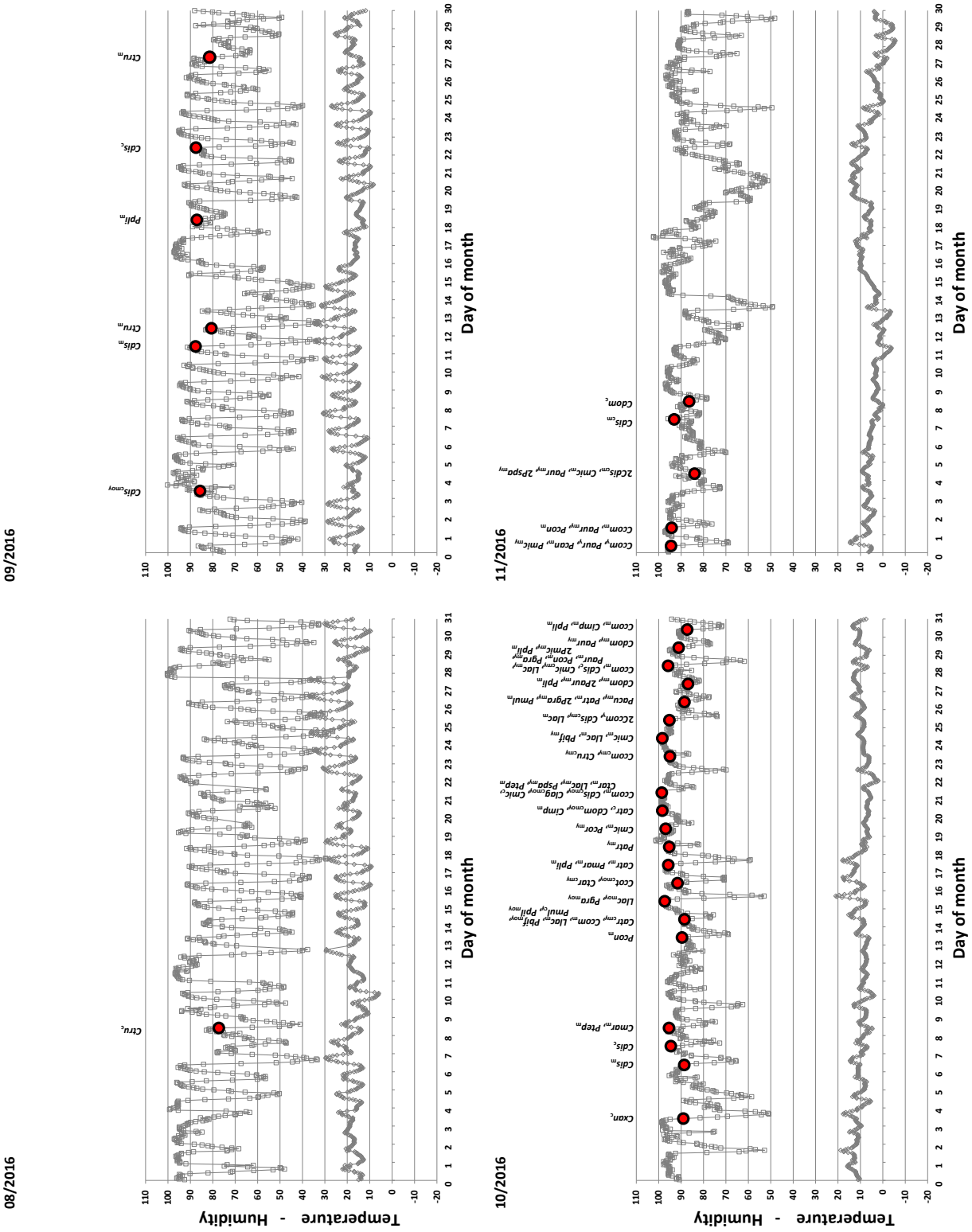

Fig. A2 continued

Chapter 3 - Appendix IV

Table A3 Occurrence of fruiting bodies of different species on a decaying elm tree (*Tilia cordata*) stump in a lawn close to the Chemistry Buildings on the North Campus of the University of Göttingen

Mushrooms of species observed				
Coprinellus micaceus	*Coprinellus xanthothrix*	*Flammulina velutipes*	Observer*	Shown in
	10.09.2011		KL	
	12.05.2012		KL	Fig. 19 A-B in Chapter 1
	17.07.2012		KL	Fig. 19 C in Chapter 1
	24.07.2012		KL	
	31.07.2012		KL	
	18.09.2012	18.09.2012	KL	
10.10.2012			KL	Fig. 19 D-G in Chapter 1
10.05.2013			KL	
	15.05.2013		KL	
30.09.2013			KL	Fig. 5A in Chapter 2
28.10.2013		28.10.2013	KL	
01.11.2013			KL	
		09.01.2014	KL	
		25.01.2014	KL	Fig. 1 K in Chapter 1
07.04.2014			KL	Fig. 5A in Chapter 2
		20.10.2014	KL	
27.10.2014			KL	
01.11.2014			KL	
		11.11.2014	KL	
20.11.2014			KL	
25.04.2015			KL	
17.10.2015			KL	
21.10.2015			KL	
30.03.2016			KL	
09.04.2016			KL	
22.10.2016			KL	
14.04.17		14.04.17	WK + UK	Fig. 5 F-H in Chapter 2
21.05.2017			WK + UK	Fig. 5 I, J in Chapter 2

* KL = Kiran Lakkireddy, WK = Werradej Khonsuntia, UK = Ursula Kües

Comment in addition: Note that after the 21.05.2017 no further fruiting body development by any of the species was observed (U. Kües, personal communication).

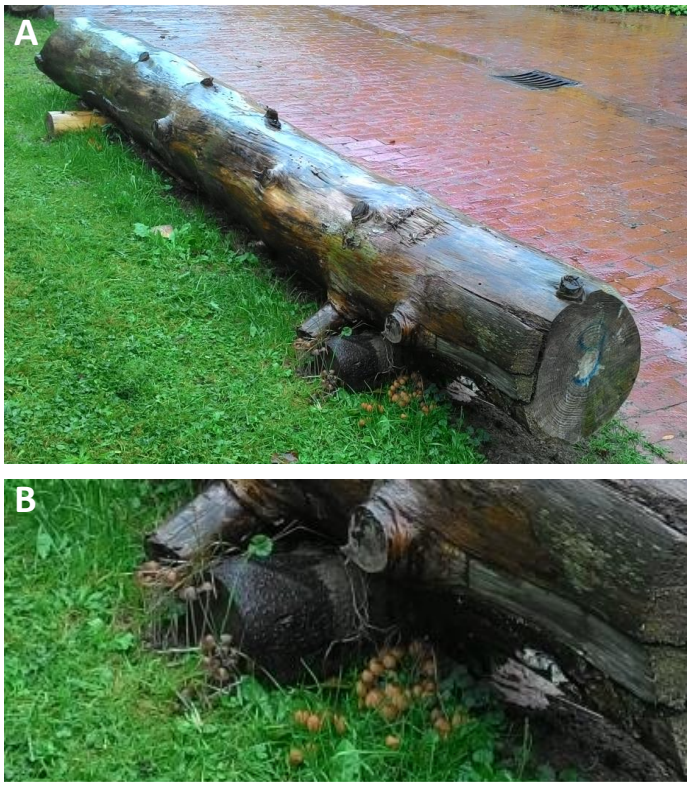

Fig. A3 *Coprinellus truncorum* fruiting bodies (code L161 in Table A1, Appendix I) growing on 24.10.2016 in the vicinity of logs of likely poplar wood underneath a stem of a *Picea abies* tree felled in 2012 or 2013 (information on tree kindly provided by Volker Meng).

Chapter 3 - Appendix V

Fig. A4 *Schizophyllum commune* fruiting bodies growing in cracks of the bark of an *Acer platanoides* tree felled in 2013.
Photos from 26.10.2016 (**A.**), from 20.5.2017 (**B.**, with the same mushroom group as in **A.**) and from 23.10.2017 with young *Trametes hirsuta* fruiting bodies replacing *S. commune* (**C.**).
Photographs kindly supplied by W. Khonsuntia and U. Kües. V. Meng kindly provided the data on tree species and the approximately felling date, correcting another earlier tree species misidentification in Fig. 2 in Lakkireddy et al. (2017).

Data of this thesis on occurrence of *S. commune* as pathogen on shrubs and trees on the grounds of the North Campus of the University of Göttingen (Fig. 18 and Table 5 of Chapter 3) have been included in the following published conference proceedings entitled:
Lakkireddy K, Zia A, Khonsuntia W, Kües U (2017) *Schizophyllum commune* as an early sapwood colonizing fungus. In: Proceeding Book. The 3rd Asia Pacific APRC 2017 Rubber Conference. 16th-17th November 2017. Prince of Songla University, Surat Thani Campus, Surat Thani, Thailand, pp 171-176
Weeradej Khonsuntia (WK) and Ursula Kües (UK) provided photos for Fig. 2 and Amjad Zia Fig. 3. WK and UK provided literature. UK wrote the manuscript under support of all authors.

The 3rd Asia Pacific Rubber Conference APRC 2017
RWR1
Rubber Agronomy, Socio-Economics and Biotechnology

Schizophyllum commune as an early sapwood colonizing fungus

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Keywords: sapwood fungi, wood decay, fungal interaction, antagonism

Abstract: *Schizophyllum commune* is a sapwood colonizing fungus of mainly broadleaf trees with restricted wood degradation abilities. We have observed mushrooms of this weak pathogen on stems and branches of 20 different living woody hosts, in 15 cases together with fruiting bodies of other wood-colonizing species. *S. commune* fruits as one of the first species regularly also on deadwood, in high numbers when the bark is still present and often also at the same time together with other wood-colonizing species. Fruiting bodies appear in cracks of the bark and may be replaced with time by basidiomes of other species. Cooperative and competing interactions might exist between different species in the wood. To understand such interactions better, we started to analyze reactions between the early sapwood fungi *S. commune* and *Trametes versicolor* in laboratory experiments in single and dual cultures on beech wood.

Introduction

Schizophyllum commune is a worldwide distributed basidiomycete which associates as a weak pathogen with many different broadleaf trees and shrubs [1,2]. Spores of the fungus are commonly found abundantly in the air [3]. When fallen into wounds in branches and stems of woody plants, they may take the opportunities for infection. More recently, *S. commune* has also been described to live in a number of plants as an endophyte, among in *Coffea arabica* and also in oil palm where the fungus may protect the host against more aggressive fungal pathogens [4,5]. As an early sapwood fungus, *S. commune* is among the first that produces fruiting bodies on felled stems and logs. Fruiting body development on over 200 different wood species has thus been reported [1]. In Thailand, *S. commune* has been detected in 16.7 to 75.0 % of all analyzed cases in decaying branches of rubber tree [6]. *S. commune* can be a severe wood destroyer in tropical regions while it acts rather as a mild rot in temperate regions [7]. Traditionally, *S. commune* was believed to be a white rot while recent genome analysis revealed features in between white and brown rot. Consistent with a reduced outfit of genes for ligninolytic enzymes, wood rotting abilities of *S. commune* in normed wood decay tests tend however to be low [8]. As a model fungus, *S. commune* is among the best studied basidiomycetes of all but overall its ecology with its seemingly contradictory behaviors in relation to wood colonization and usage is poorly understood [8]. Interrelations with other fungi living in the same substrate might offer explanations for opposing observations on *S. commune*.

Experimental

Observations in nature

Inspections for fruiting bodies of *S. commune* on living trees and on deadwood in the temperate region were done on the grounds of the North Campus of the University of Goettingen with plantings of the Forest Botanical Garden, predominantly in autumn-winter months on days after rainfall when after leave shedding a clear sight on all branches was possible and mushrooms were well expanded by uptaken moisture and well visible by their whitish color.

Wood decay tests

About 3 g of dried weighted sterile beech sapwood particles (ca. 4 x 1 mm) in a glass vial (3 cm in Ø, 7.5 cm in length) wetted with 4.5 ml of sterile H_2O were inoculated with one agar block of a freshly grown fungal culture of either monokaryons or dikaryons of *S. commune* or *Trametes versicolor*. Samples were incubated for 30 days at 25 ± 1 °C in the dark, then dried at 105 °C for 48 h and weighted, and the percentage of weight loss was calculated.

Fungal interactions

Each 2 g of sterile beech sapwood particles with 3 ml of sterile H_2O were added into two chambers of tripartite Petri-dishes and further 10 ml of sterile H_2O into the 3rd chambers for humidification during incubation. One agar plug with either fresh *S. commune* or *T. versicolor* mycelium was used to inoculate the wood particles in a Petri-dish chamber. Cultures were incubated for 16 days at 25 ± 1 °C in light.

Results and discussion

In accordance with a broad host range, fruiting bodies of *S. commune* were observed on the University North Campus on living trees and shrubs of 20 different broadleaf tree species (Fig. 1A to D, Table 1), as early colonizers on recently fallen and still barked deadwood (Fig. 1E to G; Fig. 2) and on burned charcoaled wood (Fig. 1H). Fruiting bodies on living hosts usually appeared on the sides of wooden substrates best exposed to the sun, often in S or W directions (Fig. 1A to D; Table 1). When shadows of buildings, other trees or other stems of a shrub blocked sunshine from S and/or W, mushrooms formed on other directions on the substrates (Table 1). Sun-exposed stretches of the branches with fruiting bodies increased often with time in length while the better shadowed opposite sides of the same branches were often still fully functional (see leaves in Fig. 1A and C). Where living trees and shrubs have been attacked by *S. commune* as concluded from fruiting body production, this correlated quite often with either replanting of a tree or with previous road and ground digging works in their close neighborhood, all of which likely resulted in root stress (root damage, disturbances in water supply). Fruiting bodies were often seen born in cracks of the bark or in lenticells as natural bark openings (see insets in Fig. 1A to D). Often, mushrooms of other strong wood-decaying fungal species such as polypores as from the genus *Trametes* were also found on the same branches and stems or on directly connected branches, directly below, above or next to *S. commune* fruiting bodies (Table 1). Fruiting bodies on deadwood formed also preferentially on the sunny sides of logs and branches (Fig. 1E to H; Fig. 2), again often together with mushrooms of other species of possibly stronger wood rotting abilities (e.g. see in Fig. 1F on the underside of the more distantly branch a resupinate polypore and in Fig. 1H young conks of *Trametes hirsuta*).

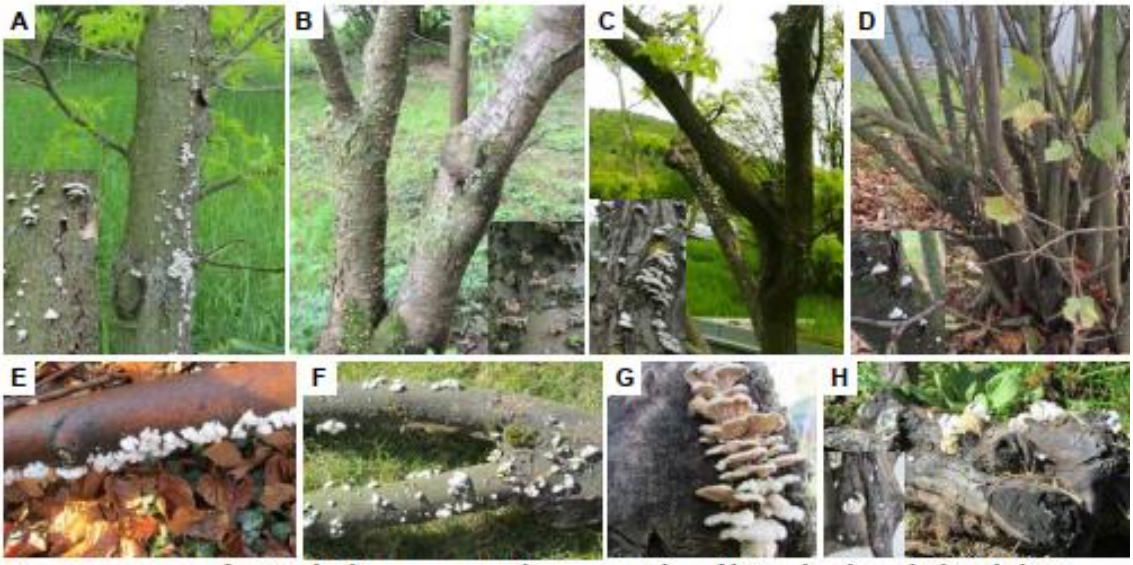

Fig. 1 *S. commune* fruiting bodies grown on the sunny sides of living hardwoods (A. *Gleditsia trecanthos* f. *inermis*, B. *Prunus incisa*, C. *Juglans ailantifolia*, D. *Syringa tigerstedtii*) and on types of deadwood (branches of E. *Fagus sylvatica* and F. *Acer pseudoplatanus*, G. cut *Quercus robur* stem, H. burned wood of unknown species)

Table 1 Mushrooms of *S. commune* and other wood-inhabiting Basidiomycetes observed on living shrubs and trees on the North Campus of the University of Goettingen in the years 2014-2016

Plant species	Place	Fruiting bodies of other species	Direction*
Acer caudatum	Living stem	*Flammulina velutipes* + *Radulomyces molaris* (below)	W (N,S)
Acer ginnala	Living stem	*Auricularia auricula-judae* (above)	E, W (S)
Alnus glutinosa	Living stem	*Ganoderma lucidum* (NE same level)	SW
Corylus tibetica	Dead branch	*Exidia glandulosa* + *Trametes pubescens* (connected dead branch)	W, S (N)
Cotoneaster moupinensis	Living branch	-	E
Crataegus monogyra	Living stem	-	W
Euonymus sanguineus	Many living branches and twigs	*Trametes versicolor* (above in 2 branches)	E, S, W
Gleditsia triacanthos f. *intermis*	Living stem	-	W
Juglans mandschurica	Living branches	*Antrodiella fragrans* (above), *Trametes* sp. (above)	S, SW
Lonicera korolkowii	Living branch	-	W, S
Lonicera maximowiczii	Living branch	*Skeletocutis nivea* (below)	W, NW
Malus angustifolia	Living stem	-	W
Malus spec. cf. *coronaria*	Living stem	*Bjerkandera agusta* (above)	w

Malus x zumi	Living stem	-	SE
Prunus incisa	Living stem	*Hypholoma fasciculare* + *Coprinellus disseminatus* + *Mycena* sp. (below at stem base)	W
Salix discolor	Living branch	-	SW (N)
Syringa tigerstedtii	Dead branch	*E. glandulosa* + *Byssomerulius corium* (connected branch)	W, E
Syringa wolfii	Living branch	-	E, NW
Viburnum burejaeticum	Living branch	*Skeletocutis subincarnata* (below)	NE, SE

* Main geographic directions of mushroom formation on the substrate are given without brackets, directions with only minor fruiting body formation are shown in brackets.

Fig. 2 Fruiting bodies of *S. commune* in 09/2016 growing out from cracks in the bark of a *Fraxinus excelsior* tree felled in 2016 (left) and young fruiting bodies of *T. hirsuta* in 10/2017 replacing the *S. commune* mushrooms (middle). Fruiting bodies of *T. versicolor* in 10/2017 replacing *S. commune* mushrooms on the cut site of a poplar log from year 2016 (right).

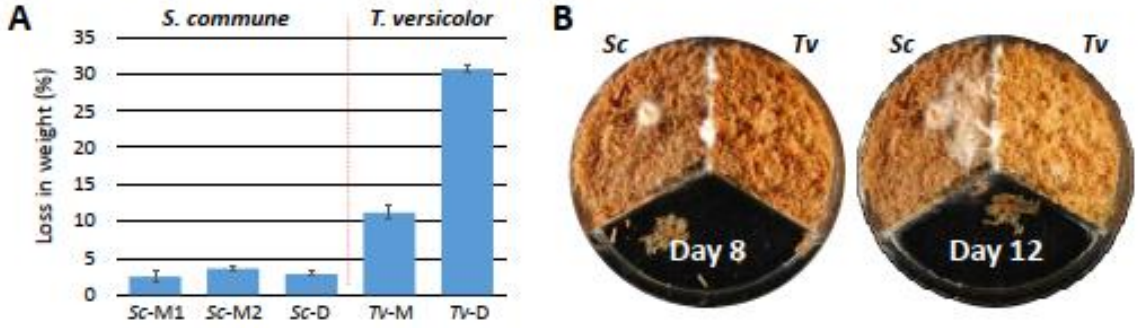

Fig. 3 A. Weight loss of beech wood after 30 days incubation with different *S. commune* strains is neglectable, unlike in cultures of *T. versicolor* where the monokaryon reduced >10% and the dikaryons >30% of the weight of the wood. B. *S. commune* (*Sc*) and *T. versicolor* (*Tv*) after 8 days in dual culture on beech wood compete with each other in the interaction zone seen as fluffy mycelium on top of the plastic border between the two wood-filled chambers. Afterwards, *T. versicolor* overgrows the *S. commune* colony as seen by the invading fluffy mycelium on the *S. commune* side in the 12-day-old culture. Note on the side of wood inoculated with the white-rot *T. versicolor* that the color of the beech particles lightened up due to wood decay.

As verified in beech wood decay tests, *S. commune* alone is only a weak degrader of wood (Fig. 3A) in accordance to literature reports [7-9]. As early colonizer of the sapwood growing underneath the barks of branches and stems (Fig. 1 and Fig. 2), the fungus may therefore live from the easily accessible nutrients in the sapwood parenchyma, the cambium and the bast, but it may also profit from the presence of other species in the same substrate [8,10], such as

Trametes species (Fig. 2; Table 1) as early sapwood colonizing species which easily degrade by white-rot the wood for fungal consumption (Fig. 3A and B). Alternatively, other fungi in the substrate may serve directly as food source for *S. commune* which can exist also as a mycopathogen [2].

With time by competition in wood, the more aggressive wood-decaying species may however replace *S. commune* in nature (Fig. 2). To better understand all the possible positive and negative interactive processes between different fungal species in wood, we have started to grow *S. commune* and *T. versicolor* on beech wood in dual culture. First *S. commune* could occupy the wooden substrate but subsequently *T. versicolor* overgrew *S. commune* (Fig. 3B). We now isolate the fungal proteome from the wood to identify by LC-MS peptide data analysis the sets of proteins produced by the two fungi for wood decay and in interaction with the other species.

Conclusions

S. commune is a worldwide early sapwood fungus with a broad host range on broadleaf tree species which grows easily underneath barks preferentially on the sunny sides of branches and stems. As a pathogen it colonizes on opportunities branches and stems of living hosts and it is regularly an early colonizer in freshly fallen deadwood. With some exceptions in the tropics, *S. commune* is however a poor wood degrader. Whether and how other fungi in the same substrate may contribute to the survival of *S. commune* in wood in nature is unclear. Modern proteomics will now allow first molecular insights into the processes.

Acknowledgements

The work is supported by the MaFoHolz PhD program through the Lower Saxony Ministry of Science and Culture and by DFG funding for a TripleTOF 5600 mass-spectrometer (DFG-GZ: INST 186/1085-1 FUGG). W.K. acknowledges a PhD fellowship from the Royal Thai Government.

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Chapter 4

Observations on Mycoparasites of Agaric Mushrooms

This chapter is subdivided into two subchapters.

Subchapter 4.1 presents a peer-reviewed conference paper entitled

"Mycoparasites Isolated from *Agaricus macrosporus* in Nature".

The paper is authored by K. Lakkireddy, W. Khonsuntia and U. Kües and was published in 2016 in: Baars, J.J.P., Sonnenberg, A.S.M. (eds.) Science and Cultivation of Edible Fungi: Mushroom Science IXX, International Society for Mushroom Science, Coatesville, PA, pp. 118-121.

This manuscript reports on initial observations on mycopathogens of *Agaricus xanthodermus* in nature. Note that at this time, we thought that the diseased mushrooms were from a related species, *Agaricus macrosporus*, by the lack of obvious *A. xanthodermus* characters (yellow tissue staining upon browsing, sharp bad smell). Later ITS-sequencing revealed the correct species identity (see subchapter 4.2).

Authors´ contributions: All authors observed mushrooms in nature. Kiran Lakkireddy (KL) photographed mushrooms in nature and described the basic steps in fruiting body production. KL isolated and cultivated mycopathogens in the laboratory and did initial basidiomycete infection tests. Weeradej Khonsuntia (WK) cultivated fungi for DNA isolation. KL and Ursula Kües (UK) collected literature. UK, KL and WK wrote the paper.

Subchapter 4.2 presents a peer-reviewed manuscript authored by K. Lakkireddy, W. Khonsuntia and U. Kües published in the open access journal AMB Express (2020) 10:141 with the DOI 10.1186/s13568-020-01085-5 under the title

" Mycoparasite *Hypomyces odoratus* Infests *Agaricus xanthodermus* Fruiting Bodies in Nature".

This manuscript reports in detail the observations on the mycoparasites on *A. xanthodermus* fruiting bodies in nature, the isolation and molecular identification of the mycoparasites and of *A. xanthodermus*, the mycelial characterization of the mycoparasites, and infection tests of fruiting bodies and mycelia of different Basidiomycetes.

Authors´ contributions and acknowledgements to others are listed at the end of the manuscript. KL and WK contributed equally to the work.

Subchapter 4.1

Science and Cultivation of Edible Fungi, Baars & Sonnenberg (ed.)

Mycoparasites isolated from *Agaricus macrosporus* in nature

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ABSTRACT

White button mushroom industry is threatened by mycoparasites such as *Lecanicillium fungicola* causing dry bubble disease and *Cladobotyrum dendroides* causing cobweb disease. The pathogens infect stages in mushroom development. They induce various symptoms on the host, such as bubbles, split stipes and spotty caps and destroy their host´s hyphae. Observations on these mycopathogens in nature are however rare, possibly because they are rather overlooked. Here, we describe mycopathogens found on *Agaricus macrosporus* in nature. Strains were isolated and we tested their behavior with different basidiomycetes in the laboratory.

Drumstick-like closed young fruiting bodies of *A. macrosporus* appear regularly each year in August to November underneath a Douglas fir on the North Campus of the University of Göttingen at sunny days of pleasant temperature. The young mushrooms open within 3 days by perforating the veil at the edges of the cap. In the next 2 days, the caps flatten in order to stretch out their pinkish-brownish gills for spore release and to then degenerate. Deformed fruiting bodies of irregular stipe and cap shapes appeared in September 2015. Fluffy mycelium covered the surface of the mis-shaped parts of the fruiting bodies. Microscopic analysis revealed ascomycetous types of conidiophores producing multiple 1-3 celled conidia. Similar structures were observed on decaying stipes of overturned mushrooms. The mycopathogens were isolated from infested mushroom tissues and used to infect in the lab commercially produced *Agaricus bisporus* and *Pleurotus ostreatus* mushrooms and mycelial cultures of basidiomycetes. The pathogens were very aggressive towards *A. bisporus* and produced huge amounts of conidiospores on the mushrooms. *P. ostreatus* in contrast was highly resistant. Slight infections were observed only with one of the isolates at stipe regions of only a few *P. ostreatus* mushrooms. Mycelium of lab strains of *Coprinopsis cinerea* was attacked by the mycoparasites but not mycelium of *P. ostreatus.* Conidiophores, conidiospores and ITS sequencing are used to determine the identity of the isolates.

Keywords: *Agaricus macrosporus*, mycopathogen, disease, ascomycete, conidia

INTRODUCTION

Commercially cultivated mushrooms such as the white button mushroom *Agaricus bisporus* can be spoiled by several pathogenic agents such as viruses, bacteria and fungi. Fungal pathogens such as *Lecanicillium fungicola* causing dry bubble disease (Berendsen et al., 2010), *Cladobotryum dendroides* causing cobweb disease (McKay et al., 1999), and *Mycogone pericosa* causing web bubble disease (Kouser and Shah, 2013) attack at any stage

of the mushroom development. Thereby, they can cause great losses to the white mushroom industry. When fruiting bodies are infected at an early stage of development (primordia or young mushrooms), they may develop into deformed mushrooms with for example split stipes and caps. Several mycopathogenic strains were previously isolated from commercially cultivated mushrooms, such as from different *Agaricus* and also *Pleurotus* species (Gray and Mogan-Jones, 1981; McKay et al., 1999). In contrast, descriptions of such infections in nature are rare. However, there are some reports on incidences of mycopathogens on mushrooms in fields and forests from species in the orders Agaricales (including *Agaricus bitorquis*), Boletales, Hymenochaetales, Russuales, and Thelephorales (Zare and Gams, 2008). Here, we observed mycopathogens attacking another *Agaricus* species in the nature and isolated the responsible strains for characterization in the laboratory.

1. Results & Discussion

1.1. Agaricus macrosporus *fruiting bodies in nature*

Drumstick-like young mushrooms of *Agaricus macrosporus* were yearly observed in 2 to 3 flushes during August to November in the needle and cone litter underneath a Douglas fir (*Pseudotsuga menziesii*) in a meadow on the North Campus of the University of Göttingen, under suitable conditions of weather, i.e. usually on sunny days with temperatures of 15-23 °C which followed after raining days with humidity levels of 50-80 % (day phase) and 80-95

Figure 1. Different stages of fruiting body development of *Agaricus macrosporus* under a Douglas fir tree at the North Campus of the University of Göttingen. A. Young mushroom coming out from the ground which has an ovate shape of the cap. B. Young still closed mushroom with stipe elongated and the upper cap surface flattened. C. and D. Convex freshly opened caps with veil remnants hanging at the edge of the cap (C.) and the main partial veil staying as an annulus around the stipe (D.). E. Fully opened flattened cap with brown gills and degenerated partial veil. F. A deformed mushroom infected by a mycopathogen found among other healthy young mushrooms.

% (night phase). In September 2015, the mushrooms came in two flushes, with a period interval of two weeks. The mushrooms come out solely or in loose groups. The mushrooms at the beginning are silky white in color and have an ovate cap shape when they are coming out of the ground (Fig. 1A). Later with growth, they get flattened on the top of the cap along with the elongation of the cylindrical stipe, giving then a drumstick-like shape (Fig. 1B,F). Within three further days, the young mushrooms open and the caps become convex with remnants of the partial veils hanging at the edge of the caps (Fig. 1C). The main parts of the partial veils will stay as an annulus around the stipes (Fig. 1D). The caps then flatten in the next two days in order to stretch out their first pinkish, then brownish gills for spore release and to afterwards degenerate (Fig. 1E).

1.2. A diseased fruiting body from nature

In September 2015, we observed in the first flush of fruiting bodies among many healthy mushrooms a nearly matured but deformed fruiting body of *A. macrosporus* which had the cap and stipe split (Fig. 1F). Foreign white fluffy mycelia covered parts of the mushroom suggesting the individual to be infected by a mycopathogen. In the second flush of mushrooms, many more structures at different stages of development were also infested by such fungal disease. Mycelia from the infestations were isolated from mushrooms, decaying tissues of overturned fruiting bodies and also from turf in the surrounding lawn. The mycopathogenic isolates were cultivated on 2% malt extract agar (MEA) and YMG/T

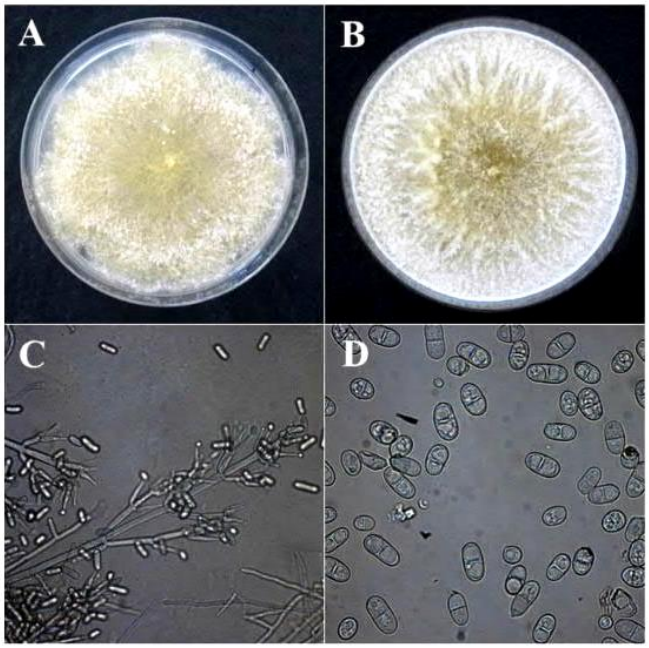

Figure 2. Morphology of a mycopathogenic fungus isolated from an *A. macrosporus* mushroom. A. Colony on 2% MEA and B. on YMG/T agar medium grown at 22 °C. C. and D. Photos of conidiophores and conidia as observed under a light microscope.

(Granado et al., 1997) on which they show different growth morphologies and colors. Initially, colonies on both media were white in color before they turned yellow (Fig. 2A,B) and on YMG/T with age pinkish. On YMG/T medium, mycelia were denser and produced more aerial hyphae and spores than on 2% MEA. Microscopic analysis revealed ascomycetous types of conidiophores which produced on the tips of phialides septated conidia with up to four cells (Fig. 2C,D). The conidia are rod-shaped with rounded edges (Fig. 2D). The morphology of isolated fungi resembles descriptions of some ascomycetes fungi belonging to the order of Hypocreales which include many known mycopathogens, among the cobweb disease causing *C. dendroides* from the family of *Cordycipitaceae* and *L. fungicola* and *M. perniciosa* from the family of Hypocreaceae causing dry bubble and wet bubble disease, respectively (McKay et al., 1999; Berendsen et al., 2010; Kouser and Shah, 2013). In order to identify the species of our fungal isolates, we are analyzing their morphologic characters in detail and we are isolating genomic DNA of the strains for ITS-sequence determination.

1.3. Infection of basidiomycetes by isolated ascomycetous strains

In order to proof that our isolates are indeed mycopathogens, we need to fulfill Koch´s postulate that the strains must cause the disease as observed in nature when inoculated onto healthy, susceptible mushrooms in the laboratory. Commercially produced *A. bisporus* and *P. ostreatus* mushrooms bought from supermarkets were therefore used to test their susceptibility to the disease in the lab when confronted with the mycopathogenic isolates. In general, the isolates were very hostile towards *A. bisporus* and produced enormous amounts of conidia on infected mushrooms. Mushrooms of *P. ostreatus,* in contrast, were better resistant to the disease. We are also testing the behavior of the isolates against mycelial cultures of a range of basidiomycetes. For example, mycelium of lab strains of *Coprinopsis cinerea* was easily colonized and attacked by the mycoparasites in contrast to those of some other species.

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Subchapter 4.2

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ORIGINAL ARTICLE Open Access

Mycoparasite *Hypomyces odoratus* infests *Agaricus xanthodermus* fruiting bodies in nature

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Abstract

Mycopathogens are serious threats to the crops in commercial mushroom cultivations. In contrast, little is yet known on their occurrence and behaviour in nature. Cobweb infections by a conidiogenous *Cladobotryum*-type fungus identified by morphology and ITS sequences as *Hypomyces odoratus* were observed in the year 2015 on primordia and young and mature fruiting bodies of *Agaricus xanthodermus* in the wild. Progress in development and morphologies of fruiting bodies were affected by the infections. Infested structures aged and decayed prematurely. The mycoparasites tended by mycelial growth from the surroundings to infect healthy fungal structures. They entered from the base of the stipes to grow upwards and eventually also onto lamellae and caps. Isolated *H. odoratus* strains from a diseased standing mushroom, from a decaying overturned mushroom stipe and from rotting plant material infected mushrooms of different species of the genus *Agaricus* while *Pleurotus ostreatus* fruiting bodies were largely resistant. Growing and grown *A. xanthodermus* and *P. ostreatus* mycelium showed degrees of resistance against the mycopathogen, in contrast to mycelium of *Coprinopsis cinerea*. Mycelial morphological characteristics (colonies, conidiophores and conidia, chlamydospores, microsclerotia, pulvinate stroma) and variations of five different *H. odoratus* isolates are presented. In pH-dependent manner, *H. odoratus* strains stained growth media by pigment production yellow (acidic pH range) or pinkish-red (neutral to slightly alkaline pH range).

Keywords: Mycopathogen, *Hypomyces*, *Agaricus*, Mushrooms, Conidiation, Microsclerotia

Introduction

Commercially cultivated mushrooms can be attacked by distinct mycoparasites such as the edible *Agaricus bisporus* by the ascomycetes *Lecanicillium fungicola*, *Mycogone perniciosa* (teleomorph *Hypomyces perniciosus*), and *Cladobotryum dendroides* (teleomorph *Hypomyces rosellus*) which cause dry bubble, wet bubble and cobweb disease, respectively (Largeteau and Savoie 2010; Berendsen et al. 2010; Carrasco et al. 2017). Such infections can result in severe crop losses, particularly in later flushes, if hygienic standards during cultivation are not high. Infections might originate from contaminated soil or spawn and the fungi might be introduced into mushroom casing in the form of spores or mycelium (Adie et al. 2006; Soković and Van Griensven 2006; Szumigaj-Tarnowska et al. 2015; Carrasco et al. 2017).

Lecanicillium fungicola not only infects the generative stage of *A. bisporus* but at all phases of fruiting body development (North and Wuest 1993; Calonje et al. 2000; Bernardo et al. 2004; Largeteau et al. 2007; Nunes et al. 2017). Depending on the developmental stage that becomes infected, disease symptoms range from totally undifferentiated spherical masses formed together by mycelia of host and pathogen ("dry bubble"), over partial disruption of stipe and cap tissues resulting in stipe deformations ("stipe blowout") to small necrotic lesions in the cap ("spotty cap") (North and Wuest 1993; Soler-Rivas et al. 2000; Largeteau et al. 2007, Largeteau and Savoie 2010; Bailey et al. 2013). Early infection of fruiting body initials by *M. perniciosa* also leads to the formation

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of undifferentiated hyphal masses ("sclerodermoid mushrooms"). These "wet bubbles" are first white and spongy. Then, they turn brownish and may be covered by amber-coloured liquid excretions. Mushroom deformations and cap spotting result from infections at later developmental stages (Fletcher et al. 1995; Umar and Van Griensven 1999; Umar et al. 2000; Glamoclija et al. 2008; Kouser and Shah 2013; Zhang et al. 2017). The soil inhabiting *C. dendroides* covers all stages of fruiting bodies in form of coarse white mycelium ("cobweb") under massive conidiospore production. Overgrown mushrooms eventually rot and collapse. Further symptoms linked to cobweb disease are brown spotting on caps instigated by germinating spores (Bhatt and Singh 2002; Potočnik 2006; Parrag et al. 2014; Carrasco et al. 2017). In recent time, other *Cladobotryum* species (mainly *C. mycophilum*, teleomorph *Hypomyces odoratus*; *C. varium*, teleomorph *Hypomyces aurantius*) have more often been reported to cause cob-web diseases including cap spotting and patching on *A. bisporus* (McKay et al. 1999; Grogan and Gaze 2000; Back et al. 2010, 2012b; Lee et al. 2011; Sharma et al. 2015; Carrasco et al. 2016, 2017; Chakwiya et al. 2019). According to McKay et al. (1999), Grogan (2006) and Tamm and Põldmaa (2013), when *H. odoratus* occurs in mushroom farms, it is quite often misidentified under the name *H. rosellus*. The sexual fruiting bodies (perithezia) cannot easily be differentiated morphologically between the species unlike their conidiophores with the asexual conidia (Rogerson and Samuels 1993, 1994). Asexual strain features together with molecular data are therefore used to define species (Kirschner et al. 2007; Põldmaa 2011; Tamm and Põldmaa 2013; Gea et al. 2019).

The different mycopathogens are not restricted to *A. bisporus* but may affect also other commercially cultivated species. Incidences of *L. fungicola* disease were reported for other *Agaricus* species (Gea et al. 2003) and *Pleurotus ostreatus* (Marlowe and Romaine 1982). *M. perniciosa* is shown to also infect *Pleurotus eryngii* and *Pleurotus nebrodensis* as well as *Volvariella volvaceae*, with the result of fruiting body malformations (Sisto et al. 1997; Sharma and Kumar 2000; Carrasco et al. 2017). Aggressive cobweb infections by *Cladobotryum* species were described for cultured *Calocybe indica* (Sharma et al. 2015), *Coprinus comatus* (Wang et al. 2015), *Flammulina velutipes* (Kim et al. 1999; Back et al. 2012b), *Ganoderma tsugae* (Kirschner et al. 2007), *Hypsizygus marmoreus* (Back et al. 2012a, b, 2015), *Pleurotus sajor-caju* (Sharma et al. 2015), *P. eryngii* (Kim et al. 1998, 2014; Gea et al. 2011, 2016, 2017; Back et al. 2012b), and *P. ostreatus* (Pérez-Silva and Guevara 1999; Gea et al. 2019).

While attention is paid on pathogen infections in commercial mushroom cultures due to the high economic interest, infection events observed in nature are scattered and usually not deeply described. In nature, an association with basidiomycete fruiting bodies and verticillium-like anamorphs (conidiophores are verticillate with whorls of few to several phialides which give rise to the phialoconidia) can help to identify potential mycopathogens (Gray and Morgan-Jones 1980; Zare and Gams 2008; Rogerson and Samuels 1989, 1993, 1994; Põldmaa and Samuels 1999; Põldmaa 2003; Tamm and Põldmaa 2013; Chakwiya et al. 2019). From the wild, *L. fungicola* has been isolated from fruiting bodies of *Agaricales* (e.g. *Marasmiellus ramealis*, *Hypholoma capnoides* and *Laccaria laccata*) and of decaying samples of *Thelephora terrestris* from the *Thelephorales*. *Lecanicillium flaccidum* from the same species complex was obtained from basidiocarps of *Coltricia perennis* of the *Hymenochaetales* and of *Gomphidius glutinosus* from the *Boletales*, and of decaying samples of *Russula nigricans* of the *Russulales* (Zare and Gams 2008). Incidences of *Hypomyces*/*Cladobotryum* infections appear to be more common. *C. dendroides* and *C. mycophilum* have a broad host range and have been isolated from mushrooms of varied species of *Agaricales*, *Boletales*, *Hymenochaetales*, *Polyporales*, *Russulales*, *Telephorales* and others. However, there are several more mycopathogens between the paraphyletic *Hypomyces*/*Cladobotryum* species group, several of which are producing yellow to red-coloured pigments and some of which have a more restricted host range (Gray and Morgan-Jones 1980; Sohi and Upadhyay 1986; Rogerson and Samuels 1989, 1993, 1994; Helfer 1991; Põldmaa and Samuels 1999; Douhan and Rizzo 2003; Põldmaa 2003; Valdez and Douhan 2012; Tamm and Põldmaa 2013; Marzuko et al. 2015; Wang et al. 2015; Zare and Gams 2016). In particular, orange-red lobster mushrooms are fruiting bodies of *Russula*, *Lactarius* and *Lactifluus* species from the *Russulales* which are infested by staining *Hypomyces lactifluorum* and are collected and commercially marketed as culinary delicacy in Mexico and Northern America (Laperriere et al. 2018).

In this report, we describe our observations on infestations of *Agaricus xanthodermus* fruiting structures in nature with strongly sporulating ascomycetous mycopathogens. We isolated mycopathogenic strains from infested material and describe their morphology and molecular identity with ITS sequences as *H. odoratus*/*C. mycophilum*. Furthermore, we performed infection studies with vegetative mycelium and fruiting structures of different basidiomycetous species.

Materials and methods

Mushroom observations, collection and fungal strain isolation

Mushrooms of *A. xanthodermus* growing underneath a *Pseudotsuga menzii* tree on the north side next to building Büsgenweg 5 of the Faculty of Forest Sciences and Forest Ecology (latitude 41.55933; longitude 9.95722) on the grounds of the North Campus of the University of Göttingen were usually observed and photographed at noon (at about 13 to 14 o'clock). Climate data (temperature and humidity) were routinely collected on the grounds through a hygro-thermo transmitter (Adolf Thies GmbH & Co. KG, Thies Clima, Göttingen, Germany). Mushrooms were identified by morphology using Breitenbach and Kränzlin (1995).

Crippled and decaying mushrooms were collected as well as rotting grass/moss samples with obvious white fungal mycelium. The samples were directly brought to a classroom laboratory and photographed by an IXUS 115 HS digital camera (Canon, Krefeld, Germany). For enlarged views, a M205 FA stereomicroscope with an integrated CF420 camera was used and the Leica Application Suite v3.8 software (Leica, Wetzlar, Germany). Samples of infesting mycelium from the cap of a crippled mushroom and mycelial samples of isolated cultures were observed with an Axioplan 2 imaging microscope (Carl Zeiss, Göttingen, Germany) equipped with a Soft Imaging System ColorView II digital camera. Digital photos taken were processed with the Soft Imaging System analySIS software (EMSIS, Münster, Germany). Size parameters were measured with the Arbitrary Distance function of the program and Excel (Microsoft, Redmond, WA) was used for calculations.

To isolate the basidiomycete, small mycelial samples were aseptically taken from the inner stipe regions of a healthy mushroom, and tissues were transferred onto MEA (2% malt extract, 1% agar; initial pH 5.0) plates with added antibiotics (ampicillin 100 µg/ml, kanamycin 50 µg/ml, tetracycline 10 µg/ml, chloramphenicol 20 µg/ml and streptomycin 100 µg/ml) as described formerly in Badalyan et al. (2011). To isolate the potential mycopathogens, foreign mycelia were taken from outer infested stipe and cap regions as well as from a grass/moss sample and transferred onto MEA plates supplemented with antibiotics. Plates were incubated at room temperature (RT) in the classroom. Growing mycelial samples were transferred for strain isolation and colony observations onto fresh MEA and YMG/T (0.4% yeast extract, 1% malt extract, 0.4% glucose, 0.001% tryptophan, 1% agar; Granado et al. 1997; initial pH 6) for growth at RT. Plastic Petri dishes (9 cm in Ø) with vents were used. Yeast extract (LP0021) and malt extract (L39) were from Oxoid (Basingstroke, UK), agar (Nr. 11396) from Serva (Heidelberg, Germany).

The isolated dikaryotic mycelium of *A. xanthodermus* (strain KKRL1) and the five different mycopathogen isolates (AscoA1, AscoB1, AscoC1, AscoD1, AscoE1) of this study were deposited in the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) strain collection in Braunschweig (Germany) under Catalog numbers DSM 111245 (KKRL1) to DSM 111250 (AscoA1 to AscoE1), respectively.

Colony characterisation

Cultures were grown at RT if not otherwise stated. Cultures were photographed with the IXUS 115 HS digital camera. pHs of culture medium were estimated with pH indicator strips which were dipped into squeezed agar pieces cut out from fresh and from mycelium overgrown medium. Mycelial samples with conidiophores, conidia or chlamydospores were observed under a Zeiss Axioplan 2 imaging microscope, digital photos were taken and size parameters measured with the analySIS software as described above. Diameters of chlamydospores as of microsclerotia and dense mycelial patches were measured crosswise in two directions and averages were calculated from all data. White mycelial patches were analysed in digital photos of complete cultures and microsclerotia using colony views of older cultures with collapsed aerial mycelium as photographed under the M205 FA stereomicroscope. Conidia from fully grown whole cultures were harvested from the culture surfaces as described in Kertesz-Chaloupková et al. (1998), spores attached to the lids of Petri dishes were washed off with sterile water and added to the spores harvested from the colony surfaces and total spores were counted using a hematocytometer.

ITS sequencing

Genomic DNA was isolated from mushroom samples taken from outside and from mycelium in culture (Zolan and Pukkila 1986). ITS sequences of basidiomycetes were PCR-amplified with primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990) and of ascomycetes with primers ITS-1* (TCCGTTGGTGAACCAGCGG) (Waalwijk et al. 1996) and ITS4 and analyzed as described before (Naumann et al. 2007). Gene sequences were deposited in GenBank under the Accession numbers KX098646-KX098654.

Mushroom infestation tests

Commercial mushrooms of *A. bisporus* (cap Ø 3.7 to 5.7 cm) and *P. ostreatus* (cap width between 2.4 and 6.7 cm) were purchased from a local supermarket. *A. bisporus* fruiting bodies were longitudinally cut into

halves and transferred into sterile crystal dishes (18.5 cm in Ø, 4.5 cm in height) with the cut side alternatively positioned to the top or to the bottom of the dish. Other *A. bisporus* mushrooms were used in whole in erect condition. *P. ostreatus* fruiting bodies were used either in whole or as halves in upside-top (lamellae oriented down) and in upside-down position (lamellae oriented to the top). Non-injured caps or cuts of caps or cut or non-cut sides of stipes of the fruiting bodies of *A. bisporus* and either caps or stipes of *P. ostreatus* were infested with small freshly grown MEA agar pieces of mycelial isolates, the crystal dishes were closed by their lids and incubated at RT. Every 12 to 24 h, mushrooms were inspected and photographed. For every isolate, at least 35 mushroom samples of *A. bisporus* and 25 mushroom samples of *P. ostreatus* were tested in at least 4 rounds of experiments.

Further, *Agaricus* mushrooms collected in September 2015 from the wild in other places in Göttingen-Weende/-Nordstadt were transferred into sterile glass jars and infested either on the cap or at the bottom of the stipe by small MEA agar pieces with freshly grown mycelial samples. Mushroom identities were determined by morphological means (Breitenbach and Kränzlin 1995) and ITS sequencing as *A. xanthodermus* (KX098653) and *Agaricus* sp. section *Arvenses* (KX098654).

Culture infestation tests

Mycelial cultures of *Coprinopsis cinerea* strain AmutBmut (*A43mut*, *B43mut*, *pab1-1*; Kertesz-Chaloupková et al. 1998), *P. ostreatus* monokaryon Pc9 (CECT20311), and of the isolated dikaryon KKRL1 of *A. xanthodermus* were prepared by inoculating one or two small freshly grown mycelial samples in the middle or at equal distances distributed on MEA or YMG/T plates and incubating them for vegetative growth at 37 °C (*C. cinerea* for subsequent grown mycelial challenge tests) or room temperature (RT, about 22 °C, used for other species in all grown mycelial challenge tests). Once a basidiomycete mycelium was fully established, a culture was challenged with two ca. 1 × 1 mm small inocula of freshly grown MEA agar pieces of a mycelial isolate to be tested by placing them onto the already grown basidiomycete mycelium 2 cm apart from the basidiomycete inoculum. The dual cultures were further incubated at RT and observed on daily basis for at least 20 days and in some instances for up to 2 months. Plates were photographed by an IXUS 115 HS digital camera. Five (*A. xanthodermus*) to six repeats (others) with two to three plates each were followed up per strain combination and MEA or YMG/T medium. Mycelial samples were observed under a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Göttingen, Germany).

In other sets of experiments (mycelial confrontation tests), basidiomycetes were inoculated on MEA or YMG/T medium 1.5 cm apart from the edge of a Petri dish (and pregrown when needed; see "Results" section), and the mycelial test strains 1.5 cm apart from the edge of the opposite side of the Petri dish. All plates were incubated at RT and observed for about a month and more after they were fully overgrown by the two mycelia. Five (*A. xanthodermus*) to six repeats (others) with two to three plates each were followed up per combination on MEA medium or YMG/T medium. Plates were regularly observed and photographed by an IXUS 115 HS digital camera and under a Zeiss Stemi 2000-C Binocular (Carl Zeiss, Göttingen, Germany). Presence of conidiophores and -spores of test isolates and hyphae of basidiomycetes were followed up by observing small mycelial samples from confrontation zones under a Zeiss Axioplan 2 imaging microscope.

Results

Mushroom development of *Agaricus xanthodermus* in nature

Since 2012, we observed every year but in 2018 and 2019 as 2 years with very dry hot summers that multiple white fruiting bodies of an *Agaricus* species appeared singly or in small loose groups variably in the months June to November in the thick layer of needle and cone litter underneath a *P. menziesii* (Douglas fir) tree and in the nearby grass of the surrounding meadow on the North Campus of Göttingen University (Fig. 1a). Initially, we noticed the conspicuous mushrooms either in still closed or in already opened conditions. Later with better attention we also saw smaller primordia (< 1 cm

(See figure on next page.)

Fig. 1 *Agaricus xanthodermus* fruiting bodies. **a** Mushrooms (marked by arrows) underneath a *Pseudotsuga menziesii* tree on the 3rd of September 2015. **b** Drum-stick-like young mushrooms: the left one is grown to full size (the arrow points to the partial veil underneath the cap). **c** Mushroom opening and **d** fully opened mushroom with vestiges of the partial veil at the edge of the opened cap and a skirt-like annulus around the stipe (marked by arrows). **e**–**l** Diseased crippled young mushroom with split stipe and cap and an infested primordium partially covered by a fluffy foreign mycelium (marked by an arrow) on 1st of September 2015 at the day of detection (**e**), 1 day after (**f**, **h**; the arrow points to partial veil still attached to cap tissues), 2 days after (**g**, **i**; note the pinkish still healthy lamellae in **i**) and 3 days after (**j**), when the mushroom was harvested (**k**; note the now brown colour of the lamellae and the white foreign mycelium which covers the crippled stipe and grows onto the lamellae). After harvest, white mycelium was seen spread over the needle and cone litter layer, the decayed primordium and a cone from the Douglas fir underneath (**l**)


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in Ø) emerging through the soil from broken ground. Mushroom production appeared to correlate with 1 to 3 prior days of high humidity triggered by good rainfall (about 80% and 95% humidity at days and nights) when temperatures reduced with the rainfall by about 5 to highest 10° from prior day time temperature values which were between 18 and to up to 30 °C at former warmer days (the actual temperatures depended on the time of the year). Spherical primordia were observed aboveground 2 to 4 days after the inducing days of high humidity and reduced temperature, still closed mushrooms with lengthened stipes ("drum-sticks") 4 to 8 days and opened mushrooms 10 to over 20 days after the rainfalls, when the days following induction were sunny and again warmer in temperature by an increase of 2 to 5° and when humidity values differed between night (ca. 80–95% humidity) and day periods (ca. 60–85% humidity). Fruiting body development continued usually in a temperature range from 15 to slightly above 20 °C. However, depending on the month there were also exceptional days encountered in later fruiting body development with temperatures up to 30 °C.

We observed round ball-like primordia (about 1.5–2 cm in Ø) on the floor and closed young white mushrooms that had a drum-stick shape and were generated from the spherical primordia by stipe growth and increase in cap size. Growth from a spherical primordium into a full-sized drum-stick-like young mushroom took several days, 2 to 3 days at warmer days (18–22 °C), while it slowed down up to 6 to 8 days at colder temperature (12–15 °C). Fully grown drum-sticks were up to 10 to 12 cm tall with a cap diameter of about 3 to 5 cm and a white partial veil at the underside of the cap that covered the lamellae (Fig. 1b). During maturation in the following 2 days, the white partial veil perforated with cap extension at the edge of the pileus. The remaining connections ripped apart with further cap opening and gave the stretched pileus a gear-wheel appearance by tooth-like vestiges (Fig. 1c, d). With the ripping, the partial veil stayed first as a well-shaped skirt-like white annulus around the stipe (about 1.0 to 1.5-cm in Ø, with the lower base somewhat swollen) at a distance of about 2 to 2.5 cm beneath the cap, but it degenerated with time over the following days. Opened caps were about 10 to 13 cm in diameter. On the upper surfaces towards the centres of the pilei were small yellowish to light brown scales. With cap opening, the densely arranged masses of initially pinkish thin lamellae (over 60 full length primary lamellae per cap with 5 to 7 secondary lamellae in between) turned quickly dark brown. Within 2 to 3 days, the cap colour turned pale-greyish and, slowly over 10 to 15 days, the open matured mushrooms grew old. The brown thick-walled smooth basidiospores (examples can be seen in Fig. 2m) measured in average $5.05 \pm 0.5 \times 3.89 \pm 0.63$ µm (n = 21).

By mushroom morphology and spore sizes, our morphological observations on the mushrooms concur with the descriptions by Breitenbach and Kränzlin (1995) for *A. xanthodermus*. However, strong yellow coloration upon injury of stems as typical for the species was first not noted; a faint yellow colour was seen on scratched freshly harvested mushroom stipes in September 2016 and again in July and more intensively in August 2017. The odour of healthy mushrooms of the colony was rather a faint mushroom scent than the typical pungent phenol odour of the species (Gill and Strauch 1984; Petrova et al. 2007) which in contrast was noticed by us for other *A. xanthodermus* colonies in the Göttingen-Weende area. Lack of both parameters together initially lead to a misidentification as *Agaricus macrosporus* by its very similar mushroom shapes and sizes (Lakkireddy et al. 2016). The species identity *A. xanthodermus* of the mushroom colony underneath the *P. menzii* tree was here confirmed by sequencing ITS DNA which was PCR-amplified from genomic DNA of a stipe of a mushroom harvested on 4th of September 2015. The established sequence (KX098652) was 99 and 100% identical to *A. xanthodermus* sequences AY484689 and DQ182529.1 from GenBank (Geml et al. 2004; Kerrigan et al. 2005).

Diseased mushrooms of *Agaricus xanthodermus* in nature

On 1st of September 2015, among several normal healthy fruiting bodies, we noticed a crippled young mushroom at the late drum-stick state that had a bended deformed stipe and a split cap (Lakkireddy et al. 2016). A directly neighboured primordial mushroom had dropped and was half-covered by a mycelial white network that extended over the stipe onto the edges of the cap of the other crippled individual (Fig. 1e). Over the next 2 days, the still healthy parts of the cap of the crippled mushroom extended in size to expose the pinkish lamellae while the primordial mushroom degenerated into an amorphous clump under actions of the foreign mycelium (Fig. 1f–i). As seen a day later, cap tissues of the crippled mushroom quickly aged, probably accelerated through the presence of the foreign mycelium. A thick mycelial layer of a fungal infestation was present at the side of the cap that was closer to the ground (Figs. 1j, 2a, b) and as cover over the stipe of the mushroom (Figs. 1k, 2c, d) from which it grew onto the lamellae (Fig. 2d–i). The harvested infected mushroom had an unpleasant smell. Sequencing of ITS DNA (KX098651) PCR-amplified from mushroom tissues again confirmed *A. xanthodermus* as the species identity.

Conidiophores with oblong spores were obvious in thick older mycelium grown on the upper side of

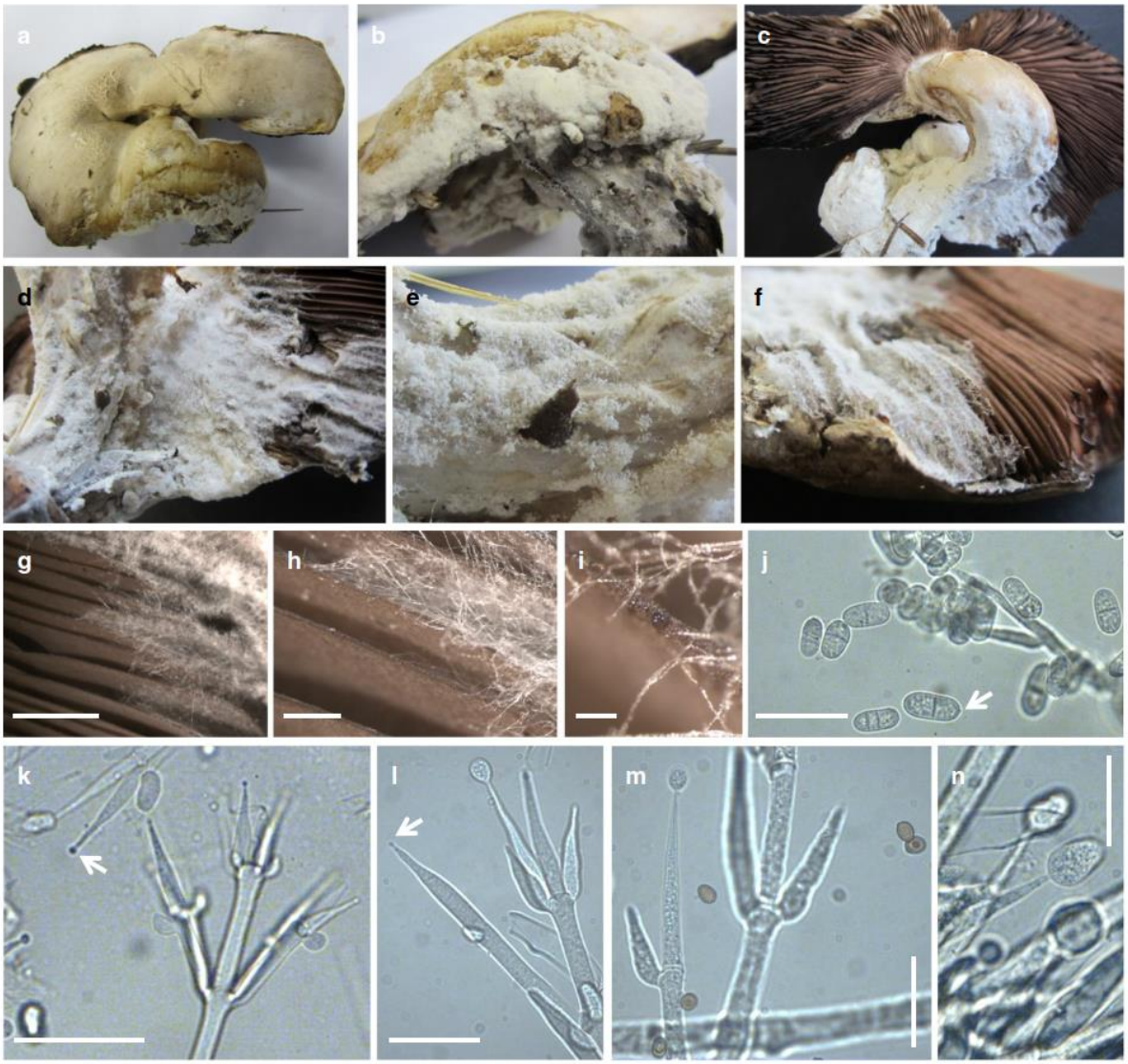

Fig. 2 Diseased crippled *Agaricus xanthodermus* fruiting body. **a**, **b** The upper surface of the cap, **c**, **d** the surface of the bended stipe, and **e**, **f** parts of the lamellae are overgrown by foreign mycelium with conidiophores visible as white flocks in the aerial mycelium (**d**). **g**–**i** For infestation of the lamellae, the foreign mycelium grew first over their edges, with conidiophore production starting about 6 to 7 mm behind the growth front (**g**; see white flocks at the right side of the photo). **j**–**n** Conidia and conidiophores in mycelial samples taken for microscopy from the lamellae. Note the blastic generation of conidiospores at the tips of phialides (arrows in **k**–**l**) and dehiscence scars (basal hilum) at the spores (see arrow in **j**) and also the small brown basidiospores of the host (**m**). Sizes bars correspond to 5 mm (**g**), 1 mm (**h**), 200 µm (**i**), 50 µm (**k**) and 20 µm (**j**, **l**–**n**)

the cap, on the stipe and the lamellae (Fig. 2b–g). We microscoped mycelial samples from the lamellae and found conidiophores and hyaline dry conidia (Fig. 2j–n) which suggested that the infestation was of the anamorphic genus *Cladobotryum* of the family of *Hypocreaceae* (*Hypocreales*, *Sordariomycetes*) of the *Ascomycota* (Cole and Kendrick 1971). Conidia were one to four-celled (18.0% one-celled, 63.9% two-celled, 9.8% three-celled; 8.2% four-celled; n = 61) with the majority being two-celled as it is typical for e.g. the mycopathogenic type species *C. varium* and *C. mycophilum* (Hughes 1958; Cole and Kendrick 1971; Rogerson and Samuels 1993; Back et al. 2012b; Tamm and Põldmaa 2013). Individual colonies were isolated from mycelium covering the

stipe (strains AscoA1 and AscoB1) and from lamellae (AscoC1) of the infested mushroom.

Upon aging, degenerating mushrooms in the meadow were also visibly attacked by similarly sporulating fungi (not further shown). Another mycelial strain (AscoE1) was thus isolated on 11th of September 2015 from a heavily infested rotting stipe of a formerly healthy *A. xanthodermus* mushroom when it was found knocked-down in course of aging on the meadow.

Following some heavy rainfall on 14th and 15th of September 2015 with a drop in temperature from the 16–21 °C at previous days, a second flush of *A. xanthodermus* mushrooms was observed in the 3rd week of September 2015, at day temperatures (noon) of 12 to 19 °C. Small spherical primordia were seen first on the 16th of September. Several structures were found 5 days later to be diseased at different developmental stages of mushroom development. Infestations started from white mycelial patches of several cm in diameter that developed first well visible on the 18th of September in the neighbourhoods on moss and decaying grass (Fig. 3), needles and cones (not shown). Sometimes these patches originated clearly from the remains of older mushrooms (Fig. 3f) but there were also multiple patches of fluffy white mycelium that did not obviously connect to a place of former mushroom production (Fig. 3n). Another fungal colony (strain AscoD1) was isolated from a decaying grass and moss sample from such a patch of sporulating white mycelium.

Mushrooms of differential developmental ages became infested by foreign mycelium, even very young primordia (Fig. 3a). Fluffy white mycelium grow onto the lower base of another young mushroom at the beginning of stipe outgrowth and, possibly as a consequence, the stipe of the young mushroom strongly bended with the mushroom cap laying down on the floor (Fig. 3a, b). Erect older drum-stick-like stages with extended stipes were also seen to be confined from the bases of the stipes (Fig. 3d–g). In some instances, heavy infestation lead to reddish-brown to lilac decolourisation of stipes (Fig. 3d, f) and also caps, and to collapse of the young mushrooms (Fig. 3f, g). Also older structures at and after cap opening were attacked by foreign mycelium (Fig. 3h–m). Caps of attacked mushrooms turned brown to blackish-brown and shrivelled, thus quickly grew old (Fig. 3i, j; l, m) and rapidly rotted (not further shown). The reactions on older fruiting bodies appeared to be more aggressive and faster than reactions on younger stages.

Mycopathogens in culture

All five isolated strains formed conidiogenous mycelium and grew well on MEA at RT (about 22 °C) with increases in colony radii of 3.7 ± 0.2, 3.8 ± 0.3, 3.6 ± 0.1, and 3.6 ± 0.1 mm/day (AscoA1, AscoB1, AscoC1, AscoE1) and of 2.4 ± 0.1 mm/day (AscoD1), respectively. On the nutrient-rich YMG/T, the colonies increased in radius by 4.3 ± 0.1, 4.3 ± 0.1, 4.2 ± 0.2, and 4.4 ± 0.1 mm/day (AscoA1, AscoB1, AscoC1, AscoE1) and 2.0 ± 0.1 mm/day (AscoD1). The odour of the fungi when grown on MEA was pleasant faint sweet aromatic (camphor-like, resembling *Eucalyptus* smell). During growth phases on YMG/T, the odour was also first pleasant faint aromatic to medicine-like but when cultures on YMG/T aged and turned wine-red it became unpleasant sharp. The mycelial scents became stronger on both media with an increase in growth temperature to 28 °C.

All five strains grow on MEA at RT as a first slightly pigmented mycelium. Growing colonies on MEA of four of the strains stained first light yellow, while cultures of strain AscoD1 were stronger yellow from the beginning. Comparably little aerial mycelium was produced by all strains resulting in overall flat colony appearances. Growing colonies had small white fringed borders due to the production of multiple conidiophores with white flocks of masses of dry hyaline conidia. Within 2 to 3 days upon production, conidia separated from conidiophores and fell in larger aggregates onto the surface of the yellowish colonies (Fig. 4a). Per fully grown MEA

(See figure on next page.)

Fig. 3 Mycoparasitic mycelium infested *Agaricus xanthodermus* mushrooms at different developmental stages in a larger disease outbreak in the 3rd week of September of 2015. **a**, **b** Foreign mycelium grew from surrounding moss to a primordium and the stipe base of a young mushroom at the stage of stipe elongation and cap growth. **c** 24 h later the stipe base was surrounded by a thick layer of foreign mycelium, the stipe and cap were enlarged but the cap laid down on the floor due to strong bending of the stipe. **d**–**m** Strong white mycelium found at multiple places in the grass and moss served as infection source of *A. xanthodermus*. Bases of elongating stipes of growing drum-stick-like young mushrooms were covered by a layer of foreign mycelium (**d**, **f**) and the same structures 24 h later photographed from different angles (**e**, **g**). While the yet less infected structure with the foreign mycelium confined only to the stipe base was still erect (**e**), the heavily infected structure with foreign mycelium reaching up to the cap already collapsed (**g**). Infested young mushrooms at the start of partial veil rupture (**h**, **i**). 24 h later, the cap of the mushroom shown in **i** coloured brownish and the rupture of the partial veil blocked. Thick white patches of the pathogen were obvious on the cap surface (see arrow; **j**). Also young stages of opened caps (the arrows mark the skirt-like annulus injured by the infestation) were attacked by mycelium growing upwards the stipe (**k**) and eventually also onto the lamellae (**l**, **m**). Rapid decolourization and mushroom collapse within 24 h resulted from strong pathogen infestation (**l**, **m**). Strong white mycelium found at multiple places in the grass and moss (**n**)


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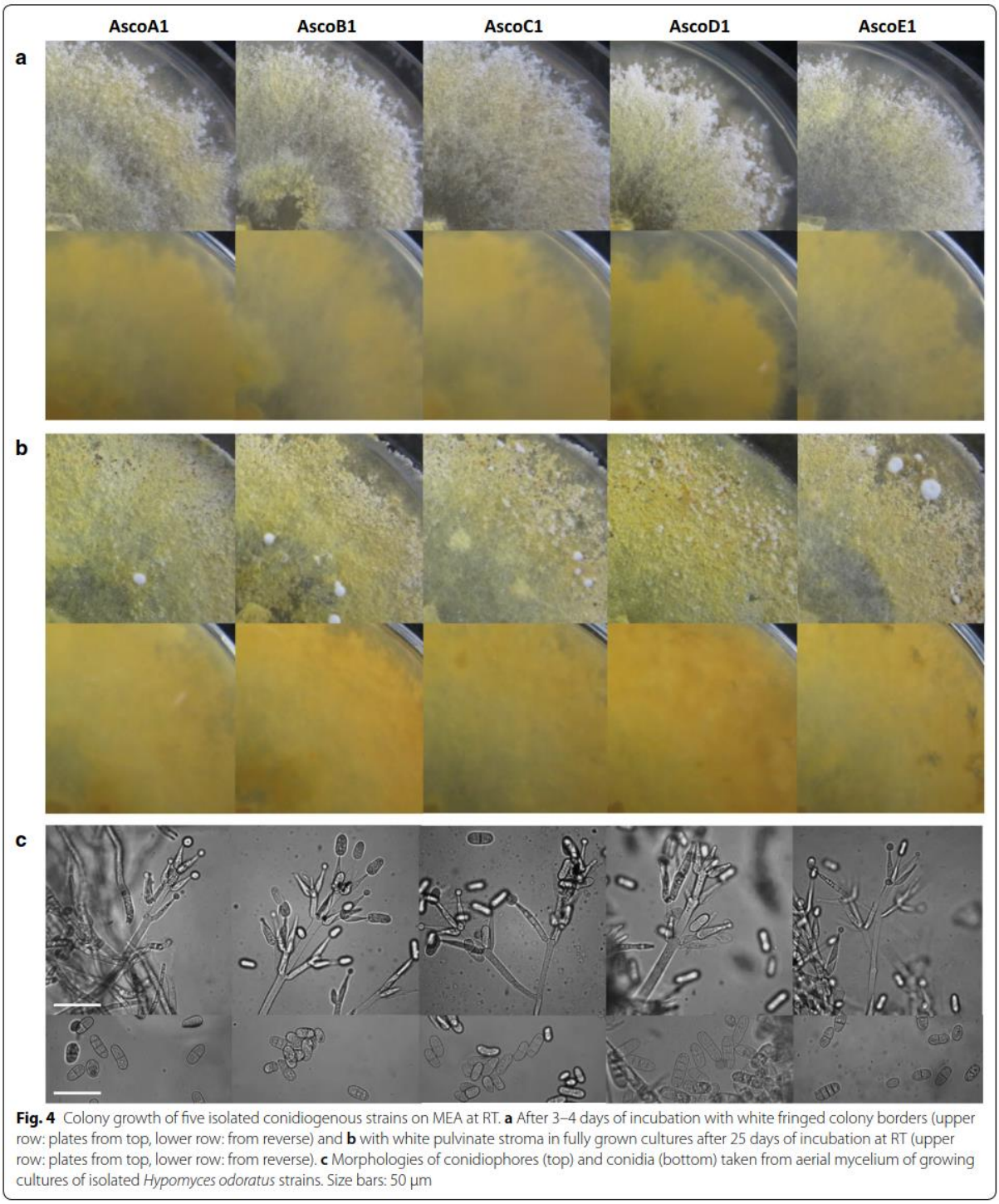

Fig. 4 Colony growth of five isolated conidiogenous strains on MEA at RT. **a** After 3–4 days of incubation with white fringed colony borders (upper row: plates from top, lower row: from reverse) and **b** with white pulvinate stroma in fully grown cultures after 25 days of incubation at RT (upper row: plates from top, lower row: from reverse). **c** Morphologies of conidiophores (top) and conidia (bottom) taken from aerial mycelium of growing cultures of isolated *Hypomyces odoratus* strains. Size bars: 50 µm

Table 1 Features of conidiophores of the five isolated strains grown at RT

Cells	Parameter	AscoA1	AscoB1	AscoC1	AscoD1	AscoE1
Conidiophores	Stem					
	1st order whorls	2–5	2–4	2–4	2–4	2–3
	Whorls with branches	0–3	1–3	0–3	1–3	0–3
	Branches per whorl	0–3	1–3	0–3	1–3	0–3
	1st order branches	0–7	1–4	0–3	1–3	1–3
	1st order branch					
	2nd order whorls	0–4	0–3	0–2	0–3	0–3
	Whorls with branches	0–2	0–1	0–1	0–1	0–1
	Branches per whorl	0–1	0–1	0–1	0–1	0–1
	2nd order branches	2	1	1	1	1
	2nd order branch					
	Whorls	2	1	1	1	1
	Branches total	0–4	0–3	0–2	0–3	0–2
	Whorls total	3–7	1–4	1–5	1–4	1–4
	n	15	9	8	14	7
Ampulliform phialides	Per whorl without branches	2–5	2–5	2–5	2–5	2–5
	Per whorl with branches	2–4	2–3	1–3	1–2	1–2
	Length (µm)	35.8 ± 7.7	35.7 ± 7.4	38.1 ± 8.3	31.6 ± 5.8	32.9 ± 5.7
	Apex width (µm)	2.7 ± 0.4	3.3 ± 0.6	3.4 ± 0.5	3.8 ± 0.6	3.1 ± 0.5
	Width broadest point (µm)	8.1 ± 1.1	8.3 ± 1.4	8.1 ± 0.8	8.7 ± 1.2	8.3 ± 1.2
	Base width (µm)	5.2 ± 1.0	4.4 ± 0.7	5.0 ± 1.0	4.6 ± 1.1	4.6 ± 0.9
	n	27	24	16	20	27
Conidia	No septum					
	Length (µm)	15.3 ± 1.2	15.1 ± 1.4	15.5 ± 1.8	15.4 ± 1.6	16.2 ± 1.0
	Width (µm)	10.7 ± 1.3	10.3 ± 1.3	9.6 ± 1.4	10.0 ± 1.0	11.0 ± 1.3
	n	21	13	16	15	13
	% of total	20.0	14.8	14.4	14.9	13.1
	One septum					
	Length (µm)	20.5 ± 3.0	20.7 ± 3.1	21.2 ± 3.3	21.1 ± 4.8	20.8 ± 3.0
	Width (µm)	10.8 ± 1.2	10.3 ± 1.2	10.0 ± 1.3	10.7 ± 1.2	10.4 ± 1.2
	n	71	64	67	64	68
	% of total	67.6	72.7	60.4	63.4	68.7
	Two septa					
	Length (µm)	24.7 ± 3.8	23.4 ± 1.5	24.4 ± 3.7	26.2 ± 4.4	24.3 ± 2.3
	Width (µm)	11.2 ± 1.4	10.5 ± 0.7	10.3 ± 0.9	11.4 ± 1.3	11.2 ± 1.3
	n	12	9	17	16	16
	% of total	11.4	10.2	15.3	15.8	16.2
	Three septa					
	Length (µm)	26.4	25.3 ± 1.4	31.7 ± 3.3	30.9 ± 4.8	26.6 ± 0.1
	Width (µm)	11.9	11.1 ± 0.3	10.5 ± 0.5	12.7 ± 1.3	12.8 ± 1.0
	n	1	2	11	6	2
	% of total	1.0	2.3	9.9	5.9	2.0
	All					
	Length (µm)	20.0 ± 4.0	20.2 ± 3.6	22.0 ± 5.2	21.7 ± 5.8	20.9 ± 3.5
	Width (µm)	10.8 ± 1.2	10.3 ± 1.2	10.0 ± 1.2	10.8 ± 1.3	10.7 ± 1.3
	n	105	88	111	101	99
Conidia per plate	MEA	$4.4 \pm 1.2 \times 10^7$	$8.3 \pm 4.9 \times 10^7$	$7.9 \pm 2.5 \times 10^7$	$1.8 \pm 1.4 \times 10^7$	$4.4 \pm 1.7 \times 10^7$
	YMG/T	$1.2 \pm 0.6 \times 10^8$	$1.2 \pm 0.2 \times 10^8$	$9.2 \pm 1.3 \times 10^7$	$2.3 \pm 0.1 \times 10^7$	$9.9 \pm 3.9 \times 10^7$

Strains were cultivated for 13 days at RT on MEA and conidiophores were taken for microscopy from the outer white growth zone characterized by flocks of conidia. n = number of structures or cells analyzed. For counting spores per plate, five plates per strains and per medium were inoculated and spores were counted after fully growth of plates at RT

plate, the strains produced between 2×10^7 and 8×10^7 hyaline 0- to 3-septate conidia (Table 1). With time, fully grown colonies turned from slighter yellow to dark yellow (after 4 to 6 days of growth at RT to after about 14 days; when cultured at 28 °C, these processes were 1 to 3 days faster) while strain AscoD1 was still darker pigmented as compared to the others. Cultures appeared to be in a final stage at RT after about 25 days of incubation, for the appearance of mycelium and colony color. The observations on plates cultivated at RT usually lasted up to 36 days as the plates became drier. Mainly the mycelium but also the agar was stained by the strains by yellow pigments. In one experiment when plates were kept for 2 months in very humid conditions, some plates of strains AscoB1 and AscoD1 adopted in the end a mixed yellow-slightly pinkish pigmentation while none of the other strains did change the color. Further in aging MEA cultures after around 3 weeks of incubation, all five strains started to produce hard white patches of dense mycelial pulvinate stroma which increased in numbers with time (between dozens to > 100 per plate). First they were small, less than 0.1 mm in Ø, but with time they could grow to patches of up to 3–4 mm in Ø (Fig. 4b). After around 30 days of cultivation with drying out medium, round dark brown microsclerotia (AscoA1: 0.33 ± 0.08 mm in Ø, n = 16; AscoB1: 0.37 ± 0.08 mm in Ø, n = 13; AscoC1: 0.35 ± 0.08 mm in Ø, n = 14; AscoD1: 0.36 ± 0.07 mm in Ø, n = 17; AscoE1: 0.36 ± 0.07 mm in Ø, n = 15) filled with large round unstained cells formed in aging colonies (not shown). In addition, masses of round chlamydospores (AscoA1: 13.3 ± 1.7 µm in Ø, n = 22; AscoB1: 13.5 ± 1.7 µm in Ø, n = 26; AscoC1: 13.9 ± 1.6 µm in Ø, n = 28; AscoD1: 13.5 ± 2.2 µm in Ø, n = 22; AscoE1: 13.9 ± 1.2 µm in Ø, n = 24) arose in chains from swelling and fragmenting of vegetative hyphal cells (not shown).

Mycelia of all five strains on YMG/T medium were first nearly unpigmented during the fresh growth at RT. The cultures were characterized by loose white fluffy aerial mycelium starting to regularly develop behind the colony growth fronts on the 1-day-old mycelium and to produce conidia over the following days. Spreading in the growing colony outwards from the inoculum, substrate mycelium with the agar began to stain yellowish 1 to 2 days after first aerial mycelium production (at 28 °C 1 or 2 days earlier than at RT), while the yellow colour intensified continuously with further mycelial age in growing. The yellow colour increased in intensity during the further incubation also after plates were fully grown (after 5 and in the case of AscoD1 7 days of incubation) while after about 15 and, in the case of AscoD1, 20 days there was a switch in colour to first light pinkish and later wine-red (Fig. 5). The final cultural stages also appeared to have been reached on YMG/T plates after culturing at RT for about 25 days, followed by only desiccation reactions with continued incubation up to 36 days.

Pink to red medium coloration has been reported before e.g. from older PDA cultures of *Hypomyces*/*Cladobotryum* strains (Back et al. 2010; Carrasco et al. 2016; Muhammad et al. 2019). *Hypomyces*/*Cladobotryum* species are known to produce aurofusarin as pigment (Rogerson and Samuels 1989; Põldmaa 2011; Tamm and Põldmaa 2013; Carrasco et al.

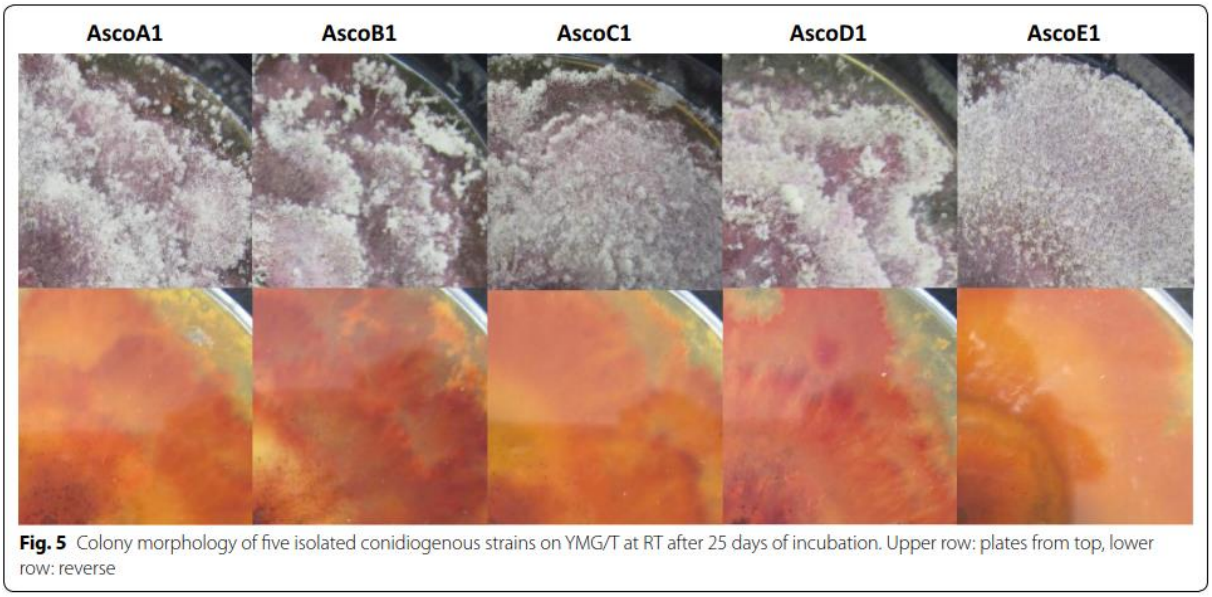

Fig. 5 Colony morphology of five isolated conidiogenous strains on YMG/T at RT after 25 days of incubation. Upper row: plates from top, lower row: reverse

2016) which changes in color from yellow to red depending on the pH (Ashley et al. 1937). We therefore checked the pH in the medium over the time of cultivation. When colonies on YMG/T plates were stained yellowish to dark yellow, the pH in the medium did not much change and was around 5.5 to 6. With onset of pinkish coloration however, the pH increased to values of 6.5 to 7. With increasing colorization when the cultures turned pink to finally wine-red, the pH rendered into the alkaline range to values of around 7 to 7.5 and then to 7.5 to 8. For comparison, the pH in the MEA cultures was in the acidic range with pH 4.5 in slightly yellow cultures and pH 4 and sometimes even pH 3 in dark yellow cultures. In 1-month-old cultures of the stronger yellow cultures of strain AscoD1, the pH raised first slightly to pH 5. In 2-month-old plates of strain AscoD1 and also of strain AscoB1, within a few days under color changes to mixed yellow-pinkish and then yellowish-pink, the pH raised further to 6 to 6.5 and then pH 7. Cultures of strains AscoE1, AscoA1, and finally AscoC1 also increased in pH to 6 but two to several days later, along with color changes into yellow-pinkish.

The pigments in YMG/T cultures stained majorly the submerged mycelial agar layer and to less part the agar beneath. Notably, the colony surfaces remained white in appearance due to the considerable amounts of whitish aerial mycelium with huge amounts of hyaline conidia produced (for spore numbers per fully grown plates see Table 1). The dry conidia assembled into larger flocks on the tips of the conidiophores. During colony growth, thick aerial mycelium arose as high as up to the lids of the Petri dishes, transferring large parts of the clumps of spores onto the plastic surface (not shown). This thick aerial mycelium was longer lasting. After mycelial growth on a plate was completed, and after the change in colour of the substrate mycelium with agar from yellow to wine-red and along with the evaporation of any humidity from the lids of the Petri dishes (after about 25 days of incubation), the aerial mycelium in undisturbed plates collapsed slowly throughout the colony. With opening the lid however, the aerial mycelium collapsed immediately. Eventually, the aggregated conidial clusters fall down from aerial mycelium and lids of Petri dishes in irregular patterns onto the surfaces of the colonies. Spore aggregates collected from agar and from lids of Petri dishes needed harsh forces to separate them into individual cells for counting (Table 1). Furthermore, all strains produced on YMG/T on the surfaces of aging cultures (after about 30 days of cultivation, mainly in the outer regions of colonies) also masses of dark brown microsclerotia which were much more in numbers but of similar sizes than those on MEA (AscoA1: 0.40 ± 0.08 mm in Ø, $n = 15$; AscoB1: 0.39 ± 0.05 mm in Ø, $n = 20$; AscoC1: 0.37 ± 0.05 mm in Ø, $n = 16$; AscoD1: 0.35 ± 0.03 mm in Ø, $n = 20$; AscoE1: 0.32 ± 0.03 mm in Ø, $n = 22$). Aging cultures on YMG/T did not produce white stromas but they gave rise to some chlamydospores resulting in chains from swellings and fragmenting of hyphal cells (AscoA1: 13.3 ± 1.4 µm in Ø, $n = 22$; AscoB1: 13.5 ± 1.5 µm in Ø, $n = 21$; AscoC1: 13.2 ± 1.8 µm in Ø, $n = 20$; AscoD1: 12.9 ± 1.6 µm in Ø, $n = 24$; AscoE1: 13.4 ± 1.7 µm in Ø, $n = 23$).

Species identification

Conidiophores with conidia were analyzed in more detail from the strains grown on MEA (Table 1). Conidiophores with conidia on mycelia of all five strains were verticillate as typical for the *Hypomyces*/*Cladobotryum* genus. Conidiophores were separated over their length into several cells. They had stems with 2 to 5 whorls with up to 5 phialides each and they were usually irregularly branched, with 1st order sidebranches arising in numbers between 1 and 3 among some phialides at the lower whorls of the stem and with some 2nd order sidebranches arising at the lower whorls of 1st order sidebranches (Fig. 4c; Table 1). The up to 5 phialides per whorl were successively produced (Fig. 4c and see also Fig. 2j–n) and grew into lengths of > 30 µm (Table 1). The ampulliform phialides tapered from broader regions (width > 8 µm) shortly above their bases (width ca. 5 µm) to slim blunt apexes of widths of around 3 µm. Conidiospores were produced at the simple tips of the ampulliform phialides in monoblastic mode (Fig. 4). First, the young blastospores were equally swelling but with increase in size, they often buckled with further growth to the lateral side (Fig. 4c and see also Fig. 2j–n). Released conidia were hyaline, oblong in shape with rounded edges, had sometimes visibly a hilum at the basal ends and different numbers of septa (Fig. 4c). The majority of conidia of all strains (60–72%) were two-celled. However, strain AscoA1 had more non-septated spores (20%, comparably to the mycelium grown on the infected cap from which AscoA1 was isolated, please see above) than the other strains. Strains AscoC1, AscoD1 and AscoE1 had higher numbers of spores with two or also three septa (in sum 18.2 to 25.2%; Table 1). Between the strains, there were some measured minor size variations of the spores (Table 1). Spore lengths ranged from 13.2 to 33.0 µm (AscoA1), 13.4 to 28.8 µm (AscoB1), 11.7 to 36.5 µm (AscoC1) and 13.6 to 37.4 µm (AscoD1), 13.8 to 28.2 µm (AscoE1). In tendency, spore lengths and widths increased with numbers of septa (Table 1). The strains AscoC1 and AscoD1 with higher percentages of both 3- and 4-celled spores had thus a bit more of the longer spores as compared to the other three strains.

The general morphological parameters of conidiophores and conidia of the five strains matched descriptions of *H. odoratus*/*C. mycophilum* in the literature (Arnold 1963; Gams and Hoozemans 1970; Cole and Kendrick 1971; Gray and Morgan-Jones 1980; Back et al. 2012b; Tamm and Põldmaa 2013; Gea et al. 2014). The occurrence of microsclerotia and presence of round chlamydospores in the aged mycelium and yellow to red stained colonies with a strong smell on nutrient-rich YMG/T medium also concur with descriptions of *H. odoratus*/*C. mycophilum* (Helfer 1991; McKay et al. 1999; Grogan 2006; Gea et al. 2014; Carrasco et al. 2017). In other instances reported in the literature, no peculiar stronger smell was noted by isolates of *H. odoratus* (McKay et al. 1999; Gea et al. 2019), similar as in this study when growing the five strains on MEA plates.

We amplified and sequenced the 530 bp long ITS rDNA regions of all five isolates (KX098646-KX098650). The sequences of the strains are identical to each other and 99–100% identical to the ITS sequences of *H. odoratus* (FN859435; Põldmaa 2011) and *C. mycophilum* strains (JF693809, JF505112, AB527074, JQ004737, Y17094, Y17095, KP267826) shown to infect mushrooms in culture (McKay et al. 1999; Back et al. 2010; Kim et al. 2012; Carrasco et al. 2016; Gea et al. 2016). In contrast, they were only 98% identical to *H. rosellus* (FN859440, FN859442; Põldmaa 2011) and *C. dendroides* ITS sequences (Y17090, Y17092; McKay et al. 1999). Three subgroups of ITS fragments of *H. odoratus*/*C. mycophilum* strains are distinguished (McKay et al. 1999; Tamm and Põldmaa 2013; Gea et al. 2016) by a 1 base pair difference in the ITS1 sequence (base A at position 80 in subgroup 1/2 versus G in subgroup 3) and 1 or 2 base pair differences in the ITS2 region (base T at position 390 in subgroup 1/2 versus C in subgroup 3; base C at position 507 in subgroup 1 versus T in subgroups 2/3). Our sequences fall into *H. odoratus*/*C. mycophilum* subgroup 3 together with strains from Ireland, Estonia, Russia and the USA (McKay et al. 1999; Tamm and Põldmaa 2013).

Fruiting body infection tests

All five isolated *H. odoratus* strains were tested on complete or halved commercial mushrooms of *A. bisporus*. All five strains regularly infected all commercial *A. bisporus* mushrooms, regardless of whether the inoculum was placed onto a non-injured stipe or cap or onto cuts of stipes and caps of sliced mushrooms (Fig. 6). After placing fresh mycelial MEA agar blocks of the ascomycetes onto a stipe or a cap region of *A. bisporus*, the hyphae started to grow (1st day). When intact pilei were inoculated, the surrounding *A. bisporus* cap region in consequence caved in with a growing pathogen, resulting in a visible dent with the inoculum in the center (2nd day). Later on, regardless of place of inoculation, the hyphae spread over all the mushrooms (3rd day) and produced huge white-coloured masses of conidia. During this time, the pathogens were very aggressive and appeared to absorb nutrients from the mushrooms, while the mushrooms reduced in sizes and weights, changed in color from white to light brownish, and became watery-rotten (5th to 6th day). All infectious strains, AscoA1 to AscoE1 gave rise to black microsclerotia on the overgrown surface of the *A. bisporus* samples (not further shown).

In contrast to fruiting bodies of *A. bisporus*, strains AscoA1, AscoB1 and AscoD1 did not grow much on commercial *P. ostreatus* fruiting bodies, neither when inoculated on the cap surface nor on the lamellae nor on the stipes. *P. ostreatus* thus showed resistance to the ascomycetes. At most, the *H. odoratus* hyphae spread

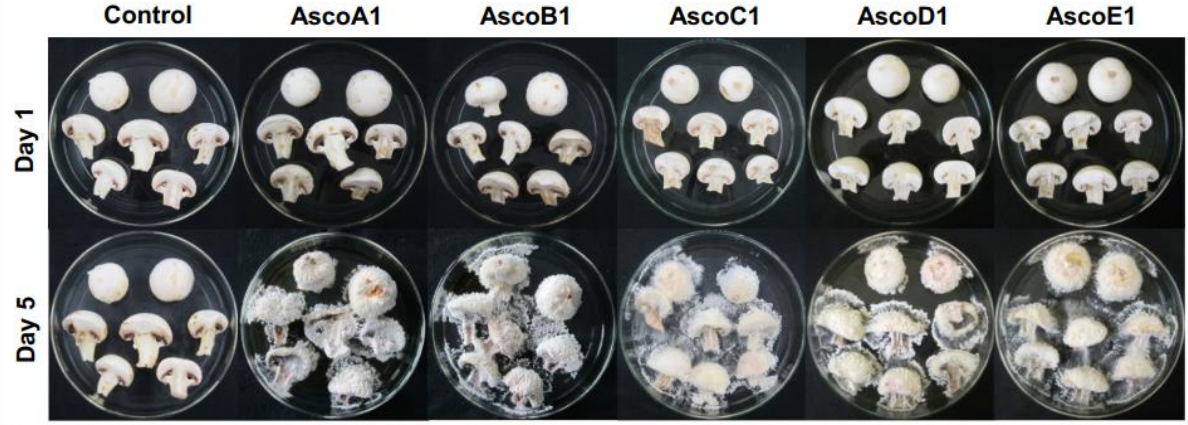

Fig. 6 Infection test with commercial *A. bisporus* fruiting bodies. Top row: day of inoculation, bottom: mushrooms after 5 days of cultivation at RT

from the inocula only over very small areas of the mushrooms without obvious symptoms of disease but not over the complete mushrooms. Importantly, when placing a mycelial agar block at the centre of mushroom caps, the *H. odoratus* mycelia started to grow out more likely on the side of the agar blocks towards the stipe region than upwards of the cap region. Only strains AscoC1 and AscoE1 showed in exceptional cases some infection by mycelial growth on the base of *P. ostreatus* stipes (noticed on each 2 of 25 in total tested fruiting bodies; Fig. 7). Still, also in these rare cases the aggressiveness towards *P. ostreatus* was comparatively low with few amounts of conidia formed by the growing mycelium. As a further interesting observation, *P. ostreatus* tissue growth (growing hyphae had clamps) occurred at the stipe margins, cap margins and the lamellae of inoculated mushrooms and also of uninfected controls. Such growth probably strengthened the mushrooms and helped in resistance against the ascomycetes.

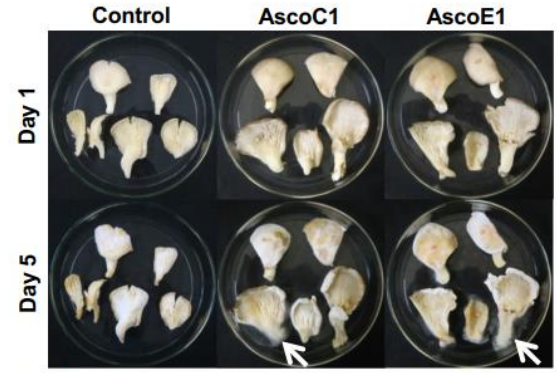

Fig. 7 Infection test with commercial *P. ostreatus* fruiting bodies. Top: day of inoculation, bottom: mushrooms after 5 days of cultivation at RT. Arrows mark infested stipe regions. Other mycelial outgrowth noticed came from *P. ostreatus*

We also tested in similar manner *A. xanthodermus* mushrooms collected from the wild (KX098653) with *H. odoratus* strains AscoA1, AscoB1 and AscoC1. Young mushrooms with still closed caps were infected by agar pieces with the ascomycetes positioned either at the stipes or the caps. Within 6 days at RT, mycelium from the inocula of the stipes grow onto the darkened gills of the matured mushrooms while stipes with the annuli degenerated whereas the caps still remained in good shape (Fig. 8). In contrast upon inoculation of pilei, tissues overgrown by the pathogens shrivelled under appearance of liquid yellow–brown droplets on the cap surface and the caps degenerated quickly (not shown). Similar observations were made, when *A. arvensis* mushrooms from the wild (KX098654) were inoculated with strain AscoC1 (not further shown).

Infection tests of growing mycelial cultures

The infection potential of the five mycopathogenic strains was further tested against mycelial cultures of *A. xanthodermus*, *P. ostreatus* and *C. cinerea*, respectively. For

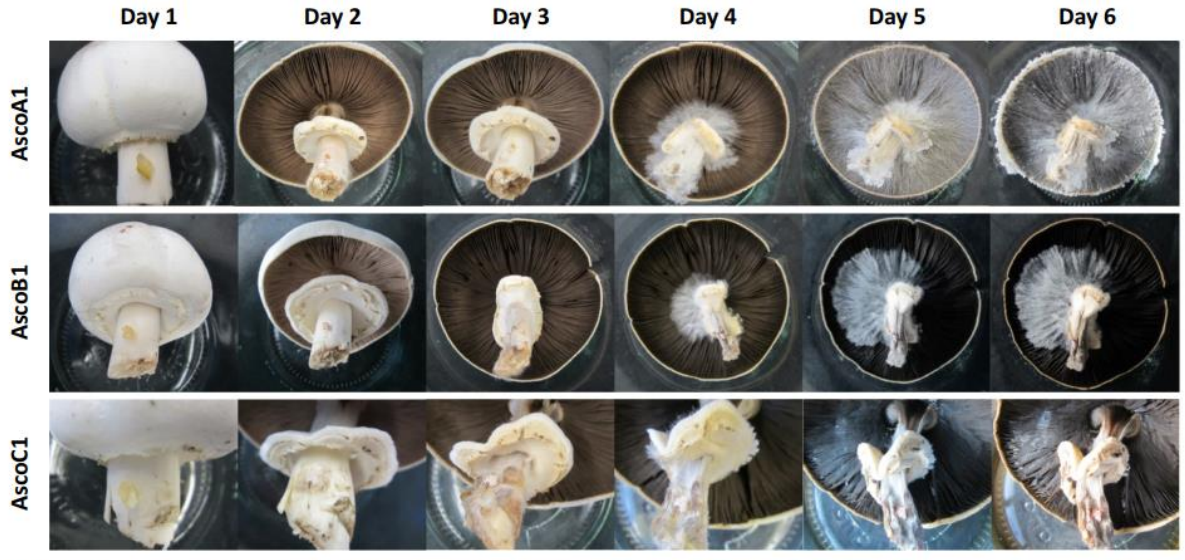

Fig. 8 Young *A. xanthodermus* fruiting bodies inoculated with *H. odoratus* strains. Upon stipe inoculation of mushrooms kept within sterile glass jars, the progress in mushroom development and pathogen infestation at RT was photographed once per day

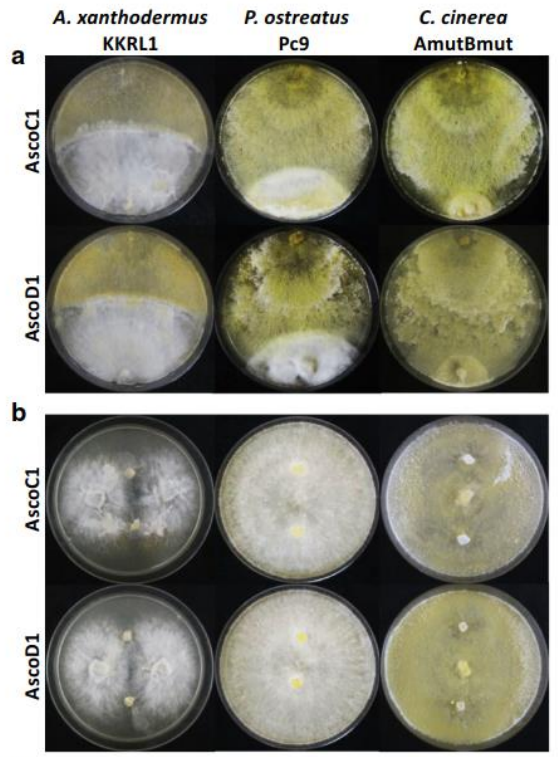

Fig. 9 Mycelial growth confrontation tests (top rows) and grown mycelium challenge tests (bottom rows) of *A. xanthodermus* KKRL1, *P. ostreatus* Pc9 and *C. cinerea* AmutBmut by *H. odoratus* isolates on MEA, photographed after 20 days of incubation at RT after inoculation of the pathogen

mycelial confrontation tests, we inoculated mycelial agar plugs from a test fungus and a respective mycopathogen onto MEA plates at opposite edges of Petri dishes (Fig. 9a).

The dikaryotic *A. xanthodermus* isolate KKLR1 grew slowly on MEA at RT (about 1.4±0.1 mm/day) with a flat cottony-dense white-colored mycelium growing in a loosely organized dense fan-strand pattern. Therefore for the mycelial confrontation tests, the species was inoculated at the edges of Petri plates and cultivated 15 days prior to the inoculation of the mycopathogens at the edges of the opposite side of the plates (see examples of AscoC1 and AscoD1 confrontation tests in Fig. 9a). Once the mycelial growth fronts of the two species reached each other in further incubation (after about 12 to 18 days of incubation), both *H. odoratus* as *A. xanthodermus* colonies were stopped in further growth at the confrontation zones with some combat reactions at the colony borders. The flat dense white mycelium of *A. xanthodermus* showed some resistance against overgrowth by the mycopathogens. However, bunches of sporulating *H. odoratus* aerial hyphae were observed to grow over the *A. xanthodermus* colonies. The mycopathogens produced huge amounts of dry aerial conidia which landed in clumps also on *A. xanthodermus* mycelium but without recognisable germination. Unevenly distributed, ribbon or thread-like mycelial aggregates appeared as deformations in the *A. xanthodermus* colonies (Fig. 9a) and, with longer incubation time, on their surfaces also faint zones of yellowish-stained aerial *H. odoratus* mycelium overlaying the basidiomycete. Further in older cultures, *A. xanthodermus* appeared to produce a new thicker white aerial mycelium at the colony borderlines which grow a few mm to over 1 cm into the zones of the opponent colonies and covered the edges of the *H. odoratus* colonies (Fig. 9a). *A. xanthodermus* in single culture on MEA plates rarely produced clamp cells at its hyphae. However, some clamp cells were also observed on growth fronts of the new white mycelium overgrowing the *H. odoratus* mycelium, supporting that the basidiomycete survived and revived in the dual cultures.

Observation with *A. xanthodermus* strain KKRL1 on YMG/T plates were in parts similar. *A. xanthodermus* colonies were for 4 weeks pregrown into colonies of ca. 3 to 3.5 cm in diameter prior to inoculation of the mycopathogens. The overgrowth of *A. xanthodermus* colonies by aerial mycelium of *H. odoratus* strains was then stronger with fast growing thick bunches of conidiogenous hyphae attracted to and overlaying densely the *A. xanthodermus* mycelium. Masses of white clumps of conidia were produced on the plate and fell over the covered *A. xanthodermus* mycelium. The areas on the plates with the grown *H. odoratum* colonies turned lilac-red unlike the unstained agar underneath the covered *A. xanthodermus* mycelium. On the reverse of the cultures, dense assemblies of many submerged brown microsclerotia filled with large round cells appeared underneath the overgrown *A. xanthodermus* colonies and often also underneath in the agar zone around the *H. odoratum* inocula. Microsclerotia were also observed above the overgrown *A. xanthodermus* mycelium. Mycelial samples from *A. xanthodermus* colonies overgrown by *H. odoratus* strains revealed under the microscope single-celled chlamydospores and many conidia of the ascomycete. No new outgrowth of mycelium was observed on plates from the *A. xanthodermus* colonies which might not have been strong enough for such activity if still alive.

In mycelial confrontation experiments with *P. ostreatus* monokaryon Pc9 on MEA, the mycopathogens grow also faster than strain Pc9 and the yellow stained colonies produced huge amounts of conidia (Fig. 9a). Where the growing species met, combat reactions resulted, leading to a margin of denser white mycelium formed by the Pc9 strain as delineation from the yellowish mycopathogens.

However, very long conidiogenous hyphae of the mycopathogens loosely overgrow the *P. ostreatus* colonies and conidia were produced in small white flocks especially at the plastic edges of Petri dishes above the *P. ostreatus* colonies. With time, some mycelial patches above the Pc9 mycelium stained yellowish. In mycelial samples from the *P. ostreatus* colonies under the microscope, no or only few mostly two-celled conidia were detected. From the reverse of plates, sometimes thinner necrotic areas became visible in the unstained *P. ostreatus* colonies of older plates (1 month) while in other areas and cases the mycelium grew denser. In mycelial confrontation tests on YMG/T medium with much more production of aerial *H. odoratus* mycelium, reactions were much stronger. Conidiogenous hyphae were strongly attracted in growth to the Pc9 colonies for covering the colonies, and masses of conidia in many very large aggregates were produced above the *P. ostreatus* mycelium. On the reverse side of cultures, production of some brown microsclerotia were observed underneath in the unstained *P. ostreatus* colonies, while all other parts of the medium covered with mycelium of *H. odoratum* strains were stained pink to lilac-red.

The third species tested, *C. cinerea* homokaryon AmutBmut, in contrast was not able on MEA to defeat any of the five mycopathogens in combat reactions. *C. cinerea* was easily overgrown by all five *H. odoratum* strains when the growing colonies were confronted with each other. *H. odoratum* strains formed regular yellow colonies with also regular conidia production over the whole plates including the growth zones of *C. cinerea* (Fig. 9a). Because *C. cinerea* AmutBmut is a self-compatible homokaryon by mutations in its mating type loci, it forms clamp cells at its hyphal septa (Swamy et al. 1984). In mycelial samples of overgrown *C. cinerea* colonies underneath the microscope, clamp cells at hyphal septa were only exceptionally seen, suggesting that at least parts of the existing mycelium probably came from the mycopathogens. White pulvinate stroma developed on top of the *C. cinerea* colonies in 1 month old MEA plates. In confrontation tests on YMG/T plates, *C. cinerea* colonies of equal growth age than the mycopathogens were also quickly overgrown by the *H. odoratus* strains through outgrowth of dense fast growing hyphal fans of *H. odoratus* mycelium being attracted to the smaller *C. cinerea* colonies. Masses of conidia were produced on top of the *C. cinerea* colonies while the edges of the colonies were less sharp and, as seen on the reverse of the plates, the pink *H. odoratus* staining diffused into the borders of the *C. cinerea* areas. In confrontation tests with larger pregrown *C. cinerea* colonies (inoculated at edges of plates and incubated 4 days at 37 °C prior to inoculation of *H. odoratus* strains and transfer to RT), defense was stronger with sharper colony borders against the mycopathogens. However, the surfaces of the *C. cinerea* mycelia were also quickly covered by fast growing conidiophores and masses of conidia.

Infection tests of grown mycelial cultures

In other experimental series to challenge a grown test fungus, mycelial agar plugs of mycopathogens were placed at 2 cm distance from inocula on the top of the completely grown basidiomycete mycelium (Fig. 9b). In the grown mycelium challenge tests with already established mycelium (grown with two inocula per MEA plate for 20 days at RT), the mycelium of *A. xanthodermus* could well resist the five mycopathogens. In the basidiomycete colonies, some white thread- or ribbon-like or in addition also globular compact mycelial aggregates were detected as reactions (Fig. 9b), similar as before in the confrontations tests on the same medium. Clamp cells were detected in the mycelium. When using slowly growing *A. xanthodermus* colonies on YMG/T medium for surface inoculation with the *H. odoratus* strains, outgrowth of the mycopathogenic strains on the basidiomycetous colonies was impeded unlike on free agar surfaces.

In mycelial challenge tests, we also noticed on both media little or no outgrowth of mycopathogens when inoculated on the top of established Pc9 mycelium (Fig. 9b). Only sometimes in closer vicinity of the inocula of *H. odoratus* strains, zones of some denser mycelium or some minor necrotic reaction were observed. Like the fruiting bodies of the species, also the vegetative mycelium of *P. ostreatus* exerts thus some but not full resistance against the mycopathogens.

No much outgrowth of the mycopathogens was then observed when inoculated on YMG/T plates that were fully grown with dense *C. cinerea* mycelium (inoculated in the middle of plates and grown for 6 days at 37 °C). When inoculated on top of established but less dense *C. cinerea* mycelium on fully grown MEA plates, the *H. odoratus* strains however could easily overgrow the *C. cinerea* mycelium and surfaces of colonies stained yellowish by the presence of the mycopathogen (Fig. 9b). Necrotic areas became visible in the *C. cinerea* colonies underneath by thinned mycelium around the inocula of the mycopathogens in mycelial challenging tests on MEA (Fig. 9b).

Discussion

In this study, we report observations on mycoparasitic infections of *A. xanthodermus* mushrooms in nature. We have observed unimpeded developing mushrooms in years 2012 to 2017, variably in the months June, August, September and, in 2015, also in November, usually after comfortably warm weather conditions. Induction of

fruiting body development of *A. xanthodermus* seems to need sufficient previous rainfall possibly to both, moisture the ground and create higher humidity in the air. Consequential to the rainfall, a drop in air temperature likely will also be favourable for induction of fruiting. Fruiting body development proceeds from ball-like primordia over drum-stick-shaped, still closed young mushrooms to mature mushrooms with open umbrellas and first pinkish and then brown lamella (Fig. 1). The speed of development from primordia to fruiting body maturation seems to depend also on the temperature and took in our observations between 10–13 and 6–8 days at colder and warmer temperature (around 12–15 °C and 18–22 °C), respectively. Mature fruiting bodies can last further 10 to 15 days.

Infections of *A. xanthodermus* fruiting bodies by *H. odoratus* cobweb in nature

Interestingly, in a first flush of mushrooms in early September 2015, one split fruiting body was visibly affected by a fungal infestation (Figs. 1e–l, 2). While we do not know whether this single mushroom was injured prior to infestation or whether infestation resulted in the injury, our observations from infections in the subsequent flush of mushrooms suggest that injury is not a premise of infection of the species in nature. Moreover, we observed that all stages of fruiting body development were susceptible for the mycopathogen (Fig. 3). The infections on *A. xanthodermus* were identified by morphological means (conidiophores and conidia) and ITS sequencing as *H. odoratus* (anamorph *C. mycophilum*). This fungus is one of a group of closely related species which can cause cobweb disease of cultivated mushrooms such as the edible species *A. bisporus*, *P. eryngii* and *P. ostreatus* (see e.g. Back et al. 2012b; Tamm and Põldmaa 2013; Gea et al. 2014, 2016, 2019; Carrasco et al. 2016; Chakwiya et al. 2019). The species proliferates also on mushroom substrates (Grogan 2006; Carrasco et al. 2016; Gea et al. 2016, 2019) and has also been encountered on the polypores *Ganoderma lucidum* (Zuo et al. 2016) and *Polyporus* sp. in culture (Rogerson and Samuels 1994). In commercial button mushroom cultures, any mushrooms encountered will be engulfed by the mycopathogen with radial outgrowth of mycelium on the substrate (Grogan 2006; Muhammad et al. 2019). We have observed similar events in nature with the mycopathogens growing from the surroundings (decayed fungal material, decaying grass/moss, soil) onto the stipes of nearby developing *A. xanthodermus* structures (Fig. 3).

Spread of *H. odoratus* cobweb clones in nature

Cobweb disease can be spread by airborne conidia. In mushroom-growing rooms, the large conidia are released from spore clusters on the colonies into the air by physical disturbances such as by watering. When subsequently landing and germinating on mushrooms, disease symptoms can be incurred (Dar 1997; Adie et al. 2006; Grogan 2006). Following initially a single infested mushroom (Figs. 1e–l, 2), we observed a larger outbreak of disease in nature after heavy rainfalls in the 3rd week of September 2015 in the 2nd flush of mushrooms of *A. xanthodermus* (Fig. 3). It is thus possible that the rainfall helped to distribute conidia from the place of the previous single mushroom infestation over the larger area, in addition to the general promotion of host and pathogen growth by providing good levels of humidity through rainfall to both. *H. odoratus* conidia do not survive long under dry conditions (Lane et al. 1991) and high humidity is needed for dispersal and germination (Carrasco et al. 2016, 2017). Attack of *A. bisporus* by *H. odoratus* in commercial cultures can happen at any stage in the fruiting body development (Carrasco et al. 2017; Chakwiya et al. 2019) while infections tend to become more severe on the crop in later flushes at longer time of cultivation and during autumn and winter cycles with increasing conidia numbers (Carrasco et al. 2016, 2017). Our observations on *A. xanthodermus* in nature resemble the reports on disease on *A. bisporus* in commercial mushroom production.

While *H. odoratum* is shown to produce perithecia with ascospores in culture (Arnold 1963; Põldmaa 2011; Tamm and Põldmaa 2013), it is not known to do so in nature. Clonality is expected to occur in nature of asexually reproducing *Hypomyces* species in course of spreading of conidia as a major mode of reproductive distribution (McKay et al. 1999; Grogan and Gaze 2000; Valdez and Douhan 2012; Tamm and Põldmaa 2013; Carrasco et al. 2017; Chakwiya et al. 2019). We isolated five *H. odoratum* strains from a close neighbourhood, three of a same infested fruiting body (AscoA1, AscoB1, AscoC1) but of different mushroom organs (from cap and stipe, respectively). Another isolate (AscoE1) came from a decaying stipe of a later infested mushroom. Their properties were very similar, in measurements only distinct in some minor details. The 5th strain (AscoD1) isolated from grass/moss was more different from the other four such as by slower growth speed, a stronger yellow colony colour and by lower spore production. This might suggest that they are not (all) clonal in relation to each other. Larger population field studies on *H. odoratus* in nature are currently missing in order to know how much genetic diversity exists in natural populations and whether sexual reproduction and recombination occurs in nature. Nearly identical clones have been isolated from commercial *A. bisporus* cultivations in different European countries and other continents. Using worldwide

the same *A. bisporus* production strain and spawn and casing soils from same sources, this could however relate to human activities in mushroom cultivations if hygienic conditions were not strictly kept. Further alternative sources of primary infections in commercial mushroom cultures were by human movements and other material transport (Carrasco et al. 2017; Chakwiya et al. 2019). In contrast, clones from mushroom farms have in some instances been interlinked to local populations in nature (Tamm and Põldmaa 2013). There is thus also a possible danger for introduction of the pathogens into mushroom farms newly from the nature.

Outbreaks and host range of *H. odoratum* cobweb

Most of the present knowledge on the species *H. odoratus/C. mycophilum* comes from cobweb outbreaks experienced in newer time in commercial mushroom cultivations (see "Introduction"; Grogan 2006; Tamm and Põldmaa 2013). In essence, cobweb disease in mushroom cultures is caused by different species and up to recently, there was much confusion on species identities. *H. odoratus/C. mycophilum* was often mistaken by *H. rosellus*, a related species with similar disease symptoms. *H. rosellus* has however distinct conidiophores with a rachis at the apex of phialides, produces only two-celled conidia, has a more confined host-range and appears to be less often prevalent in the wild. In addition, the two species differ in their ITS sequences allowing to distinguish the two species further by molecular data why several misidentified strains were later reassigned to *H. odoratus/C. mycophilum* (McKay et al. 1999; Tamm and Põldmaa 2013). Our morphological and molecular data define the five strains isolated in this study from the wild clearly as *H. odoratus*.

H. odoratus has a very broad host range on mushrooms growing in nature in temperate regions (Tamm and Põldmaa 2013). Incidences of infections on agaric fruiting bodies in the wild have sporadically been recorded before for *A. xanthodermus*, *Armillaria mellea*, *Calocybe gambosa*, *Cortinarius collinitus* var. *mucosis*, *Enteloma clypeatum*, *Hebeloma* sp., *Hygrophorus camarophyllus*, *Inocybe* sp., *Lycoperdon pyriforme*, *Megacollybia platyphylla*, *Mycena galericulata*, *Oudemannsiella platyphylla*, *Pholiota* sp., *Pseudoclitocybe cyathiformis*, and *Tricholoma terreum* as well as occurrence on soil, leaf litter and rotting wood (Arnold 1963; Gams and Hoozemans 1970; Helfer 1991; Rogerson and Samuels 1994; Tamm and Põldmaa 2013). *H. odoratus* is considered to be agaricicolous (Rogerson and Samuels 1994) whereas other *Hypomyces*/*Cladobotryum* species are specified as boleticolous and polyporicolous (Rogerson and Samuels 1989, 1993; Tamm and Põldmaa 2013). However, *Coniophora* sp., *Suillus aeruginascens* and *Suillus bovinus* from the *Boletales* (Arnold 1963; Rogerson and Samuels 1994; Tamm and Põldmaa 2013), *Albatrellus* sp., *Lactarius mitissimus*, *Lactarius deliciosus*, *Lactarius quietus*, *Lactarius* cf. *vellereus*, *Russula virescens*, *Russula* sp., and *Stereum sanguinolentum* from the *Russuales* (Arnold 1963; Gams and Hoozemans 1970; Helfer 1991; Tamm and Põldmaa 2013), and *Trametes versicolor* from the *Polyporales* (Gray and Morgan-Jones 1980) are further named as potential hosts for *H. odoratus* in nature, as well as *Cantharellus cibarius* from the *Cantharellales*, *Gloeophyllum sepiarium* from the *Gloeophyllales*, and *Clavariadelphus truncatus* from the *Gomphales* (Helfer 1991). Newer observations on the species in nature with molecular identification would be helpful to unambiguously confirm these claims.

Other than the many incidences in commercial mushroom cultivations and the mostly older reports on occasional fungal collections in the wild, little is so far known on the ecology of necrotrophic *Hypomyces* species such as *H. odoratus* in nature. Our observations in nature and the infection tests in the laboratory confirm *A. xanthodermus* fruiting bodies to be susceptible to *H. odoratus*. The host range of the five isolated strains does not restrict to *A. xanthodermus* but include further *Agaricus* species. The strains grew on and quickly decayed commercial fruiting bodies of *A. bisporus*, in accordance with the various reports in the literature on occurrence of the species on the white button mushroom in cultivation (see "Introduction"; Grogan 2006; Tamm and Põldmaa 2013). The host range of the five strains extends also onto mushrooms of an *Agaricus* sp. from the section *Arvenses* but not particularly to fruiting bodies of *P. ostreatus*. Resistance against *H. odoratus* has been reported from infection tests for *Hypsizygus marmoreus* fruiting bodies (Back et al. 2012a, b, 2015) whereas *F. velutipes* (Back et al. 2012b), *G. lucidum* (Zuo et al. 2016), *P. eryngii* (Back et al. 2012b; Kim et al. 2014; Gea et al. 2011, 2014, 2016, 2017) and *P. ostreatus* (Pérez-Silva and Guevara 1999; Gea et al. 2019) were found to be (partially) susceptible. However, the place of inoculation can play a role. Upper parts of intact caps of *P. eryngii* were thus relatively resistant against *H. odoratus* infection while the pathogen could effectively attack mushrooms of the species through cuts (Gea et al. 2014, 2016). A recent report on infestation of *P. ostreatus* by *H. odoratus* revealed further that the bases of fruiting bodies of this species can be more sensitive against infections by the pathogen (Gea et al. 2019), similarly to our own observations on rare events of overgrowth of mushroom stipes of *P. ostreatus* (Fig. 9).

Mycelial proliferation of *P. eryngii* is hindered by *H. odoratus* and the species is attacked by the pathogen at any cultivation stage (Kim et al. 2014). Differently to

the fruiting bodies, mycelium of *A. bisporus* has been reported to be resistant e.g. for the wet bubble disease inducer *H. perniciosus* (Zhang et al. 2017) while the dry bubble inducer *L. fungicola* has variably been found to attack or not attack host mycelium (Dragt et al. 1996; Calonje et al. 2000; Shamshad et al. 2009) and the cobweb inducer *Cladobotryum varium* overgrow with time cultures of the basidiomycete and caused necrosis (Gray and Morgan-Jones 1981). Furthermore shown in this study, in mycelial confrontations with growing or grown *A. xanthodermus* and *P. ostreatus* cultures, the five *H. odoratus* isolates here were not or not very aggressive with both species. In contrast, the strains more strongly attacked mycelial *C. cinerea* colonies. This latter species is a dung fungus that likes higher temperatures around 37 °C best for growth (Kües 2000) while it is poorly growing at lower temperature ranges such as RT (Fig. 9). Strains of the temperate species *H. odoratus* grow in temperature ranges of 5 to 25 °C and only very poorly at warmer temperatures up to 28 °C (Back et al. 2012b). As seen in Fig. 3, strains of *H. odoratus* proliferate in nature from soil, plant litter and former mushroom residues onto their hosts. The two fungi *C. cinerea* and *H. odoratus* may live under quite different environmental circumstances and ecological niches why a species like *C. cinerea* with higher temperature preferences might not have developed a mycelial growth resistance at lower temperature towards this particular pathogen. As also seen in this study, *H. odoratus* does not generally infect all Agarics (Fig. 5) although the mycopathogen has an apparent preference for them. Other parasitic *Hypomyces* species appear to preferentially attack polypores and boletes (Rogerson and Samuels 1989, 1993). The broader host range is one criterium to distinguish *Hypomyces* species, temperature preferences another. *H. odoratus* and the also agaricicolous *H. rosellus* are adapted to temperate regions, whereas other *Hypomyces* species are found on mushrooms in the tropics and subtropics (Põldmaa 2011; Tamm and Põldmaa 2013). *C. cinerea* is an edible mushroom cultivated in some tropical countries including Thailand (Kües et al. 2007) and it could be of interest to test whether the species is better resistant against tropical *Hypomyces* species.

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Authors' contributions
KL, WK and UK observed mushrooms in nature, designed experiments, performed mycelial confrontation tests and analyzed data. KL performed most outdoor research, mushroom infection tests and strain isolations. WK did ITS analyses and provided most of the cell measurements. KL wrote a first draft of the paper. UK, KL and WK revised the manuscript. All authors read and approved the final manuscript.

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All authors declare that they have no conflict of interest associated with this work.

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Publisher's Note

Chapter 5

Bulk Isolation of Basidiospores from Wild Mushrooms by Electrostatic Attraction with Low Risk of Microbial Contamination

This chapter represents a peer-reviewed original paper authored by K. Lakkireddy and U. Kües published in the open access journal AMB Express (2017) 7:28 with the DOI 10.1186/s13568-017-0326-0.

The paper describes that ballistic basidiospores from 66 different species of *Agaricomycetes* are attracted against gravity to electrostatic charged plastic surfaces. Using this feature, a new method for basidiospore formation was developed that helps to harvest bulks of spores from mushrooms collected from the wild which rarely contain contaminations by cells of other fungi or bacteria.

Authors´ contributions are listed in the paper under the declarations that follow the discussion section of the work.

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AMB Express

ORIGINAL ARTICLE

Open Access

Bulk isolation of basidiospores from wild mushrooms by electrostatic attraction with low risk of microbial contaminations

Kiran Lakkireddy[1,2] and Ursula Kües[1,2*]

Abstract

The basidiospores of most *Agaricomycetes* are ballistospores. They are propelled off from their basidia at maturity when Buller's drop develops at high humidity at the hilar spore appendix and fuses with a liquid film formed on the adaxial side of the spore. Spores are catapulted into the free air space between hymenia and fall then out of the mushroom's cap by gravity. Here we show for 66 different species that ballistospores from mushrooms can be attracted against gravity to electrostatic charged plastic surfaces. Charges on basidiospores can influence this effect. We used this feature to selectively collect basidiospores in sterile plastic Petri-dish lids from mushrooms which were positioned upside-down onto wet paper tissues for spore release into the air. Bulks of 10^4 to $>10^7$ spores were obtained overnight in the plastic lids above the reversed fruiting bodies, between 10^4 and 10^6 spores already after 2–4 h incubation. In plating tests on agar medium, we rarely observed in the harvested spore solutions contaminations by other fungi (mostly none to up to in 10% of samples in different test series) and infrequently by bacteria (in between 0 and 22% of samples of test series) which could mostly be suppressed by bactericides. We thus show that it is possible to obtain clean basidiospore samples from wild mushrooms. The technique of spore collection through electrostatic attraction in plastic lids is applicable to fresh lamellate and poroid fruiting bodies from the wild, to short-lived deliquescent mushrooms, to older and dehydrating fleshy fruiting bodies, even to animal-infested mushrooms and also to dry specimens of long-lasting tough species such as *Schizophyllum commune*.

Keywords: *Agaricomycetes*, Fruiting bodies, Basidiospores, Isolation, Buller's drop, Electrostatic attraction

Introduction

Fruiting bodies of *Agaricomycetes* may serve in food supply (Kües and Liu 2000) and for medicinal purposes (Wasser 2011), why isolation of mycelial cultures for mushroom cultivation is of high interest. Also, mycelia and produced enzymes might be applied in diverse fields of biotechnology (e.g. Pointing 2001; Hofrichter et al. 2010; Kües 2015a; Eibes et al. 2015; Masran et al. 2016) and further use is made of e.g. biologically active fungal polysaccharides (Cohen et al. 2002; Schmidt et al. 2011; Wasser 2011), non-enzymatic proteins (Wösten and Scholtmeijer 2015) and a broad range of secondary metabolites (Xu et al. 2010; Degenkolb and Vilcinskas 2016; Schmidt-Dannert 2016). Last but not least, Agaricomycetes have also their distinct position in fungal research, for example in studies on mating-type control of sexual reproduction (Kües 2015b), development of fruiting bodies as most complex multicellular fungal structures (Ohm et al. 2010; Stajich et al. 2010; Kües and Navarro-Gonzaléz 2015), and specific ecological functions such as by decay of lignocellulose (Floudas et al. 2012, 2015) and in mycorrhizal symbiosis (Martin et al. 2008; Kohler et al. 2015).

The typical life cycle of *Agaricomycetes* is heterothallic. It starts with meiotic basidiospores with one (1n) or two identical haploid nuclei (2 × 1n) which germinate into primary mycelia. Such a primary mycelium is called

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homokaryon because of the genetically identical nuclei in its cells. The more specific term monokaryon is used when primary mycelia harbour only a single haploid nucleus in their cells (1n). Primary mycelia are sterile but may fuse and, if of different mating type, will then form a fertile dikaryon. The dikaryon has binucleate cells and contains one haploid nucleus from each mating partner (1n + 1n). Mushrooms are produced on such secondary mycelia, with hymenia as specialized mycelial tissue covering their gills, ridges or pores. Dikaryotic hyphae of the hymenial layer give rise to the initially binucleate basidia with still two distinct haploid nuclei (1n + 1n). Karyogamy (2n) and meiosis (1n + 1n + 1n + 1n) occur in the basidia. Eventually, the resulting four haploid nuclei migrate individually into the four basidiospores (1n) which bud off from sterigmata at the apex of a basidium. A postmeiotic mitosis can lead to presence of two identical haploid nuclei (2 × 1n) in a spore (Kües 2000; Kües and Navarro-Gonzaléz 2015). Basidiospores of heterothallic species have different mating type specificities required for control of dikaryon, fruiting body and basidiospore development (Kües et al. 1998, 2002). However, there are also self-fertile homothallic species which form mushrooms without mating to another strain and produce basidiospores which are genetically identical to the parental strain. Pseudohomothallic species in contrast take up haploid nuclei of different mating type into their only two basidiospores (1n + 1n) per basidium, based on a nuclear mating type recognition reaction within the basidium. These heterokaryotic spores germinate then directly into fertile mycelia able to produce fruiting bodies without any further mating (Kües 2015b).

Isolation of pure mycelia from mushrooms collected from the wild may target at obtaining homokaryons or dikaryons. Basidiospores and vegetative fruiting body tissues may serve as sources for mycelium isolation (Ainsworth 1995). An obvious problem in the isolation of mycelia from the non-sterile wild mushrooms is the danger of contamination by bacteria and particularly by other fungi born from the air, soil and other surroundings. Attraction of a multitude of small animals (beetles, flies and other insects and their larvae, mites, nematodes, etc.) to the mushrooms bring in further sources of microbial contamination and very much enhances the problem. Ainsworth (1995) in his technical bulletin on methods for the isolation of basidiomycetes therefore advices to collect wild mushrooms as young as possible.

Obtaining a fertile mycelium from vegetative mushroom tissues can be difficult, depending on the size and consistency of a collected fruiting body. The risk of contamination can be reduced by semi-sterile surgery of inner parts from stipes or pilei of more compact larger mushrooms while for older, bug-ridden and small fragile mushrooms this is often impossible. Removed mushroom tissues are laid onto sterile agar medium but mycelial outgrowth can be hindered by other faster growing organisms, both bacteria and other fungi (Schuytema et al. 1966; Snelling et al. 1996; Lodge et al. 2004). In our hands, we failed in an estimated 20% of cases in isolation of mycelia from fruiting body tissues, mostly due to overgrowth by other competing microbes, especially by filamentous *Ascomycetes* or sometimes also by *Mucoromycotina* (unpublished data). Suitable antibiotics for suppression of bacteria and possibly benomyl for suppression of more sensitive *Ascomycetes* might be used. Outer tissue sterilisation prior to surgery by NaOH, ethanol or H_2O_2 might also reduce outgrowth of unwanted organisms. However, if isolation from mushroom tissues still fails, there might still be the possibility for heterothallic species to generate a new dikaryotic mycelium from poly-spore germination on agar plates or by mating of isolated germinated basidiospores (Schuytema et al. 1966; Snelling et al. 1996; Lodge et al. 2004). Isolation and germination of basidiospores from pseudohomothallic and homothallic species on the other hand will be sufficient to directly obtain desired fertile mycelia (Kües and Liu 2000; Kües 2015b).

Basidiospores are often collected from mushrooms as spore prints. The mushroom cap might be laid directly onto filter paper, aluminium foil or (water) agar. Alternatively, mushrooms might be fixed into the lids of Petri-dishes by agar, petroleum jelly (Vaseline) or other suitable gluing agents, or positioned by toothpicks or glass rods above the respective surfaces so that the mature spores drop down from the mushrooms onto these. Repeated spore prints might be taken from a mushroom with the hope that the number of contaminants is lower in later prints. Of advantage for many species is that basidiospores can be stored for some time. When they are plated for germination, antibiotics are then used in growth medium in order to reduce outgrowth of unwanted microorganisms as much as possible (Snelling et al. 1996; Choi et al. 1999; Lodge et al. 2004; Kropp 2005).

Basidiospores are ballistospores which are catapulted at maturity from their sterigmata at the basidia. The motion forces result from the hygroscopic Buller's drop which grows within seconds by condensation at the hydrophobic hilar spore appendix and its rapid fusion with an also hygroscopic liquid film which arises in a dent on the adaxial side of the spore (McLaughlin et al. 1985; Webster and Davey 1985; Webster et al. 1989; Ingold 1992; Money 1998; Pringle et al. 2005). The spores are propelled into the free air space between lamellate hymenia or of a pore, fall out of the caps by gravity (Ingold 1957, 1992; Pringle et al. 2005; Money and Fischer 2009; Noblin et al. 2009; Fischer et al. 2010a), and might then be transported by air streams further to new substrates (Galante et al. 2011; Horton et al. 2013; Halbwachs and Bässler 2015; Dressaire et al. 2015, 2016).

Mushrooms with ballistospores are naturally opened to the ground which helps that the spores are falling down by gravity out of the cap (Ingold 1957, 1992). When culturing *Schizophyllum commune* dikaryons on agar medium in plastic Petri-dishes under fruiting body-inducing conditions in the lab (Ohm et al. 2010), we however repeatedly observed that masses of basidiospores accumulated against gravity in the plastic lids of Petri-dishes incubated in upright position (unpublished observations). This observation motivated experiments with wild mushrooms collected in nature. Here we demonstrate that spores can be transported against gravity out of mushrooms when these lie upside-down facing up their lamellae, ridges or pores and when electrostatic forces act on the spores. We use this observation and present a new technique of bulk basidiospore isolation from mushrooms with reduced risk of contamination by unwanted microbes. Basidiospores are attracted and attach to electrostatically charged plastic lids of sterile Petri-dishes positioned in short distance above the reversed mushrooms. Subsequently, the spores can be harvested from the lids in sterile solution for further use.

Materials and methods

Mushroom collection and identification

Wild mushrooms were collected as found on the North Campus of the University of Göttingen and neighbouring areas of the village of Göttingen–Weende from 09.2011 to 09.2014. Mushrooms were photographed prior to harvest using a Cannon IXUS 115 H5 digital camera (12.1 megapixels; Canon, Krefeld, Germany). Harvested mushrooms were transported into the lab and photographed again, using a ruler as size marker to allow cap size estimations. Ecological parameters of mushrooms' growth (biotope, substrate, host trees) and morphological characters of the mushrooms (of stipes, caps, veils, lamellate hymenia or pores and, crucially in the identification process, of spores) were recorded for species determination. Basidiospores (sizes, colour and shapes) were observed under an Axioplan 2 imaging microscope (Carl Zeiss, Göttingen, Germany), photographed by a computer-linked Soft Imaging ColorView II Mega Pixel digital camera and analysed in size with the AnalySIS® software program (Soft Imaging System, Münster, Germany). Averages of spore sizes of collected specimens were determined from usually 5–20 spores. The field guides of Breitenbach and Kränzlin (1986, 1991, 1995), Bresinsky and Besl (1990), Flück (1995), Dähncke (2001) and Gerhardt (2010) and the *Coprinus* pages by Uljé (http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/Coprinus.htm) were used in species identifications and the MycoBank database (http://www.mycobank.org/) was considered for current species names and higher classification.

Coprinopsis cinerea mushrooms were all of strain AmutBmut (FGSC25122) and produced in the lab on artificial YMG/T medium (4 g yeast extract, 10 g malt extract, 100 mg tryptophan, 10 g agar) under standard fruiting conditions (Granado et al. 1997). A coincidental mushroom of *Leucocoprinus birnbaumii* was collected from a flowerpot in a student office of the institute.

Basidiospore harvests

Any dirt and noticed animals were removed from collected mushrooms. Caps were carefully separated from stipes with a sterile razor blade and forceps. Depending on their diameter (abbreviated by Ø throughout this work) or on the cap height of mushrooms which never fully open their umbrellas (i.e. *Coprinopsis picacea*, *Coprinus comatus*), caps were kept intact or sliced into 2, 4 or more equally sized portions. In cases of mushrooms with thick fleshy pilei (*Amanita strobiliformis*, *Coprinopsis atramentaria*, all *Boletales* but *Hygrophoropsis aurantiaca*) the upper gill-less or tube-free pileus parts of caps were sliced off in order to generate sufficient free air space above mushrooms during the experiments in the Petri-dishes. Caps up to ca 4–5 cm in diameter or parts of caps in case of larger mushrooms (Ø > 4–5 cm) were laid upside-down onto sterile wet paper tissue in individual sterile plastic Petri-dishes (polystyrene, 9 cm Ø, with cams, REF 82.1473; Sarstedt, Nümbrecht, Germany), sterile standard glass Petri-dishes (9 cm Ø) or higher sterile glass dishes (9 cm in Ø, 3.2 cm in height; used for *Armillaria solidipes*, *Pholiota squarrosa* and *H. aurantiaca* mushrooms) covered by a lid of a plastic Petri-dish. Care was taken to ensure that there was at least 0.5 mm free airspace in Petri-dishes above the mushrooms, while the airspace between mushrooms and plastic lids on higher glass dishes were between 1 and 2 cm. Dishes were stored for a few hours to overnight (up to 18–20 h) on a bench at room temperature (RT) for spore ejection. Patterns of spores adhering to the plastic lids were photographed using a Stemi 2000-C binocular (Carl Zeiss, Göttingen, Germany) connected to the Soft Imaging ColorView II Mega Pixel digital camera. Spores attached to the lids were washed off with 200 µl sterile water or with 200 µl of sterile 0.1% Tween 80 and counted using a hematocytometer.

Directional effects of mushrooms on spore release were observed in experiments of two distinct set-ups. Fruiting bodies of a same size and age or defined parts of fruiting bodies of a species were incubated in parallel in Petri-dishes on wet tissue paper in either natural direction or in upside-down position (Experimental Set-up 1). In

other experiments in order to avoid a direct contact with the wet paper tissue, mushroom samples were attached to the bases of plastic Petri-dishes by sticking them with their cap surface into a layer of sterile hand-hot water agar (1%) so that the cap surfaces touched the bottoms of the respective Petri-dishes. Petri-dishes were then incubated either in up-right position or upside-down (Experimental Set-up 2). Spores were harvested after 18 h incubation at RT either from the wet paper tissues (Set-up 1) or from the surface of the plastic lids of Petri-dishes (Set-up 2).

Two different strategies were also followed up to observe spore release over the time. First, equally sized and aged mushrooms or parts of mushrooms of a species were in parallel incubated upside-down on wet tissues in Petri-dishes at RT. At defined time points, spores were harvested from selected individual samples and counted. Data from the different individual samples for the different time points were compared (Experimental Set-up 1). In a second approach, spores were consecutively harvested in different lots per distinct mushroom sample at distinct time points of incubation. Counted spore numbers per harvest points were added together in order to obtain total spore numbers for different lengths of incubation of a given mushroom (Experimental Set-up 2).

The standard experimental set up (caps or pieces of caps laid upside down onto wet paper with plastic lids above) was changed in some experiments by using plastic Petri-dishes without tissue paper, by using plastic Petri-dishes with a layer of Vaseline smeared onto the inner lid surfaces, by using thin glass Petri-dishes with and without a layer of Vaseline smeared onto the inner lid surfaces, and by using thin transparent plastic rings from 1 to 10 cm in height and 8.95 cm in diameter as spacers between Petri-dish bases and lids in order to adjust the relative distances between reversed mushrooms positioned on wet paper tissue in the dishes and the plastic lids above.

Evaporating dishes (9 cm Ø 4.6 cm in height) with plastic lids above were used in experiments with gasteroid mushrooms.

Germination tests

Spore suspensions (50 µl) as harvested were plated onto 2% MEA agar (20 g malt extract, 10 g agar) or LB agar (5 g yeast extract, 10 g tryptone, 5 g NaCl, 1 ml 1 N NaOH, 10 g agar) and incubated at 25 °C for up to 15 days. Mixtures of antibiotics (AB) were added to media as needed (end-concentrations: ampicillin 100 µg/ml, kanamycin 50 µg/ml, streptomycin 100 µg/ml, tetracycline 20 µg/ml, chloramphenicol 20 µg/ml). Plates were checked on daily basis for growth and nature of microbes. Where possible, colonies grown on a plate were counted.

Results

Collection of basidiospores against gravity in plastic lids of Petri-dishes

In first experiments, accumulations of spores in lids were observed when complete or bisected mushrooms of different species were overnight incubated upside-down in plastic Petri-dishes. Spores from complete or bisected mushrooms in natural orientation collected contrariwise in the bottom of plastic Petri-dishes (Table 1). In tendency after 18 h incubation at RT, spore prints of mushrooms of *Coprinellus domesticus*, *C. atramentaria*, *Lacrymaria* spec., *Paneolus papilionaceus*, *Pluteus* spec. and *S. commune* (fresh and dry specimens) incubated in natural orientation contained about tenfold more spores than harvests from spores in lids obtained after incubation of reversed mushrooms (Table 1). Thus, a considerable part of all spores in these species reacted against gravity and moved upward in the dishes. Only *Coprinellus micaceus* differed from the other species in that spore yields in both directions were comparable both after 18 h incubation and after shorter 8 h incubation tested also for this fungus (Table 1).

Effects of humidity

We found that the amounts of spores present in lids after upside-down incubation of complete mushroom caps were influenced by humidity. There were between 2.4× and 62.1× less spores when mushrooms of the species *Coprinellus disseminatus* (50× less; 5 tested), *C. domesticus* (28.5× less; 3 tested), *Psathyrella conopilus* (40.3× less; 3 tested), *Pluteus* spec. (8.5× less; 1 tested), *Tubaria hiemalis* (2.4× less; 5 tested which were winter mushrooms soaked from snow cover) and *S. commune* (7× less; 3 tested) or halves of *C. atramentaria* (62.1× less; 1 tested) and eighths of *C. comatus* mushrooms (17.6× less; 3 tested) were incubated upside-down overnight (18 h) in dry plastic Petri-dishes as when similar sized and aged fruiting bodies or mushroom parts of a species (tested in parallel in same numbers) were laid upside-down onto wet tissues placed at the bottoms of the plastic Petri-dishes (absolute data from incubations on wet tissues were included in Table 2, absolute data from dry Petri-dishes incubations not shown). Because high humidity is required for basidiospore discharge (Webster et al. 1984b, 1989; Webster and Davey 1985; Money 1998; Noblin et al. 2009) and evaporation by the mushrooms from own tissues can provide required humidity only for a limited time (Turner and Webster 1991; Husher et al. 1999), the results indicate that transfer of basidiospores into the lids of the plastic Petri-dishes depended on an active ballistospore discharge mechanism.

Table 1 Spore harvests in lids of plastic Petri-dishes after incubation of mushrooms in upside-down or upside-top position relative to the lids

Species	No of mushrooms per situation	Part used	No of samples	Incubation time (h)	Spore harvests	
					Mushroom upside-down	Mushroom upside-top
Experimental set-up 1: mushroom caps laid onto wet paper						
Coprinellus domesticus	3	1	3	18	$4.8 \pm 0.3 \times 10^6$	$6.7 \pm 0.3 \times 10^7$
Panaeolus cinctulus	2	1	2	18	$4.5 \pm 0.9 \times 10^6$	$4.0 \pm 0.8 \times 10^7$
Schizophyllum commune	3	1	3	18	$7.3 \pm 1.0 \times 10^6$	$8.3 \pm 0.7 \times 10^7$
Tubaria hiemalis	5	1	5	18	$3.5 \pm 1.2 \times 10^4$	$8.1 \pm 0.9 \times 10^4$
Experimental set-up 2: mushroom caps stuck into water agar						
Coprinellus domesticus	1	1	1	18	2.4×10^6	2.1×10^7
Coprinellus micaceus[a]	2	1/2	3	8	$1.1 \pm 0.1 \times 10^5$	$0.9 \pm 0.2 \times 10^5$
				18	$1.2 \pm 0.1 \times 10^5$	$4.0 \pm 0.2 \times 10^5$
Coprinopsis atramentaria	2	1/2	3	18	$2.1 \pm 0.7 \times 10^6$	$2.2 \pm 1.0 \times 10^7$
Lacrymaria spec.	2	1/2	3	18	$2.7 \pm 1.2 \times 10^5$	$2.8 \pm 1.1 \times 10^6$
Paneolus papilionaceus	1	1	1	18	4.1×10^5	3.5×10^6
Pluteus spec.	1	1/2	1	18	6.7×10^4	2.7×10^5
Schizophyllum commune[b]	3	1	3	18	$4.7 \pm 1.8 \times 10^6$	$6.1 \pm 2.1 \times 10^7$
					$5.6 \pm 0.8 \times 10^4$	$2.6 \pm 0.2 \times 10^5$

[a] In one series of experiments, the incubation time was stopped at 8 h to avoid faults through onset of cap autolysis. In all other instances where inkcaps were used, there was no obvious cap autolysis

[b] Spore data in the upper line are from freshly collected mushrooms, data in the lower line are from revived dry fruiting bodies after 15 days open storage incubation at RT

Spores and droplets in plastic lids

Under standard incubation conditions (mushrooms laid upside-down on wet paper tissue in dishes covered with plastic lids), fine droplets usually developed overnight in the zones of the lids directly above the incubated reversed caps (Fig. 1a–d). Observations under the binocular revealed spores to be present in the droplets or, most common, spread at the surfaces of the droplets (Fig. 1e–h). Droplets with spores tended to spread somewhat irregularly flat over the plastic surfaces (Fig. 1) which suggests that they may contain some kind of surfactants. Regularly, patterns of lamellae were reflected in the lids due to the preferred positions and sizes of droplet formation. For species with dark spores, this was further visibly emphasized by their colour (Fig. 1a–f). The clear patterns of lamellae seen printed in the lids imply that spores fly straight up from their place of release toward the plastic lids where they collect with the growing droplets. Droplets however evaporated very fast upon opening of dishes. A clearly visible film of dried material was regularly left behind on the plastic surfaces (not further shown).

Spores were initially collected from the lids in 200 µl sterile water. Spores did however not easily transmit into the water but showed an affinity to stick to the plastic lid of a Petri-dish. Quick wiping with the plastic tip of a micro-pipette was required to transfer the spores into the liquid. Spores tended to clump and quickly sink to the bottoms of sterile Eppendorf cups into which spore solutions were transferred. Addition of mild detergent such as Tween 80 can help to suspend clumped basidiospores (Dhawale and Kessler 1993; Rincón et al. 2005). Therefore in later experiments, we used 200 µl sterile 0.1% Tween 80. With the detergent, spores were easily taken up from the lids and suspended.

Species range with open hymenia tested

Caps or parts of caps of mushrooms of a broad taxonomic species range were incubated upside-down on wet paper tissue in dishes covered with plastic lids. In nearly all cases (i.e. for mushrooms of 66 distinct species), basidiospores collected in large numbers in the lids (Table 2). Fresh mushrooms with open hymenia were collected over the time of species with gills (in total 59 species from 12 different families of the *Agaricales*—i.e. 7 species from the *Agaricaceae*, 2 species from the *Amanitaceae*, 2 species from the *Bolbitiaceae*, 4 species from the *Inocybaceae*, 3 species from the *Hygrophoraceae*, 3 species from the *Marasmiaceae*, 1 species from the *Mycenaceae*, 2 species from the *Physalacriaceae*, 1 species from the *Plutaceae*, 25 species from the *Psathyrellaceae*, 5 species from the *Strophariaceae*, and 3 species from the *Tricholomataceae*; 1 species from the *Repetobasidiaceae*, *Hymenochaetales*; 1 species from the *Russulaceae*, *Russulales*),

Table 2 Spore harvests from plastic lids as covers of Petri-dishes or 3 cm-high glass jars after 18 h or 20 h (marked by *) incubation of mushrooms in upside-down position on wet paper tissues

Species	No of mushrooms	Cap Ø (cm)	Part used	No of samples	Spore size (µm)[a]		Spore harvests[b]
					Length	Width	
Mushrooms with gills							
Agaricus augustus	1	13.9	1/4	2	7.4 ± 0.5	5.0 ± 0.4	$5.4 \pm 2.2 \times 10^6$
*Agaricus bitorquis**	1	6.1	1/4	4	6.0 ± 0.4	4.5 ± 0.3	$4.0 \pm 1.1 \times 10^6$
Agaricus campestris	1	4.5	1/4	4	nd	nd	$4.1 \pm 1.6 \times 10^7$
*Agaricus subfloccosus**	1	8.7	1/2	1	5.9 ± 0.4	4.8 ± 0.5	2.1×10^7
Agaricus subperonatus	1	15.6	1/4	4	6.4 ± 0.2	4.6 ± 0.3	$6.9 \pm 2.1 \times 10^6$
Agrocybe dura	4	3–3.8	1	4	12.8 ± 0.2	7.3 ± 0.1	$2.6 \pm 0.6 \times 10^6$
Amanita excelsa	1	5.2	1	1	nd	nd	3.3×10^6
Amanita strobiliformis	1	14.8	1/4	1	11.5 ± 0.7	8.5 ± 0.3	5.1×10^5
Armillaria solidipes	5	3.2–4.6	1	5	8.5 ± 0.7	5.2 ± 0.2	$2.2 \pm 1.0 \times 10^6$
Conocybe tenera	1	3.3	1	1	12.5 ± 0.1	7.2 ± 0.7	1.7×10^5
Coprinellus disseminatus	13	1.1–1.2	1	13	8.4 ± 0.3	6.2 ± 0.2	$3.3 \pm 2.6 \times 10^5$
Coprinellus domesticus	14	3.0–4.4	1	15	7.6 ± 0.4	4.5 ± 0.3	$4.1 \pm 1.8 \times 10^6$
Coprinellus micaceus	6	3.4–5.6	1/2	9	8.1 ± 0.4	4.9 ± 0.1	$3.4 \pm 1.8 \times 10^5$
Coprinellus subimpatiens	1	1.8	1	1	11.5 ± 0.3	6.6 ± 0.4	2.4×10^5
Coprinellus tardus	1	3	1	1	10.5 ± 0.9	5.0 ± 0.3	4.1×10^5
Coprinellus truncorum	1	5	1	1	8.9 ± 0.3	5.2 ± 0.1	5.3×10^5
Coprinellus xanthothrix	2	3.8–4.0	1/2	2	7.9 ± 0.3	4.8 ± 0.2	$3.1 \pm 2.0 \times 10^6$
Coprinopsis atramentaria	9	3.8–7.6	1/2	13	8.3 ± 0.6	4.8 ± 0.3	$4.9 \pm 2.3 \times 10^6$
Coprinopsis cinerea	5	4.2–5.4	1	5	10.7 ± 0.4	6.8 ± 0.5	$1.9 \pm 0.6 \times 10^5$
Coprinopsis picacea	1	11.6 (5.8 height)	1/2	2	14.2 ± 0.4	9.9 ± 0.4	$5.6 \pm 0.1 \times 10^6$
Coprinus comatus	9	2.6–5 (4.5–9 height)	1/8	17	12.2 ± 0.5	7.6 ± 0.4	$5.3 \pm 2.6 \times 10^6$
Hygrocybe conica	1	3.8	1	1	nd	nd	3.5×10^6
Hygrophorus olivaceoalbus	1	3	1	1	11.9 ± 0.1	8.2 ± 0.2	3.9×10^6
Hypholoma fasciculare	7	2.5–3.2	1	7	6.1 ± 0.4	3.5 ± 0.3	$3.9 \pm 2.6 \times 10^5$
Inocybe erubescens	1	3.3	1	1	10.7 ± 0.3	6.8 ± 0.7	1.8×10^5
Inocybe fraudans	1	4	1	1	9.8 ± 0.6	6.2 ± 0.2	6.4×10^5
Kuehneromyces mutabilis	3	4.8–5.6	1/2	6	7.3 ± 0.2	4.3 ± 0.2	$4.4 \pm 1.7 \times 10^6$
Lacrymaria lacrymabunda	2	5.0–5.8	1/4	4	8.9 ± 0.5	6.2 ± 0.2	$4.3 \pm 1.3 \times 10^4$
Lacrymaria spec.	7	3–3.8	1	7	10.9 ± 0.3	6.0 ± 0.4	$3.8 \pm 1.4 \times 10^5$
Lepista nuda	2	4.5–6.2	1/4	4	6.8 ± 0.4	4.7 ± 0.2	$4.4 \pm 2.0 \times 10^5$
Leucocoprinus birnbaumii	1	5.6	1/2	2	8.8 ± 0.6	6.7 ± 0.5	$2.4 \pm 0.8 \times 10^6$
Marasmius cohaerens	1	2.9	1	1	8.8 ± 0.2	4.9 ± 0.5	4.0×10^5
Marasmius oreades	1	2.8	1	1	9.6 ± 0.6	6.3 ± 0.4	4.8×10^5
*Marasmius wynneae**	4	3–3.7	1	4	7.1 ± 0.3	4.2 ± 0.1	$5.3 \pm 0.8 \times 10^6$
Panaeolus ater	2	2.6–3.2	1	1	12.8 ± 0.6	7.4 ± 0.5	$3.8 \pm 2.2 \times 10^4$
Panaeolus cinctulus	6	3.5–3.7	1	6	13.3 ± 0.1	7.4 ± 0.3	$3.1 \pm 1.3 \times 10^6$
Panaeolus olivaceus	1	3.1	1	1	12.8 ± 0.6	7.4 ± 0.5	2.3×10^5
Panaeolus papilionaceus	4	3.6	1	4	13.6 ± 0.3	8.3 ± 0.5	$3.0 \pm 1.2 \times 10^5$
Panellus serotinus	1	5.5	1	1	5.8 ± 0.1	1.9 ± 0.2	3.1×10^5
Parasola plicatilis	2	1.4–2.6	1	2	12.1 ± 0.9	6.6 ± 0.9	$4.5 \pm 2.1 \times 10^4$
Pholiota squarrosa	2	10–13	1/4	3	6.8 ± 0.1	4.0 ± 0.2	$6.7 \pm 2.8 \times 10^5$
Pholiota spec.	1	6.2	1	1	nd	nd	3.4×10^4
Pholiotina vestita	2	3.2–3.6	1/2	2	7.4 ± 0.5	5.2 ± 0.2	$3.0 \pm 1.2 \times 10^6$
Pluteus spec.	2	8	2	1	nd	nd	$7.1 \pm 0.4 \times 10^4$
Psathyrella atrolaminata	4	1.8–2.2	1	4	12.5 ± 0.3	7.2 ± 0.2	$5.1 \pm 2.4 \times 10^5$

Table 2 continued

Species	No of mushrooms	Cap Ø (cm)	Part used	No of samples	Spore size (µm)[a]		Spore harvests[b]
					Length	Width	
Psathyrella candolleana	6	3.1–5.8	1	6	7.0 ± 0.3	4.3 ± 0.4	$6.2 \pm 2.0 \times 10^5$
Psathyrella conopilus	7	4–4.8	1	7	12.7 ± 0.9	6.4 ± 0.8	$3.2 \pm 2.3 \times 10^5$
Psathyrella microrhiza	5	1.6–3	1	5	11.7 ± 0.3	6.5 ± 0.1	$0.8 \pm 0.4 \times 10^4$
Psathyrella pseudogracilis	7	4–4.8	1	7	13.7 ± 0.8	6.6 ± 0.4	$8.8 \pm 2.1 \times 10^5$
Psathyrella spadiceogrisea	1	3.8	1	1	7.9 ± 0.3	4.9 ± 0.4	8.2×10^6
Psathyrella tephrophylla	3	4–4.4	1	3	10.5 ± 0.4	5.8 ± 0.3	$2.9 \pm 0.2 \times 10^5$
Russula exalbicans	1	6.2	1	1	6.6 ± 0.2	4.7 ± 0.1	1.4×10^6
Stropharia caerulea	1	2.5	1	1	8.6 ± 0.5	5.2 ± 0.3	1.5×10^5
Tubaria furfuracea	3	4–4.8	1	3	7.5 ± 0.4	4.8 ± 0.2	$8.8 \pm 0.6 \times 10^5$
Tubaria hiemalis	5	2.5–3.2	5	5	nd	nd	$6.2 \pm 1.4 \times 10^4$
Xerula spec.	1	9.3	1	1	14.4 ± 0.7	10.9 ± 0.6	1.3×10^6
Mushrooms with gill-like ridges or pseudolamellae							
Hygrophoropsis aurantiaca	1	5.5	1	1	5.7 ± 0.6	3.6 ± 0.4	2.4×10^5
Schizophyllum commune	14	1.8-2.3	1	14	6.2 ± 0.3	2.2 ± 0.1	$4.9 \pm 2.0 \times 10^6$
Mushrooms with pores							
*Boletus luridus**	2	4.6-5.9	1/4	2	12.8 ± 0.5	5.6 ± 0.5	$2.8 \pm 1.9 \times 10^5$
*Boletus rhodoxanthus**	1	13.2	1/4	1	12.2 ± 0.5	4.7 ± 0.3	1.2×10^5
*Boletus splendidus**	1	15.3	1/4	1	12.7 ± 0.5	5.2 ± 0.4	3.7×10^5
*Chalciporus piperatus**	1	14.7	1/4	1	10.1 ± 0.5	3.5 ± 0.9	1.1×10^6
Laetiporus sulphureus	1	5.8	1	1	5.8 ± 0.1	4.4 ± 0.4	7.8×10^5
*Suillellus queletii**	1	12	1/4	1	12.6 ± 0.5	6.4 ± 0.4	1.0×10^4
Suillus spec.*	1	10.6	1/4	1	12.6 ± 0.5	5.5 ± 0.6	1.6×10^5
*Xerocomellus chrysenteron**	1	9.4	1/2	1	12.5 ± 0.7	5.3 ± 0.5	1.2×10^6

[a] nd = not determined

[b] Averages of spore numbers included the data shown individually in Tables 1 and 3, the data for 20 h incubation of some mushroom species presented in Fig. 2a and b, and the 1 cm height values of mushrooms presented in Fig. 3 and the 18 h data for mushrooms presented in Fig. 6

species with gill-like ridges (1 species from the *Hygrophoropsiceae*, *Boletales*) and pseudolamellae (1 species from the *Schizophyllaceae*, *Agaricales*), and species with pores (in total 7 species from the *Boletales*, i.e. 6 species from the *Boletaceae* and 1 species from the *Sulliaceae*; 1 species from the *Polyporaceae*, *Polyporales*). Nearly all tested species are characterized by four-spored basidia but *Agaricus bitorquis*, *Agaricus subperonatus*, *L. birnbaumii* and *Suillellus queletii* which can have both heterokaryotic and homokaryotic spores in their caps by mixed formation of bi- and four-spored basidia (Breitenbach and Kränzlin 1991, 1995). The only four exceptions of fleshy mushrooms which failed in spore collection in our experiments were single mature individuals of *Hygrocybe virginea* and *Hygrophorus eburneus* (both *Hygrophoraceae*), *Lepista saeva* (*Tricholomataceae*) and *Rickenella fibula* (*Repetobasidiaceae*). Since other species of the same or a closely related family gave spores in the lids (Table 2), these four failures did not relate to any specific taxonomic position of mushrooms but possibly to that spore shedding ended by fruiting body age (Haard and Kramer 1970; Li 2005; Saar and Parmasto 2014; not further analysed).

Spore solutions from mushrooms incubated upside-down overnight in Petri-dishes typically contained

(See figure on next page.)

Fig. 1 Basidiospores of *Coprinopsis domesticus* (**a**, **b**), *Schizophyllum commune* (**c**, **d**), *Coprinellus micaceus* (**e**, **f**), and *Psathyrella conopilus* (**g**, **h**) accumulated in droplets in lids of plastic Petri-dishes after 18 h incubation of full mushroom caps or halves of caps (*C. micaceus*) positioned upside-down on wet tissues in the base of the Petri-dishes. **a** and **c** show overviews on lamellar patterns recognized by the distribution of larger droplets with spores. Lamella positions are also reflected in the photos in **b**, **d**–**f** by the distinct accumulation of spores in the liquid droplets. The milky appearance of droplets in **d** is due to hyaline spores of *S. commune*. *Brown* spores of the other species can be recognized as dark spots in the droplet areas. Liquid droplets with spores spread irregular flat on untreated surfaces of plastic lids (**a**–**e**, **g**) while droplets of some species flattened more and those of others rounded more up when lids were covered with Vaseline (**f**, **h**)

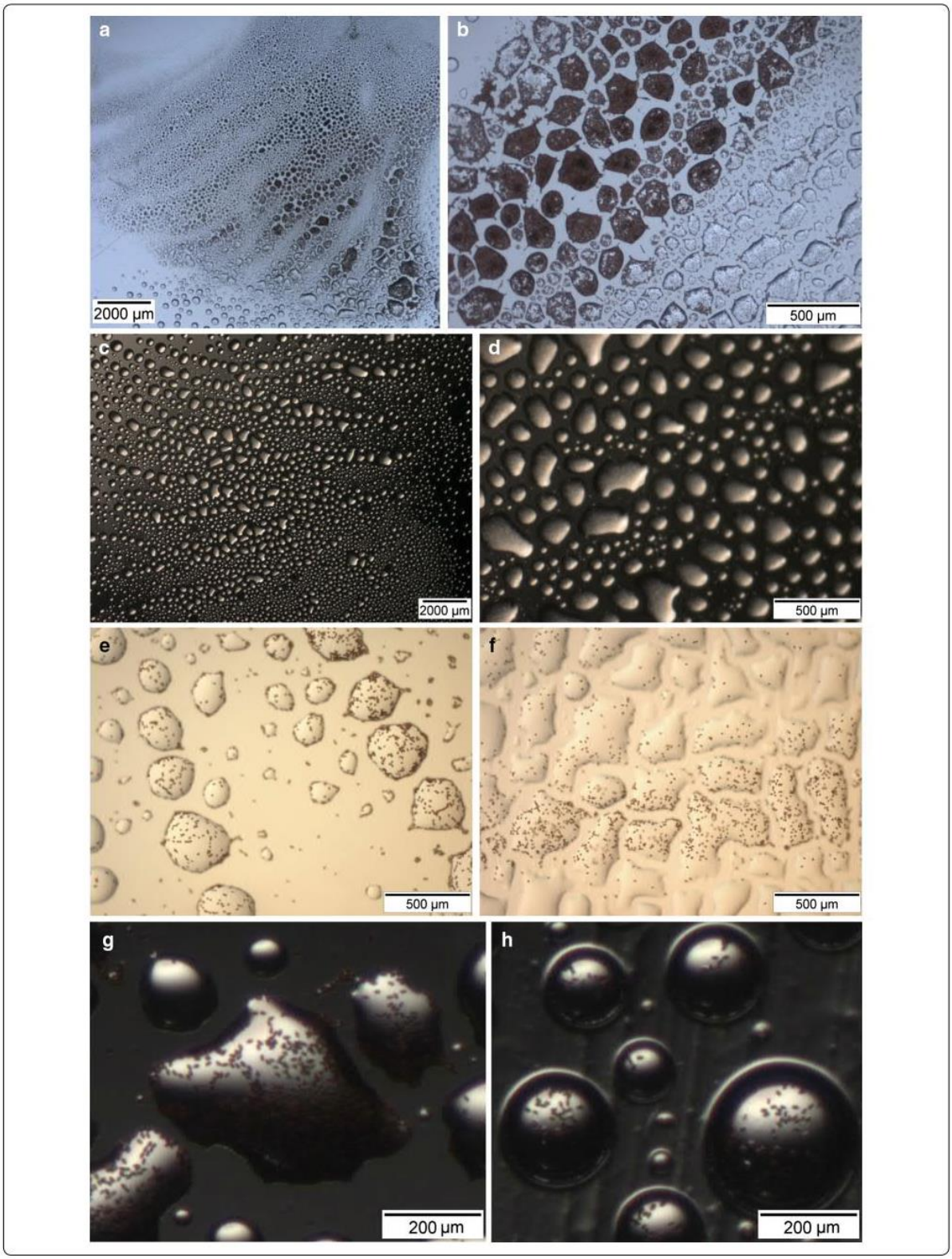
a
2000 µm
b
500 µm
c
2000 µm
d
500 µm
e
500 µm
f
500 µm
g
200 µm
h
200 µm

Table 3 Spore harvests in lids after 18 h incubation of mushrooms in upside-down position in Petri-dishes on wet paper tissues

Species	No of mushrooms per situation	Part used	No of samples	Spore harvests			
				Plastic lid		Glass lid	
				Plain	With Vaseline	Plain	With Vaseline
Coprinellus micaceus	2	1/2	3	$5.1 \pm 3.0 \times 10^5$	$2.1 \pm 1.0 \times 10^5$	6.0 ± 3.3	7.3 ± 5.3
Coprinellus domesticus	3	1	3	$4.9 \pm 1.4 \times 10^6$	$1.6 \pm 0.5 \times 10^6$	11.0 ± 6.5	20.0 ± 13.1
Coprinellus disseminatus	5	1	5	$2.1 \pm 0.9 \times 10^5$	$4.5 \pm 1.1 \times 10^5$	11.7 ± 9.4	24.0 ± 19.6
Coprinopsis atramentaria	1	1/2	2	$5.9 \pm 0.4 \times 10^6$	$4.1 \pm 0.4 \times 10^6$	0	0
Coprinus comatus	1	1/8	2	$9.2 \pm 0.4 \times 10^6$	$5.7 \pm 0.5 \times 10^6$	0	0
Hypholoma fasciculare	3	1	3	$1.0 \pm 0.5 \times 10^5$	$0.9 \pm 0.5 \times 10^5$	0	0
Psathyrella candolleana	1	1	1	9.4×10^5	7.6×10^5	0	0

between about 10^4 up to in highest cases $>10^7$ total spores (compare Table 2). Spore yields obtained from different mushroom samples of a same species were usually very similar, even when harvested and incubated at different days (details not further shown but see the averaged data in Table 2 and compare the data for individual species also with those from specific experiments presented in Tables 1, 3; Figs. 2, 3). This suggests in coincidence with earlier reports on spore releases of different species (Fischer and Money 2009; Saar and Salm 2014) that species-specific parameters determine the frequency of spore release and the actual spore capture in the plastic lids. However, spore yields did not plainly depend on a single simple parameter such as cap size, spore sizes, structures of hymenia (lamellae, ridges or pores) (Table 2), or gill numbers per cap and pore diameters (see the cited field guides for the individual parameters of species for comparison with the spore collection data in Table 2). Cap age and speed of younger cap maturation, lengths and mode (consecutive or synchronous) of spore production and maturation periods (Kües and Navarro-Gonzaléz 2015; Halbwachs and Bässler 2015) might also be needed to be considered on individual species level as parameters of potential influence on spore harvests.

Effects of mushroom conditions on spore harvests

We collected mostly fresh fleshy mushrooms of Agarics (Table 2) and many were still young, in the stage of opening or close to be fully opened. During overnight incubation in reversed orientation, younger caps of most of the fleshy species further opened and cap diameters (measured at the time of harvest from nature, see Table 2) tended to further extend by stretching out the umbrellas. Of the lamellate species, the single fruiting bodies of *Agaricus campestris*, *Agaricus subfloccosus*, *Hygrophorus olivaceoalbus*, *Inocybe fraudans*, *Marasmius oreades*, and *Psathyrella spadiceogrisea*, the two mushrooms of *Pluteus* spec., all four of *Marasmius wynnei* and all six of *Psathyrella candolleana* were already fully open at their harvest. There were no problems with any of the younger and the here listed mature mushrooms to obtain high numbers of spores transferred to the plastic lids positioned above (Table 2).

Notably, also younger (*P. conopilus*), mature (the single fruiting bodies of *Agaricus augustus*, *Pholiota* spec. and *Stropharia caerulea*; one mushroom each of *P. candolleana* and *S. commune*, two mushrooms each of *Panaeolus ater* and *Psathyrella atrolaminata*, and a series of mushrooms of *C. domesticus*) or aging mushrooms (*Conocybe tenera*, one mushroom each of *Agrocybe dura*, *Marasmius cohaerens* and *Panaeolus cinctulus*) which were to different extend desiccated were successfully appointed in collecting spores in numbers of 10^4–10^6 in plastic lids by reversed incubation on wet paper tissue (Table 2; Fig. 2b). The shrivelled mushrooms refreshed in shape by taking up humidity from the wet paper tissue. For *A. dura*, we had an older dry fruiting body and young fresh mushrooms. Spore harvests were similar (2.6×10^6 spores versus from 2.0 to 3.5×10^6 spores). Spore numbers for dehydrated mushrooms of *C. domesticus* were only somewhat reduced ($0.9 \pm 0.1 \times 10^6$; Fig. 2b) as compared to most mushrooms harvested in fresh stage ($4.1 \pm 1.8\ 1 \times 10^6$; Table 2). Moreover, mushrooms of the durable species *S. commune* which were dried on purpose at RT for 15 days gave still considerable numbers of spores in lids ($5.6 \pm 0.8 \times 10^4$; n = 3; tested with the water agar system) over the 18 h of reversed incubation although these were 100× reduced as compared to mushrooms which were used directly at the day of harvest (Table 1). *S. commune* in active phases produces continuously new spores (Kües and Navarro-Gonzaléz 2015) by which fresh mushrooms might distinguish from revived specimens that will require time for full physiological recovery.

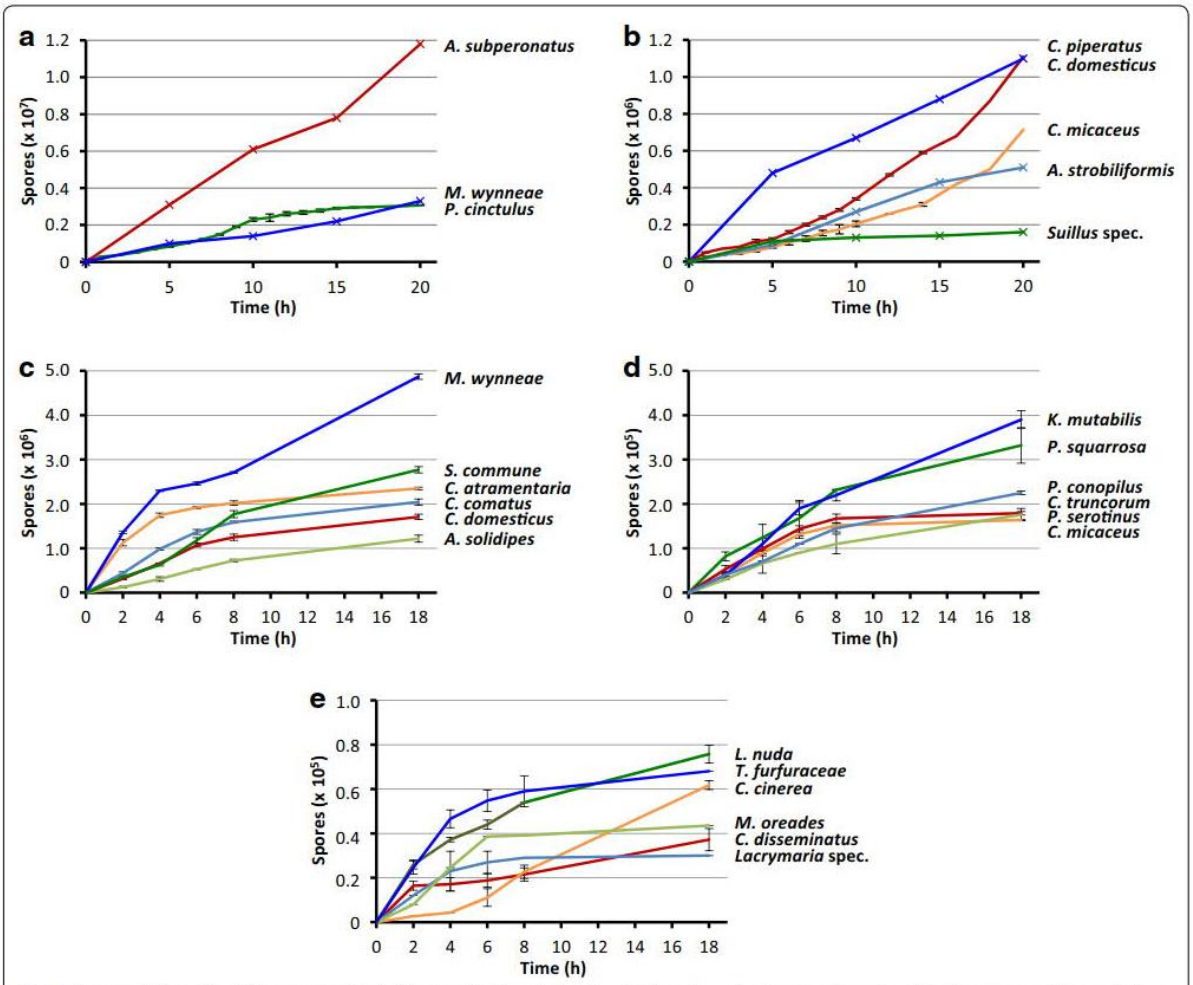

Fig. 2 Accumulation of basidiospores in plastic lids over the time during incubation of mushrooms positioned upside-down on wet tissues in the base of Petri-dishes or glass jars. **a**, **b** Experimental Set-up 1: The two halves of different mushroom caps of *Paneolus cinctulus* (in total from 20 caps) and *Coprinellus micaceus* (in total from 15 caps) and whole caps of *Coprinellus domesticus* (in total 30 caps, all slightly dehydrated) were incubated in parallel in distinct Petri-dishes for fixed times to collect their spores in plastic lids, count the harvests and calculate from each two respective values the averages presented in the figures. Four complete caps were incubated in different dishes for *Marasmius wynneae*, the four quarters of single mushrooms for all other species. At distinct times (indicated in the figures by ×), the spores of an individual mushroom or an individual quarter were harvested for counting. **c**–**e** Experimental Set-up 2: Spores from whole fruiting bodies or parts of fruiting bodies were harvested at distinct time points and lids were then put back onto the Petri-dishes for further incubation and spore collection. Spores per point of harvest were counted and the different values for an individual sample were added together in order to calculate total spore numbers over the whole length of an incubation period. Average values with standard deviations were calculated for all species from each three distinct samples run in parallel. Halves of caps for *C. micaceus*, *Coprinellus truncorum*, *Coprinopsis atramentaria*, *Kuehneromyces mutabilis*, *Lepista nuda*, and *Panellus serotinus* were used, quarters of caps for *Pholiota squarrosa*, eighths of caps for *Coprinus comatus*, and complete mushrooms for all other species (for their full names see Table 2)

Further of importance, also mushrooms infested with small animals (Fig. 4) can be used in collecting spores in lids. Of the lamellate mushrooms mentioned above, *Pluteus* spec. for example carried small slugs (Fig. 4a, b), on *P. spadiceogrisea* and *A. campestris* were rove beetles (Fig. 4d) and red mites and internally some larvae (Fig. 4g, h), while the ones of *A. campestris* (Fig. 4h), *A. subperonatus* (Fig. 4i–k) and *M. wynneae* (Fig. 4e, f), one of six of *P. cinctulus* (Fig. 4l, m) and one of two mushrooms of *Pholiotina vestita* (Fig. 4c) had grubs. Particularly the fleshy pilei of the *A. campestris* (Fig. 4h) and *A. subperonatus* fruiting bodies (Fig. 4i–k) and also all

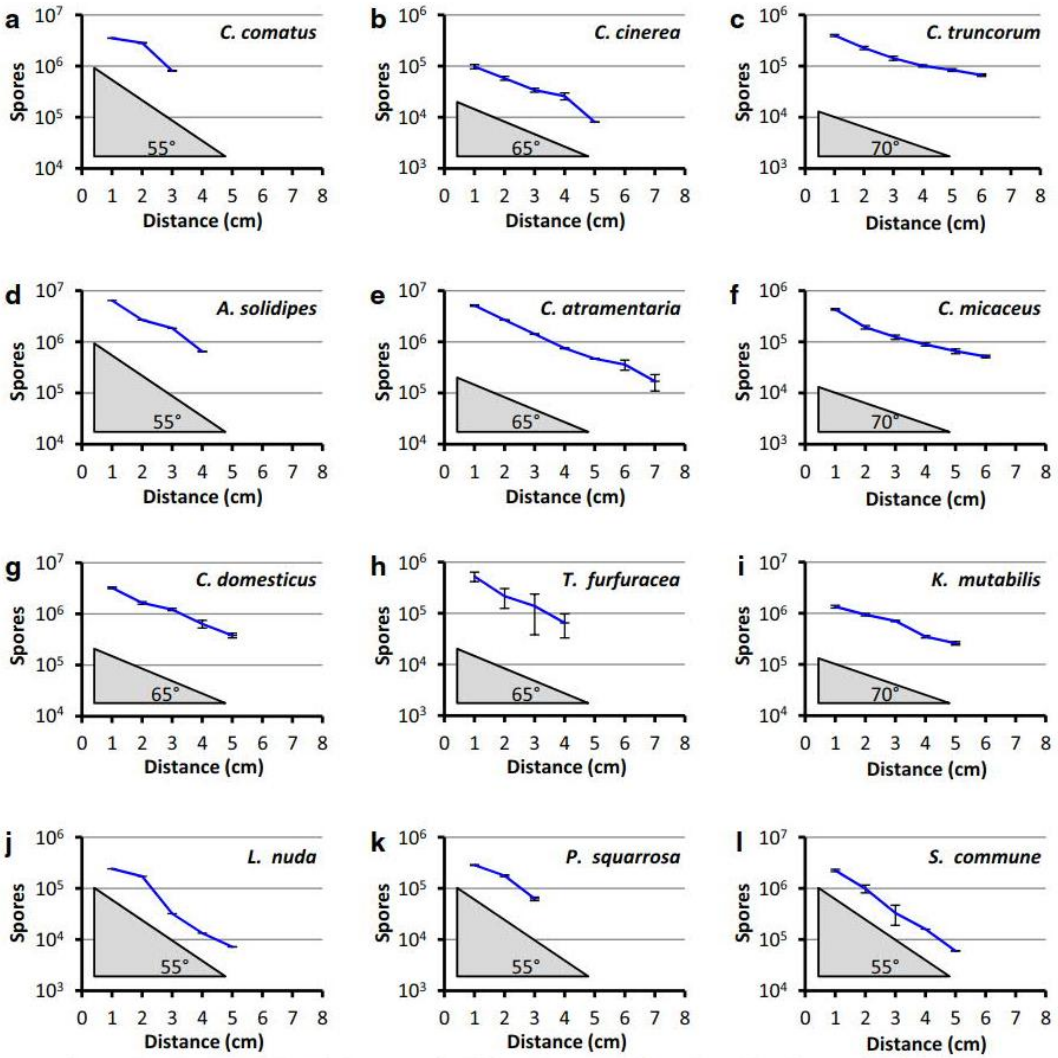

Fig. 3 Accumulation of basidiospores in plastic lids positioned at different distances to the surfaces of mushrooms placed upside-down onto wet tissues in the base of Petri-dishes or glass jars. Spores were collected after 18 h incubation. Spore values were calculated as averages with standard deviations from each three distinct samples (*Kuehneromyces mutabilis*: each 1/2 of a mushroom; *Coprinopsis atramentaria*, *Lepista nuda*: each 1/4 of a mushroom; *Coprinus comatus*: each 1/8 of a mushroom; all others: complete mushrooms; for their full species names see Table 2). Values are shown for all distances where spores were found in the lids; lack of spores in lids (0 spores) after incubation is documented in the graphs by no value entry. In all instances, distances up to 10 cm were tested in 1-cm steps. Subfigures are arranged by spore lengths of the species, from longest (**a**) to shortest (**l**). **a**–**c**: 12.2–8.9 µm; **d**–**f**: 8.5–8.1 µm; **g**–**i**: 7.6–7.3 µm; **j**–**l**: 6.8–6.2 µm (for detailed data compare the entries in Table 2). *Grey shaded triangles* with angle values given for the right corner (55°, 65° or 70°) indicate grouping by similar steepness of logarithmic decrease in spore numbers with increasing distance to the lids

mushrooms of the *Boletales* (Fig. 4n and not shown) were much infested with many grubs. Insect larvae had eaten tunnels into the pilei of the *S. queletii* (Fig. 4n) and the other *Boletales*' mushrooms. However, there was still much surface area with intact pores (Fig. 4n and not shown) in order to obtain bulks of basidiospores in plastic lids above (Table 2). Only for an aged, slug-eroded and fully grub-populated decaying *Xerocomellus chrysenteron* fruiting body, it was not possible to obtain spores from. Where we had infested and animal-free fruiting bodies, harvested spore numbers were still similar (infested decaying *C. domesticus* fruiting body:


a
b
c
d
e
f
g
h
i
j
k
l
m
n

(See figure on previous page.)
Fig. 4 Examples of mushrooms infested by small animals. **a**, **b** *Pluteus* spec. mushroom with small slugs. Different insect larvae were found on mushrooms of **c** *Pholiotina vestita*, **e**, **f** *Marasmius wynneae* (one of four animal-infested mushrooms tested for sporulation is shown), **h** *Agaricus campestris*, **i**–**k** *Agaricus subperonatus*, **l**, **m**, *Paneolus cinctulus* (one of in total six mushrooms tested for sporulation was visibly animal-infested), and **n** *Suillellus queletii* (note the still intact pores and tubes at the upper area of the photo). **d** An adult beetle of *Oxyporus rufus* (*Coleoptera*, *Polyphaga*) on *Psathyrella spadiceogrisea*. **g** *A. campestris* with red mites (same mushroom as in **h**). *White* and *black arrows* point to positions in the photos of animals less easy to detect. All mushrooms shown in the figure were used in reversed position on wet tissues to collect spores in plastic lids above and all gave high numbers of spores (data are included in Table 2)

0.9×10^6 spores, animal-free mushrooms: from 1.3 to 6.9×10^6 spores; infested *P. cinctulus* fruiting body: 0.9×10^6 spores, animal-free mushrooms: from 2.9 to 5.4×10^6 spores).

Spore release over the time

Mushrooms started quickly to propel off spores when incubated upside-down on wet tissues in closed dishes (Fig. 2). Already after 2 h of incubation, considerable amounts of spores ($>10^4$–10^5 for most species or for somes species even 10^6) could be harvested from mature fruiting bodies in plastic lids. Fast spore release continued for a few hours (4–6 h) but the speed of spore release usually decreased for several of the species with time of incubation to eventually level off to a maximum amount of spores which possibly can be discharged by a single specimen under the experimental conditions applied.

In many cases, we used in our experiments fleshy mushrooms of limited life-time such as of the ephemeral inkcaps (species of *Coprinellus*, *Coprinopsis*, *Coprinus*) that after a transitory period of ballistospore ejection release a majority of their spores in liquid droplets by autolysing their caps (McLaughlin et al. 1985; Kües 2000; Redhead et al. 2001; Nagy et al. 2013). The time of spore release by ballistospore ejection up to onset of cap autolysis was always sufficient to obtain high spore numbers in lids of plastic Petri-dishes, in amounts of $>10^5$ to $>10^6$ (Tables 1, 2, 3; Figs. 2, 3). In case of *C. cinerea* fruiting bodies from laboratory cultures, spore release was delayed by 4 h from the start of incubation of young

Table 4 Contaminations of basidiospore solutions (from Experimental set-up 1 in Table 1) with other microbes

Fruiting body	Classic spore print (mushroom upside-top)						
	Basidiospores in 50 µl	Colonies on plate/50 µl spore solution plated					
		MEA			MEA + AB		
		Basidiomycete	Contamination[a]		Basidiomycete	Contamination[a]	
			Bacteria	Other fungi		Bacteria	Other fungi
Coprinellus domesticus	1.6×10^7	68	Uncountable	2 yeasts, 34 molds	51	–	122 yeasts, 14 molds
Panaeolus cinctulus	8.0×10^6	–	5.4×10^3	3 yeasts, 38 molds	–	–	8 yeasts, 14 molds
Schizophyllum commune	2.1×10^7	2563	Uncountable	34 yeasts, 4 molds	2326	–	544 yeasts, 8 molds
Tubaria hiemalis (1st mushroom)	1.9×10^4	–	1.7×10^5	14 yeasts, 6 molds	–	–	42 yeasts, 10 molds
T. hiemalis (2nd mushroom)	2.4×10^4	–	4.3×10^5	6 yeasts, 5 molds	–	–	64 yeasts, 21 molds
	Spore print in plastic lid (mushroom upside-down)						
	Basidiospores in 50 µl	Colonies in plate/50 µl spore solution plated					
		MEA			MEA + AB		
		Basidiomycete	Contamination		Basidiomycete	Contamination	
			Bacteria	Other fungi		Bacteria	Fungi
C. domesticus	1.2×10^6	4660 (0.33% germination)	–	–	4852 (0.34% germination)	–	–
P. cinctulus	9.0×10^5	1352 (0.15% germination)	–	–	1280 (0.14% germination)	–	–
S. commune	1.8×10^6	Uncountable	–	–	Uncountable	–	–
T. hiemalis (1st mushroom)	7.8×10^3	2458 (31.5% germination)	–	–	2873 (36.8% germination)	–	–
T. hiemalis (2nd mushroom)	9.0×10^3	2213 (24.5% germination)	–	–	2437 (27.0% germination)	–	3 yeasts

[a] Numbers of fungi in different media did not clearly correspond to each other since fast bacterial growth suppressed fungal growth and fast growing molds suppressed that of other molds and yeasts

opening caps with still pale gills (Fig. 2e). This time coincided well with the known time schedule of basidiospore maturation with corresponding black gill staining after light-induced synchronized karyogamy in the basidia (Kües and Navarro-Gonzaléz 2015).

Spore attachment to lids of different material

In preliminary experiments, mushrooms of *S. commune* and of *C. atramentaria* were incubated upside-down in dishes with plastic lids or in dishes with glass lids. Spore collected overnight in plastic lids whereas spores were not present in glass lids (data not shown). The results suggested that an electrostatic disposition possibly helped to attach ejected spores to the surface of the plastic lids.

We smeared sticky Vaseline onto glass and plastic lids of Petri-dishes to support attachment of spores to the surfaces. Experiments with parallel sets of overnight upside-down incubated mushrooms or parts of mushrooms of *Hypholoma fasciculare*, *P. candolleana* and five different inkcap species showed that Vaseline in plastic lids had no incisive negative effect on spore yields (Table 3; Fig. 1e–h). Spores stuck well in droplets to the Vaseline (Fig. 1f, h) although it was harder to harvest them from Vaseline and bring them into solution than without. However, Vaseline did not lead to considerably increased numbers of spores that attached to glass lids. With and without Vaseline, there were always either no or only negligible few spores in glass lids (Table 3). We conclude from the experiments that different affinities between the glass and plastic surfaces of tying spores is not the primary cause for the unequal spore harvests from lids of different material but a distinct effect of the plastic on upward spore transfer. Likely, static electricity of the plastic (Woodland and Ziegler 1951; Kuo 2015) will force spores to fly upward onto the lids where the same forces will then support attachment of the spores to the plastic surface.

Spore transfer to plastic lids over different distances

The data in Table 1 implicate for several species that not all basidiospores released from reversed mushrooms are attracted to plastic lids. Experiments in which the distance between the upside-down laid mushrooms and the plastic lids above for several species were varied further support an influence by spore properties on spore yields in the lids. For all species, numbers of spores attached to the lids decreased in linear trends on the log scale with increasing distance to the reversed mushrooms (Fig. 3). Roughly, three groups in line steepness might be distinguished (Fig. 3a, d, j–l, steepest: *C. comatus*, *A. solidipes*, *Lepista nuda*, *P. squarrosa*, *S. commune*; Fig. 3b, e, g, h, medium: *C. cinerea*, *C. atramentaria*, *C. domesticus*, *Tubaria furfuracea*; Fig. 3c, f, i, flattest: *C. truncorum*, *C. micaceus*, *Kuehneromyces mutabilis*). Break-offs of all lines (lowest distances where no spores detected in lids) were abrupt. Between species, break-offs occurred at different heights, following different orders of magnitudes (ranging from $<10^4$ up to nearly 10^6) of absolute spore numbers at the last positive height. Absolute numbers of spores collected in lids at low distances did not correspond throughout to the possible longest distance over which spores of a species were found to be attracted to plastic lids (Fig. 3). *C. atramentaria* as a species with highest spore numbers in lids at short distance ($>10^7$ at 1 cm distance) also yielded reasonable amounts of spores in lids at highest distances ($>10^5$ at 7 cm distance; Fig. 3e), followed by *Coprinellus truncorum* and *C. micaceus* with both ca. 4×10^5 spores in lids at 1 cm distance and ca. 5 to 7×10^4 spores at 6 cm distance (Fig. 3c, f). In all other cases, maximum heights of spore detection varied between 3 cm and 5 cm distance. Spore sizes (lengths, see Table 1) or volumes (as calculated for an ellipsoid $V = 4/3\ \pi \times ½$ length $\times 2 \times ½$ width; data not shown) did also not correlate with maximum heights at which spores were attracted to plastic lids (Fig. 3). Attraction between higher up-flying spores and the plastic lids might be expected to be stronger than between less far up-flying spores and the lids. Basidiospores do also have electric charges (Buller 1909, 1922; Gregory 1957; Swinbank et al. 1964; Webster et al. 1988; Saar 2013; Saar and Parmasto 2014; Saar and Salm 2014). Individual differences in charging of spores remain as explanation for the observations.

Capturing spores from gasteroid basidiomycetes

We concluded before that spore collection in plastic lids positioned above mushrooms with open hymenium depended on their forcible ballistospore discharge mechanism mediated at high humidity by the fusion of Buller's drops with liquid films at the adaxial sides of the spores. Gasteroid species have lost this active basidiospore discharge mechanism, produce their basidiospores within closed fruiting bodies and disperse them from openings or cracks passively with wind or through pushing the spore sacs by rain drops (Hibbett et al. 1997; Wilson et al. 2011; Kües and Navarro-Gonzaléz 2015). Accordingly, basidiospores did not accumulate during overnight windstill incubation in evaporating dishes (18–24 h) in the plastic lids above mature puffballs (*Lycoperdon perlatum*, *Agaricaceae*) which were either already cracked or of which the inner gleba with the basidiospores was opened by cutting the fruiting body into two equal halves. Similarly, there were no spores in lids after 18 h incubation when we positioned two different mature earth-stars (*Geastrum rufescens* and *Geastrum striatum*; *Geastraceae*, *Geastrales*) with an ostiole as a natural

opening and unrolled segments of the exoperidium into windstill evaporating dishes covered by plastic lids. However, when we hit 2× with caution the spore sacs from the side with a glass rod through the spouts of the evaporating dishes, clouds of spores escaped through the ostioles from the spore sacs into the air and attached to the plastic lids above (not further shown). We harvested and counted in the lids then 6.9×10^6 basidiospores of *G. rufescens* (plating proofed them to be contamination free) and 4.4×10^6 for *G. striatum*. We conclude that the spores are moved into the air is decisive for spore attraction to the plastic lids but not the particular initial mode of the spore release.

Spore plating

The reliable yields of spores from upside-down positioned wild mushrooms in the plastic lids of Petri-dishes evoked the further idea to test whether just basidiospores were released onto the lids. First, 50 µl aliquots of spore solutions from individual mushrooms from the Experimental Set-up 1 in Table 1 were analysed on MEA media. Spore solutions from mushrooms incubated in Petri-dishes in natural direction gave rise to massive bacterial and also numerous fungal contaminations, in contrast to aliquots from spore suspensions which were harvested from plastic lids from upside-down incubations of mushrooms (Table 4). Generally, sizeable bacterial colonies appeared on media after 1 day at 25 °C incubation, colonies of yeasts and molds after 2 days and basidiospore germlings visible to the naked eye for all tested mushrooms after 3 days, respectively. Both bacterial and fungal contaminants (molds, mostly *Ascomycetes*) hindered by overgrowth other organisms in growth, including germination of the basidiospores and growth of germlings of the respective mushroom plated (Table 4). Addition of antibiotics suppressed bacterial growth but fast growing molds present in the samples of classic spore prints still overgrow the basidiospores (Table 4; Fig. 5a). Colonies from germinated basidiospores of spore suspensions from classical prints among contaminations were therefore only observed in the cases of *C. domesticus* and *S. commune* but at much lower frequency (≥1000 fold less) than on plates onto which samples of basidiospores were plated from harvests from plastic lids located above upside-down incubated mushrooms (Table 4). In absence of contaminants, germination of basidiospores from samples harvested from plastic lids was unhindered for all mushrooms of the four species tested. There were no bacterial contaminations and fungal contamination occurred only in one instance (=10% of all tested cases) with just 3 yeast colonies (Table 4; Fig. 5b). While the germination rates of basidiospores were different between the species used (Table 4), possible effects by densities of the own basidiospores in the solutions were not further tested.

Second, spore suspensions from classical prints of *C. domesticus* and *P. papilionaceus* mushrooms fixed to dishes by agar during downward spore shedding were compared on LB medium at 25 °C with spore suspensions obtained by harvests from plastic lids from agar-fixed mushrooms incubated upside-down (solutions from the Experimental Set-up 2 in Table 1). In case of the classical *C. domesticus* spore print (5.3×10^6 spores/50 µl), >360 large slimy and blurred bacterial colonies grew over the surface of the plate and five yeast colonies on medium with antibiotics while the plates with spore suspensions from the upside-down incubated fruiting body (6.0×10^5 spores/50 µl) were all free of any contaminations (but also of germinated basidiospores; not further shown). Judging by colony morphologies, multiple bacterial species (>10) grew in high density of a plated basidiospore suspension from the classical spore print of *P. papilionaceus* (8.8×10^5 spores/50 µl) and still three types of bacteria (338, 15 and 3 colonies, respectively) when antibiotics were added to the growth medium. This contrasted the situation with the spore solution from the upside-down incubated mushroom (1.0×10^5 spores/50 µl) where no bacteria were found on LB without or with antibiotics while germination of basidiospores at 0.72% frequency was observed (not further shown).

From the time course experiment presented in Fig. 2, we had each two samples for *C. domesticus* (Fig. 2b), *C. micaceus* (Fig. 2b) and *P. cinctulus* (Fig. 2a) per tested time point of spore release into lids of plastic Petri-dishes. We tested also these for presence of contaminations by cultivation on MEA at 25 °C. In case of *C. domesticus* 26 of 30 basidiospore samples tested (87%) were free of contaminants, in case of *C. micaceus* 22 of 30 samples tested (73%), and in case of *P. cinctulus* 30 of 40 samples tested (75%). When contaminants were observed (in 22% of all these cases together), these were always bacteria (usually 1 or 2 types, rarely 3 types of bacteria) while the number of bacteria per 50 µl plated spore suspensions differed between 3 and 407 for *C. domesticus*, 3 and 14,400 for *C. micaceus* and between 2 and $>10^6$ for *P. cinctulus* (Fig. 6). There was a tendency in likelihood for samples obtained from longer mushroom incubation to contain contaminations but there was no continuous increase in bacterial numbers over the different samples with length of mushroom incubation applied for spore release. Moreover, samples from two different halves of a same mushroom often differed in that bacteria were observed in only one of the two (Fig. 6). Furthermore, spore samples (50 µl) of the each four analysed time periods (5, 10, 15, 20 h) of

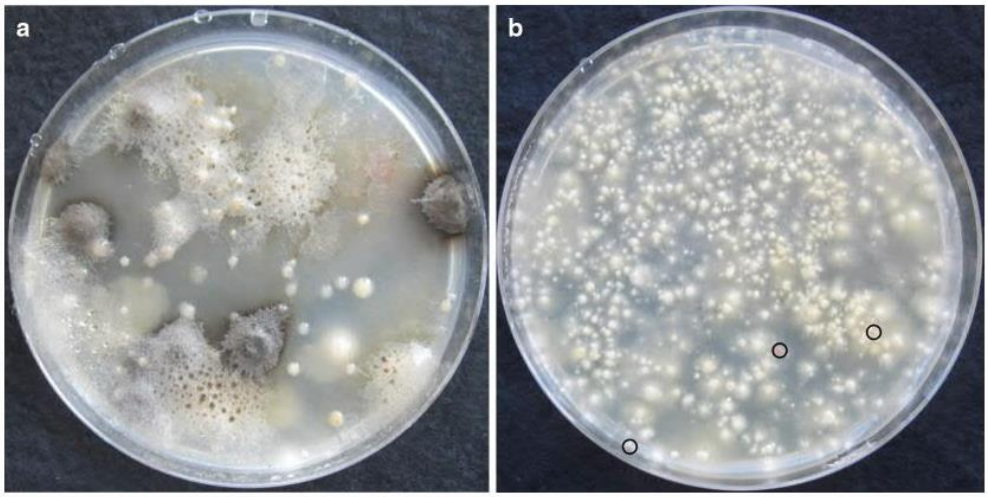

Fig. 5 Outgrowth of microbes from 50 µl basidiospore solutions of *Tubaria hiemalis* on MEA + AB during incubation at 25 °C. **a** Spore solution from a classic spore print (from the 2nd mushroom incubated in upside-top condition in Table 4). Different species of molds have overgrown the *T. hiemalis* basidiospores as well as colonies of different yeast species. **b** Spore solution from a spore print collected in the plastic lid of a Petri-dish (from the 2nd mushroom incubated in upside-down condition in Table 4). Colonies of 2437 germinated basidiospores are seen (27% germination rate). Encircled are three red yeast colonies found as the only contaminations grown on the plate

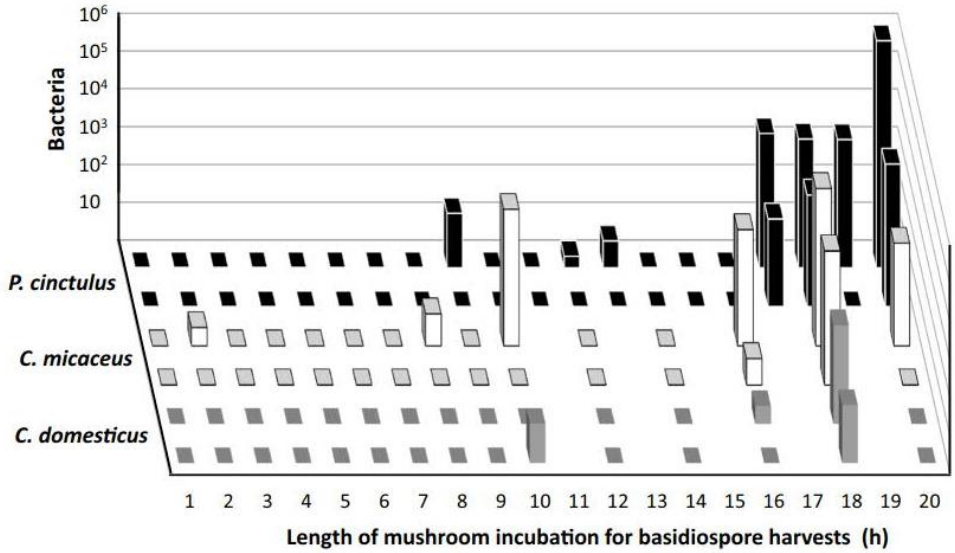

Fig. 6 Bacterial contaminations in spore solutions of *Paneolus cinctulus* (*black columns*), *Coprinellus micaceus* (*white columns*), and *Coprinellus domesticus* mushrooms (*grey columns*). After upside-down incubation of mushrooms for basidiospore harvests from plastic lids (Fig. 2a, b), each 50 µl of spore solutions were plated onto MEA medium, incubated at 25 °C for 3 days and bacterial colonies were counted. Two different basidiospore solutions were always tested per time point for a species. For *P. cinctulus* and *C. micaceus*, these came always from the two halves of a same mushroom which were incubated for spore collection in parallel in different Petri-dishes. For *C. domesticus*, the each two parallel spore collections came from different mushrooms. Bacterial colonies in the *P. cinctulus* sample B, 20 h (shown in the figure at furthest distance to the observer) were uncountable why 10^6 bacteria was used in the figure as an arbitrary estimate

spore release of *A. strobiliformis* (Fig. 2b), *A. subperonatus* (Fig. 2a), *C. piperatus* (Fig. 2b), *M. wynneae* (Fig. 2a), and *Suillus* spec. (Fig. 2b) were all free of bacteria but the 15 h sample of *C. piperatus* (1 bacterial colony), the 20 h sample of *M. wynneae* (1124 bacterial colonies) and the 20 h sample of *Suillus* spec. (2 yeast colonies). Thus, also in these series of samples, when contaminations were found (in 15% of all cases; 10% bacterial contaminations; 5% fungal contaminations) they came from longer mushroom incubations appointed for spore shedding.

Finally, spore solutions (50 µl) from 18 or 20 h incubation of mushrooms in upside-down position of a broader range of 19 randomly collected species (from Table 2) were tested on MEA. No contaminants were found in spore suspensions of individual fruiting bodies or of parts of fruiting bodies of *A. augustus*, *A. bitorquis*, *A. campestris* (grub infested; Fig. 4g, h), *A. subfloccosus*, *A. subperonatus* (grub infested; Fig. 4i–k), *Amanita excelsa*, *Hygrocybe conica*, *L. sulphureus*, *L. birnbaumii*, *Pholiota* spec., *P. vestita* (of two tested, one was grub infested; Fig. 4c), *Psathyrella spadiceogrisea* (with beetle; Fig. 4d), and *X. chrysenteron* (grub infested). Contaminations were found in one of two tested *A. dura* spore solutions (203 colonies, 1 type of bacteria), in one of the solutions from two tested grub-infested *Boletus luridus* fruiting bodies (20 colonies of yeasts), and in spore solutions of *H. olivaceoalbus* (59 bacteria, 1 type), *Inocybe erubescens* (3 yeast colonies), and grub-infested mushrooms of *Boletus rhodoxanthus* (312 colonies, 2 types of bacteria) and *S. queletii* (2128 colonies, 3 types of bacteria); i.e., contaminations in spore solutions in this series of platings were discovered in solutions of 27.3% of all tested mushrooms (i.e. in 20.7% of all individual samples tested) with 18.2% (13.8%) bacterial infections and 9.1% (6.9%) fungal contaminations. As a further important aspect, it should be recalled that in total 9 of these 22 mushrooms tested (Fig. 4d and not further shown) were infested with animals while only 3 spore solutions of these were contaminated.

Considering all plating test series together, 19 mushrooms infested with animals were analysed (from 15 different species; 28 different samples). Spore solutions of only 7 of these (25.0% in total; 17.9% with bacteria, 7.1% with fungi) were found contaminated.

Discussion

Classically, when spore prints are to be produced from mushrooms with open hymenia such as for species identification or when individual basidiospores are to be isolated for germination, mushroom caps are laid facing down onto a surface (see "Introduction" section), in compliance to their orientation in nature. Under saturating levels of humidity, matured basidiospores are propelled off from the sterigmata on their basidia by fast hygroscopic development and subsequent actions of Buller's drop. The surface energy obtained from the Buller's drop by its fusion with the also hygroscopic liquid adaxial spore film is calculated to be sufficient for the spores to just reach the middle in between two lamellae or ridges or of a pore in order to then fall down out of the mushroom by gravity (Ingold 1939, 1957, 1992; Webster et al. 1984a, 1989; Turner and Webster 1991; Pringle et al. 2005; Noblin et al. 2009; Fischer et al. 2010a, b). In this study, we show indirectly by a dependence on high humidity that ejection of ballistospores from their sterigmata is a prerequisite for later collection of basidiospores from plastic lids positioned above upside-down incubated mushrooms with open hymenia. This is however not all.

Driving forces for spore flight

If only the catapulting energy and gravity will be the acting driving forces for the spores to move upon release, accumulation of basidiospores against gravity in the lids of plastic Petri-dishes would not be possible. The energy of catapulted basidiospores is quickly used up during the propelling into the free airspace by the braking effect through the viscosity of the air (Turner and Webster 1991; Pringle et al. 2005; Stolze-Rybczynski et al. 2009; Fischer et al. 2010a, b). Once fully braked, the spores should then drift downward by action of gravity, which in case of upside-down positioned mushrooms would be down into the free gaps between hymenia. This reasonable prospect made us wondering what in our experiments might be the reason for about 10% to up to 100% of the basidiospores from a mushroom to instead fly up and attach to the plastic Petri-dish lids (Table 1).

The air space in the closed Petri-dishes can be expected to be motionless. Concerted release of basidiospores by a mushroom and evaporate cooling of the air surrounding the cap can principally help that the spores are whirled up in clouds by created airflows (Buller 1934; Deering et al. 2001; Dressaire et al. 2015, 2016). However, the clear lamellar patterns of mushrooms reproduced in our experiments by the spore repositories in the plastic lids speak against any generation of influential eddies in the closer head space of the mushrooms. Our investigations revealed that electrostatic charges by plastic are the likely reason for spores to move straight up against gravity and also for them to attach to the attracting plastic (Table 1). However, charges innate to the spores should also have their part in this process (Fig. 3). Basidiospores when ballistically discarded from mushrooms are indeed electrically charged. Charges of endogenous origin are possibly connected to the mass transfer when Buller's drop suddenly fuses with the liquid film on the adaxial side of a spore. In addition, airflow will create charges on spores

by triboelectric effects (Buller 1909; Webster et al. 1988; Saar and Parmasto 2014; Saar and Salm 2014) which could give an extra stimulus to the spores to leave the caps against gravity by electrostatic attraction to plastic lids.

In species-specific manner, spores of an individual mushroom can differ in type of charge (+ or −) and in strength of charging. The charges cause in horizontal electric fields that spores shed from mushrooms will drift away to one side. In many species, there are populations of positively and of negatively charged spores, while spores in some species are unipolar-positively and in some others unipolar-negatively charged (Buller 1922; Gregory 1957; Webster et al. 1988; Saar 2013; Saar and Parmasto 2014; Saar and Salm 2014). Of the species that we tested in this study (Table 2), absolute charges of basidiospores have been estimated before for *A. campestris* in different experimental series between 1.55/0.39/0.39 and 3.10/3.11/2.16 $\times$ 10^{-17} C (mean 2.23/1.28/0.93 $\times$ 10^{-17} C), for *C. micaceus* between 0.85 and 4.22 $\times$ 10^{-17} C (mean 2.14 $\times$ 10^{-17} C), for *L. nuda* 1.88 $\times$ 10^{-17} C, for *M. oreades* between 1.25 and 10.12 $\times$ 10^{-17} C (mean 4.37 $\times$ 10^{-17} C), for two *Pholiota* spec. strains between 1.65 and 2.03 $\times$ 10^{-17} C, and for *S. commune* between 1.70 and 9.55 $\times$ 10^{-17} C (mean 4.10 $\times$ 10^{-17} C), (Webster et al. 1988; Saar and Salm 2014). Populations of basidiospores of *L. nuda* were found to be 97% negatively charged, whereas populations of *A. campestris* and *Pholiota* species were bipolar with more numerous positively charged spores and populations of *C. micaceus* bipolar with more negatively-charged spores (Buller 1909; Gregory 1957; Saar 2013; Saar and Salm 2014).

Depending on individual spore charges and on relative distances (Fig. 3), electrostatic charged plastic lids of the Petri-dishes may thus differentially attract the also loaded spores. This can then explain why in our experiments mostly only parts of the total spores released from a fruiting body are directed toward the plastic lids positioned above mushrooms (Table 1) and why with higher distances between lids and mushrooms the number of spores attached to the lids decreased in logarithmic fashion (Fig. 3). Overall charges of spores correlate little with spore sizes and actual spore charges are also independent on spore emission rates from mushrooms (Saar and Salm 2014). This is also reflected in our results on different species as shown in Fig. 3. Spore sizes have in contrast been shown to influence sizes of Buller's drops, the spore velocity upon ejection from the sterigmata, the length of the move vertically through a mushroom airspace before being braked (Stolze-Rybczynski et al. 2009; Fischer et al. 2010a), and the distance of spore deposition from the source (Norros et al. 2014), parameters which appear all be not of primary relevance to the electrostatic attraction and attachment of the spores to plastic lids reported here.

Incidence of basidiospore charging in Agaricomycetes

In this study, basidiospores of in total 66 species with open hymenia and ballistospore propulsion mechanism (from 36 genera, 19 families and 5 orders) did accumulate against gravity in plastic lids and we failed only for four species with each one mushroom tested (*H. virginea*, *H. eburnus*, *L. saeva*, *R. fibula*) to proof such effect. Charging of ballistospores appears thus to be a widely distributed property in the *Agaricomycetes*. This confirms former observations by Saar and Salm (2014). These authors concluded from own and literature data for 43 species, 33 genera, 23–25 families and 9 orders of the *Agaricomycetes* that their ballistospores exhibit electric loads. While we used in our studies in the majority species of the *Agaricales* and to less extend species from the *Boletales* and other orders (Table 2), Saar and colleagues analysed the electric properties of larger species ranges of *Polyporales* and *Boletales* with poroid hymenia, in addition to species from the *Agaricales*, *Russulales* and others (Saar 2013; Saar and Parmasto 2014; Saar and Salm 2014). Considering shared species between the different studies, evidence for ballistospore electric charging is now available for over 100 different species (i.e. 103 or 104 species) of the *Agaricomycetes*.

As we have shown here, charging is not only confined to ballistospores of *Agaricomycetes*. The spores of earthstars which lost the mechanism of ballistospory (Hibbett et al. 1997; Wilson et al. 2011) were also attracted by plastic lids once they were manually pushed into the air. They attached even stronger to the plastic lids than the spores of other *Agaricomycetes*. When trying to brute-force them into solution, they repeatedly formed a dense layer as a cover over the surface of a sphere of 200 µl water. The water ball with the spores on the outside repeatedly burst to slip away from underneath the spore layer which remained then as dry dark brown spot (about 7 mm in Ø) of densely packed spores strongly attached to the plastic surface of the lid (our unpublished observations).

Microbial contaminations in spore solutions

We have demonstrated that basidiospore transfer from upside-down incubated mushrooms with open hymenia to the plastic lids depends on an active ballistospore discharge mechanism (Table 1). This suggested that producing spore prints from up-flying spores would be more selective to the basidiospores than the usual spore prints collected underneath wild mushrooms which tend to be mixed with fallen spores and cells of other microbes. This assumption was tested by plating spore solutions from classical prints and spore solutions collected from

plastic lids positioned above upside-down incubated mushrooms (Table 4; Figs. 5, 6). In contrast to conventional spore prints, presence of yeasts or spores from other filamentous fungi which need animal vectors or air flow for their movements was rarely detected in plating tests of spore solutions which were collected from plastic lids positioned above reversed incubated mushrooms (at maximum 10% of samples were contaminated). Bacteria were in somewhat higher frequency present (in up to 22% of total samples of an individual test series), possibly because they may at times be flying directly with a basidiospore. However, there are possible measures against bacteria through addition of suitable antibiotics to the growth media (Fig. 5; Table 4). Further, accumulation of larger numbers of bacterial cells can be precluded by shorter incubation times applied for basidiospore collection (Fig. 6). Already 2–4 h of incubation are sufficient to collect 10^4–10^5 spores from mushrooms in the plastic lids (Fig. 2) with no or exceptionally a few bacteria being present in the spore solutions (Fig. 6). Spore ejection rates in our study (Fig. 2; see Table 1 for total ejection figures) correlate with ratios of ballistospore ejection/h from mushrooms reported by Buller (1909, 1922) and to ratios which can be calculated from the spore emission data/s × cm^2 compiled in Saar and Salm (2014).

Basidiospores secrete hygroscopic hexoses and alcohols (mannitol) localized at their hilar appendices for the assembly of Buller's drops and at their adaxial shallow dents as hygroscopic compounds for the formation of liquid films, both of which are required for rapid spore catapulting upon their fusion (Goates and Hoffmann 1986; Webster et al. 1989, 1995; Turner and Webster 1995). These organic metabolites together with inorganic ions like phosphate, sodium and potassium are transferred with the liquids spread over the propelled-off spores onto the lids of the Petri-dishes where they might act further hygroscopically to attract more water to the spores (Elbert et al. 2007; Hassett et al. 2015). Eventually, the metabolites might be used for growth of any microbial contaminants happened to be present in the liquid droplets in which basidiospores amplify on the plastic lids during mushroom incubation (Fig. 1). Such microbial growth effect can explain the explosive sudden increase in bacterial cells which was observed more often in spore samples collected after longer incubation times of mushrooms (Fig. 6).

Accordingly, earlier spore harvests can reduce the chance for unwanted bacterial transfer and the time for possible proliferation of contaminants. For later use after a time of storage, drying off the liquid and storing the basidiospores under dry conditions on the sterile plastic lids is also a possible measure of avoidance. Earlier harvest times furthermore reduce the danger of undetected small animals to creep out of mushrooms and to crawl over and contaminate with other microbes the spore collections assembled over the time above the mushrooms on the plastic lids. Such crawling of initially overlooked insect larvae over the spore prints in the plastic lids has indeed been noticed by us on two occasions (not further documented).

Spore charges in applications

Charging of fungal spores and any potentially connected functions (e.g. in support for spores to serve as nuclei for raindrops; Elbert et al. 2007; Hassett et al. 2015) are understudied and generally little understood (Webster et al. 1988; Wargenau et al. 2011, 2013; Saar 2013; Saar and Parmasto 2014; Saar and Salm 2014). In line, practical use of charging of spores and spore behaviour in electric fields has so far only seldom been made of. However, guarding of bookshelves with an electric field screen has been tested to successfully protect old valuable books in library stack rooms against mold infection by airborne spores (Takikawa et al. 2014) and electrostatic dust collectors were applied in French archives to study their fungal allergenic potentials (Roussel et al. 2012). Spores in buildings and rooms distribute unevenly, among as factors behind by being influenced by electric and magnetic devices (Anaya et al. 2015, 2016). Knowledge on fungal spore distributions in buildings as in outside environments can come through application of electrostatic dust collectors (Normand et al. 2016) and electrostatic precipitators might be used (Han et al. 2011). An electrostatic nursery shelter and an ozone-generative electrostatic spore precipitator were reported to protect tomato plants in hydroponic culture and in open-window greenhouses against fungal pathogens (Shimizu et al. 2007; Kakutani et al. 2012; Takikawa et al. 2016) and an electrostatic spore collector has been applied to collect conidia of the barley pathogen *Blumeria graminis* (Moriura et al. 2006). While our experimental set-up here is simple and easy to apply by using electrostatic features intrinsic to the plastic lids of Petri-dishes to selectively collect basidiospores from mushrooms, our observations offer the possibility to also develop technically more sophisticated devices for basidiospore collection.

Abbreviations

AB: antibiotics; Ø: diameter; RT: room temperature.

Authors' contributions

UK and KL designed research. KL performed research. UK and KL analysed data and wrote the paper. Both authors read and approved the final manuscript.

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Competing interests
Both authors declare that they have no competing interests.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

Ethics approval
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Chapter 6

Proteins Expressed during Hyphal Aggregation for Fruiting Body Formation in Basidiomycetes

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The paper concentrates on families of small secreted proteins with sugar-binding and/or haemolytic activities reported in previous work to link to hyphal aggregation and initiation of fruiting body development. Genes for different proteins were usually only found in a few species independent of fungal relationships which makes a global and central function for such proteins in hyphal aggregation and fruiting body initiation unlikely.

Authors´ contributions: Rajesh Velagapudi (RV) and Monica Navarro-Gonzaléz (MN) observed fungal development and supplied photos on primary hyphal knot formation and a secondary hyphal knot. Kiran Lakkireddy (KL) analyzed fungal genome databases for presence of fungal lectin and haemolysin genes. KL together with Ursula Kües (UK) produced alignments and the phylogenetic tree of fungal fruiting body lectins. UK wrote the paper. MN and KL provided literature and gave input to paper writing.

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PROTEINS EXPRESSED DURING HYPHAL AGGREGATION FOR FRUITING BODY FORMATION IN BASIDIOMYCETES

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ABSTRACT

The first visible step in fruiting body development in basidiomycetes is the formation of small hyphal knots by localized intense branching of hyphae of restricted length accompanied by hyphal aggregation. In *Coprinopsis cinerea*, the first not yet fruiting-specific step of hyphal branching occurs in the dark, the second step requires a light signal. Hyphal aggregation implies cell-cell contacts and protein interactions on the outer cell walls are anticipated. Few protein candidates were identified and discussed in the past for such function. Amongst were the galectins of *C. cinerea* and the Aa-Pri1 protein (aegerolysin) of *Agrocybe aegerita* that are specifically expressed during the step of hyphal aggregation as well as during subsequent primordia development. In this study, we follow up the distribution of genes for proteins with lectin and/or hemolysin function in the steadily growing number of available genomes of basidiomycetes. Neither galectin genes nor genes for other lectins nor *Aa-pri1*-like genes nor other hemolysin genes are present in all mushroom species, making an essential role for such functions in hyphal aggregation unlikely.

Keywords: Lectin, hemolysin, mushroom formation, hyphal knots, predator defence

INTRODUCTION

Vegetative mycelial growth of filamentous fungi basically consists of tip growth of leading hyphae with sporadic subterminal initiation of a sidebranch that then also undergoes tip elongation for further growth [1]. Such simple mycelial growth can thus locally be considered as just two-dimensional. Fruiting body development in contrast is a complex process which changes from simple two-dimensional vegetative growth of the mycelium to formation of a compact three-dimensional aggregated structure in which differentiation of specific cap and stipe tissues takes place [2]. The first visible structure is the hyphal knot generated by intense localized formation of stunted, growth-restricted sidebranches that interweave and eventually aggregate with each other. In *Coprinopsis cinerea*, we distinguish primary from secondary hyphal knots (Figs. 1, 2) which form within dark and subsequently upon (blue) light illumination, respectively [3, 4]. Initiation of hyphal aggregation is controlled by the mating type genes [5, 6] and environmental factors – which in addition to light are temperature, nutrients, humidity, and aeration [2, 7] –, but little is yet known on the cellular processes leading to aggregation. Different proteins have however been implicated in basidiomycetes in functioning in hyphal aggregation, based on observations of coincidental expression of their genes with initiation of fruiting and subsequent primordia development [7, 8].

Many different types of sugar moieties-binding lectins are known to occur in mushrooms as candidate proteins for mediating cellular aggregation [8, 9]. Galectins are β-

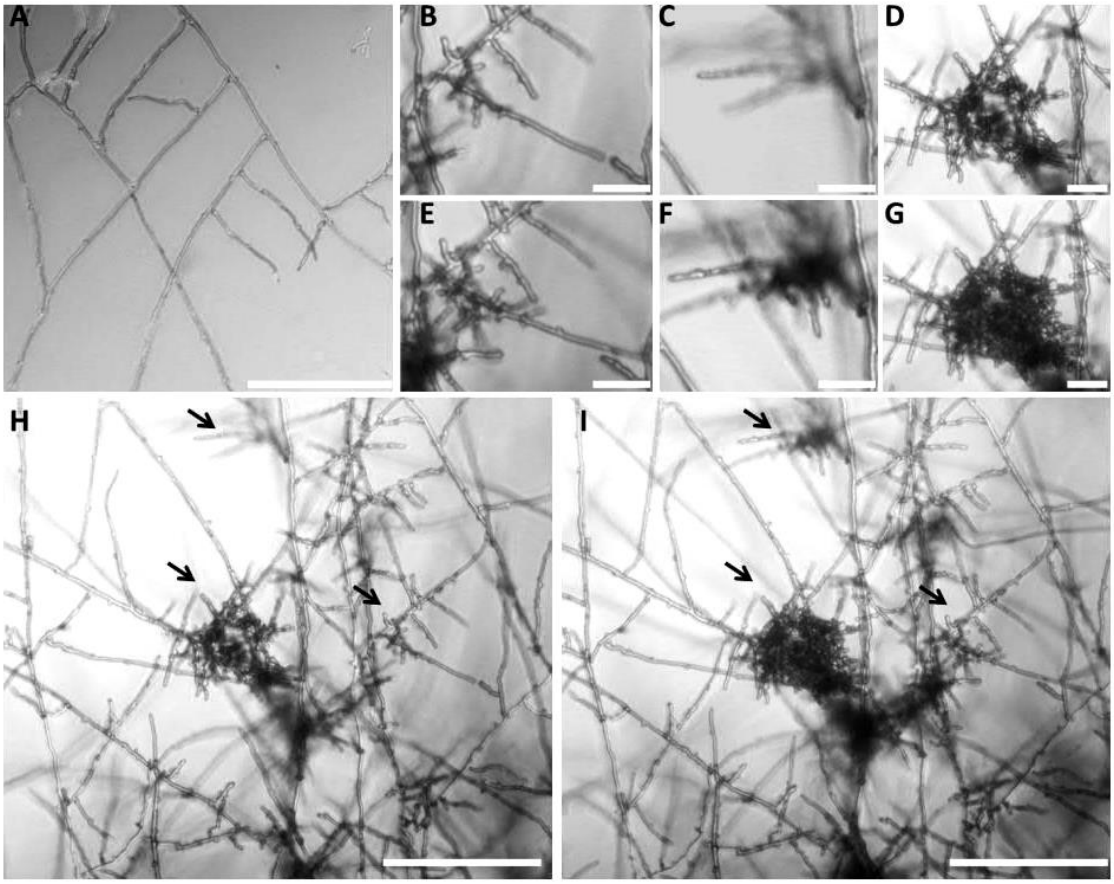

Figure 1: Primary hyphal knot formation of *C. cinerea* homokaryon AmutBmut in an YMG/T agar culture: Mycelial lattice after 24 h of growth (A). Primary hyphal knots within the lattice after 40 h (B-D,H) and 60 h of growth (E-G,I). Arrows in I and H point to structures shown enlarged in B-D and E-G, respectively. Size bar = 20 µm (B-G), = 100 µm (A,H,I).

galactoside binding lectins characterized by a specific sugar-binding domain [10]. In *C. cinerea*, expression of galectins (CGL1, CGL2) and a galectin-related lectin (CGL3) starts at the stages of primary, respectively secondary hyphal knot formation and continues throughout primordia formation. Galectins are secreted and localize to cell walls and the extracellular matrix (ECM) of mushroom tissues. Highest expression is found in the outer cap and outer stipe tissues [4, 11-13]. Although a function in cell-cell aggregation had been postulated [4], more recent studies showed that the proteins are not essential and point to a role in protection against grazing arthropods and nematodes [14-16]. Related mushroom-specific galectins exist in *Agrocybe aegerita* (syn. *cylindracea*) [17, 18], *Heterobasidion annosum* (*irregulare*) [8], *Laccaria bicolor* and *Laccaria amethystina* [12, 19]. Application of isolated *A. aegerita* galectin AAL in fresh cultures reduced mycelial growth rates and induced mycelial cord formation. Most interestingly, application on own and on also foreign (*Auricularia polytricha*) established mycelium resulted in formation of aggregates and primordia differentiation [20, 21]. Also *Agrocybe* lectins have anti-nematode activities [22].

Members of another family of β-galactoside binding lectins [FB (fungal fruit body) lectin super-family] occur in *Athelia* (*Sclerotium*) *rolfsii* (SRL; SLR-like), *Agaricus bisporus* (ABL), *Xerocomus chrysenteron* (XCL), *Pleurotus cornucopiae* (PCL-M, PCL-F), *Boletus edulis* (BCL) and *Paxillus involutus* [23-30]. Functions in aggregation in sclerotia formation and in inhibition of sclerotia germination have been reported for SRL in *A. rolfsii* [31].

Figure 2: View on an about 100 µm sized secondary hyphal knot (center) grown on horse dung and neighbouring primary hyphal knots to the left and right.

PCL-F is envisaged to contribute to aggregation during fruiting although lectin-deficient *P. cornucopiae* mushrooms of normal shape appear to exist [32, 33], SRL, SLR-like and XCL showed anti-nematodal and insecticidal activities [24,26,34]. Structurally, ABL and XCL resemble actinoporins [27, 35], a family of membrane-integrating pore-forming toxins that act hemolytic [36]. LSLa is one of three closely related lectins (LSLa to LSLc) from *Laetiporus sulphureus* and represents another small characterised mushroom protein with combined lectin and pore-forming activities. This hemolysin divides into an N-terminal lectin-domain and a C-terminal porin domain of the haemolytic aerolysin protein family [37]. Further mushroom lectins are represented by ricin B-type proteins from *Clitocybe nebularis* [38], *Pleurotus squamosus* [39], *Pleurocybella porrigens* [40], and *Marasmius oreades* [41], and the immunomodulatory lectin FIP-fve from *Flammulina velutipes* [42]. Lectin PVL from *Psathyrella* (*Lacrymaria*) *velutina* is an integrin-like protein with seven internal repeats expressed both in mycelium and in mushrooms. There is a homolog in *C. cinerea* [43].

Aegerolysins (for which *A. aegerita* Aa-Pri1 = aegerolysin was name-giving) belong to another family of pore-forming hemolysins. Lectin-like interactions are not described but these proteins interact with lipid rafts in cellular membranes [44]. *Pleurotus ostreatus* ostreolysin and pleurotolysin A (with pleurotolysin B from a two-component system), *Pleurotus eryngii* erylysin A (interacting with erylysin B) and possibly *Pleurotus nebrodensis* nebrodeolysin are other closely related members of this family [45-48]. Postulated to be aggregation factors [49, 50], application of ostreolysin to *P. ostreatus* mycelium was found to be fruiting inducing [45]. In coincidence, aegerolysins in *Agrocybe* and *Pleurotus* species are expressed at initiation of fruiting and during fruiting body development [49, 50]. Also in *Moniliophthora perniciosa*, aegerolysin genes expressed along with fruiting body formation have been described [51]. Flammutoxin from *F. velutipes* is another type of pore-forming hemolysin specifically expressed during fruiting [52].

MATERIALS AND METHODS

Strain, culture conditions and microscopy. The self-compatible *C. cinerea* homokaryon AmutBmut able to form fruiting bodies due to mutations in both mating type loci [4] was cultivated on YMG/T complete medium or on horse dung as described [53]. For microscopy of hyphal development, observation windows were made within fully grown fungal YMG/T cultures by cutting out agar pieces of about 1 cm^2. Cultures were further incubated at 28°C in the dark for about 50 h and hyphal growth within the windows was monitored at intervals of 3 to 4

hours, using an inverse Axiovert (Zeiss, Göttingen, Germany) microscope in a dark room with a yellow filter placed into the light beam of the microscope.

Sequence analysis. *L. velutina* PVL (GenBank ABB17278), *C. cinerea* CGL1 (AAB04141; CCG1_05003), *F. velutipes* FIP-fve (ADB24832), ricin B-like lectins of *C. nebularis* (ACK56062), *P. squamosus* (BAC87876), and *L. sulphureus* (LSLa; 1W3A_A), FB lectins of *A. bisporus* (ABL; Q00022), *P. cornucopiae* (PCL-F1; AB056470), and *X. chrysenteron* (XCL; AAL73235), *A. aegerita* aegerolysin (AAC02265), *P. eryngii* erylysin A (BAI45247) and B (BAI45248), and *F. velutipes* flammutoxin (BAA76510) were used in tblastn searches (expect 1.0E-0; word size 3; no filter) of basidiomycete genomes available in July 2011. From the MycoCosm page (http://genome.jgi-psf.org/programs/fungi/index.jsf) of the Joint Genome Institute (JGI) were used Pucciniomycotina *Melampsora laricis-populina* v1.0, *Puccinia graminis*, *Rhodotorula graminis* strain WP1 v1.1, and *Sporobolomyces roseus* v1.0, Ustilagomycotina *Malassezia globosa* and *Ustilago maydis*, Agaricomycotina *A. bisporus* var. *bisporus* (H97) v2.0, *A. bisporus* var. *burnettii* JB137-S8, *Auricularia delicata* SS-5 v1.0, *Ceriporiopsis subvermispora* B, *Coniophora puteana* v1.0, *C. cinerea* Okayama 7, *Cryptococcus neoformans* var. *grubii* H99, *Dacryopinax* sp. DJM 731 SSP-1 v1.0, *Dichomitus squalens* v1.0, *Fomitiporia mediterranea* v1.0, *Fomitopsis pinicola* SS1 v1.0, *Ganoderma* sp. 10597 SS1 v1.0, *Gloeophyllum trabeum* v1.0, *H. annosum* (*irregulare*) v2.0, *L. bicolor* v2.0, *Phanerochaete carnosa* v1.0, *Phanerochaete chrysosporium* v2.0, *Phlebia brevispora* HHB-7030 SS6 v1.0, *P. ostreatus* PC9 v1.0 and PC15 v2.0, *Postia placenta* MAD-698, *Punctularia strigosozonata* v1.0, *Schizophyllum commune* v1.0, *Serpula lacrymans* S7.3 v2.0 and S7.9 v1.0, *Stereum hirsutum* FP-91666 SS1 v1.0, *Trametes versicolor* v1.0, *Tremella mesenterica* Fries v1.0, and *Wolfiporia cocos* MD-104 SS10 v1.0, from the fungal site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) of the National Centre for Biotechnology Information (NCBI) *Microbotryum violaceum* p1A1 Lamole, *Puccinia triticina* 1-1 BBBD Race 1, *Mixia osmundae* IAM 14324, *Melassezia restricta* CBS 7877, *Cryptococcus gattii* R265 and WM276, and *M. perniciosa* FA553, and from the Munich Information Center for Protein Sequences (MIPS) *Sporisorium reilianum* (http://mips.helmholtz-muenchen.de/genre/proj/sporisorium/). Where required, computer-defined gene coordinates were manually corrected and genes annotated on respective pages at JGI (except *S. lacrymans* 1627212: delete N-terminal 54 aa). *M. perniciosa* ABRE01005301 (NCBI) was also amended: join 834-1117,1172-1310. For other accession numbers see figures. Using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc/), sequences were aligned Phylogenetic trees were calculated by neighbour joining by MEGA version 4.0 [54].

RESULTS AND DISCUSSION

Hyphal knot formation and early hyphal aggregation. Fig. 1A shows at first two-dimensional vegetative mycelial growth (known as lattice formation [3]) of *C. cinerea* homokaryon AmutBmut when entering a new surface. About 20-30 hours later, hyphal knots of different developmental stages can be found at distinct places within the mycelium (Fig. 1B-D, H). Clearly, intense initiation of formation of sidebranches occurs highly localized over a restricted length (about 50 µm) at one leading hypha or, in closest distance, at two or three neighbouring hyphae. Although the sidebranches stop growing after a few µm (up to about 30 µm as estimated from Fig. 1B-G), the close vicinity of areas of intense branching at neighbouring hyphae allow sidebranches from different leading hyphae to intermingle (Fig. 1D,E).

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	F. velutipes FIP-fve	*L. velutina* PVL	*C. cinerea* CGL1	*C. nebularis* ricin B-like lectin	*P. squamosus* ricin B-like lectin	*L. sulphureus* LSL.a	*D. squalens* 158296 *G. trabeum* 129557	FB lectins ABL, PCL-F1, XCL	*A. aegerita* aegerolysin, *P. eryngii* erylysin A	*P. eryngii* erylysin B	*F. velutipes* flammutoxin
A. bisporus var. *bisporus*	-	-	-	-	-	-	-	75698 194888 194894	-	-	-
A. bisporus var. *burnettii*	-	-	-	-	-	-	-	114704	-	-	-
A. delicata	57009	-	-	-	-	-	-	67476 85006 115017 115035 117944	-	-	199645
C. subvermispora	172151[§] 172155[§] 172156[§]	-	-	-	117217 117225 125532	172144[+]	-	-	172089	-	-
C. puteana	-	-	-	-	-	-	-	-	-	-	-
C. cinerea	-	CC1G _03091*	CC1G _00723 _05003 _05505	CC1G _10077 _10083	CC1G _10075 _10077 _10083	CC1G _08369[+] _10318 _11805	CC1G _08369[+] _10318 _11805	-	-	-	-
Dacryopinax sp.	-	-	-	-	-	-	-	-	-	-	-
D. squalens	53695 101883 125142	-	-	-	148933 172700 172722 172724 172726 172744 201382 201385 201389[Δ]	158296	158296	-	69680	-	90817
F. mediterranea	-	-	-	-	-	-	-	-	-	-	160286 160275 187205 187206
F. pinicola	-	-	-	-	82284 82401 82402 82405 82406 82407 82435 82645 89059	124282 281656[+]	124282	-	-	-	82673 95762
Ganoderma sp.	-	-	-	-	-	-	-	-	-	-	-
G. trabeum	-	-	-	-	-	129557	129557	-	-	-	-
H. irregulare	-	-	58543	-	-	-	-	-	148469	38497	65755
L. bicolor	-	692684	236913 312069 723752	-	-	576524[+] 461940	461940	185716	-	-	-
M. perniciosa	-	-	-	-	EEB99847[Δ] EEB94816[Δ]	ABRE01 005301*	-	-	EEB90416 EEB92328[Δ] EEB93043[Δ] EEB93315[Δ] EEB95579[Δ] EEB96271	EBB89936 ABRE01 017070[§]	-
P. carnosa	192435	-	-	-	211794 261044	-	-	257886 263561	-	-	-
P. chrysosporium	-		-	-	-	-	-	6917 140897	-	-	-
P. brevispora	79844 117676	-	-	-	-	-	-	71190	-	-	-
P. ostreatus PC9	-	-	-	-	122379	-	-	107763	72745	133806	67050 117864
P. ostreatus PC15	-	-	-	-	1119533[+]	-	-	1044138	1090164	1090161	168572 1091975
P. placenta[#]	135177 135180	-	-	92379 135173	92379 135173 135175 135176[Δ] 135181 135182 135188 135191	135167 135168	135167 135168	46158 46169 57081 135165	-	-	135146 135148

* model corrected; [+] model from gene with frameshift/deletion/early stop codon; [§] gene remnant?; [#] contains hits from alleles; [Δ] incomplete gene; [¶] putative family 5 glycoside hydrolase with two N-terminal ricin B-like motifs

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina (continued)

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	F. velutipes FIP-fve	*L. velutina* PVL	*C. cinerea* CGL1	*C. nebularis* ricin B-like lectin	*P. squamosus* ricin B-like lectin	*L. sulphureus* LSLa	*D. squalens* 158296 *G. trabeum* 129557	FB lectins ABL, PCL-F1, XCL	*A. aegerita* aegerolysin *P. eryngii* erylysin A	*P. eryngii* erylysin B	*F. velutipes* flammutoxin
P. strigosozonata	-	-	-	-	-	-	-	134444 143781 154836 154837[Δ]	101965	-	-
S. commune	-	-	-	-	103548	-	-	-	-	-	74780
S. lacrymans S7.3	-	-	-	-	162712* 173380 187490	-	-	-	-	-	-
S. lacrymans S7.9	-	-	-	-	442012 457021 477093	-	-	-	-	-	-
S. hirsutum	-	-	-	-	-	-	-	153353 182934	-	-	-
T. versicolor	121721 184741	-	-	-	-	-	-	-	52920	52921	-
T. mesenterica	-	-	-	-	73633[¶]	-	-	-	-	-	-
W. cocos	-	-	-	-	-	-	-	81600	-	-	-
Total	**12**	**2**	**7**	**4**	**46**	**13**	**9**	**28**	**13**	6	**16**

Formation of further primary and also higher order side branches occurs so that within the developing primary hyphal knot first hyphal aggregation becomes possible (Fig. 1D, E, G-I). When this happens, it becomes difficult to follow up further processes of development by simple light microscopy since the three-dimensional structures are more and more impervious to light. How the step from primary hyphal knot to the secondary hyphal knot and compact aggregation happen is thus still to be clarified. Fig. 2 shows an impression of a secondary hyphal knot with primary hyphal knots growing in the neighbourhood.

Candidate proteins for hyphal aggregation. Proteins proposed to act in hyphal aggregation for mushroom formation (see Introduction) were used to search the genomes of in total 40 different species (7 Pucciniomycotina; 4 Ustilagomycotina; 29 Agaricomycotina). Allelic genomes of two different monokaryons were available for analysis of *P. ostreatus* (PC9, PC15) and *S. lacrymans* (S7.3, S7.9) and, due to dikaryon sequencing, also for *P. placenta* [55]. Strikingly, none of the tested proteins detected candidate genes in the tblastn searches with any of the Pucciniomycotina, the Ustilagomycotina, and the yeast-like Agaricomycotina, suggesting that these proteins are specific to the mushroom-forming Agaricomycotina (see results in Table 1). Also remarkable, none of the species had genes for all types of proteins but most had one or more genes for lectins and/or hemolysins. Species from different orders share types of proteins and closely related species in contrast do not. The gene distribution limited to always only a few and often even unrelated species does not argue for an essential function of any of the tested proteins in hyphal aggregation and fruiting body formation.

FIP-fve-like lectins and integrin-like proteins. Genes for FIP-fve-like lectins were found in seven wood-rotting species from the Auriculariales, Polyporales and Corticiales (Table 1). In contrast, genes for lectins with integrin-like repeats such as PVL of the saprotroph *L. velutina* were only detected in the dung fungus *C. cinerea* and the ectomycorrhizal *L. bicolor* from the Agaricales (Table 1, Fig. 3). PVL binds N-acetylglucosamine and N-acetylneuromic acid in dependence of calcium and this may help in defence of bacteria [43]. However, it might not be by accident that this type of lectin is not present in any of the many wood-rotting species analysed, raising the question whether occurrence of this type of lectin is restricted to saprotrophic and mycorrhizal species for example to help, as suggested [43], in colonisation of humic soil containing pectins and polygalacturonic acid from decomposing plant material.

Galectins and FB lectins. Genes for galectins were only found in the Agaricales *C. cinerea* and *L. bicolor* and in *H. irregulare* from the Russulales (Table 1), all of which were reported before [8,13]. In contrast, FB lectins are wider distributed and genes were found in Agaricales (4 of 7 species), Auriculariales (1 of 1 species), Corticiales (4 of 4 species), Polyporales (2 of 7 species) and Russulales (1 of 2 species), irrespectively of whether *A. bisporus* ABL, *P. cornucopiae* PCL-F1 or *X. chrysenteron* XCL were used in tblastn searches (Table 1). The β-galactoside binding galectins and FB lectins are distantly related [8]. Galectins have a carbohydrate recognition domain (CRD) consensus of H-3x-R-(7-11)x-N-(6-7)x-W-2x-E-x-R. The *C. cinerea* galectin-like CGL3 (CC1G_00723) contains an R instead of the sugar-recognizing W. Due to this R, CGL3 binds chitooligosaccharides but, unlike galectins, not lactose [13]. Of the members of the fungal galectin family, only LBG3 has also this residue while others possess the classical galectin CRD motif or have 1-2 changes at other positions (Fig. 4). The W residue of the conserved amino acids in the CRD of the galectins is also found in the FB lectins [8] but other amino acids make contact with the sugars (Fig. 4, [57]). The sugar-binding residues (Fig. 4) are highly conserved in all proteins of the FB lectin super-family analysed in this study, although only the residue G was found in all 28 of them and only 8 of them had a perfect central HNY-4x-D-I/V/L-x-T motif (Fig. 4; not further shown).

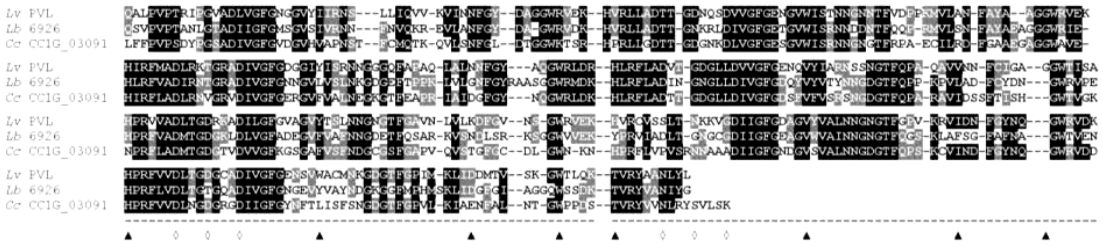

Figure 3: Integrin-like repeats (underlined, 2x per line) from *L. velutina* (*Lv*), *C. cinerea* (*Cc*) and *L. bicolor* (*Lb*) lectins. N-termini of proteins are not shown due to ambiguities in the protein models. Amino acids of sugar and calcium binding are indicated by▲ and ◊, respectively [43].

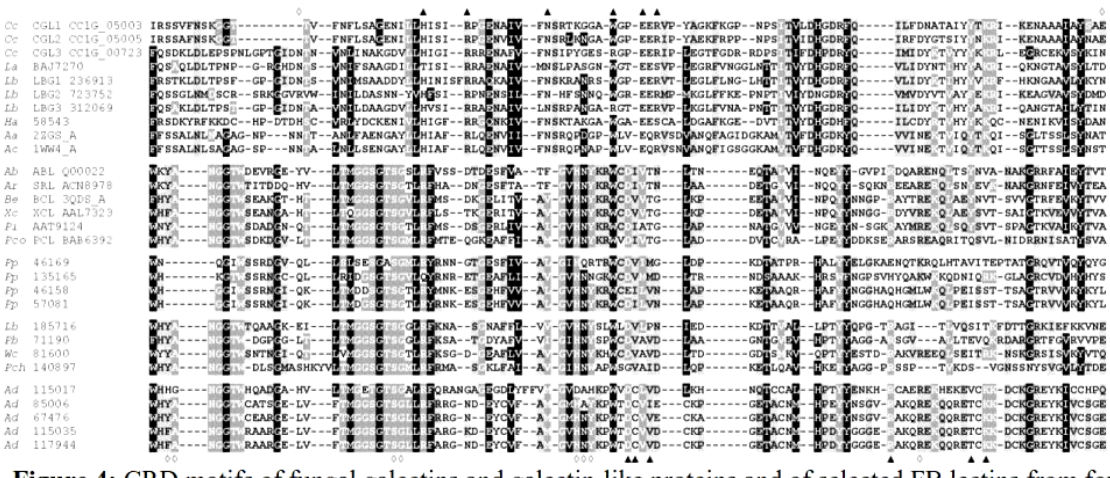

Figure 4: CRD motifs of fungal galectins and galectin-like proteins and of selected FB lectins from four different phylogenetic clades (see Fig. 5). ▲marks primary sugar binding sites for galectins and FB lectins, ◊ extra sites for CGL3 and secondary sites for FB lectins [13, 56].

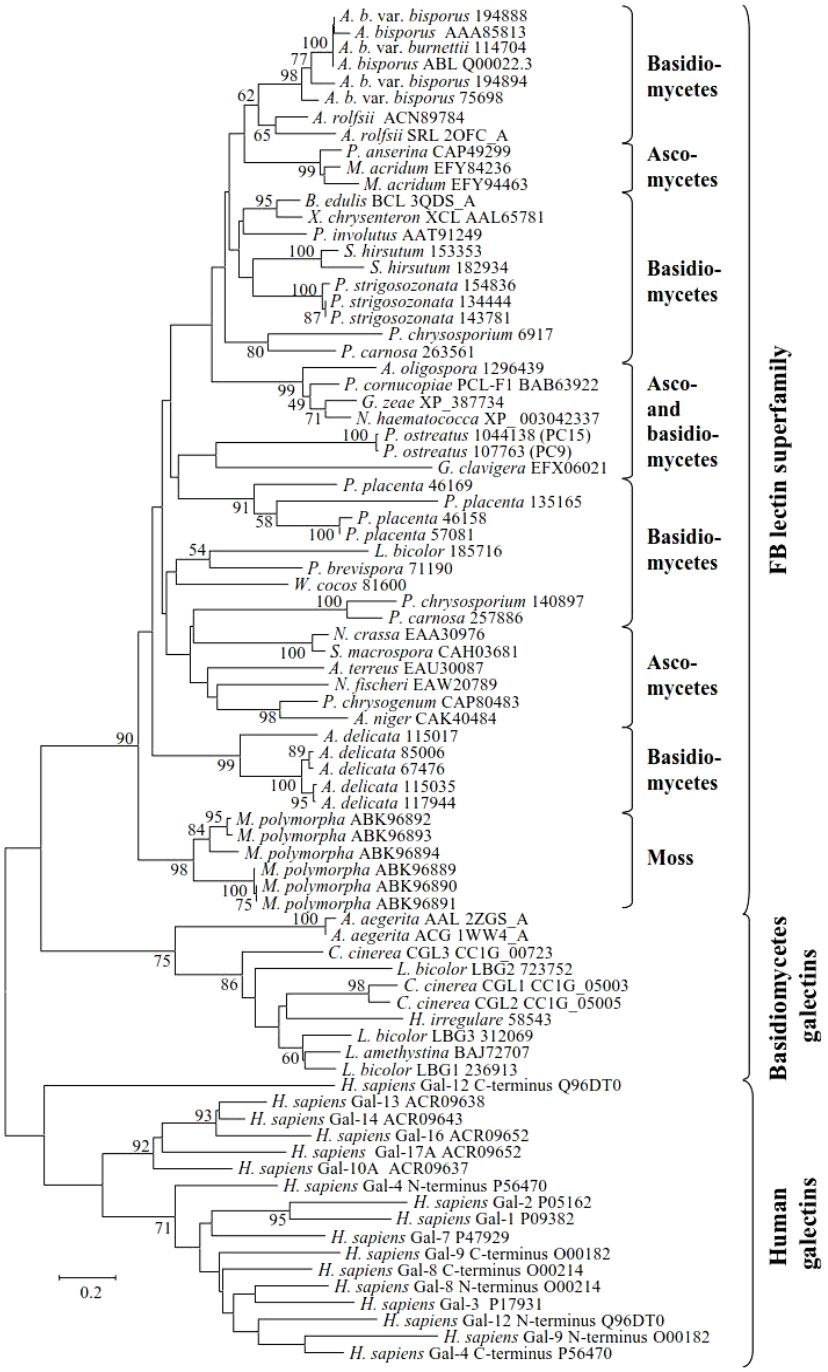

Figure 5: Phylogenetic tree of fungal FB lectins, galectins and galectin-related lectins, FB lectins from the liverwort *Marchantia polymorpha* and human galectins (where present duplicated CRDs were included as N- and C-terminal domain). Fungal species other than those with genomes analysed in study were from the basidiomycetes *A. aegerita, A. rolfsii*, *Boletus edulis*, *L. amethystina*, *P. involutus*, *P. cornucopiae*, and *X. chrysenteron*, and from the ascomycetes *Arthrobotrys oligospora*, *Aspergillus niger*, *Aspergillus terreus*, *Gibberella zeae*, *Grosmannia clavigera, Metarhizium acridum*, *Metarhizium anisopliae*, *Nectria haematococca*, *Neosatorya fischeri*, *Neurospora crassa*, *Podospora anserina*, and *Sordaria macrospora*. JGI protein IDs and GenBank accession numbers are given in the figure. Bootstrapping values (500 replications) above 50 are shown at tree branchings. Scale bar = number of nucleotide substitutions per site.

A phylogenetic tree was produced from all fungal galectins and FB lectins, using the human galectins and FB lectins found recently in a liverwort as foreign proteins (Fig. 5). The tree suggests that FB lectins might have been evolved from ancestors common to the fungal galectins. The position of the group of moss FB lectins is interesting since it might point to a split of galectins and FB lectins prior to the split of plants and fungi. In support of this, there are also genes for galectin-like proteins in plants [10]. Interesting is further to note that ascomycete and basidiomycete FB lectins intermingle with each other. Duplications of FB lectins happened frequently late in evolution close to speciation. There are four major clades of fungal proteins within the FB super-family (Fig. 5) which correspond largely to differences in the sugar-interacting residues in the CRD motifs (Fig. 4 and not shown). Whether these go along with alterations in sugar binding (efficiencies or sugar types) remains to be elucidated.

Ricin B-like lectins. Of the known fungal ricin B-like lectins, two were used in this study in genome searches. Hits to genes were rare for the *C. nebularis* protein unlike the *P. squamosus* lectin that obtained wider distributed hits, including multiple genes in some of the species and the hits by the *C. nebularis* protein. Genes were found in 4 of 7 Agaricales, 1 of 2 Boletales, 1 of 4 Corticiales, 4 of 9 Polyporales, and in the Tremellales species (Table 1). The products divide in four subgroups of simple ricin B-like lectins plus three other ricin B proteins. Subgroups of the ricin B super-family may have little amino acid identity but they share a β-trefoil structure and contain a conserved Q/NxW motif for sugar binding [38, 40]. All 46 proteins listed in Table 1 had this motif in one or more copies. Of the putative simple lectins, 2 had 1, 14 had 2, 3 had 3, 13 had 4, and 1 had 6 copies [proteins of subgroups I (132 to 182 aa), II and III (256 to 336 aa)] and 6 had 6 to 8 copies [proteins of subgroups III [774 to 838 aa)], respectively. Searching the JGI pages with the keyword ricin revealed many more potential genes for ricin B-like lectins in the fungi than found in our current tblastn searches (also in species with so far no detected gene of interest; Table 1). It apparently will be a major but also a fascinating task to resolve the complete ricin B super-family in the basidiomycetes.

Hemolytic LSL-like lectins. *L. sulphureum* LSLa is special by its N-terminal lectin and C-terminal aerolysin domains [37]. Evidence for such dual proteins was found in 8 different species of the Agaricales and Polyporales (Table 1). These proteins divide into four different groups with highly conserved aerolysin domains and less conserved N-terminal domains (Fig. 6). The N-terminal domain in LSLa adopts a β-trefoil structure exposing specific sugar- contacting residues [37]. These residues are not much conserved between the proteins except *F. pinicola* 124282 (Fig. 6), suggesting that the N-termini of the new proteins have either no lectin function or that they developed novel sugar-binding sites. The N-terminal domains of *L. bicolor* 461940, *C. cinerea* CC1G_11805, and *P. placenta* 135167 were individually used in tblastn searches. Neither gave any further meaningful hits, implying unique evolutionary developments for these domains.

Aegerolysins and flammutoxins. Like for the analysed types of lectins, genes for different types of hemolysins were only found in some species (Table 1). In *D. squalens*, *H. irregulare*, and *P. ostreatus*, genes for different types of hemolysins were detected; other species had only one type in one or more gene copies or no gene for any of the tested kinds of hemolysins. The distribution was independent of the fungal order. Aegerolysin genes occurred in Agaricales, Corticiales, Polyporales, and Russulales and orthologues for flammutoxin in Auriculariales, Agaricales, Hymeochaetales, Polyporales, and Russulales (Table 1).

The closely related *A. aegerita* aegerolysin and the *P. eryngii* erylysin A detected the same set of putative aegerolysin genes (Table 1). Some species contained aegerolysin genes and, in addition, genes for erylysin B-like proteins (Table 1). The latter type of gene never occurred

without an aegerolysin gene. Moreover, where both present, the genes come together in divergently transcribed pairs (not shown), emphasizing a common functional role such as has been suggested in *P. eryngii* and *P. ostreatus* by the experimental finding of dimerization of their products [46, 47]. In *D. squalens*, an erylysin B-like gene was missing but there was a footprint of a former gene upstream to the aegerolysin gene 69680 (not shown).

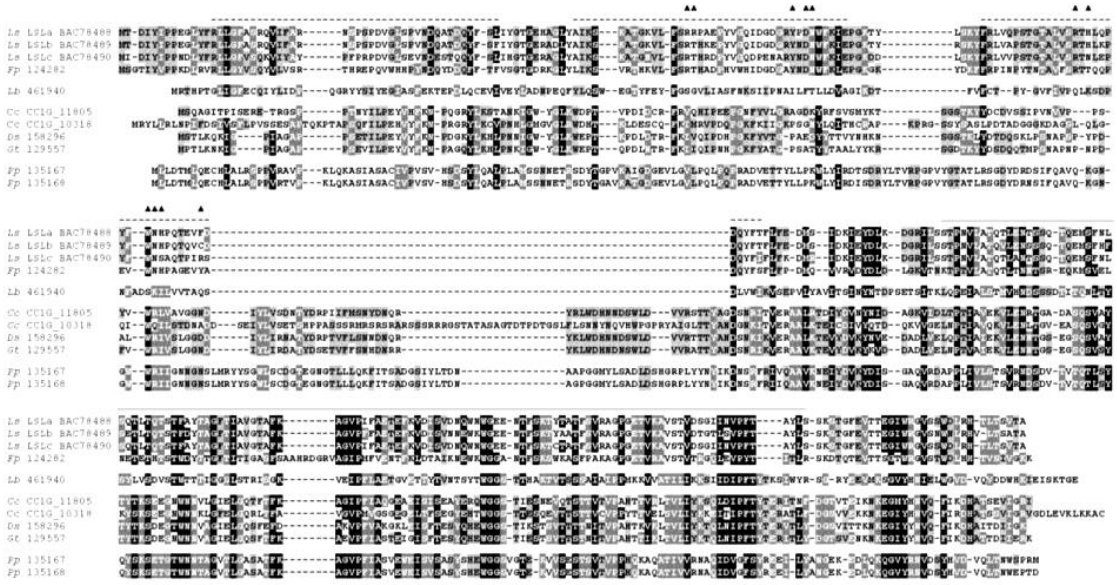

Figure 6: Alignment of putative lectins with aerolysin domains. The β-trefoil module and the sugar-binding residues at the N-terminus of *L. sulphureum* LSLa are marked above the sequence by dashed lines and ▲ and the aerolysin region is indicated by a solid line [37]. Note that from Table 1 only proteins from complete and from intact genes are shown.

CONCLUSIONS

Different lectins and hemolysins have been forwarded as candidate proteins to act in hyphal aggregation, often evidenced by their expression correlating to fruiting (see Introduction). Genome analysis in this study revealed that genes for the tested types of proteins were specific to the mushroom forming species. However, genes for none of the various specific types of proteins occur in all mushroom forming species, although presence of one or more types of lectins and of hemolysins was not rare. The results imply that either these proteins may not be essential for fruiting body initiation and hyphal aggregation or that in evolution different routes have in parallel been developed for the course of events to initiate and continue the fruiting process. Since fruiting body development stands as an essential step at the beginning of sexual spore formation, it appears unlikely that a process central to initiation has been independently invented several times. Experimental evidences we have so far from studies in the literature suggest neither for the different lectins nor for the pore-forming hemolysins a direct function in aggregation.

Pore-forming hemolysins may influence membrane signalling in specific interaction with lipid rafts [44] and, by this, they may indirectly influence the hyphal aggregation process by for example modifying the frequency of fruiting or the environmental conditions under which fruiting occurs. Such effect could explain the observation of induction of fruiting upon application of ostreolysin (in excess?) to vegetative *P. ostreatus* mycelium [45]. Is this a true biological function or an experimental artefact by just exceeding the normal threshold of the

protein? What might be another function of fungal hemolysins? Do they possibly (also) act in defence? Lectins linked to fruiting body development have been seen to act toxic against small putative predators [15, 16, 22, 24, 26, 34]. This may reflect an adoption of a secondary function of these proteins, if there is any in hyphal aggregation. *F. velutipes* LSL-type lectins have an extra pore-forming domain for membrane interaction [37]. FB lectins resemble in structure the bacterial pore-forming haemolytic actinoporins [27, 35]. Lectins may thus interact with membranes [57], possibly via binding to glycolipids [12, 31], thereby effecting signalling [57]. Application of fungal galectins to vegetative mycelium resulting in initiation of fruiting [20,21] might reflect such effect possibly mediated by unnatural high protein concentrations. The importance of membranes in fruiting of Agaricomycotina is supported by the fruiting-inducing effects of various membrane-interacting substances and surfactants when added to vegetative mycelium [2,7] and by the finding of a gene for a cyclopropane fatty-acid synthase being essential for fruiting body initiation in *C. cinerea* [58].

ACKNOWLEDGEMENTS

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Chapter 7

Thiamin Biosynthesis in Fungi and Fruiting

Most of the *Agaricomycetes* are thiamin auxotrophs. Known proteins from ascomycetous yeasts were used to elucidate the thiamin bionsynthesis pathways in *Agaricomycetes*. Inventories of sequenced genomes showed that thiamin auxotrophy is due to loss of the thiamin biosynthesis gene *thi5* [for HMP (hydroxymethyl pyrimidine) phosphate synthase] early in the development of the class *Agaricomycetes* in the subdivision *Agaricomycotina*, after the split of the *Wallemiomycetes* from the other orders of the subdivision and also after the split from the *Tremellomycetes* but probably prior to the split from the *Dacrmycetes*. Species of the order *Gloeophyllales* of the *Agaricomycetes* appear to have remained or newly obtained a *thi5* gene. This chapter further describes experiments to detect possible stimulating effects of thiamin addition on growth and fruiting body development of the model basidiomycete *Coprinopsis cinerea*. In an own experiment, strains on different media with added thiamin (20 mg/L) grow faster than without. The results of experiments on fruiting body development using media containing already thiamin however remained not fully conclusive by variable data between different researchers. More attention has to be given to the question by carefully performed experimental repeats on growth and fruiting in the future.

Authors´ contributions: Kiran Lakkireddy (KL) performed laboratory experiments and the tblastn searches of complete genomes. Ursula Kües (UK) helped in blasting and with Fig. 2 and provided similarity/identity data of proteins, Fig. 1, introduction and discussion material. Shanta Subba provided a fruiting experiment on *C. cinerea*. KL and UK analyzed data. KL provided a first draft of the chapter and finalized with UK the chapter.
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Thiamin Biosynthesis in Fungi and Fruiting

Thiamin is a vitamin (B1) required as coenzyme for important enzymes in the catabolic carbohydrate metabolism, such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase. However, not all fungal organisms produce thiamin. In this work, we have analyzed genomes of *Agaricomycetes* and other fungal and further eukaryotic clades for the presence genes for thiamin production and also for thiamin uptake. Unlike many *Ascomycetes*, most *Agaricomycetes* have lost the *thi5* gene required for production of the pyrimidine moiety HMP-PP of thiamin and are therefore thiamin-auxotrophs. Such species require thiamin-uptake from the environment for good growth and eventually also for fruiting. Laboratory tests in this work suggest that this could possibly also be the case for both, mycelial growth and fruiting in *Coprinopis cinerea*. In an own series of experiments, strains on different media with extra added thiamin (20 mg/l) grow faster than without such addition. Results of fruiting experiments using media containing already thiamin had variable outcomes, with controls that in part were not fruiting indicating suboptimal culturing conditions in experiments. Experiments have to be repeated in future studies.

Introduction

Thiamin (vitamin B1, aneurin) is produced by bacteria, a range of lower eukaryotes, plants, and many fungi but not by animals and humans. The major bacterial pathway is long understood and 16 different enzymes have been defined to comprise the whole biosynthetic pathway (Jurgenson et al. 2009; Hazra et al. 2010). The fungal pathway of thiamin synthesis differs profoundly from the bacterial pathway by unique mechanisms which produce the two precursors of de novo thiamin synthesis, the pyrimidine heterocycle 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) diphosphate (HMP-PP) and the thiazole heterocycle 5-(2-hydroxyethyl)-4-methylthiazole phosphate (HET-P) (Kowalska and Kozik 2008; Jurgenson et al. 2009; Begley et al. 2012; Fig. 1). In *Saccharomyces cerevisiae*, the TMP diphosporylase/HET kinase THI6p couples HMP-PP and HET-P to the thiamin monophosphate ester (TMP; Nosaka et al. 1994). Upon dephosphorylation of TMP by an unknown phosphatase, the thiamin pyrophosphokinase (diphosphokinase) THI80p converts the free thiamin to the diphosphate ester (TDP or TPP for thiamin pyrophosphate) representing the main biologically active thiamin derivative (Nosaka et al. 1993).

The biochemical description of several of the enzymatic steps in production of the precursors has only recently been disclosed in *Saccharomyces cerevisiae* (Chatterjee et al. 2011; Coquille et al. 2012; see Fig. 1 for involved proteins). Production of the pyrimidine moiety HMP-PP requires a histidine and pyrodoxal phosphate (PLP) as substrates from which the iron-binding HMP-P synthase THI5p (from a family of in total four proteins – THI11p, THI12p, THI13p; Fig. 1) forms the HMP monophosphate (HMP-P) (Coquille et al. 2012). Studies with the homologous THI5p of *Candida albicans* suggest that the active-site His66 of THI5p is the source of the required histidine and that THI5p is a single-turnover suicide enzyme similar as THI4p (Lai et al. 2012). Upon formation, HMP-P is then phosphorlyated by the redundant HMP-P kinases THI20p or THI21p (Fig. 1; a third related protein THI22p is inactive; Kawasaki et al. 2005; Haas et al. 2005). The thiazole moiety HET-P in the baker´s yeast is synthesized in several steps from nicotinamide adenine dinucleotide (NAD), glycine and a cysteine of the unusual suicide enzyme THI4p (Fig. 1) functioning as co-substrate. First, ADP-ribose is yielded from NAD and forms with glycine an adenylated intermediate. In subsequent iron-dependent steps, sulfur will be incorporated into a further intermediate and finally upon additional conversions, adenylated thiazole (ADT) results. Remarkedly, the

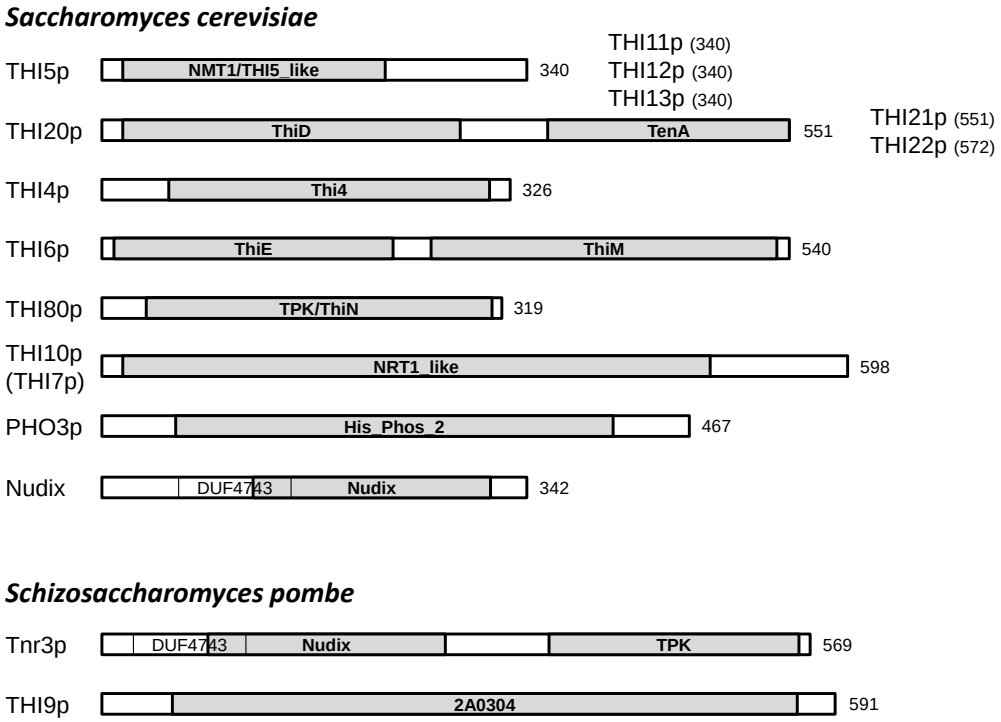

Fig. 1 Schemes on domain structures of proteins from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* involved in thiamin biosynthesis, metabolism and transport. NMT1/THI5-like: domain required for biosynthesis of the pyrimidine moiety of thiamin; ThiD: HMP kinase domain; TenA: domain cleaves thiamine and related compounds to produce HMP; Thi4: Thi4 family thiazole biosynthesis protein; ThiE: thiamin-phosphate diphosphorylase; ThiM: hydroxyethylthiazole kinase; TPK/ThiN and TPK: thiamin pyrophosphokinase; NRT1_like: nucleobase-cation-symport-1 (NCS1) transporter domain; His_Phos_2: histidine phosphatase superfamily (branch 2); DUF4743: domain of unknown function associated with Nudix domain; Nudix: domain for hydrolysis of nucleoside diphosphates linked to other moieties; 2A0304: amino acid permease (GABA permease). Numbers give the length of proteins in amino acids. Proteins named at the right side are extra *S. cerevisiae* proteins of a same family. THI10p and THI7p are alternative names for the same protein of *S. cerevisiae*.

thiamin thiazole synthase is the sulfur source for the formation of ADT. Transfer of a sulfur atom from the active-site Cys205 yields a dehydroalanine residue in the protein and thus inactivates the enzyme why every THI4p molecule can undergo only a single turnover (Chatterjee et al. 2007, 2011; Roach 2011; Zhang et al. 2016). A yet to be identified phosphohydrolase from the family of Nudix (for nucleoside diphosphatases linked to other moieties, X) hydrolases might then act to replace the ADP on the site chain of ADT by a phosphate to form HET-P (Lai et al. 2012). One Nudix-enzyme (YJR142W; Fig. 1) has so far been identified in thiamin metabolism in *S. cerevisiae* but it functions on diphosphate forms of thiamin and thiamin analogs similar as the bimodular protein TNR3p in *Schizosaccharomyces pombe* and has a protective role as damage control enzyme of toxic thiamin degradation metabolites (acts on TDP and 60-fold higher on the damaged thiamin forms oxy- and oxo-thiamin) rather than being active in *de novo* thiamin biosynthesis (Goyer et al. 2013; Srouji et al. 2016). TNR3p of *S. pombe* (Fig. 1) is a fused protein with an N-terminal Nudix hydrolase and a C-terminal thiamin pyrophosphokinase domain and likely has repair functions on errorously pyrophosphorylated thiamin-based molecules in TTP production (Fankhauser et al. 1995; Goyer et al. 2013).

Notably, there is not a unique eukaryotic pathway of thiamin synthesis. In plants, synthesis of the precursors HET-P and HMP-P and their coupling to TMP occurs in the plastids whereas maturation to TTP occurs in the cytoplasm (Gerdes et al. 2012). Plants follow the fungal pathway by using the THI4p homolog THI1 in ADT synthesis (Belanger et al. 1995; Machado et al. 1996) and the bacterial pathway in production of HMP-P by appointing enzyme THIC (Raschke et al. 2007) which is a homolog of the bacterial hydroxymethyl pyrimidine synthase ThiC (Jurgenson et al 2009). Strikingly, the plant THI1 enzyme is bifunctional and also acts in production of the pyrimidine moiety of thiamin. THI1 acts as HMP-P kinase in the formation of HMP-PP and catalyzes also in the condensation of HMP-PP to HET-P to TMP (Ajjawi et al. 2007; Rapala-Kozik et al. 2007). Differences in thiamin requirements are reported for species of the different algal phyla (Croft et al. 2006; McRose et al. 2014) and from the fungus-like *Oomycota* from the *Heterokonta* (Guarro et al. 1999; Judelson 2012). Evidence for a *THI4*-like gene has been provided for *Saprolegnia parasitica* (Torto-Alalibo et al. 2005).

Presence of *THI4* orthologs have already been reported for the filamentous ascomycetes *Acremonium chrysogenum*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Fusarium oxysporum*, *Neurospora crassa*, and *Verticillium dahliae* (Kubodera et al. 2000, 2003; Faou and Tropschg 2003, 2004; Ruiz-Roldan et al. 2008; Hoppenau et al. 2014; Liu et al. 2015; Shimizu et al. 2016), *THI5* orthologs are known *in A. nidulans*, and *A. oryzae* and a *THI80* is known in *A. oryzae* (Tokui et al. 2011; Shimizu et al. 2016). However, there are some thiamin-auxotrophic yeasts among the *Saccharomycetes* that lack THI5p (Wightman and Meacock 2003) and various species in other lines of *Ascomycetes* show also deficiencies in thiamin production (Fries 1943; Bereston 1953). As early as in the first half of the last century it had been established that many lines of *Basidiomycota* and also of the *Mucoromycotina* are auxotrophic for thiamin and do not grow or grow only very reluctantly without addition of thiamin (Burgeff 1934; Schopfer 1937, 1943; Bonner and Erickson 1938; Fries 1938; Melin 1939; Melin and Nyman 1940; Schopfer and Blumer 1940; Kühlwein and Zoberst 1953; Lyr 1954; Jennison 1955; Madelin 1956; Sommer and Halbsguth 1957; Rawald 1962; Wessels 1965; Volz and Beneke 1969; Volz 1972; Eul and Schwantes 1985; Gramss 1990; Beguin 2010). In *Agaricomycetes*, also fruiting body development can negatively be affected by lack of sufficient thiamin in a culture medium (Wessels 1965). Thiamin is required as coenzyme by various enzymes in catabolic carbohydrate metabolism, such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase, and resulting energy generation (Bunik et al. 2013; Kochetov and Solovjeva 2014). Thiamin auxotrophy might be overcome by addition of thiamin or by addition of the thiazole and pyrimidine precursors or by pyrimidine alone (Volz and Beneke 1969). Thiamin is synthesized by auxotrophic *Agaricomycetes* from the two precursors HMP-P and HET-P (Melin and Norkrans 1942; Melin 1939; Fries 1955; Schopfer and Blumer 1940) and further results suggested that the thiazole precursor can also be produced by these fungi (Volz and Beneke 1969). The rust *Uromyces viciae-fabae* from the *Pucciniomycotina* in contrast has functional genes for production of both precursors (Sohn et al. 2000) while other species in the phylum need outside supply of thiamin (Beguin 2010). In the *Mucorales Phycomyces blakesleanus*, both precursors are required to overcome a thiamin deficit whereas in other *Mucorales* either one or the other precursor are reported to be required for growth in absence of thiamin (Leonian and Lilly 1940; Bartnicki-Garcia and Nickerson 1961; Ebringer 1960).

Uptake of thiamin or its precursors by thiamin-dependant organisms implies presence of suitable transporters. In *S. cerevisiae*, THI10p (= THI7p; Fig. 1) transports thiamin (Enjo et al. 1997) and likely also HMP (Ishibashi et al. 1973). A functional ortholog has been described in *V. dahlia* (Qi et al. 2016) while in many other ascomycetes such protein is missing (Donovan et al. 2018). Moreover, acid phosphatase PHO3p (Fig. 1) has high affinity

for thiamin phosphoesters and hydrolyses these in the periplasm to thiamin in order to promote thiamin transport (Nosaka et al. 1989, 1990, 2005). Recent evidence in *Candida parapsilosis* revealed DUR31 as an ortholog to the urea transporter family protein NCU01977 of *N. crassa* to have a thiamin transporter activity. In *S. cerevisiae* and *S. pombe* however and in a number of other ascomycetous yeasts, a *DUR31* gene is missing (Donovan et al. 2018) as well as in most *Pucciniomycota*, in the *Tremellomycetes*, *Dacrymycetes* and most *Agaricomycetes* but not in the *Ustilagomycotina* and the *Wallemiomycetes* (our own observations, not further shown). *S. pombe* has an unrelated high-affinity thiamin transporter THI9p (Fig. 1) with an amino acid permease (GABA permease) domain and protein Bsu1 of the Major Facilitator Superfamily which functions as a B6 (pyridoxal 5′-phosphate) transporter and contributes on a minor scale to thiamin and possibly also to HMP uptake (Niederberger et al. 1996; Stolz et al. 2005; Vogl et al. 2008). All fungal transporters differ from the known bacterial and from the known mammalian thiamin transporters. Thiamin transporters have thus evolved several times convergently (Vogl et al. 2008; Donovan et al. 2018).

Fungal genome projects allow now to mine deeper into the possibilities of thiamin production (Kuo et al. 2014). Here, we show by genome screenings that the ability to produce thiamin was lost in early evolution of *Agaricomycotina* and the *Agaricomycetes* by loss of a *thi5* gene. Other data show that similar loss of thiamin production occurred in various fungal lineages also at later times. This bioinformatics work is supported by experimental work with the inkcap *Coprinospis cinerea* elucidating effects of thiamin on growth and development.

Material and Methods

Strains, media, and culture conditions. *C. cinerea* homokaryon AmutBmut (Swamy et al. 1984; Kertesz-Chaloupková et al. 1998) and its fruiting-defective mutant Proto159 (Granado et al. 1997) were grown on three different media (BSM, MEA and YMG/T), in presence and absence of thiamin in order to observe hyphal and fruiting body development. The basidiomycete standard medium BSM contained per l: 0.5 g yeast extract, 0.65 g L-asparagine monohydrate, 1 g KH_2PO_4, 0.5 g $MgSO_4$ x 7 H_2O, 0.5 g KCl, 50 ml Stock I (per l: 0.2 g $Fe(II)SO_4$ x 7 H_2O), 50 ml Stock II [per l: 0.16 g $Mn(CHCOO)_2$ x 4 H_20), 0.04 g $Zn(NO_3)_2$ x 4 H_2O, 1 g $Ca(NO_3)_2$ x 4 H_2O, 0.06 g $CuSO_4$ x 5 H_2O], and 5 g glucose (separately autoclaved in 50 ml H_20) (Hüttermann and Volger 1973). MEA as general fungal growth medium contained 2 % malt extract (Badalyan et al. 2011), and the specific *C. cinerea* medium YMG/T per l: 4 g yeast extract, 10 g malt extract, 4 g glucose (separately autoclaved in 50 ml H_20), and 100 mg tryptophan (Granado et al. 1997). To solidify the media, 1 % agar (Nr. 11396, Serva, Heidelberg. Germany) was added. When required, 0.5 ml from a filter-sterilized stock solution (20 mg thiamin HCl solved in 500 ml bidest. H_2O, = 40 mg thiamin/l) were added per l autoclaved hand-warm medium (corresponding in the media to an end-concentration of added 20 mg/l). Strains were precultured at 37°C on YMG/T medium in dark ventilated boxes on wetted tissues to ensure high humidity during the growth. Small agar pieces (ca. 2 x 2 mm) of fully grown YMG/T cultures were cut out and are placed onto the middle of fresh agar plates of appropriate media. Inoculated plates were incubated at 37°C in the dark under humid conditions in dark ventilated boxes. Growth per day was daily marked on the bottoms of the plates and the increase in colony radius per day measured with a ruler once plates were fully grown. Per plate, six different measurements per day were taken. Per medium and strain, six different plates were incubated and measured. Average values with standard deviations of growth increase in mm per day were then calculated from in total 18 different values per day. Fully grown plates were transferred into standard fruiting conditions

(12 h light/12 h dark, 25°C, 90 % humidity; Granado et al. 1997), to further observe any effects of thiamin on the fruiting ability of the strain. In a repeat of the fruiting experiments on YMG/T medium by S. Subba, 0.5 ml of the thiamin HCL stock solution (40 mg/l) was added prior to autoclaving of medium (S. Subba, personal communication). Note that it is therefore possible that in this run of the eperiments, the actual thiamin-concentration was lower in the autoclaved medium than in the experiments with the filter-sterilized thiamin added after autoclaving to the medium.

Sequence analysis. Accession numbers of *S. cerevisiae* proteins (see schematic domain structures of proteins in Fig. 1) used for blasting the genome pages at the JGI fungal web sites (http://genome.jgi-psf.org/programs/fungi/index.jsf; Grigoriev et al. 2014) or the NCBI databases (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi; Robbertse and Tatusova 2012) are as follows: THIp5 (NP_116597) - [THI11p (NP012690), THI12p (NP_014067) and THI13p (NP_010037) are alternatives with 99/99-100 % identity/similarity to THIp5, of which THI13p was used in blasting for crosschecking], THI20p (NP_014586) - [THI21p (NP_015065) and THIp22 (NP_015446) are alternatives with 86/93 % and 76/87 % identity/similarity, respectively to THI20p, of which THI22p was used in blasting for crosschecking], THI4p (NP_011660), THI6p (CAA97929), THI80p (NP_014786), THI10p (BAA09504) = THI7p. Thiamin transporter Thi9p (CAB57428), thiamin pyrophosphokinase Thi80p (CAA59135), and thiamine-phosphate dipyrophosphorylase Thi4p of *S. pombe* (CAA55402), HMP-P synthase Thi1p of *U. viciae-fabae* (O00057), chloroplastic thiazole synthase Thi1p of *A. thaliana* (AED96539) and *Homo sapiens* thiamin pyrophosphokinase Tpk1 (AAH68460) were also used in crosschecks. Of these, *S. cerevisiae* THIp5 and *U. viciae-fabae* Thi1p are related proteins with 61/76 % identity/similarity to each other, *S. cerevisiae* THIp4 and *A. thaliana* Thi1p have 51/64 % identity/similarity to each other and *S. cerevisiae* THIp6 and *S. pombe* Thi4p 40/54 % identity/similarity, while *S. cerevisiae* THI80p and the human Tpk1 show 27/43 % identity/similarity to each other, *S. cerevisiae* THI80p and the C-terminal Thi80p-like thiamin pyrophosphokinase domain of *S. pombe* protein Tnr3p 29/22 % identity/similarity and human Tpk1 and the C-terminal thiamin pyrophosphokinase Thi80p-like domain of *S. pombe* 37/57 % identity/similarity, respectively. Accordingly in the blast searches, the same hits were encountered with any related proteins but with the exception when using the complete *S. pombe* Tnr3p protein with its both domains. Further searches were therefore performed with only the N- terminal Nudix-domain and only the C-terminal thiamin pyrophosphokinase Thi80p-like domain of *S. pombe* Tthi80p, respectively. The N-terminal Nudix-domain of *S. pombe* had 39/59 % identity/similarity to an uncharacterized *S. cerevisiae* Nudix protein (NP_012676) which also hit the same proteins from the genomes of other fungi than the S. pombe Nudix-domain.

We did tblastn (protein against DNA) searches with fully sequenced genomes of fungal species of the *Ascomycota* (68 strains from in total 67 species), *Basidiomycota* (72 strains from in total 69 species), and *Mucoromycotina* (4 strains of in total 4 species), of the fungal sister clade *Microsporidia* (*Anncaliia algerae* PRA339, GCA_000385875; *Antonospora locustae* HM-2013, GCA_007674295; *Edhazardia aedis* USNM 41457, GCA_000230595; *Encephalitozoon cuniculi* GB-M1, GCA_000091225; *Encephalitozoon hellem* ATCC 50504, GCA_00027781; *Encephalitozoon intestinalis* ATCC 50506, GCA_00014646; *Encephalitozoon romaleae* SJ-2008, GCA_000280035; *Enterocytozoon bieneusi* H348, GCA_000209485; *Hamiltosporidium tvaerminnensis* OER-3-3, GCA_0001808; *Nematocida parisii* ERTm1, GCA_000250985; *Nematocida* sp. 1 ERTm2, GCA_000250695; *Nosema ceranae* BRL01, GCA_00018298; *Trachipleistophora hominis*, GCA_000316135; *Vavraia culicis 'floridensis'*, GCA_000192795; *Vittaforma corneae* ATCC 50505, GCA_000231115), and of species of the *Oomycota* (*Phytophthora ramorum*, GCA_000149735), slime molds (*Dictyostelium purpureum*, GCA_000190715), plants (alga:

Chlamydomonas reinhardtii, GCA_000002595; lycophyte: *Selaginella moellendorffii*, GCA_000143415; moss: *Physcomitrella patens* subsp. *patens*, GCA_000002425, monokots: *Aegilops tauschii*, GCA_00034733; *Brachypodium distachyon*, GCA_000005505; *Oryza sativa* Indica Group, GCA_000004655; *Oryza sativa* Japonica Group, GCA_000005425; *Sorghum bicolor*, GCA_000003195; *Zea mays*, GCA_000005005: dikots: *A. thaliana*, GCA_000001735; *Carica papaya*, GCA_000150535; *Citrus sinensis*, GCA_000317415; *Cucumis sativus*, GCA_000004075; *Fragaria vesca* subsp. *vesca*, GCA_000184155; *Glycine max*, GCA_00000451; *Lotus japonicas*, GCA_000181115; *Populus trichocarpa*, GCA_000002775; *Ricinus communis*, GCA_000151685; *Solanum lycopersicum*, GCA_000188115; *Vitis vinifera*, GCA_000003745) and animals (*Caenorhabditis elegans*, GCF_000002985; *H. sapiens*, GCA_000001405). Using ClustalX (http://wwwigbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and GeneDoc Version 2.6.002 (http://www.psc.edu/biomed/genedoc/), sequences from fungi were aligned and phylogenetic trees were generated by neighbor joining appointing MEGA version 4.0 and 6.0 (Tamura et al. 2007, 2013).

Results and discussion

Analysis of genomes for definition of thiamin biosynthesis pathways and putative thiamine transporters. In a first general overview of all blasting results, we encountered that none of the *Microsporidia* and the oomycete *P. ranorum* had any of the genes searched for. The slime mold *D. ramorum*, the worm *C. elegans* and *H. sapiens* had homologs of *S. cerevisiae* THI80 but missed homologs of other biosynthesis genes. All these species are thus thiamin auxotrophs and depend thus on uptake of outer thiamin sources. Of the analyzed plants, none had a *THI5* gene, in agreement that they use the bacterial pathway in production of HMP-P by appointing enzyme THIC as a homolog of the bacterial hydroxymethyl pyrimidine synthase ThiC (Raschke et al. 2007; Hanson et al. 2018). In agreement with the fact that the typical plant can produce thiamin (Jurgenson et al. 2009), only *C. papaya* was missing in our searches a *THI80* homolog in the genome and the water plant *L. japonicas* was missing a *THI4* and a *THI6* homolog, respectively.

We continued with a tblastn screen on a range of fungal genomes from the phyla *Mucoromycotina*, *Ascomycota* and *Basidiomycota* (Table 1). The most striking observation was a loss of the *thi5* gene in the majority of analyzed *Basidiomycota* species and in part also of the *Ascomycota* and *Mucoromycotina*. Loss of other thiamin biosynthesis genes in contrast appeared to be less frequent (Table 1). Similar observations can be deduced by readers from a diagram on presence of thiamin biosynthesis genes in a range of fungi shown in the publication of Jiang et al. (2018) while the authors in their publication left the found gene distribution patterns uncommented. Furthermore, Nudix-enzymes for potential damage control of toxic thiamin degradation metabolites are common and all tested fungi appear to variably have candidate genes for thiamin transporter functions (Table 1).

Protein sequences were collected from the NCBI databases (https://www.ncbi.nlm.nih.gov/) and the JGI Mycocosm site (https://mycocosm.jgi.doe.gov/mycocosm/home) for further analysis. For all proteins (note that numbers of proteins were in part much higher than numbers of analyzed organisms because sometimes 2 strains of a same species were analyzed and some of the organisms had two or more copies for a protein type, in particular candidates for Nudix enzymes and, variably, for potential transporters), we generated first phylogenetic trees with all collected

Table 1 Screening of genomes of different fungal species for candidate genes acting in thiamin biosynthesis, damage repair or potentially in thiamin transport

Phylum (species numbers)		Species with potential homologs acting in								
		Biosynthesis					Repair	Transport		
		Thi5[1]	Thi20	Thi4	Thi6	Thi80	Nudix[2]	Thi10	Pho3	Thi9
Mucoromycotina (4)	+	3	4	2	4	4	4	0	0	4
	-	1	0	2	0	0	0	1	4	0
Ascomycota (67)	+	53	67	62	67	66	67	66	60	67
	-	14	0	5	0	4	0	1	7	0
Basidiomycota (69)	+	20 (21)	66	62	66	57	68	65	61	65
	-	49 (48)	3	7	2	11	0	4	7	3

[1]Of all tested species, Thi5 is missing in *Phycomyces blakesleanus* from the *Mucoromycotina*, in *Candida glabrata*, *Candida temuis*, *Clavispora lusitaniae*, *Dekkera bruxellensis*, *Kazachstania africana*, *Lodderomyces elongisporus*, *Naumovospora castellii*, *Naumovospora farinosa*, *Tetrapisispora phaffii*, *Vanderwaltozyma polyspora*, and *Yarrowia lypolytica* from the *Saccharomycotina* and in *Ascobolus immersus*, *Exophiala dermatitidis*, and *Tuber melanosporum* from the *Pezizomycotina* of the *Ascomycota*, in *Microbotryum violaceae* and *Mixia osmundae* from the *Puccinomycotina*, in *Cryptococcus gattii* and *Cryptococcus neoformans* from the *Tremellomycetes*, and in *Agaricus bisporus* var. *bisporus*, *Agaricus bisporus* var. *burnettii*, *Amantia muscaria*, *Amanita thirsii*, *Auricularia subglabra*, *Bjerkandera adusta*, *Boletus edulis*, *Botryobasidium botryosum*, *Ceriporiopsis subvernispora*, *Cerrena unicolor*, *Coniophora puteana*, *Coprinopsis cinerea*, *Daedalea quercina*, *Dichomitus squalens*, *Fibroporia radiculosa*, *Fistulina hepatica*, *Fomitiporia mediterranea*, *Fomitopsis pinicola*, *Galerina marginata*, *Gymnopus luxurians*, *Heterobasidion irregulare*, *Hydnomerulius pinastri*, *Jaapia argillaceae*, *Paxillus involutus*, *Paxillus rubicindulus*, *Phanerochaete carnosa*, *Phanerochaete chrysosporium*, *Piloderma eroceum*, *Piriformospora indica* (*Serendipita indica*), *Pisolithus microcarpus*, *Pisolithus tinctorius*, *Phlebia brevispora*, *Postia placenta*, *Ramaria rubella*, *Scleroderma citrinum*, *Schizophyllum commune*, *Sebacina vermifera*, *Serpula lacrymans*, *Stereum hirsutum*, *Suillus luteus*, *Trametes versicolor*, *Tulasnella calospora*, and *Wolfiporia cocos* from the *Agaricomycetes*.
Protein sequence from a gene model for a Nmt1-/Thi5-like protein of likely bacterial origin newly deduced from a chromosomal region (scaffold_2120:4380-5360) of *Serpula himantioides* (not counted in the open number presented in the table while counted in the number presented in brackets in the table):
MKKRIYLYAVMITVIVLFAAGCGGRKVNPGHIRVVLDWTPNTNHTGLFVAREKGWFAEEGLTVEITQPPEEGALL
MVGSGIAQFGVDTQEVVGPGIAKEHDAVPATTVAAIISHNTTGILSLAKHNIKRPRDLMGKRYEYWETPMVTALI
KEIVENDGGKFEDVIMVPNYSTDPITALQTDMDAIWIYYAWEGLEAEVLGLDTNYIDLRELGPQFDFYTMIITAN
TDWLNKNPETGRKFMRAVSRGYNFAIENPAEAGEILLKCAPELDRELVMRSQEYLASRYRQDSPRWGEIDPERWA
AFYXWMYERGLLEKDIGAGGFTNEFLP
[2]Only in the four *Schizosaccharomycetales* that were analyzed (*Schizosaccharomyces cryophilus*, *Schizosaccharomyces japonicus*, *Schizosaccharomyces octosporus*, *Schizosaccharomyces pombe*), the Nudix unit was fused to the Thi80 unit whereas other fungal species have free-standing Nudix homologes (for further information see also Goyer et al. 2013).

fungal proteins and, where applicable, with proteins from other species groups as mentioned above. By the lack of the protein in other eukaryotic groups, only the Thi5 tree represented just fungi. Clear differentiation between fungi and plants as well as between different fungal phyla were seen in trees of Thi20-, Thi4-, Thi6-, Thi80-, Nudix-, Thi10-, Pho3- and Thi9-like proteins (not further shown here) supporting divergent evolutionary developments betweenorganismal clades for all the proteins. However, all analyses need further careful detailed sequence polishing (such as in some cases correcting predicted gene models and defining and focusing on core sequences throughout groups of proteins) to optimize the alignments to possibly then give more stringent phylogenetic trees. This effort was not further performed in this PhD study. In the following text, we focused then further on the most interesting case of the HMP phosphate synthase Thi5.

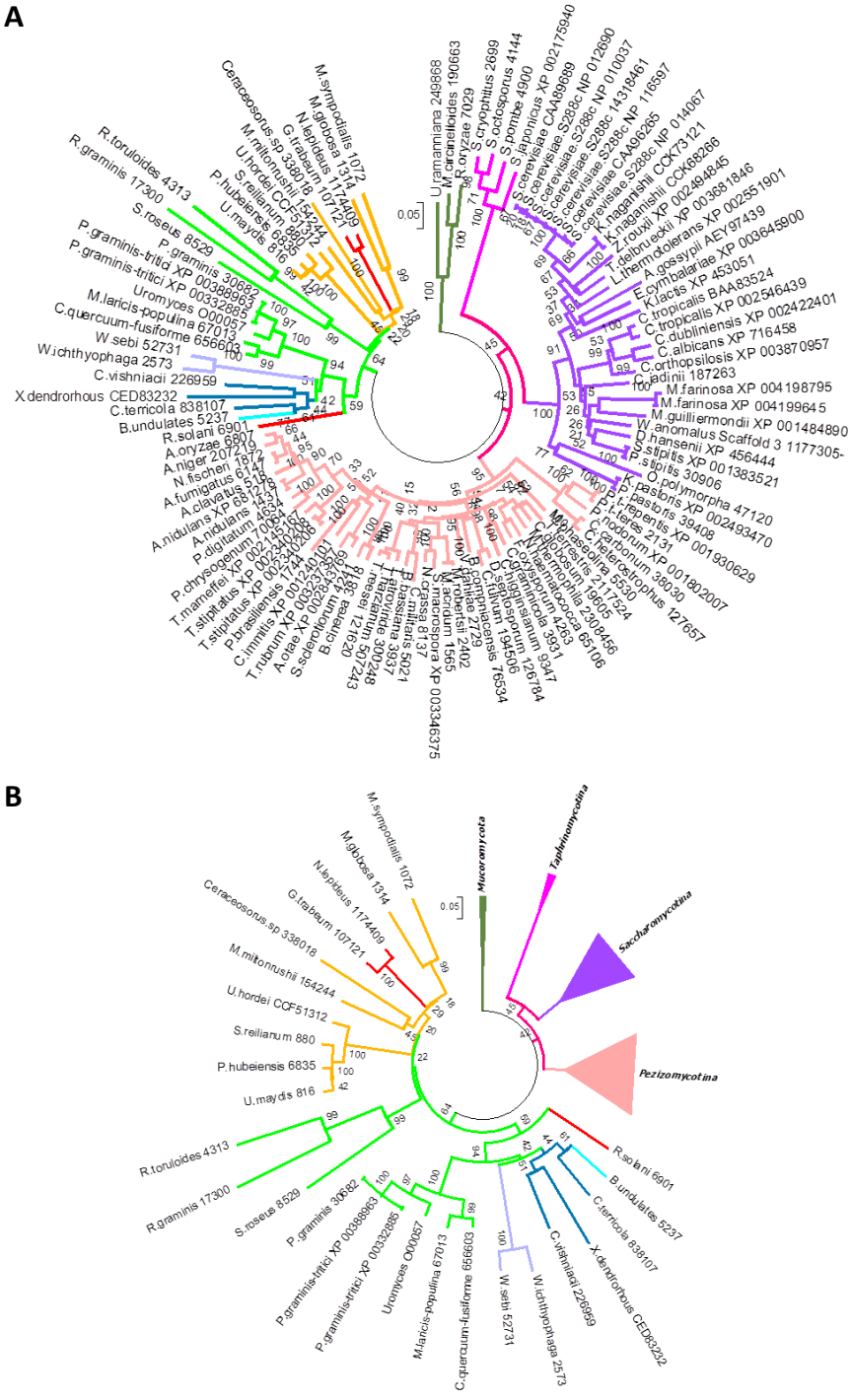

Fig 2. Joint neighbor analysis of fungal Thi5 proteins shown in A. with all individual fungal proteins collected in this study and in B. with the clades of the *Mucoromycotina* and the three subdivisions from the *Ascomycota* being comprised.

Thi5 sequences collected from fungal genome projects (with GeneBank accession numbers or JGI protein IDs) were aligned with ClustalX. The phylogenetic tree was constructed with the alignment in MEGA6 (bootstrap value: 500). The tree was rooted at the *Mucoromycotina*. *Mucoromycotina* are in olive-green, the subdivisions *Taphrinomycotina*, *Saccharomycotina* and *Pezizomycotina* from the *Ascomycota* in magenta, mauve and dusky pink, respectively. Line colors indicate taxanomic clades. Of the *Basidiomycota*, green represents species assigned to *Pucciniomycotina*, yellow *Ustilagomycotina*, grey blue *Wallemiomycetes*, light blue *Basidioascus undulates* from the order *Geminibasidiales* tentatively classified to the *Wallemiomycetes* (Nguyen et al. 2015), blue *Tremellomycetes*, and red *Agaricomycetes*.

The* thi5 *gene in fungal evolution. Fungal species without a *thi5* gene appeared in different fungal clades on phylum, subphylum, order, family or even genus levels (see list of species in the footnote of Table 1) suggesting that loss of the gene appeared repeatedly in fungal evolution. Interesting is then to see in which fungi *thi5* genes were found (Fig. 2). With only four analyzed cases, reasonable conclusions on timings of loss of *thi5* are not possible to provide for the *Mucoromycotina*. However, in the *Ascomycota* with many *thi5* losses in clades combined with many parallel *thi5* maintenances as observed within the *Saccharomycotina* and the *Pezizomycotina*, losses of *thi5* appeared to be common and recurrent at later times in divergent evolution of clades. Similarly, later losses seem to have occurred in some lines of the earliest *Basidiomycota* phylum *Puccinomycotina* (21 of the 47 sequenced species present in May 2020 in the JGI Mycocosm had a *thi5* copy; U. Kües, personal communication) and in the phylum of *Ustilagomycotina* (only three of the 27 sequenced species present in May 2020 in the JGI Mycocosm lacked a *thi5* copy, i.e. *Moesziomyces aphidis*, *Pseudozyma antartica*, *Tilletiopsis washingtonensis*; U. Kües, personal communication). In contrast in the evolution of the *Agaricomycetes* within the *Basidiomycota*, a main loss of *thi5* roots probably occurred very early within the subphylum *Agaricomycotina*, i.e. probably after the split of the *Agaricomycetes* from the clades of *Wallemiomycetes* and *Tremellomycetes* which both contain many species with *thi5* genes (Fig. 2) and possibly prior to the split of the *Dacrymycetes* of which all genomes provided so far are all without *thi5* genes (none of the nine sequenced species present in May 2020 in the JGI Mycocosm had a *thi5* copy; U. Kües, personal communication).

It is then interesting to discover which of the few species in the *Agaricomyetes* (only 3 in total in our analysis in Table 1) carry a fungal *thi5* gene (Fig. 2) and further to note that with *Serpula himantioides* there is a species without a typical fungal *thi5* gene but with a sequence for a related NMT1/THI5-like putative periplasmic N-formyl-4-amino-5-aminomethyl-2-methylpyrimidine (FAMP) substrate-binding ThiY protein (see footnote to Table 1) of likely bacterial origin (e.g. 64 % identity with HCC37805, *Treponema* sp.; 60 % identity with HHY51696, *Clostridiales* bacterium). Bacterial ThiY proteins as structural homologs of Thi5 proteins are parts of an ATP-binding cassette transport system ThiXYZ for transport of the thiamin salvage pathway intermediate FAMP for conversion into HMP (Bale et al. 2010).

The Thi5 sequence of *Rhizoctonia solani* from the *Cantharellales* as most basic clade of the *Agaricomyetes* situates in our constructed phylogenetic tree at the base of the *Basidiomycota* and may still present an ancient *thi5* gene as originally present within the *Agaricomycetes*. The enzymes of the two species of the *Gloeophyllales*, *Gloeophyllum trabeum* and *Neolentinus lepidus*, however nest within sequences of *Ustilagomycotina* (Fig. 2), which suggestd different origins of their *thi5* genes.

In the meantime, with more genomes of the *Agaricomyetes* sequenced (genomes of >300 species present at JGI in May 2020), *thi5* genes were further detected by blasting only in two more species of the *Ceratobasidiaceae* of the *Cantharellales* (*Ceratobasidium* sp., *Thanetophorus cucumeris*), in two more *Gloeophyllales* (*Borreostereum radiatum*, *Heliocybe sulcata*), in a species in the relatively basic clade of the *Auriculariales* (*Exidia glandulosa*) and in *Fomes fomentarius* from the *Polyporales* and in *Lyophyllum atratum* from the *Agaricales*. The three distinct *Ceratobasidiaceae* Thi5 proteins relate close to each other (≥95 % identity) and the four *Gloeophyllales* Thi5 proteins also with each other (≥95 % identity) and then best with the *E. glandulosa* Thi5 protein (≥82 % identity).

In direct consequence, if all these would present the ancient situation of the *thi5* gene content in *Agaricomycetes*, *thi5* gene losses must have occured multiple times at many

different taxon roots in the generation of many of the *thi5*-less clades of the *Agaricomycetes*. This however appears more elaborate than a single early loss of *thi5* in the evolution of the *Agaricomycetes* (He et al. 2019) and a later uptake in specific clades of a new *thi5* copy through horizontal gene transfer from the *Pucciniomycotina* (in the case of *Cantharellales*) and from the *Ustilagomycotina* (in the cases of *Auriculariales* and *Gloeophyllales*), respectively. Strong support for evolutionary events of horizontal gene transfer comes not only from the one gene of likely bacterial origin found in *S. himantioides* (see footnote in Table 2) but also from the fact that the Thi5 copies of *F. fomentarius* and that in *L. atratum* had their closest relatives within the *Pezizomycotina* of the *Ascomycota*, i.e. in the *Botryosphaeriaceae* of the *Dothideomycetes* (86 % identity) and in the *Pyronemataceae* (≥90 % identity) in the *Pezizomycetes*, respectively (U. Kües, personal communication). In sum of all observations, at least 4 or possibly 5 events of horizontal gene transfer of *thi5* and *thi5*-like genes might have been encountered so far to explain the current genetic *thi5* situation within the *Agaricomycetes*.

In summary, it appears from all results and especially from the very rare observation of a presence of a *thi5* gene in species of the *Agaricomycetes* that the *Agaricomycetes* are in total better off without a *thi5* gene while occasionally a *thi5*$^{+}$ situation could be restored by horizontal gene transfer from different fungal or also bacterial sources. However, functionality of all found genes remains to be tested.

Why is it then that i. the *Agaricomycetes* are usually better off without a complete thiamin biosynthesis pathway and that ii. it is mostly the *thi5* gene that is gone lost not only in the *Agaricomyctes* but also in clades of other fungi (Table 1)? Some thoughts for potential reasons are presented in the following.

Potential answer to question i. Since thiamin is involved in functions of several central enzymes of carbohydrate degradation and of energy production, e.g. in glycolysis and the citrate acid cycle (Jurgenson et al. 2009; Kraft and Anfert 2017), it could be best for fungi when growing on typical carbon-rich and otherwise nutrient-poor plant substrates to counteract any harmful carbohydrate (glucose) overflow through mechanisms that reduce unneeded energy accumulations that would be provided through oxidative metabolic reactions. Without much thiamin in the cells, carbohydrates will not be fully degraded and resulting energy levels would thus be lower.

Potential answer to question ii. Thi5 as well as Thi4 are both suicidal proteins, i.e. they undergo reactions in which they act themselves as substrate providers and thereby inactivate themselves (Chatterjee et al. 2007, 2010, 2011; Coquille et al. 2012; Lai et al. 2012; Fitzpatrick and Thore 2014; Song et al. 2017; Hanson et al. 2018). These enzymes are thus biosynthetic costly as exemplified with e.g. Thi4 turnover by consuming an estimated minimum of 2 to 12% of the maintenance energy budget of a plant, because for every new reaction, a new enzyme has to be produced (Hanson et al. 2018; Sun et al. 2019). If the thiamin biosynthesis pathway has somehow to be inactivated by mutation, either of the genes of these two energy-costly functions might thus be best destroyed. The resulting thiamin-auxotrophs then rely on uptake of the instable thiamin or pathway intermediates by suitable transporters from the outside environment as provided from other organismal origins and, alternatively, on thiamin salvage possibilities achieved through restoration of downstream pathway intermediates from own thiamin breakdown products (Jurgenson et al. 2009; French et al. 2011; Gonçalves and Gonçalves 2019).

There is however another apparently independent aspect into this: The Thi4 proteins for de novo-synthesis of in *S. cerevisiae* and plants have been shown to provide a secondary function to cells in UV protection, DNA damage tolerance and mitochondrial stability,

independently of the presence of thiamin. The exact function of Thi4 in mitochondrial DNA repair however is so far not clarified (Machado et al. 1996, 1997; Medina-Silva et al. 2006; Hoppenau et al. 2014). Further as studied in *Verticillium dahlium*, the function of Thi20 also increases tolerance to UV (Qin et al. 2020). In addition to the HMP-P kinase activity of Thi20 on HMP-P in de novo synthesis of thiamin, Thi20 proteins might confer a salvage HMP kinase activity to form HMP-P of a rescued precursor HMP (Kawasaki et al. 2005; Nosaka 2006; Onozuka et al. 2008; French et al. 2011) while there are also salvage pathways to the thiazole moiety of thiamin from degradation products of thiamin (Yazdani et al. 2013). Currently it appears to be open whether deletion of *thi5* for *de novo* HMP synthesis might thus be more favorable for an organism than deleting the gene *thi4* for *de novo* HET production (Hanson et al. 2018). Notably however, there are also various protists and bacterial groups which are HMP auxotrophs and depend on thiamin or thiamin precursor uptake mechanisms in order to obtain the essential thiamin. The chemically unstable thiamin is usually scarce in habitats, unless conditions are acidic or thiamin is bound to protective material such as clay. Accumulating biotic and abiotic degradation products of thiamin include the better stable while potentially toxic thiamin precursor HMP (Kraft and Anger 2017). Thiamin biosynthesis and degradation processes with effects such as providing thiamin precursors and toxic compounds can confer strong selection forces and complex competition advantages to organisms. Interconnected outcomes in ecological interactions can influence survival and abundances of ranges of organisms such as exemplified e.g. by the repeated observation that pathogenicity of plant and animal antagonists is fostered through thiamin depletion or in other cases by syntropism effects between organisms being unable to exist alone in their environments. Ecological coincidences, reasons and consequences of genetic thiamin pathway decay are only at the beginning of recognition (Helliwell et al. 2013; Kraft and Anger 2017). For the endophytic agaricomycete *Piriformospora indica* (*Serendipita indica*) that lives in soil and colonizes plant roots, an auxotrophic interaction via thiamin-feeding with the plant-growth-promoting soil bacterium *Bacillus subtilis* has been reported (Juang et al. 2018).

Effects of added thiamin on mycelial growth, developmental processes, and metabolite production in various fungal species. Exogenously added thiamin itself can help to improve the resistance response of yeasts to oxidative, osmotic and thermal stress by supporting the maintenance of the redox balance, partly independent of the TDP-dependent enzymes in basic cytoplasmic and mitochondrial biochemical energetic carbon pathways (glycolysis, citrate acid cycle, cytoplasmic-mitochondrial malate-aspartate shuttle), through generation of free radicals and oxidation of macromolecules (Wolak et al. 2014, 2015; Kartal and Palabiyik 2019). Overexpression of *thi4* or of a regulator of gene expression for thiamin synthesis increases in *S. cerevisiae* glucose metabolism and ethanol production (Shi et al. 2018). Thiamine synthesis upregulates branched amino-acid synthesis and ethanol production under hypoxic conditions in the filamentous ascomycete *Aspergillus nidulans* (Shimizu et al. 2016) when NADH:NAD^+ ratios are high as an adaption to environmental stress (Shimizu 2018). Analogously, thiamine increases production of the antibiotic cephalosporin in *Acremonium chrysogenum* (Liu et al. 2015). Thiamin synthesis genes are involved in stress responses of also other filamentous ascomycetes (Ruiz-Roldan et al. 2008; Hoppenau et al. 2014; Qi et al. 2016). The natural deletion of the *thi5* gene such as in the oleaginous yeast *Yarrowia lipolytica* impairs growth in absence of adequate amounts of thiamin by decreases in energy metabolism and reduction of the lipid biosynthesis pathway (Walker et al. 2020). In basidiomycetous wood decay fungi that usually lack the gene *thi5*, addition of thiamin may increase production of ligninolytic enzymes (Levin et al. 2010). Further, energy-consuming fruiting body development considered as a fungal stress response on environmental signals including running short in nutrients (Kües and Liu 2000) was reported to be positively influenced in presence of thiamin by the example of the *thi5*-lacking basidiomycete

Schizophyllum commune (Wessels 1965). Overall, the observations on metabolic and developmental effects of thiamine in fungi are so far only sporadic while very disperse over types of fungal species and focus of study. A first picture of high complexity emerges with interplays between providing carbon for nutrition, the energy metabolism and cytological redox stress with secondary metabolisms and developmental differentiations to which thiamin contributes as an important metabolic cofactor of enzymes in energy-producing pathways. It was therefore of imminent interest to study effects of thiamin addition in growth and development of *C. cinerea*.

Effects of added thiamin on mycelial growth of* C. cinerea *strains. Two *C. cinerea* strains, the self-fertile homokaryon AmutBmut (Fig. 3 to Fig. 5A; Table 2) and its fruiting-defective mutant Proto159 (Fig. 5B and 6; Table 2), have been cultivated in Petri dishes with agar media as described in the section Material and Methods, with and without extra thiamin (at 20 mg/l end concentration) added to a culture medium. Homokaryon AmutBmut grew fastest on the synthetic basidiomycete medium BSM (Hüttermann and Volger 1973), followed by

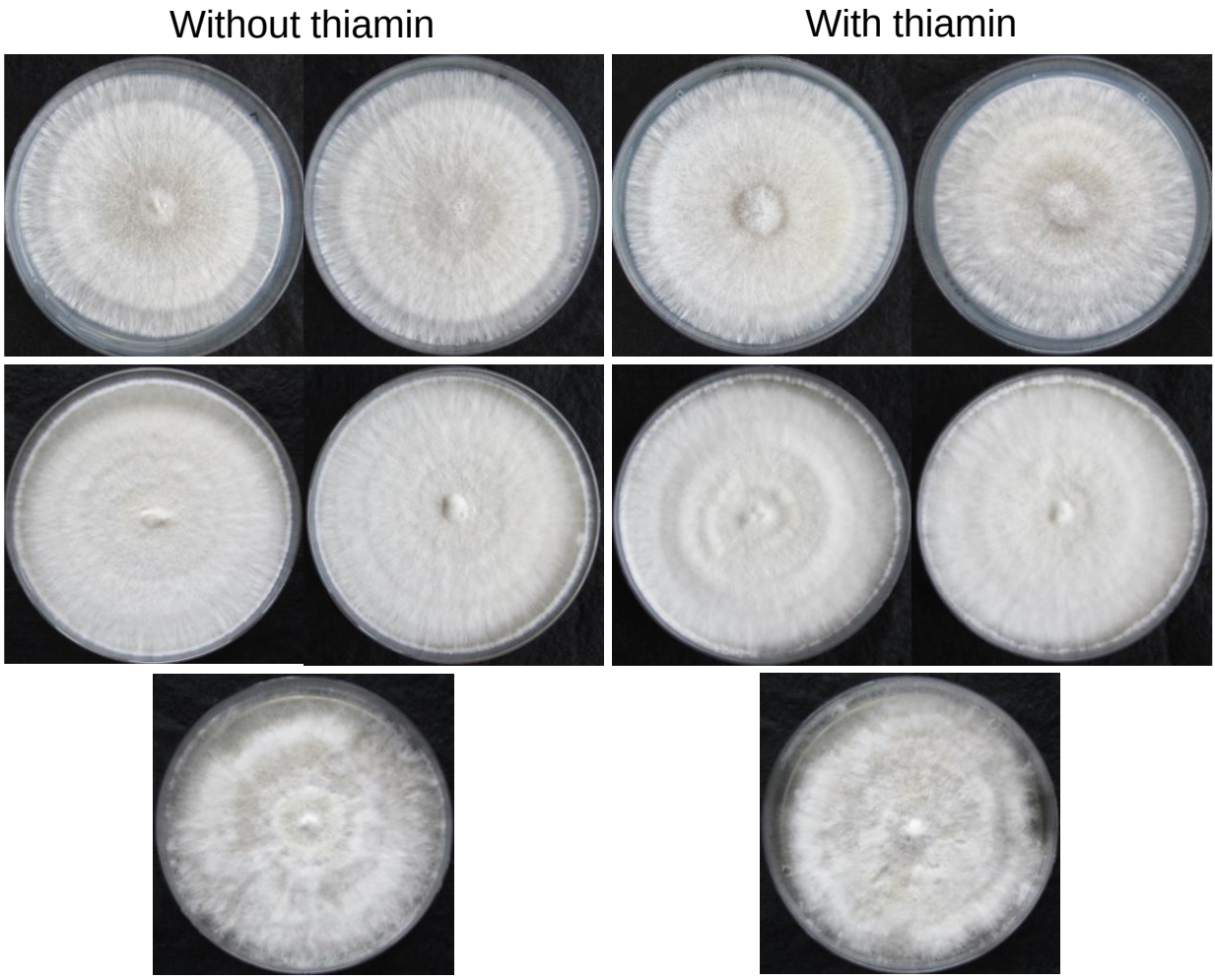

Fig. 3 Views on the aerial mycelium of fully grown colonies of the *C. cinerea* homokaryon Amut Bmut grown at 37°C in the dark on synthetic basidiomycete medium BSM (upper row), complete medium YMG/T (middle row) and malt extract medium MEA (bottom row) without (plates at the left) or with extra 20 mg/l added thiamin (plates at the right).

Note: Plates are from the same experiments than the plates shown in Fig. 4. Growth in dark was every day once interrupted by marking daily growth on the plates.

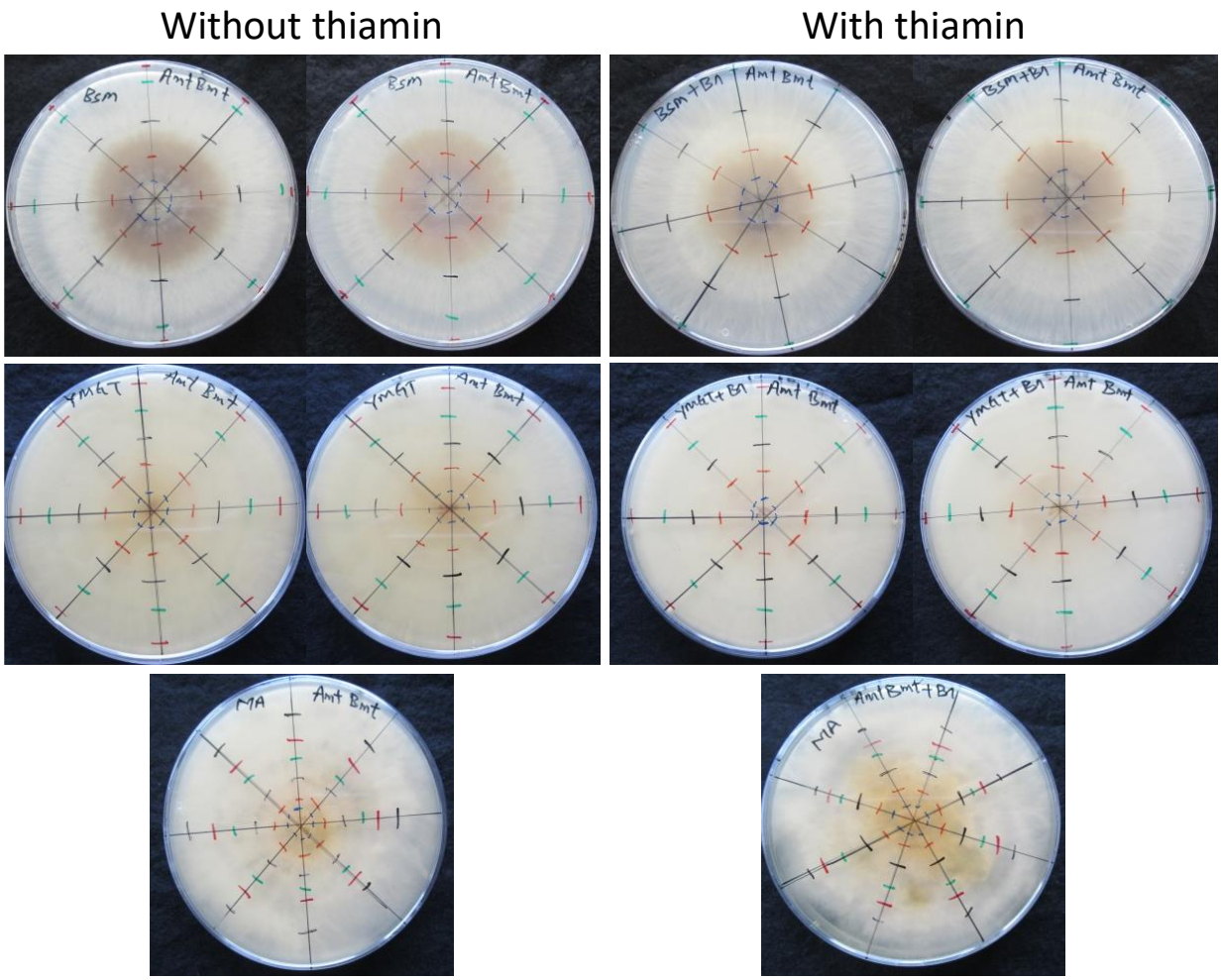

Fig. 4 Growth of *C. cinerea* homokaryon Amut Bmut at 37°C in the dark on synthetic basidiomycete medium BSM (upper row), complete medium YMG/T (middle row) and malt extract medium MEA (bottom row) without (plates at the left) or with 20 mg/l thiamin (plates at the right).

Same experiment as shown in Fig. 3. Every 24 h, growth fronts were marked at 6 places on the reverse of a plate under changing the pen color from day to day (day 1 = blue, day 2 = red, day 3 = black, day 4 = green, day 5 = red, day 6 = black; the number of marks per radius indicate the number of days which the mycelium needs for growth to cover a whole plate) to better allow comparisons between different plates and different media (same color on a circle = same day). Every 24 h, the plates therefore obtained a light signal.

YMG/T as the favoured nutrient-rich *C. cinerea* complete medium (Rao and Niederpruen 1972; Granado et al. 1997), and then by the nitrogen-poor fungal MEA medium (Fig. 3 to Fig. 5A; Table 2). The aerial mycelial appearance of homokaryon AmutBmut were on MEA plates less regular than on BSM medium and on YMG/T plates (Fig. 3, and see also through the reverses of the plates shown in Fig. 4).

For mutant Proto159 however, growth on YMG/T plates was faster than on BSM medium while growth on MEA was slower (Fig. 5B and Fig. 6; Table 2). Proto159 seems to have severe metabolic defects that suppress formation of much aerial mycelium (Fig. 6) and that cause probably as an overstrong stress response that the strain produced abundant amounts of a brown pigment believed to be melanin. The pigment stained the agar and submerged mycelial layers of the mutant dark-brown, variably intense in dependency of the

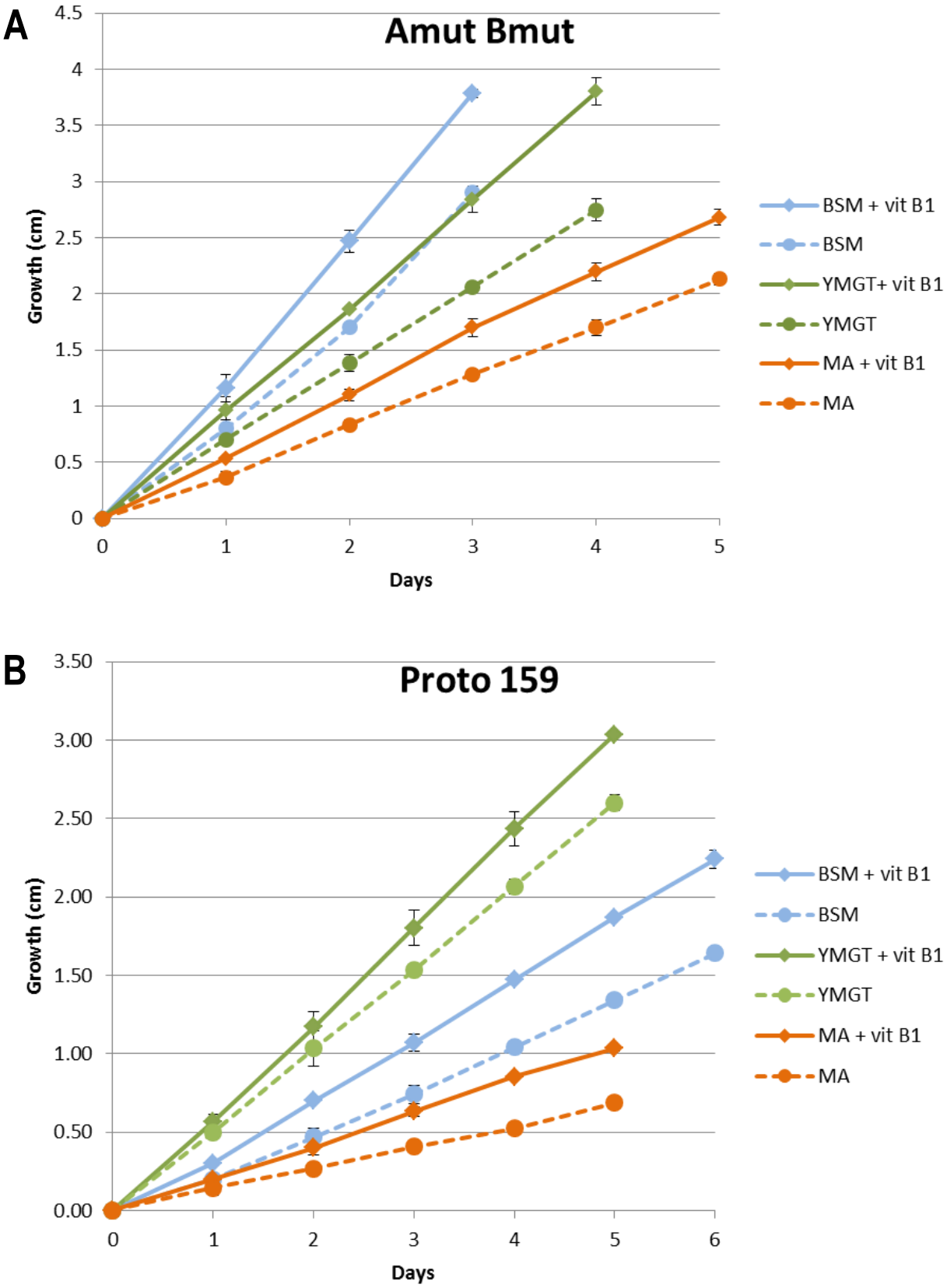

Fig. 5 Colony growth (increase of radius in mm/d) of A. *C. cinerea* homokaryon AmutBmut (top) and B. its mutant Proto 159 (bottom) cultivated on different media with or without extra added thiamin (20 mg/ml) at 37°C in the dark

Data are from the same experiment as shown in Figs. 3, 4 and 6. Note that light signals were received every 24 h when marking the colony growth increases per day. n = 6 analyzed plates per strain with n = 6 marks per day per plate.

Table 2 Average daily growth rate (mm/d) of *C. cinerea* strains AmutBmut and Proto159 at 37°C on different media with or without extra added thiamin (20 mg/l)*

Medium	Growth at 37 °C in cm/d			
	AmutBmut		Proto 159	
	Standard	+ thiamin	Standard	+ thiamin
BSM	0.97 ± 0.21	1.26 ± 0.09	0.27 ± 0.04	0.37 ± 0.04
YMG/T	0.69 ± 0.01	0.95 ± 0.03	0.52 ± 0.02	0.61 ± 0.03
MEA	0.43 ± 0.04	0.54 ± 0.05	0.13 ± 0.01	0.21 ± 0.02

* Data are from the same experiment as shown in Fig. 3 to Fig. 6.

medium used (Fig. 6A) and was strongests in the agar in aged YMG/T plates with and without extra added thiamin (Fig. 6B). Staining of submerged mycelia at the agar interface was strongests in aged cultures on BSM (Fig. 6A). This staining in crusteous submerged mycelium could explain the observed reduction in growth speed as compared to the YMG/T medium (Fig. 5B and Fig. 6; Table 2).

Addition of extra thiamin to BSM and YMG/T medium had then apparently positive effects on the growth speeds of both fungal strains (Fig. 3 to Fig. 6A; Table 2). The overall nutrient composition of a medium (such as available carbon but possibly also available nitrogen and C/N ratios) seems to have an effect on growth behavior. Excluding the first more variable increase in colony growth in the first 24 h from day 0 to day 1 which is delayed by a lag phase because of a need of physiological adaptation (Fig. 4 and Fig. 6A), the calculated average increases in growth in cm/d with standard deviations are presented in Table 2. In average in the experiment, homokaryon AmutBmut grew 2.9 mm/d, 2.2 mm/d and 1.1 mm/d faster on BSM, YMG/T and MEA, respectively with extra added thiamin than on plates without. In average in the experiment, mutant Proto159 grew 1.0 mm/d, 0.9 mm/d and 0.8 mm/d faster on BSM, YMG/T and MEA, respectively with extra added thiamin than on plates without.

Effects of added thiamin on fruiting body development of* C. cinerea *strains. *C. cinerea* strain AmutBmut is a self-competent homokaryon obtained by mutations in both mating type loci and can therefore produce fruiting bodies with sexual basidiospores (Swamy et al. 1984; Boulinanne et al. 2000). Strain Proto159 however is a derived mutant of strain AmutBmut which has lost the ability to produce fruiting bodies (Granado et al. 1997). Mycelial plates (with and without extra added thiamin) of both strains on all three media were transferred into fruiting conditions (12 h light/12 h dark, 25°C) once plates were fully grown.

In two first small experiments with single plates, in the case of the fruiting defective mutant Proto159, addition of thiamin did not restore the fruiting ability in any medium (not further shown). Notably strain AmutBmut initiated fruiting on BSM medium with formation of secondary hyphal knots as still undifferentiated fungal aggregates in a first committed step in fruiting (Majcherczyk et al. 2019; Subba et al. 2019) like the YMG/T medium, but development did not complete on BSM plates (not further shown). Secondary hyphal knot formation was 4 to 5 times higher on BSM with added thiamin (263 and 284 were counted on plates) as compared to without (72 and 58 were counted on plates).

Subsequently, two experiments were performed with more plates of only homokaryon AmutBmut and only medium YMG/T, with and without extra added thiamin (20 mg/l). For homokaryon AmutBmut on YMG/T, the age of transfer was after ca. 5 ½ days incubation at 37 °C (see Fig. 7 upper row for appearance at the transfer to fruiting conditions; same

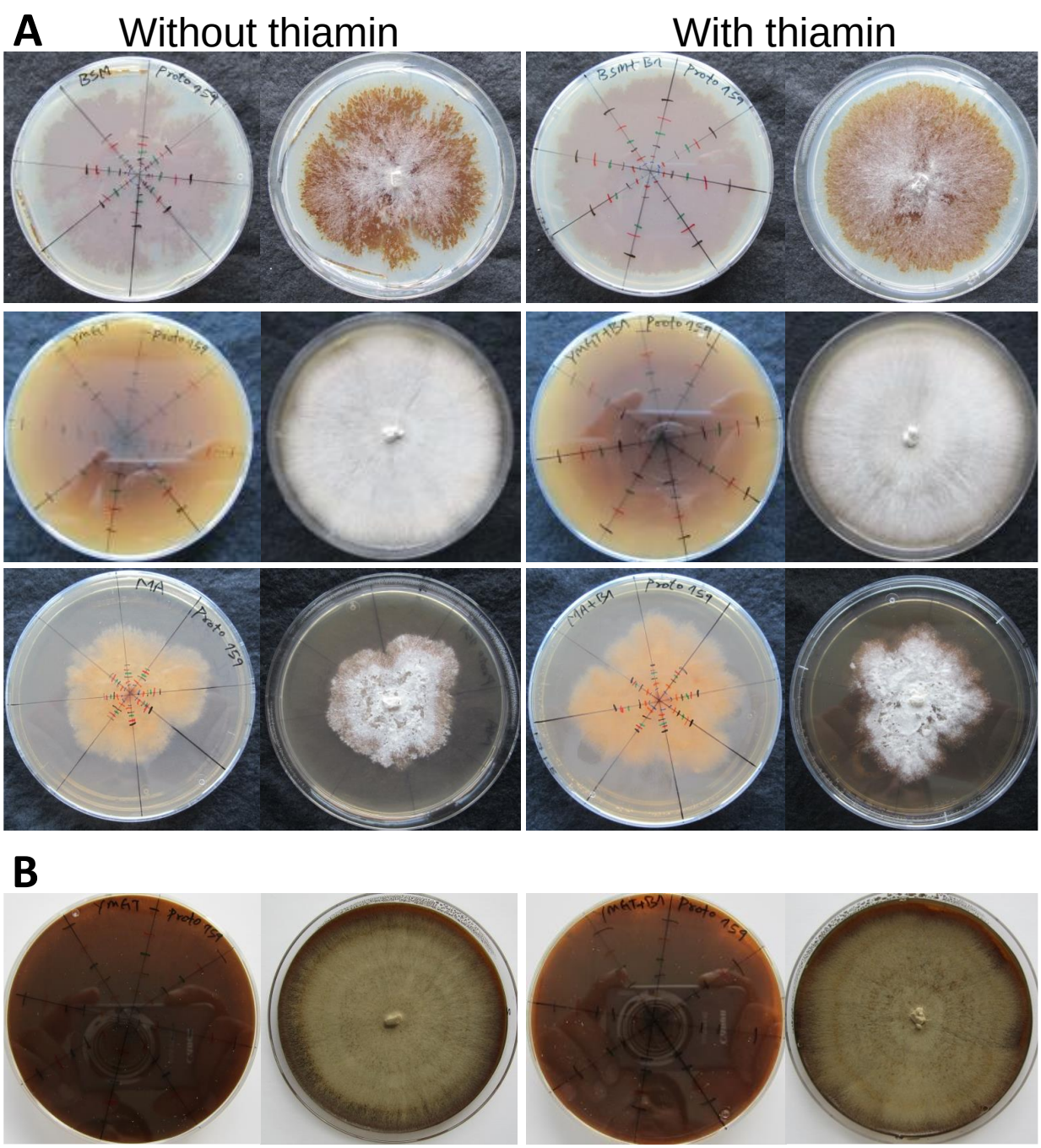

Fig. 6 Appearance of aerial mycelium on agar plates and mycelium at the reverse of plates of *C. cinerea* mutant Proto159 grown at 37°C in the dark on synthetic basidiomycete medium BSM (upper row), complete medium YMG/T (middle row) and malt extract medium MEA (bottom row) without (plates at the left) or with 20 mg/l extra added thiamin (plates at the right).

A. documents plates after mycelial growth and **B.** fully grown YMG/T plates further aged 2 ½ weeks later. Note that every 24 h, the plates obtained a light signal when marking growth fronts on the reverse of the plates (between the different media, same color on a circle = same day of incubation in the series of colors starting in all cases with blue) as described in the legend of Fig. 4 for homokaryon AmutBmut grown in parallel to Proto159. The number of marks of growth per radius indicate the number of days which the mycelium needs for growth to cover a whole plate (YMG/T medium) ir as long as growth was daily observed on media of poor growth (BSM, MEA).

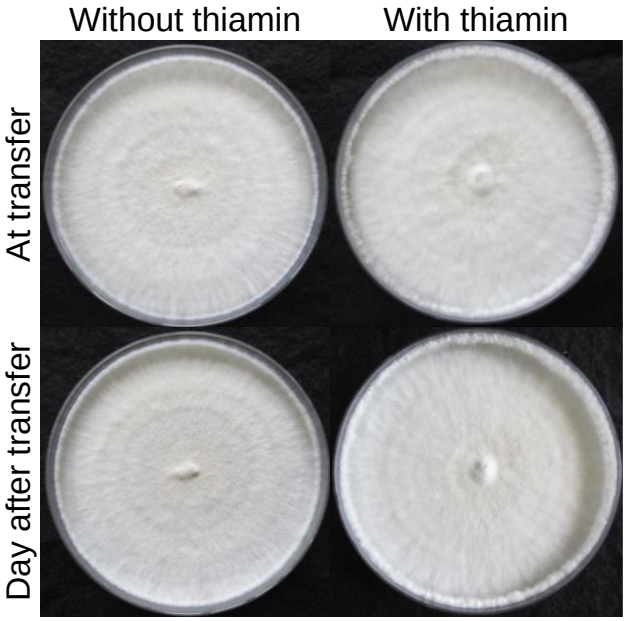

Fig. 7 Appearance of aerial mycelium of homokaryon AmutBmut on YMG/T agar plates with and withoput extra added thiamin (20 mg/l) in a fruiting experiment after 5 ½ day of growth at 37 °C in the dark (top row) and one day after transfer of the plates into standard fruiting conditions (bottom row).
Plates were from the same experiment than those shown in Fig. 3 and 4. Note the more fluffy mycelial edges in the faster grown plates with extra added thiamin.

experiment as shown in Fig. 3 to Fig. 5) and plates were then daily observed for further development (see Fig. 7 lower row for appearance 1 day after the transfer). Primordia development and fruiting body maturation was significantly better on AmutBmut YMG/T plates with extra added thiamin in the two distinct rounds of experiments performed (Fig. 8; Table 3), suggesting that adding thiamin could have a positive effect on fruiting abilities of *C. cinerea*, such as has been observed before for *S. commune* (Wessels 1965).

Fruiting body production by strain AmutBmut on YMG/T control plates in both experimental sets was however not perfect with 100% fruiting in all plates, differently as described for the strain when all cultivation parameters were most perfect (Kües et al. 2016; Majcerczyk et al. 2019; Subba et al. 2019). Among the decisive factors, fruiting body development of homokaryon AmutBmut is very sensitive to the stage of transfer of plates from growth at 37 °C in the dark into the standard fruiting conditions. Standard YMG/T plates should be nearly fully grown but the growth front should not have yet entered the plastic edges of the Petri dishes (Kües et al. 2016; Majcerczyk et al. 2019; Subba et al. 2019) such as seen in Fig. 7 (upper row, left) in the experiments performed here. A close distance of about <1 mm from the edges of fresh YMG/T plates usually ensures up to 100 % fruiting of homokaryon AmutBmut on all transferred plates. Earlier transfer and especially later transfer (when the aerial mycelium grows up or is already grown up onto the plastic walls of the Petri dishes as in seen in Fig. 7) reduces reliability of initiation of fruiting or abolishes it fully (Kües et al. 2016; Majcerczyk et al. 2019; Subba et al. 2019). Of further negative effect is when cultures during the growth phase at 37 °C are not constantly kept in dark. Repeated light signals from day to day such as experienced in the experiments here when marking the growth fronts on the plates can block fruiting (S. Subba, personal communication).

Fig 8. Mature fruiting bodies of *Coprinopsis cinerea* (Amut Bmut) on YMG/T plates with (20 mg/l) and without exta added thiamin, after 7 days of cultivation under standard fruiting conditions (Experiment 1 in Table 3)

In the two own experiments reported here, only 50 % of the standard YMG/T plates serving as control of fruiting of homokaryon AmutBmut resulted in formation of mature fruiting bodies (Fig. 8; Table 3), indicating suboptimal cultivation conditions in the two experiments. In contrast, the better fruiting behavior on YMG/T plates with extra added thiamin could not have been based simply on a better providing of the ideal cultivation conditions, because the mycelium on these plates were even further developed onto the plastic edges of the Petri dishes albeit with a more fluffy appearance (Fig. 7).

S. Subba as best experienced colleague in setting exact conditions of fruiting body development therefore performed for comparison another growth and fruiting experiment with homokaryon AmutBmut on YMG/T medium with and without extra added thiamin (20 mg/l; however, differently from the own experiments, autoclaved together with the medium) and with cultures that without any disruptions have been kept constantly at 37 °C in the dark from the time of inoculation up to the moment of transfer into standard fruiting conditions (Fig. 8).

Views of a selection of plates of S. Subba (in both series of the experiment those numbered 1 to 4) at the moment of transfer from 5-days growth at 37 °C in the dark into

Table 3 Development of primordia and fruiting bodies by homokaryon AmutBmut upon growth at 37°C on YMG/T and YMG/T with 20 mg/l extra added thiamin and transfer into standard fruiting conditions (12 h light/12 h dark, 25 °C).

Plate	YMG/T		YMG/T + thiamin	
	No of secondary hyphal knots	No of fruiting bodies	No of seconadary hyphal knots	No of fruiting bodies
Experiment 1				
1	158	5	236	3
2	134	6	264	3
3	163	1	243	7
4	124	0	288	0
5	84	0	220	3
6	92	2	280	14
7	86	9	234	7
8	104	0	253	9
9	82	0	264	4
10	122	0	201	4
Average	**114.9 ± 30.2**	**2.3 ± 3.2**	**248.3 ± 2 6.9**	**5.4 ± 4.0**
Experiment 2 (same as shown in Fig. 3 to Fig. 5 and Fig. 7)				
1	3	1	138	6
2	3	0	141	4
3	48	0	203	2
4	8	0	156	8
5	84	2	183	5
6	24	1	112	3
Average	**28.2 ± 32.4**	**0.7 ± 0.8**	**155.5 ± 32.9**	**4.7 ± 2.2**

standard fruiting conditions are shown in Fig. 8A and, later after seven days incubation under standard fruiting conditions with matured fruiting bodies in Fig. 8B. In the whole experiment performed by S. Subba, 9 of 11 plates without extra thiamin and 11 of 12 plates with the extra thiamin added fruited normally, with 2.2 ± 1.3 and 2.4 ± 1.2 mature fruiting bodies per plate, respectively. Accordingly, there were no clear differences then in the fruiting behavior without and with extra added thiamin. Neither the growth speed of cultures in the dark nor the average number of fruiting bodies per plate seemed to substantially differ. However, because thiamin is heat-instable (Kraft and Angert 2017), the actual difference in thiamin content in the plates in this experiment is uncertain with autoclaving of the added thiamin.

What can further be deduced from the results? In contrast to when all cultivation conditions are at best or close to best as in S. Subba´s experiment, more stress during cultivation has been given to the fungus in the own experiments by a light signal in daily marking of plates and by growth of the mycelium onto the plastic edges of the plates. Because the cultures with extra added thiamin did not negatively reacted on this in fruiting, did the

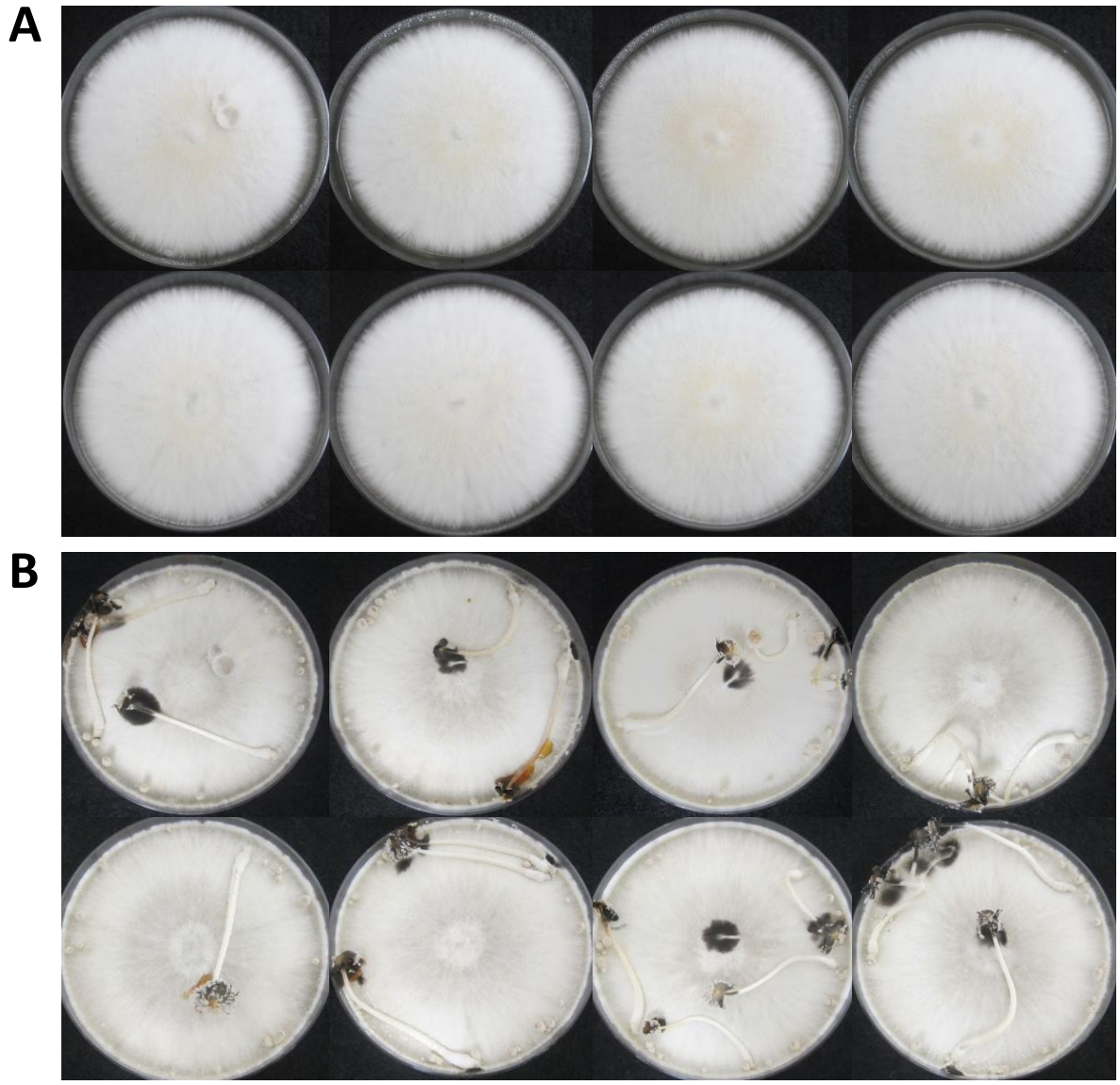

Fig. 8 Plates of homokaryon AmutBmut A. at the time of transfer from growth at 37 °C in the dark into stnadrd fruiting conditions and B. with fruiting bodies after seven days of cultivation under standard fruiting conditions.
Upper rows: plates with YMG/T medium; lower rows: plates with YMG/T medium plus 20 mg/l thiamin (added prior to autaclaving). The plates numbered 1 to 4 in both series of the experiment are shown. Photos and experiment by courtesy of S. Subba.

additional thiamin present in YMG/T plates possibly helped to better counteract the extra stress? This question will need to be clarified in the future with more experiments.

Conclusions

In this study, we unraveled that the long known thiamin deficiency in many of the *Agaricomycetes* bases primarily on loss of the *thi5* gene. The species are thus mostly thiamin-auxotrophs and need to obtain thiamin or relevant precursor molecules from their environments and from thiamin salvage pathways. Thiamin is an essential cofactor of various enzymes in the carbohydrate and energy metabolism of cells with effects in redox homeostasis (Jurgenson et al. 2009 et al. 2009; Kraft and Angert 2017). Certainly therefore, thiamin thus functions in fungal growth and development.

In summary from the growth and fruiting experiments with the model fungus *C. cinerea*, results from this study appear promising and indicative of possible effects of thiamin at higher added concentrations on growth and fruiting probably under stress conditions but more experiments are needed in the future to substantiate any differential effects of thiamin on growth and fruiting of the fungus. The media BSM, YMG/T and MEA used in this study will contain already some thiamin by ingredients such as yeast extract (in YMG/T 8-times more present than in BSM) and malt extract (in YMG/T 5-times more present than in MEA), respectively. Unfortunately for reliable fruiting, the fungus needs YMG (or YMG/T) as complete medium (Rao and Niederpruem 1969; Granado et al. 1997), making it more difficult to encounter additive effects of thiamin amounts in growth and development. Fully synthetic media without any natural nutrient mixtures (as provided for example by the yeast and malt extracts) such as presented by the standard *C. cinerea* minimal medium (Granado et al. 1997) could however serve in the future at least for to study the growth behaviour the fungus under exactly defined nutritional conditions and additions of differently defined amounts of added thiamin.

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Chapter 8

A Cyclopropane Fatty Acid Synthase Gene of *Coprinopsis cinerea* Involved in Fruiting

This chapter is subdivided into two subchapters.

Subchapter 8.1 presents a book chapter written by K. Lakkireddy (KL) and U. Kües (UK) on behalf of the Chinese Academy of Engineering on the occasion of the 7th International Medicinal Mushroom Conference on 25-29 August, 2013 in Beijing (China). The chapter entitled

"An Essential Gene in Fruiting Body Development of *Coprinopsis cinerea* Encodes a Cyclopropane Fatty Acid Synthase"

was published in: Xunjin, Y., Weilai, J.Y. (eds.) (2014) Medicinal Mushroom – Health and Future (International Top-level Forum on Engineering Science and Technology Development Strategy), Higher Education Press, Bejing, China, pp. 302-316 and in Chinese translation in copy on pp. 118-129. Unfortunately, the Chinese publisher modified the correct authors' list of the two authors Lakkireddy and Kües into Kües et al. in the published book. Note also the mistake in the published Fig. 4 of which a corrected version is given at the end of the chapter.

The paper describes the background of the fruiting-defective *cfs1* mutant of *Coprinopsis cinerea*, the enzymatic mechanism of bacterial cyclopropane fatty acids synthases, the subcloning of cyclopropane fatty acid genes into a bacterial expression vector and first bacterial expression experiments.

Authors´ contributions: KL performed research and wrote the paper. UK and KL revised and finalized the manuscript.

Subchapter 8.2 presents further experiments performed to define the protein function of gene *cfs1* using an acid stress assay with *Escherichia coli* as a host. KL designed experiments, conducted most of the experimental research and analyzed the data on acid resistance tests. Mojtaba Zomorrodi is acknowleged for technical help in protein gel electrophoresis and proteomics, UK and Sevda Haghi Kia for data analysis in protein identification. UK helped in writing up the proteomics work and by revision of other writing by KL. All colleagues are sincerely thanked for their kind support.

Subchapter 8.1

MEDICINAL MUSHROOM——HEALTH AND FUTURE

An Essential Gene in Fruiting Body Development of *Coprinopsis cinerea* Encodes a Cyclopropane Fatty Acid Synthase

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Abstract: *Coprinopsis cinerea* is an inkcap that is cultured in some Asian and African countries on agricultural wastes for human consumption. In science, the fungus serves as an ideal model fungus to study fruiting body development in basidiomycetes since *C. cinerea* completes its whole life cycle within two weeks on artificial media in the laboratory. Fruiting body initiation is controlled by environmental conditions such as light, temperature, nutrients, and humidity as well as genetically by the *A* and *B* mating type genes as the master regulators of sexual development. Fruiting bodies therefore form normally only on the dikaryon. However, specific mutations in the two mating type loci (*Amut* and *Bmut*) allow mushroom development also on mutant homokaryons without a need of previous mating. In consequence, *Amut Bmut* homokaryons are used for the generation and screening of dominant as well as of recessive mutations in the fruiting process. In our laboratory, we possess a large collection of mutants from homokaryon AmutBmut (ca 1200 different clones) that have been generated either by UV-or by REMI mutagenesis. As a first characterized strain from our collection, UV-mutant 6 – 031 was found to be defective in formation of hyphal aggregates (secondary hyphal knots) as an early step in fruiting body development. Previously, complementation studies of the recessive mutant gene with a wildtype library detected a gene *cfs*1 whose protein product has strong similarity with bacterial cyclopropane fatty acid synthases. In *Escherichia coli*, cyclopropane fatty acid synthase (referred to as CFA synthase, CFAS

or shortly Cfa) is synthesized when a bacterial culture enters the stationary phase. The enzyme alters the alkyl chains of membrane phospholipids in response to changing environmental conditions. As a result, a cyclopropane ring is formed on the alkyl chain. Production of cyclopropane fatty acids helps to make cells better resistant to acids. In this study, we want to exploit this bacterial system to characterize the enzymatic function of our *C. cinerea* protein.

Keywords: hyphal aggregates; fruiting body development; cyclopropane fatty acid synthase; acid resistance

1 Introduction

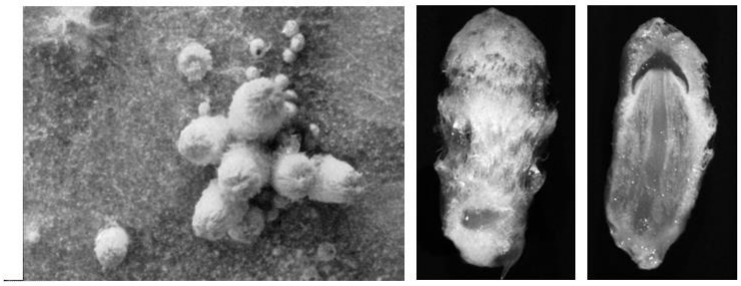
Coprinopsis cinerea is a typical multicellular basidiomycete that forms mushrooms for sexual reproduction. In some Asian and African countries (e. g. in Thailand and in Tanzania), it is nowadays cultivated on agricultural wastes for human consumption[1]. By the ease to culture this fungus on artificial media it has been introduced long ago as a model species for studying developmental processes in the higher basidiomycetes[2-5]. The fungus completes its sexual cycle under laboratory conditions within two weeks on artificial media such as YMG (yeast extract, malt extract, glucose) or YMG/T (with tryptophan) complete medium[6,7]. Fruiting bodies normally develop only on the fertile dikaryons. Within the fruiting bodies, in the basidia at the surface of the gills, karyogamy happens and is directly followed by meiosis and the formation of basidiospores as sexual propagules. These can then germinate on suitable substrates into the sterile haploid monokaryons. If two compatible monokaryons meet they can fuse to form a fertile dikaryon with two haploid nuclei in its cells, one from each mating partner. Monokaryons are compatible when they differ in mating type. *C. cinerea* has two mating type loci, *A* and *B*, and their different alleles together define the different mating types of monokaryotic mycelia[4]. Mating type genes furthermore control the formation of mushrooms on the dikaryon as master regulators of developmental processes[8]. Fruiting body development is moreover highly influenced by environmental factors such as light, temperature, nutrients and humidity[4,9].

The importance of mating type genes for mushroom production and sexual development is further seen in specific self-fertile mutants that have defects in both

mating type loci. Such genetic defects are referred to as *Amut* and *Bmut*. Respective *Amut Bmut* mutants can form fruiting bodies without mating to another strain[10,11]. Furthermore, such mutants produce haploid single-celled spores (oidia)[12,13], which can be used for easy mutagenesis and selection of defects in the haploid stage[7]. This is of special benefit when screening for recessive mutants in the process of fruiting body development. As stated above, fruiting happens normally only in the dikaryotic stage. With two distinct sets of chromosomes, the dikaryon is functionally equivalent to the diploid situation of plants and animals. Therefore, on the dikaryon recessive mutations can be difficult to be found[14,15]. Mutagenesis of spores of homokaryon AmutBmut (*A*43*mut*, *B*43*mut*; numbers refer to the distinct wild type mating type alleles mutations occurred in) was done by UV exposure and by transformation of plasmids in presence of restriction enzymes, a molecular technique which is known under the name REMI (restriction enzyme-mediated integration)[7,16]. A large collection of mutants with defects in fruiting body development (ca 1200 different strains) is thus available in our laboratory (Kües et al. unpublished).

UV-mutant 6 – 031 derived from homokaryon AmutBmut by UV mutagenesis. This mutant has a defect at a very early step in fruiting body development[16]. By intense localized formation of short hyphal branches it can form small loose mycelial aggregates[16], the primary hyphal knots that in the wildtype might either develop into small sclerotia for duration or may give rise to secondary hyphal knots[11,14]. Secondary hyphal knots are compact hyphal aggregates that are formed in a first specific step of fruiting body development. In the secondary hyphal knots, differentiation of tissues for mushroom caps and stipes start[11]. Mutant 6 – 031 does not give rise to secondary hyphal knots[16]. However, the defect in mutant 6 – 031 has been complemented by transformation with a genomic cosmid library of *C. cinerea* AmutBmut (Fig. 1). Subcloning of DNA fragments from the complementing cosmid revealed that a mutation in the gene *cfs*1 was responsible for the loss of ability to enter the fruiting pathway. Sequencing uncovered that the encoded protein has high similarity to bacterial cyclopropane fatty acid synthases[16].

The cyclopropane fatty acid synthase (CFA synthase, CFSA or shortly Cfa) in the Gram-negative bacterium *Escherichia coli* is a cytoplasmic enzyme that transfers a methylene group from an Sadenosyl-L-methionine (SAM) to the double bond in the chain of an unsaturated fatty acid (UFA). As result, a cyclopropane ring is formed and



(a) (b) (c)

Fig. 1 Fruiting defective mutant 6 – 031 after transformation with a *cfs*1-containing cosmid[16]

(a) primordia are formed on the mycelium under standard laboratory fruiting conditions[7] on complete medium at 25°C in a 12h dark/12 h light cycle; (b) and (c) within the primordia, cap and stipe tissues developed. Photos taken by Dr. Y. Liu

S-adenosyl-L-homocysteine (SAH) is released (Fig. 2). In *E. coli*, UFAs are major components of phospholipids shaping the cellular membrane. During enzymatic conversion of UFAs to cyclopropane fatty acids (CFAs), the enzyme transiently interacts with the cellular membrane. CFAs are typically produced at the onset of the stationary phase of bacterial growth. Importantly, the enzyme also alters the alkyl chains of membrane phospholipids in response to changing environmental conditions[17]. Stress situations leading in bacteria to CFA production might by mediated by cold or by high temperature, by high Mg^{2+} concentrations, by high salinity, by high osmolarity, by extreme pH values, by high pressure and by unfavorable conditions of aeration[17-21]. Production of CFAs alters membrane properties by a decrease in fluidity, changes membrane permeability and can inhibit membrane pumps and all this helps to defeat stresses[19-22]. *E. coli* strains lacking CFAs are thus more sensitive to environmental stresses. For example, they react more sensitive to acid shock[23].

In this study, we make use of a *cfa* mutant of *E. coli* in an acid-resistance test system. The defect in acid-resistance in the mutant can be complemented by transformation of the *E. coli cfa* wild-type gene[23]. In this work, we cloned an isolated cDNA from *cfs*1 of *C. cinerea* behind a bacterial promoter and transformed the construct into the *E. coli cfa* mutant for complementation tests.

2 Materials and methods

E. coli strains, DNAs and molecular techniques. Isogenic *E. coli* strains ZK126 (*cfa*$^+$) and YYC1272 (*cfa*$^-$)[23] were kindly provided by Prof. Dr. J. E. Cronan. Vector pET-16b (5.7 kb; Novagen, Darmstadt, Germany) was used in subcloning *E. coli cfa*

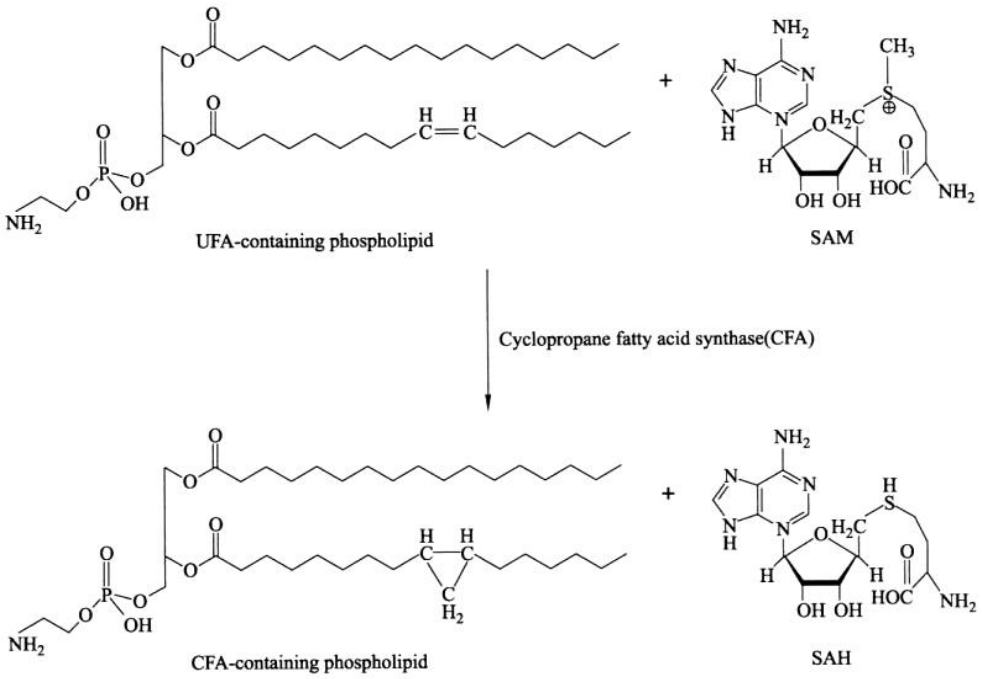


Fig. 2 Substrates and products of CFA synthase in bacteria[17]

(P30010) and *C. cinerea cfs*1 cDNA (AAL73238)[16,24]. The pET-16b expression vector has an inducible T7 promotor, a *lacI* gene and an ampicillin resistance marker gene (Novagen). Specific primers (Bam-cfaF 5′CACCGACCAGTGATGGAGAAACT*GGATCC*G ATGAGTTCATCGTGTATAG3′ and Bam-cfaR 5′ CTTGGCGCACGCGTAGGAT*GGATC* CAACATTGAAATCGATCCAC3′; gene-specific sequences are underlined; sites for restriction enzyme *Bam*HI are shown in italic) were used to amplify the 1.3 kb-long *cfa*$^+$ wildtype gene from genomic DNA of *E. coli* DH5α[25] by colony PCR[26]. The amplified fragment was digested and subcloned into *Bam* HI digested pET-16b and transformed into *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) using standard techniques[27]. Constructs of both directions of insertion were obtained (T7 promoter and gene in same direction = pET-16b *cfa*, in inverse direction = pET-16b *cfa*-inv). Similarly, the *C. cinerea cfs*1 cDNA was amplified from the pBluescript KS (−)-derivative pYL29[24] with primers PETcfs1 (5′ TGATA *GGATCC*ATGCCGGCCCACCAC3′; gene-specific sequences are underlined; the *Bam* HI site is shown in italic) and M13 (−20) (5′ GTAAAACGACGGCCAG3′) (Microsynth, Baldach, Switzerland). The amplified cDNA was digested with *Bam* HI making use of a second site originating from the multiple-cloning site of the *E. coli* vector and then ligated into *Bam* HI-digested pET-16b and

transformed into *E. coli* DH5α to give pET-16b *cfs*1[24] . After verification of the constructs, the plasmids and the naked vector pET-16b were transformed into *E. coli* ZK126 and YYC1272 for expression studies.

For expression, transformed *E. coli* strains were grown overnight in 25 mL LB medium[27] plus 100 μg/mL ampicillin at 37°C on a shaker at 200 r/min speed. 4 mL culture (2. 0 OD_{600nm}) was transferred into 25 mL fresh medium to give an OD_{600nm} of 0. 3 to 0. 4. Cultures were incubated up to 4 h, either in presence of 1 mmol/L ITPG (isopropyl β-D-1 – thiogalactopyranoside) or in absence of ITPG, until an OD_{600nm} of 1. 5 was reached. 1 mL aliquots were harvested and centrifuged and pelleted cells were solved either in neutral media (pH 7. 0) or in acidic media (pH 3. 0) and treated further as described by Chang and Cronan[23]. Survival of cells after 1 h acidic treatment were calculated as described[23].

Using *C. cinerea* Cfs1 as a query protein, searches were done in the NCBI blastp page: http://blast. ncbi. nlm. nih. gov/Blast. cgi? PROGRAM = blastp&BLAST _ PROGRAMS = blastp&PAGE_TYPE = BlastSearch&SHOW_DEFAULTS = on&LINK_LOC = blasthome) against proteins of specific sequenced fungi in the NCBI database. Accession numbers of proteins homologous to the cyclopropane fatty acid synthase of *C. cinerea* were collected.

3 Results and discussion

1) Sequence comparison and protein structure. *C. cinerea* Cfs1 and *E. coli* Cfa have lengths of 469 amino acids (aa) and 382 aa, respectively. *C. cinerea* Cfs1 is somewhat longer than Cfa of *E. coli* due to an extended N-terminal sequence. However, the two enzymes share 31% aa identity and 47% aa similarity with each other over a length of 378 aa (*C. cinerea*) and 333 aa (*E. coli*), respectively (Fig. 3).

More than half of this region of similarity is occupied by an S-adenosylmethionine-dependent methyltransferase (AdoMet-MTase)[28] domain. N-terminal and C-terminal to the SAM-binding domain are stretches of 29 and 32 aa, respectively (Region I and Region II in Fig. 3) that have been implicated in cyclopropane fatty acid synthase function[16,29-32]. The non-functional Cfs1 in *C. cinerea* mutant 6 – 031 had been found to carry an aa exchange from Y441 to D441 in Region II. This sequence is predicted in the wildtype to adopt a helical structure with the potential ability to anchor in the cellular membranes. The mutation in strain 6 – 031 replaces a residue with a hydrophobic side

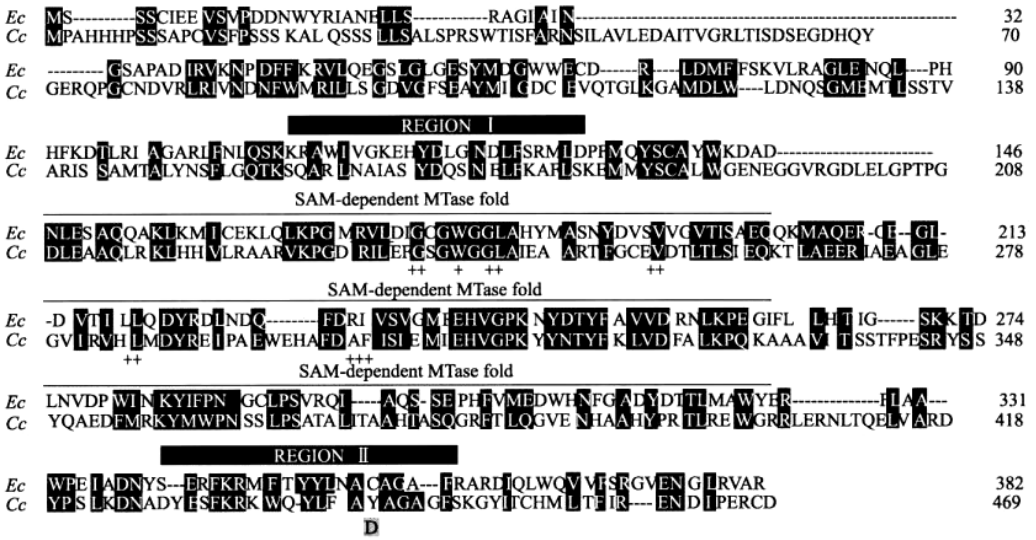


Fig. 3 Sequence alignment of *E. coli* Cfa (*Ec*) and *C. cinerea* Cfs1 (*Cc*)

A line above sequences denotes the structurally conserved SAM-dependent MTase fold with amino acids shown in other proteins to contact SAM marked underneath the sequences by a "+"[28]. Region I denotes a protein region specific to CFASs, Region II a helical domain with potential catalytic or membrane anchoring functions. Letter D beneath Region II indicates the position of a Y to D replacement in *C. cinerea* mutant 6-031 that lead to loss of function

chain by a negatively charged aa. In consequence, the C-terminus is less hydrophobic and does likely not anymore adopt a helical structure that could anchor in the membrane[16]. However, other studies have shown that the Region II may contribute in the folded protein to the active-site of the enzyme[31,32]. The N-terminal Region I is specific to bacterial enzymes with cyclopropane fatty acid synthesis activity, fungal enzymes of the structure of Cfs1 and some protozoa and plant cyclopropane fatty acid synthases[16,32-34] (our unpublished observations). Fungal genes for such enzymes exist in filamentous ascomycetes. For example, each two different proteins are encoded in the genomes of *Aspergillus nidulans* (XP_663292, XP_680644), *Neurospora crassa* (XP_962215, XP_963120) and *Sordaria macrospora* (XP_003347329, XP_003350421), respectively. Also filamentous basidiomycetes can have more than one gene for a cyclopropane fatty acid synthase such as *C. cinerea* that has in total two genes for CFASs including gene *cfs*1 (XP_001841224 = Cfs1; XP_001836055), *Laccaria bicolor* that has four genes for CFASs (XP_001881602, XP_001884269, XP_001875675, XP_001887832) and *Schizophyllum commune* that has three genes for CFASs (XP_003028767, XP_003036753, XP_003034568). The encoded enzymes clearly distinguish in Region I from another group of related fungal membrane-interacting SAM-dependent

methyltransferases to which protein ERG6 of the ascomycetous yeast *Saccharomyces cerevisiae belongs*[16]. ERG6 is a membrane-associated Δ24-sterol-C-methyl-transferase that acts in the ergosterol biosynthesis pathway and transfers a methyl group to a C-C-double bond at position C-24 in the ergosterol precursor zymosterol for its conversion into the precursor fecosterol[35,36]. While the targeted substrate is different, the overall biochemical reaction is similar. Accordingly, Region I has been postulated to present a domain that might be decisive for which specific substrate is converted by an enzyme of the larger membrane-interacting SAM-dependent C-methyl-transferase family[16]. Mutagenesis studies together with a good test system for function will be essential for deeper characterization of all the domains. Principally, transformation of *C. cinerea* mutant 6 −031 is possible to do[16] but the efforts for obtaining solid results by analyzing sufficient amounts of transformants in fruiting behavior are considerable. Therefore, an easy bacterial test system would suit better an analysis for biochemical enzyme characterization. In addition, bacterial expression systems such as the pET-vector systems (Novagen) do allow production of proteins in large amounts which will be further helpful for protein characterization.

2) Heterologous expression of *C. cinerea* Cfs1 in *E. coli*. The similarity between Cfs1 and Cfa in sequence and structure (Fig. 3) prompted us to express the *C. cinerea* protein for functional analysis in *E. coli*. A cDNA for the protein was at hand[16,24] and thus amplified to insert it into the *E. coli* expression vector pET-16b to obtain construct pET-16b *cfs*1 (Fig. 4)[24]. A parallel construct pET-16b *cfa* was generated with the *E. coli cfa*$^+$ gene (Fig. 4) and a construct pET-16b *cfa*-inv in which gene *cfa* was inserted in opposite direction to the bacteriophage T7 promoter. All three constructs and the empty vector pET-16b were transferred into *E. coli* strains ZK126 (*cfa*$^+$) and

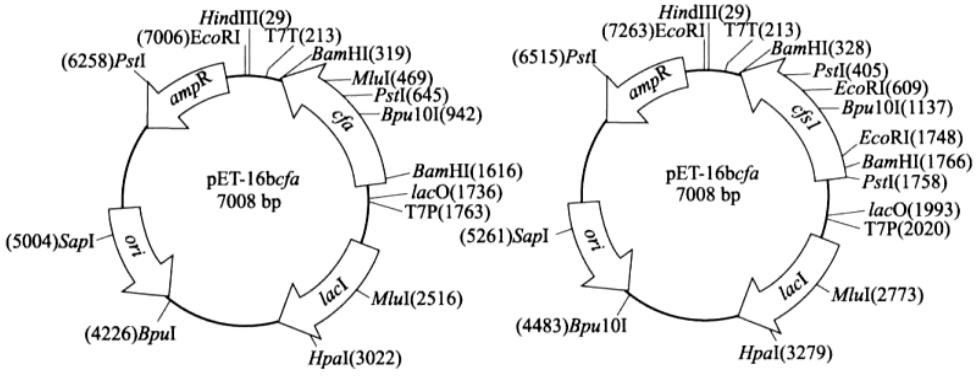


Fig. 4 Structures of pET-vectors for expression of *C. cinerea cfs*1 and *E. coli cfa* in *E. coli*

YYC1272 (*cfa*$^-$), respectively.

In first test experiments with cells treated in presence or absence of ITPG, about 40% of mutant YYC1272 control cells with pET-16b survived, whereas the survival rates of the wildtype strain with pET-16b were above 90%. Similar results were obtained for vector pET-16b *cfa*-inv. In contrast, *cfa*$^-$ mutant cells with either pET-16b *cfa* or pET-16b *cfs*1 showed better survival rates of 60% ~70% and more. An effect of ITPG was not obvious. However, this was also not to be expected. In the pET-system of Novagen, ITPG is used to induce transcription of a T7 RNA-polymerase gene provided from another source (such as from a gene insertion into the chromosome or from a phage) in the *E. coli* cells than the pET-vector (Novagen). The T7 RNA-polymerase induces then transcription of the gene of target at the T7 promoter localized upstream of the respective cloned gene (compare Fig. 3). However, a T7 RNA-polymerase gene was not yet present in our system to induce high-level production of the two types of cyclopropane fatty acid synthases. Nevertheless, our results suggest that both enzymes were produced at effective levels and that the product of the *C. cinerea cfs*1 can indeed replace the native *E. coli* enzyme in UFA modification to CFA (see Fig. 2 for the reaction scheme). The T7 promoter on a high copy number plasmid can be leaky[37] and such effects can account for our observations on better survival of mutant YYC1272 cells with pET16b *cfa* or pET16b *cfs*1 than mutant YYC1272 cells with pET-16b or pET16b *cfa*-inv, respectively. The latter construct has in fact the functional *cfa* gene but it is on the opposite strand to the T7 promoter why in this situation a leaky promoter cannot give a transcript that could be translated to a functional Cfa protein. If our interpretations of the available results are right, in further experiments we should find the expressed enzymes on protein gels. More importantly, we should find in the bacterial membranes of cells with pET16b *cfa* and of cells with pET16b *cfs*1 sufficient levels of CFAs that should be detectable by GC/MS analysis[33,34,38]. Finally, in presence of the T7 RNA polymerase we should obtain high levels of proteins for further biochemical characterization.

4 Conclusions

In this study, we cloned the *C. cinerea cfs*1 gene and for control also the *E. coli cfa* gene into an expression vector of *E. coli*. Our first results in *E. coli* seem to indicate that the fungal enzyme can replace the bacterial Cfa in conferring acid resistance to growing cells. This indirectly suggests that CFAs are produced by the fungal enzyme in the

bacterium. Little is yet know on CFAs in eukaryotes. Some protozoan animal parasites have recently been shown to produce CFAs by an own enzyme believed to be obtained through horizontal gene transfer from bacteria[33]. Similar, a few plants have been reported to have cyclopropane fatty acid activities and to produce CFAs[34,39,40]. Other eukaryotes take up bacterial CFAs from the environment[41,42]. In bacteria, CFA production is linked to survival under stress conditions[17-21]. In the pathogenic protozoa, CFA formation is of advantage for stronger virulence, membrane transport activities and defeat of oxidative stress and the host's defense systems[33]. Draught resistance in plants and desiccation tolerance in certain animals (millipedes) have been connected to presence of CFASs[43,44]. In the millipedes, there appears to be a link to egg formation for reproduction[45] and in the slime mold *Polysphondylium pallidum*, levels of CFAs fluctuate with developmental transitions from the amoebal to the aggregation stage[41]. Functional expression of *E. coli* Cfa in *S. cerevisiae* shows that the fungal membranes and unsaturated lipids within are in principle accessible by the bacterial enzyme for biochemical reactions[38]. Likewise, plant CFASs can be functionally expressed in yeast[40]. As confirmed by a tblastn search with Cfs1 of the *S. cerevisiae* genome, the yeast has no own CFA synthase. Our unpublished observations from multiple fungal genome analyses show that one or more *cfs*1-like fungal genes are more likely to be found in filamentous ascomycetes and basidiomycetes that produce multicellular structures for sexual development. The finding that *cfs*1 is essential for mushroom production in *C. cinerea*[16] suggests that CFA production could be part of the process of environmental adaptation for the initiation of fruiting. This suggests further that initiation of fruiting requires changes in membrane structure and function. And this postulate agrees well with experimental findings in several Agaricomycotina that addition of membrane-interacting substances and surfactants to mycelium can cause fruiting body induction. Sucrose esters of fatty acids, saponins, cerebrosides and other surface- and membrane-interactive compounds were reported to induce fruiting body development in various basidiomycetes[46-50]. However, it is not only application of lipid-interactive compounds that point to importance of membranes in fruiting body initiation. Application of membrane pore-forming hemolysins and other (glyocolipid-binding) lectins can also induce fruiting, possibly due to taking influence on membrane signaling[51,52].

Acknowledgements

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Corrections

1. The sequence of primer PETcfs1 presented in the manuscript accidently missed one base T directly after the *Bam*HI restriction site which was required for insertion into the correct reading frame. The PETcfs1 sequence reads thus correctly ´5 TGATA*GGTACC*T**ATG**CCGGCCCACCAC 3´. The *Bam*HI site in the primer is shown in italic, the formerly missing T for achieving the correct reading frame is underlined and the native ATG startcodon of gene *cfs1*in bold. By in frame gene insertion of the *Bam*HI-digested PCR product into the *Bam*HI site of *Escherichia coli* expression vector pET-16b (Novagen, Darmstadt, Germany), a resulting expressed fusion protein should thus be slightly bigger than the native *C. cinerea* protein, by ca 3.2 kDa extra obtained through the N-terminal addition of a 10xHis-Tag and a Factor Xa site and the amino acids HMLEDP (full extra sequence: MGHHHHHHHHHHSSFHIEGRHMLEDP) generated from the vector DNA sequence positioned directly prior to the native *cfs1* ATG codon (see pET system manual. 11th edition. TB055 11th edition 01/06. Novagen, Madison, WI). The sequence of primer Bam-cgaF with a base G after the *Bam*HI site and prior to the ATG of gene *cfa* in contrast was correct. A resulting fusion protein with *E. coli* Cfa contains then the same extra N-terminal sequence MGHHHHHHHHHHSSFHIEGRHMLEDP than the respective Cfs1 fusion protein.

2. Inadvertently, Fig. 4 of the published chapter contained a mistake in plasmid labelling. The corrected figure is given below.

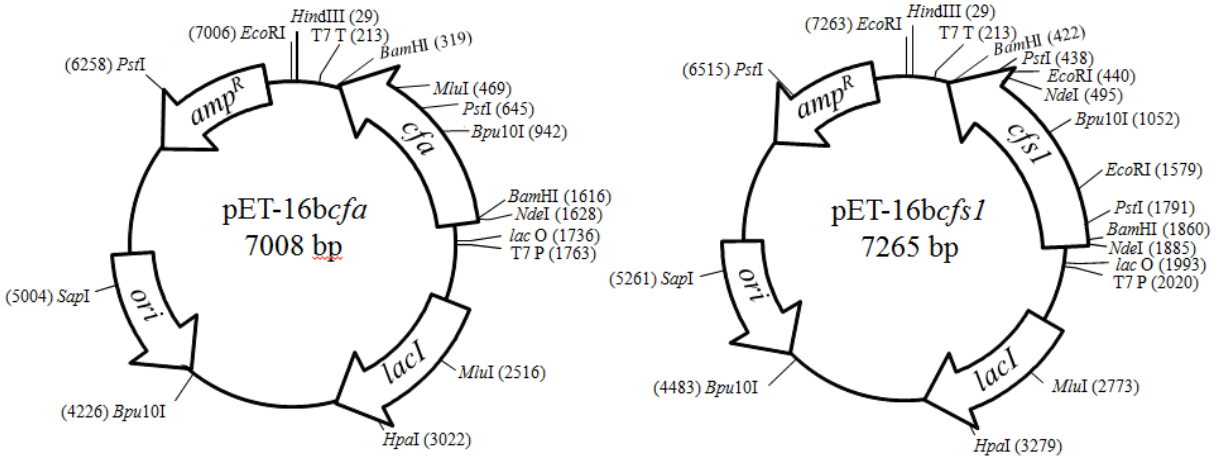

Fig. 4 (corrected) Structure of pET-16b vectors for expression of *C. cinerea cfs1* and *E. coli cfa* in *E. coli*.
Note: T7 plus *lacO* = T7*lac* promoter.

Subchapter 8.2

A Potential Fungal Cyclopropane Fatty Acid Synthase

Escherichia coli cyclopropane fatty acid (CFA) synthase Cfa and the similar *Coprinopsis cinerea* enzyme Cfs1 were recombinantly produced as N-terminal 10xHis-tag-fusion proteins in *E. coli* strain BL21(DE3), using the T7 RNA-polymerase-specific T7*lac* promoter in the vector pET-16b for expression. Both proteins were relatively stable in the strain and were detected by mass-spec analysis of peptides in trypsin-digested bands of the intracellular proteome of transformed clones. Cfa and Cfs1 were decteted in the bacterial proteomes, independently of whether gene expression at the T7*lac* promoter was induced by addition of isopropyl-ß-D-thiogalactopyranoside (IPTG) or not. Accordingly, the non-induced T7*lac* promoter allowed leaky gene expression in strain BL21(DE3) by a background production of the T7 RNA polymerase. Surprisingly, a peptide of Cfs1 was detected that started with the native methionine as first amino acid and indicated that some of protein molecules were produced from a cryptic internal ribosome binding site located close to the native *cfs1* ATG startcodon.

As described in literature, the *E. coli* enzyme Cfa cyclopropanates C double bonds in unsaturated fatty acids (UFAs) localized in the cellular membrane phospholipid bilayer. CFA production alters membranes more stress resistant, such as against low pH. The *cfa* and *cfs1* genes cloned in pET-16b were thus further used in acid resistance-tests with transformed *E. coli* strains ZK126 (*cfa*$^+$) and YYC1272 (*cfa::kan* of ZK126), by comparing survival of cells incubated at pH 3.0 with cells incubated at pH 7.0, respectively. Results variably but not always suggested for pET-16b*cfa* and for pET-16b*cfs1* to confer some acid resistance to the *cfa*$^-$ mutant YYC1272. The mutant has no T7 RNA-polymerase gene and any *cfa* or *cfs1* expression would require leaky activity at the T7*lac* promoter in absence of T7 RNA polymerase, but probably in dependency of added IPTG. In a first proteomics analysis for full length proteins in absence of IPTG, neither Cfa nor Cfs1 was detected in the transformed strain ZK126 and its transformed mutant YYC1272. More sensitive techniques in proteomics and IPTG in cultures might need to be applied in the future to find evidence for existence of the proteins in *E. coli* ZK126 and YYC1272 transformants. For better proof of Cfa and Cfs1 activity in conferring acid-resistance in *E. coli cfa*$^-$ mutants, either the YYC1272 strain should be equipped with a T7 RNA polymerase gene for high protein expression or, probably better, the *cfa*$^+$ gene must be deleted from the BL21(DE3) strain in order to create a new *cfa*$^-$ mutant in which Cfs1 is stable for acid tests. A further alternative with overexpressing BL21(DE3) cells is to change to an easier new growth test system in liquid culture under applying e.g. osmotic stress.

Introduction

Coprinopsis cinerea is a well established model fungus used to dissect functions in fruiting body development of *Agaricomycetes* (Kües 2000, Kües and Navarro-Gonzaléz 2015), such as by genomics (Stajich et al. 2010), transcriptomics (Cheng et al. 2013, 2015; Muraguchi et al. 2015; Krizsán et al. 2019), and proteomics (Majcherczyk et al. 2019), through developmental mutants combined with DNA transformation techniques (Kamada 2002; Kamada et al. 2010; Nakazawa et al. 2011) and by microscopy techniques (Subba et al. 2019). A range of genes acting in the fruiting process have been identified (for examples see Muraguchi and Kamada 1998, 2000; Liu et al. 2006; Muraguchi et al. 2008; Nakazawa et al. 2010, 2011, 2016; Kuratani et al. 2010; Ando et al. 2013; Masuda et al. 2016) while many

hundreds more are candidates for yet undefined functions participating in fruiting body development (Cheng et al. 2013, 2015; Muraguchi et al. 2015; Krizsán et al. 2019).

As an example, *cfs1* is a gene essential in *C. cinerea* for the initiation of fruiting body development at the stage of formation of primary hyphal knots (Liu et al. 2004) which are loose hyphal aggregates that are produced in the dark localized on selected parental hyphae in freshly grown fungal mycelium by formation of multiple stunted branches (Boulianne et al. 2000; Lakkireddy et al. 2011 - Chapter 6 of this thesis; Kües et al. 2016). Among the sequenced *Agaricomycotina*, all species but the two *Tulasnella calospora* strains UAMH and AL13 and the yeast *Cryptococcus neoformans* H99 have at least one, but usually several genes for related proteins (22 in the highest case). Genes are also found in all sequenced species of *Ustilagomycotina* and *Pucciniomycotina*. Furthermore in the *Ascomycota*, many while not all species of the *Pezizomycotina* contain *cfs1*-like genes (e.g. *Xylariacae* and *Aspergillus* species miss such a gene copy). In contrast, species in the *Taphrinomycotina* (ca 50 % of the species) and the *Saccharomycotina* (< 25 % of the species) possess less often such *cfs1*-like genes (Blast search results in the JGI Mycocosm in May 2020; U. Kües, personal communication). The broad distribution of genes especially in filamentous fungal species might indicate an importance of the encoded functions in fungal multicellularity.

As deduced from sequence conservation to bacterial enzymes such as *Escherichia coli* cyclopropane fatty acid synthase Cfa (Grogan and Cronan 1997; Hari et al. 2018), *C. cinerea cfs1* encodes a putative cyclopropane fatty acid synthase Cfs1 but the actual enzymatic function remains still to be shown (Liu et al. 2006). The native Cfs1 protein of *C. cinerea* is 469 amino acids (aa) long (Liu et al. 2006). It has a ca 150 aa-long N-terminal region which is poorly conserved with the N-terminal domain of bacterial enzymes (Liu et al. 2006) shown to serve for dimer formation (Hari et al. 2018). At the catalytic C-terminus, the Cfs1 protein of *C. cinerea* carries a conserved 313 amino acid long domain 'Cfa superfamily' [cyclopropane fatty-acyl-phospholipid synthase and related methyltransferases (lipid transport and metabolism)]. In more detail, the Cfa superfamily domain consists of a short REGION I (CFA synthase signature sequence) specific for cyclopropane-fatty acid synthases and likely for substrate catalysis reactions, a structurally conserved S-adenosyl-L-methionine (SAM)-dependent C-methyltransferase (Mtase) fold and a short REGION II also specific for cyclopropane-fatty acid synthases with a likely membrane-anchoring function (Liu et al. 2006; Lakkireddy and Kües 2014 - Subchapter 8.1) and, in partial sequence overlap with the ultimate C-terminus, a function as potential dimerization interphase with the N-terminus of another enzyme unit (Hari et al. 2018). In a newest model for *E. coli*, only one of the units in a Cfa dimer is believed to contact a phospholipid membrane leaflet of the lipid bilayer for dimer anchoring, whereas the other unit is catalytically active with unsaturated fatty acid (UFA) chains of a phospholipid flipped out of the membrane leaflet for enzymatic cyclopropanation of C double bonds (Hari et al. 2018) such as at C16:1 and C18:1 fatty acid positions (Chen and Ganzle 2016), in order to form cyclopropane fatty acids (CFAs).

In the fruiting-defective UV-mutant 6-031 of *C. cinerea*, it is in the C-terminal putative dimerization and membrane-anchoring domain where a missense mutation resulted in an aa change Y441D (Liu et al. 2006; Lakkireddy and Kües 2014 - Subchapter 8.1). Mutations have artificially been introduced into all three recognized domains of the *C. cinerea* Cfs1 protein. Replacements of Y168A and Y185A in REGION I and replacements of G237A and G239A of SAM-contacting residues of the Mtase fold caused loss of protein function in complementation tests of the *cfs1* defect in mutant 6-031 in fruiting body development, unlike to an exchange of the sequence just upstream, ILEF of *C. cinerea*, by the *E. coli* motif VLDI at positions 232 to 236 (complementation frequeny was 6.0 % and comparable to 8.7 to 11.5 % of control plasmids with the unmutated gene). The results

support the importance of both protein domains for the Cfs1 function. A larger replacement of the *C. cinerea* sequence SFKRKWQYLFAYAGAGFSKGY in REGION II (aa 430 to 450) by the respective *E. coli* sequence RFKRMFTYYLNACAGAFRARD resulted in a 10fold reduction of the complementation frequency (0.6 %) of fruiting body initiation of mutant 6-031 (Ciardo 2001). In light of a potential dimerization function of REGION II with the fitting N-terminus of a second protein, a block in dimerization could be a feasible reason for the reduction in overall complementation activity. In *E. coli*, Cfa variants unable to dimerize have an >150-fold lower enzymatic activity than the wildtype enzyme (Hari et al. 2018).

In *E. coli* and some other bacteria, the Cfa enzyme has been implicated in conferring acid resistance to cells by altering the membrane fluidity through cyclopropanation of C double bonds (cyclopropane ring formation) in unsaturated phospholipids, by an enzymatic process in which a methylene group is transferred from SAM to the C double bond in the UFA chain for CFA formation (Grogan and Cronan 1997; see also Lakkireddy and Kües 2014 - Subchapter 8.1). This has prompted the development of an acid stress test for elucidation of enzymatic function for studying the functionality of the native enzyme in *cfs* defective *E. coli* mutant cells by conferring acid-resistance to the cells (Chang and Cronan 1999) and also of enzymes of other bacteria and even of plants by trials of complementation of the defective *E. coli* gene by respective cloned genes (Grandvalet et al. 2008; Dong et al. 2018).

Here, we wanted to make use out of a cloned cDNA of the *C. cinerea* wildtype *cfs1* gene from strain AmutBmut (Loos 2002; Liu 2004) and the wildtype *E. coli cfa* gene (Wang et al. 1992; Chang and Cronan 1999) in expression studies of an *E. coli cfa*$^-$ mutant and its isogenic wildtype strain, in order to elucidate whether the fungal protein has also the predicted cyclopropane fatty-acid synthase function.

Material and Methods

Strains, plasmids, media and standard culture conditions. The Lon and OmpT protease-free *E. coli* B (naturally *lon*) strain BL21(DE3) [F^- *omp*T *hsd*S_B (r_B^-, m_B^-) *gal dcm*] with a genomic insertion of a copy of the phage T7 RNA polymerase gene driven by the artificial *LacUV5* promoter was used for protein overexpression by the *E. coli* pET expression system with the vector pET-16b harboring an isopropyl-ß-D-thiogalactopyranoside (IPTG)-inducible phage T7*lac* promoter (= phage T7 promoter-*lacO* operator fragment; Novagen, Darmstadt, Germany; Novagen 2006). *E. coli* K12 strains ZK126 (*cfa*$^+$) and YYC1272 (*cfa::kan* mutant of ZK126) were kindly provided by Prof. Dr. J. E. Cronan (Chang and Cronan 1999) and used after plasmid transformation for the acid-resistance tests, respectively. Plasmids used in this study are listed in Table 1 and were transformed into competent *E. coli* cells using standard chemical transformation methods (Sambrook et al. 1989). *E. coli* cells were routinely cultivated at 37°C in shaken liquid LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 100 µl 10 N NaOH) or on solid medium with agar added (10 g/l; Serva, Heidelberg, Germany), either with 100 mg/l ampicilin (LB/amp) added after autoclaving or without any antibiotic, as needed.

Minimal medium E (MME, 1x) of a pH of 7.0 was prepared by 50-fold dilution from a 50 x salt stock solution (10 g $MgSO_4$ x 7 H_2O, 100 g citric acid x H_2O, 500 g K_2HPO_4 and 175 g $NaNH_4HPO_4$ x 4 H_2O that were solved in 670 ml H_2O to give 1 l stock solution with a pH of 7.0) and 4 g glucose per l medium, separately autoclaved in 100 ml H_2O (Vogel and Bonner 1956). When needed for acid tests, a pH of 3.0 was achieved by adding about 2 ml of 6 N HCl to 1 l medium (without glucose) to adjust the pH (Chang and Cronan 1999). Rich broth (RB) medium contained per l: 10 g tryptone, 5 g NcCl, and 1 h yeast extract; 100 µl 10 N NaOH was added to a l medium for pH adjustment (Chang and Cronan 1982). When

Table 1 Plasmids used in this study

Plasmid	Cloned gene	Comment	Reference
pET-16b	-	Low copy 5.7 kb *E. coli* expression vector with an IPTG-inducible T7 promoter and a downstream *lacO* operator for LacI repression (T7*lac* promoter), a *lacI* gene for LacI repressor production and an *amp*^{r} resistance gene	Novagen, Darmstadt, Germany
pET-16b*cfa*	*E. coli cfa* wildtype gene	*cfa* gene cloned in the *Bam*HI site of pET-16b site in sense direction downstream to the T7*lac* promoter	Lakkireddy and Kües (2014) – Subchapter 8.1
pET-16b*cfa*-inv	*E. coli cfa* wildtype gene	*cfa* gene cloned in the *Bam*HI site of pET-16b site in antisense direction downstream to the T7*lac* promoter	Lakkireddy and Kües (2014) – Subchapter 8.1
pET-16b*cfs1*	*C. cinerea cfs1* wildtype cDNA	*cfs1* cDNA cloned in the *Bam*HI site of pET-16b site in sense direction downstream to the T7*lac* promoter	Loos (2002)
pET-16b*cfs1*-inv	*C. cinerea cfs1* wildtype cDNA	*cfs1* cDNA cloned in the *Bam*HI site of pET-16b site in antisense direction downstream to the T7*lac* promoter	Lakkireddy and Kües (2014) – Subchapter 8.1

needed, 1 % Serva agar was added to the media for solidification and/or after autoclaving 100 mg/l ampicillin yielding MME/amp or RB/amp.

Protein expression. Selected BL21(DE3) clones were picked from freshly grown LB/amp plates and cultivated for 2.5 h in 3 ml LB/amp medium at 37 °C and 200 rpm (round per min) to an optical density (OD_{600nm}) of 0.2. Cells were transferred into 100 ml LB/amp in 500 ml flasks for 3 hr growth at 37 °C and 200 rpm until an OD_{600nm} of ≈0.5. Cultures were then split into two, with transferring 50 ml LB/amp each in new sterile 100 ml flasks. One culture was induced by 1 mM IPTG (final concentration, added from a 100 mM stock) as described in the pET-vector manual (Novagen 2006) and the other remained non-induced during further growth of all cultures for 2 h at 37 °C and 200 rpm up to an OD_{600nm} of ≈1.0. Flasks were placed on ice for 5 min. Each one ml of the well-mixed cultures was centrifuged at 10,000 × g for 1 min. Supernatants were removed and pellets drained by inversion of tubes under taping onto a paper towel to remove any excess medium. Pellets were completely resuspended by mixing in 100 µl of 1x phosphate-buffered saline (PBS). Then, for lysis of cells 100 µl of 4x SDS (sodium dodecyl sulfate) sample buffer [250 mM Tris-HCl pH 6.8, 6% SDS, 300 mM DTT (dithiothreitol), 30% glycerol, and 0.02% Bromophenol blue; Cat. No. 70607-3, Novagen, Darmstadt, Germany] was added and sonicated with a microtip until the pellet was completely dissolved. Immediately after, the sample was heated for 3 min at 85°C to denature the proteins and then stored at –20°C until SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis.

For total cytoplasmic protein isolation from ZK126 and YYC1272 clones incubated at either pH 3.0 or pH 7.0 in acid tests, strains were cultured (IPTG non-induced) and acid-treated as described below in Protocol A. Proteins from 1 ml culture for SDS-PAGE were harvested from collected cells as described above.

***Protein gelelectrophoresis*.** SDS-PAGE was performed as described by Laemmli (1970) in a Multigel-Long chamber (Biometra GmbH, Göttingen, Germany) using 4 % stacking gels

[2.5 ml of 0.5 M Tris pH 6.8, 1.3 ml 37 % acrylamide, 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS (ammonium peroxodisulfate, Sigma-Aldrich, Steinheim, Germany), 10 µl TEMED (N,N,N',N'-tetramethylethylenediamine, Amersham Bioscience AB, Uppsala, Sweden), filled up to 10 ml with dH_2O] and 12 % resolving gels [2.5 ml of 1.5 M Tris pH 8.8, 4.0 ml acrylamide (37%), 100 µl 10% (w/v) SDS, 50 µl 10% (w/v) APS, 5 µl TEMED and filled up to 10 ml with dH_2O]. Protein samples (25 or 30 µl) with Bromophenol blue were loaded into wells. Protein size marker #SM0431 from Fermentas (Vilnius, Lithuania) was used. Electrophoresis was first conducted at a constant current of 15 mA until the samples reached the resolving gel and then at 25 mA for migration of proteins into the resolving gels. Afterwards, gels were fixed in 12 % trichloroacetic acid (TCA) for at least 1 h, stained overnight in colloidal Coomassie solution [10 % phosphoric acid (v/v), 10 % ammonium sulfate (w/v), 0.12 % Coomassie Brilliant Blue G250 (w/v; Serva, Heidelberg, Germany) in a 4:1 water/methanol solution]. Gels were washed with water until an optimal contrast for bands was achieved. Gels were scanned for documentation.

Protein identification. Bands in SDS-PAGE gels of interest were excised, in-gel digested by trypsin (Roche Diagnostics, Penzberg, Germany), separated on a 12 cm capillary column packed with 3 µm particles of Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) for peptide separation to obtain electrospray ionization tandem mass spectrometry (ESI-MS/MS) spectra using an LC 1100 series high performance liquid chromatograph (Agilent, Waldborn, Germany) coupled to an Esquire 3000 plus ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) as previously described (Kilaru et al. 2006; Rühl et al. 2013). Each sample was analyzed in a mass range of the ion trap from 200 to 1500 m/z. Resulting raw data were then analyzed with Data-Analysis v. 3.0 software (Bruker Daltonik GmbH) and proteins identified by searches against a database of the annotated genome of *C. cinerea* Okayama 7 #130 (Stajich et al. 2012) combined with the SwissProt database (http://us.expasy.org/sprot/) and against NCBIprot, using a local installation of Mascot 2.2 software (www.matrixscience.com; Matrix Science, Boston, MA, USA). Settings for database search were C-carbamidomethylation as fixed modification, M-oxidation as variable modification, peptide mass tolerance 1.4 Da, peptide charge 1+, 2+, and 3+, MS/MS tolerance 0.4 Da, and 1 missed cleavage allowed.

Acid test: Protocol A (modified after Chang and Cronan 1999). Single colonies of ZK126 and YYC1272 clones were picked from freshly streaked LB/amp agar plates and inoculated for a first liquid culture into 25 ml LB/amp in 100 ml Erlenmeyer flasks for growth overnight at 37 °C and 200 rpm on a shaker to reach OD_{600nm} of 2.0 of the overnight preculture for the following acid test. For growth of main cultures, appropriate amounts of preculture (4 ml) were transferred into 25 ml LB/amp [either with IPTG (1 mM final concentration) or without inducer] in 250 ml volume Erlenmeyer flasks. Inoculated main cultures were then incubated at 37 °C for 2 to 3 h at 200 rpm on a shaker to reach an OD_{600nm} of approximately 1.2 (late logarithmic phase) for the actual acid stress test of the main culture. For the acid stress test, one ml aliquots per test sample were centrifuged for 10 s at full speed and the supernatants were discarded. The pellets were washed one time with LB medium (pH 7.0). 1 ml LB/amp per sample was added and cells were resuspended at appropriate pH (control cells at pH 7.0 and acid-shocked cells at pH 3.0) for incubation for 1 h on a shaker at 37 °C. One other set of untreated control cells (pH 7.0) were immediately diluted and plated onto RB/amp agar for colony formation. After 1 h incubation of all other samples on a shaker at 200 rpm speed at 37 °C, the samples of acid-shocked cells and the samples of control cells at pH 7.0 were diluted (0.5 ml bacterial culture in 4.5 ml LB/amp pH 7.0) and 100 µl per sample were plated on RB/amp agar for colony formation during overnight incubation at 37 °C. Colonies were counted and ratios of survival calculated by the formula: acid-shocked cells/control cells = yield of cells surviving the acid stress.

Acid test: Protocol B (modified after Chang and Cronan 1999). Different from protocol A, cells were grown overnight at 37 °C and 200 rpm in a shaker in 100 ml flasks in 25 ml MME/amp (0.4 % glucose, pH 7.0) to reach an OD_{600nm} of 1.5. For the main cultures, four ml of overnight preculture (corresponding to an initial OD_{600nm} of 0.3 to 0.4 in the diluted medium) were added into 25 ml MME (pH 7.0) without any glucose, with or without 1 mM IPTG. Main cultures were incubated on a shaker (200 rpm) at 37 °C for 2 to 3 h until an OD_{600nm} of 1.2 (late logarithmic phase) was reached. For the acid stress test, cells from each 1 ml culture were pelleted, washed 1x with 1 ml MME lacking glucose and resuspended at the appropriate pH (control cells at pH 7.0, acid-shocked cells at pH 3.0) in 1 ml MME lacking glucose for incubation for 1 h as standard, or also 2 h or 3 h in first test phases for definition of ideal incubation times, at 37 °C on a shaker at 200 rpm. Then, control cells from pH 7.0 samples were immediately diluted by adding 0.5 ml bacterial cells into 4.5 ml MME (pH 7.0) without glucose and plated in 100 µl aliquots from serial dilutions (10^{-6} to 10^{-8}) onto RB medium for colony formation. Similarly, acid-shocked cells from pH 3.0 treatments were immediately diluted by adding 0.5 ml cell samples into into 4.5 ml MME (pH 7.0) without glucose and in 100 µl aliquots from serial dilutions (10^{-3} to 10^{-7}) plated onto RB/amp medium for colony formation. Grown colonies were counted and ratios of survival calculated by the formula: acid-shocked cells/control cells = yield of cells surviving the acid stress.

Results and discussion

Expression of the cloned fungal cfs1 gene in* E. coli – *data from earlier work. In previous experimental work, heterologous expression in *E. coli* of *C. cinerea cfs1* cDNA cloned into vector pBluescriptKS(-) behind the native bacterial *cfa* promoter did not result in a measured increase in YYC1272 mutant cell survival in an acid resistance test. The results were like in YYC1272 cells which were transformed with the empty *E. coli* vector pBluescriptKS(-) and unlike when the YYC1272 cells were transformed with plasmid pAYW19 containing the cfa^+ gene (Loos 2002). This latter plasmid is a derivative of the vector pGEM5Tf(+) with the *E. coli* cfa^+ gene inserted with its natural promoter (Wang and Grogan 1992). Under normal unstressed growth conditions, the *E. coli cfa* promoter is naturally active late in cultivation at the transfer from the late logarithmic into the stationary growth phase, and this is stimulated by the sigma factor RpoS (Wang and Cronan 1994; Eichel et al. 1999).

Next therefore, in order to be independent of the natural *cfa* promoter and the bacterial growth phase, Loos (2002) cloned the *cfs1* cDNA into the *E. coli* expression vector pET-16b behind the IPTG-inducible phage T7 promoter-*lacO* operator fragment. In a preliminary acid-resistance test performed by Loos (2002) with this contruct, YYC1272 mutant cells transformed with either pAYW19 or pET-16b-*cfs1* both resisted acid stress better than ZK16 cells (wildtype) and much better than pBluescriptKS(-)-transformed YYC1272 mutant cells. In a second experiment however, there was no recognizable difference in survival between the four different cell types. In two further rounds of experiments, with and without IPTG addition, pAYW19 and pET-16b-*cfs1* transformed YYC1272 mutant cells survived slightly better than the wildtype ZK16 cells whereas YYC1272 cells with pBluescriptKS(-) or pET-16b were much more sensitive against acid (Loos 2002). Similar preliminary positive results were reported by Lakkireddy and Kües (2014) – Subchapter 8.1, for vector pET-16-*cfs1* and also pET-16-*cfa* for probable mediation of acid-resistance in YYC1272 mutant cells. In summary, the results appeared to be promising to unravel a CFA synthase function for *C. cinerea* Cfs1 in resistance against acid stress similar as described before for *E. coli* Cfa. However, due to the experimental variability in the results obtained, the data were also not without any doubt.

IPTG application in the *E. coli* pET-16b expression system releases any T7 promoter repression blocked directly at the downstream interlinked *lacO* sequence (= at the artificial T7*lac* promoter) by binding of the compound to the *lac* repressor LacI which in turn cannot bind anymore to *lacO*. However, induction by IPTG of gene expression at the T7*lac* promotor of the vector in *E. coli* BL21(DE3) is mainly indirectly. It occurs via a direct induction by IPTG at the articifial LacI-controlled *LacUV5* promoter in front of the phage T7 RNA-polymerase gene as it was cloned into the genome of the strain. This induction results in T7 RNA-polymerase production and the enzyme then initiates transcription at the T7*lac* promoter in the pET-16b vector (Novagen 2006). On the contrary, strains ZK16 and YYC1272 contain no phage T7 RNA-polymerase gene copy. Any start of transcription at the T7 RNA-polymerase-specific T7*lac* promoter of the pET-16b vector in these strains cannot become much induced by IPTG addition to the cells via release of any LacI repression at the T7*lac* promoter region. Accordingly, if there is any actual Cfa or Cfs1 production from the vector in strains ZK16 and YYC1272 in presence of IPTG mediated through transcription from a sequence upstream of the *lacO* sequence, either the non-repressed T7*lac* promoter should have some low leaky basal transcription activity even in absence of the T7 RNA-polymerase or another upstream sequence recognized by the normal *E. coli* RNA polymerase could have some pseudo-promoter activity (Loos 2002; Lakkireddy and Kües 2014 – Subchapter 8.1).

In the literature, leakiness of the T7 or the T7*lac* promoter in cells of specific *E. coli* expression strains has variably been reported to be based on leakiness in expression of the T7 RNA-polymerase gene which is not fully prevented by LacI repressor control on the *LacUV5* promoter (Kelly et al. 1995; Mertens et al. 1995; Novy and Morris 2001; Novogen 2006; Kang et al. 2007; Rosano and Ceccarelli 2014; Briand et al. 2016). Despite, certain media components (availabilty of glucose) have also an effect on better suppressing background expression from the T7 and T7*lac* promoters in the pET system, in order to avoid stimulation of T7 RNA-polymerase gene expression by cAMP through derepression at the *LacUV5* promoter (Novy and Morris 2001; Novogen 2006; Braind et al. 2016). However, in any *E. coli* strains that lack a phage T7 RNA-polymerase gene, the T7 promoter sequence might be expected to be inactive or, if at all, perhaps be very poorly used by the normal *E. coli* RNA polymerase (von Gabain and Bujard 1979; Loos 2002; Briand et al. 2016; Rotchanapreeda et al. 2018).

Successful expression of* C. cinerea *protein Cfs1 in* E. coli *BL21(DE3). Because results of former acid-resistance tests with the cloned *C. cinerea cfs1* gene in *E. coli* as well as with the native *cfa1* gene were promising but not fully free of doubt, mass-spec proteomics techniques were used to detect the expressed proteins in cellular extracts of transformants.

Transformed *E. coli* BL21(DE3) clones were grown at 37 °C overnight in 50 ml shaken LB/amp culture to inoculate main cultures for protein expression as described in Material and Methods. After 3 h of growth, main cultures were divided into two subsets of which each one subset was induced for T7 polymerase induction by 1 mM IPTG. Cultures were further incubated for 2 h, cells were then harvested by centrifugation and intracellular proteins as total protein fraction isolated from the bacterial pellets as described by Novagen (2006) and in the Material and Methods section above. Protein samples were loaded onto a 12 % SDS gel and separated by gel-electrophoresis (at a constant current of 15 mA until the samples reached the resolving gel and then at 25 mA for migration of proteins in the gel until the Bromophenol blue stain reached the lower edge of the gel). The gel was subsequently stained by Coomassie-blue and then scanned (Fig. 1).

Intensities of most protein bands in the gel were comparable between the different probes, including in the samples of clones that were transformed with the antisense constructs

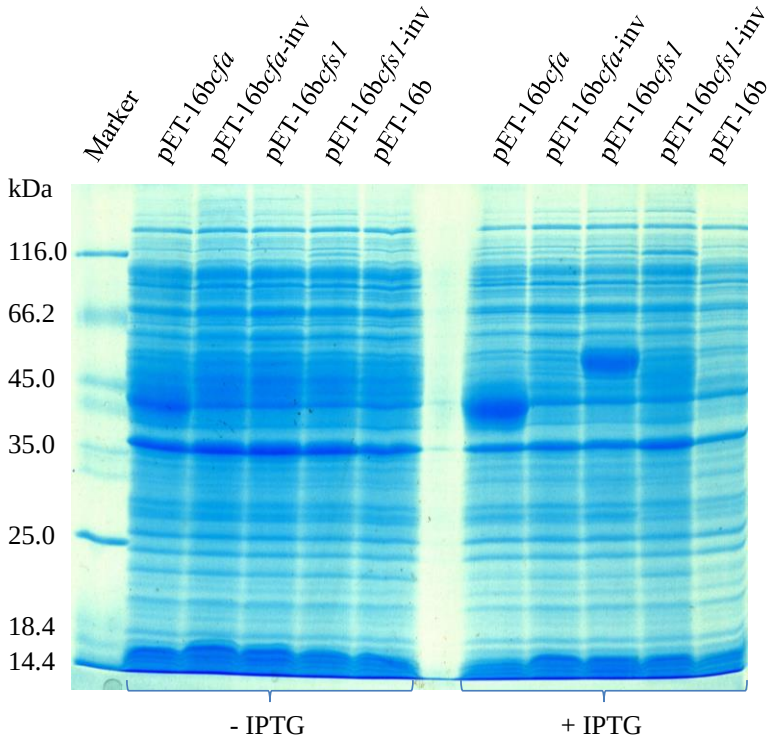

Fig. 1 12 % SDS gel with protein extracts isolated from *E. coli* BL21(DE3) cells transformed with pET-16b expression plasmids as indicated above the gel lanes.

Note the strong bands in the IPTG-induced samples of BL21(DE3) carrying pET-16b*cfa* and pET-16bcfs1, respectively. Marker: Protein size marker #SM0431 from Fermentas. Left: Protein extracts (30 µl per lane) from cultures non-induced by IPTG. Right: Protein extracts (30 µl per lane) from cultures induced by IPTG.

(Fig. 1). However, in the samples with the sense-plasmids pET-16b*cfa* and pET-16b*cfs1*, stronger blue bands were clearly visible in the gel at positions of around 45 kDa and 55 kDa, respectively. A slightly stronger band of corresponding size was also detected in the IPTG-non-induced sample of BL21(DE3) with the pET-16*cfa* plasmid but not in the sample of BL21(DE3) with the pET-16*cfs1* plasmid (Fig. 1). The native *E. coli* protein Cfa has a calculated mass of 44.3 kDa and the native *C. cinerea* protein a calculated mass of 54.8 kDa (see Table 2). These calculated sizes correspond to the two strong bands seen in Fig. 1 in the samples induced by IPTG. The proteins recombinantly produced from the pET-16b vector however should be slightly bigger than the native protein, by ca 3.2 kDa extra through the N-terminal addition of a 10xHis-Tag and a proteolytic Factor Xa site (Novagen 2006), as expressed fusion proteins resulting from an in frame gene insertion into the *Bam*HI site of pET-16b. The fully N-terminal added amino acid sequence in both proteins should be MGHHHHHHHHHHSSFHIEGRHMLEDP (for details see Corrections to Subchapter 8.1).

The stronger bands of IPTG-induced samples of BL21(DE3) produced with the pET-16b*cfa* plasmid and with the pET-16b*cfs1* plasmid, respectively were cut from the gel, as well as band regions of same sizes from the non-induced samples of the same clones. Excised bands were used for protein identification by tryptic digestion and mass-spec analysis of resulting peptides as described in Material and Methods. *E. coli* Cfa and *C. cinerea* Cfs1 were then found with very high scores as main proteins in these bands from IPTG-induced cells (Table 2). This indicated that the two proteins were produced in high amounts from the respective genes cloned in the vector pET-16b behind the T7*lac* promoter and being expressed under IPTG induction of the T7 polymerase gene. Moreover, the two proteins were

Table 2 Mascot analysis of trypsin-digested protein bands cut from the gel shown in Fig. 1 with separated proteins isolated from recombinant *E. coli* BL21(DE3) cells

Hit	Protein	Parameter	Sequence with peptide hits (marked in yellow)*
45 kb band from non-induced pET-16*cfa* transformed cells			
1	Elongation factor Tu	Score 250 Mass 44860 Matches 20 (12) Sequences 12 (8) Coverage 39%	MLSPEGESTIVRNIAVSKEKFERTKPHVNVGTIGHVDHGK*TTLTAAITTV* *LAK*TYGGAARAFDQIDNAPEEKARGITINTSHVEYDTPTRHYAHVDCPGH ADYVKNMITGAAQMDGAILVVAATDGPMPQTREHILLGRQVGVPYIIVFL NKCDMVDDEELLELVEMEVRELLSQYDFPGDDTPIVRGSALKALEGDAEW EAKILELAGFLDSYIPEPERAIDKPFLLPIEDVFSISGRGTVVTGRVERG IIKVGEEVEIVGIKETQKSTCTGVEMFRKLLDEGRAGENVGVLLRGIKRE EIERGQVLAKPGTIKPHTKFESEVYILSKDEGGRHTPFFKGYRPQFYFRT TDVTGTIELPEGVEMVMPGDNIKMVVTLIHPIAMDDGLRFAIREGGRTVG AGVVAKVLG
2	Cfa	Score 114 Mass 44337 Matches 25 (12) Sequences 9 (6) Coverage 27%	MSSSCIEEVSVPDDNWYRIANELLSRAGIAINGSAPADIRVKNPDFFKRV LQEGSLGLGESYMDGWWECDRLDMFFSKVLRAGLENQLPHHFKDTLRIAS ARLFNLQSKKRAWIVGKEHYDLGNDLFSRMLDPFMQYSCAYWKDADNLES AQQAKLKMICEKLQLKPGMRVLDIGCGWGGLAHYMASNYDVSVVGVTISA EQQKMAQERCEGLDVTILLQDYRDLNDQFDRIVSVGMFEHVGPKNYDTYF AVVDRNLKPEGIFLLHTIGSK*KTDLNVDPWINKY*IFPNGCLPSVRQIAQS SEPHFVMEDWHNFGADYDTTLMAWYERFLAAWPEIADNYSERFKRMFTYY LNACAGAFRARDIQLWQVVFSRGVENGLRVAR
3	Citrate synthase	Score 68 Mass 48383 Matches 2 (2) Sequences 2 (2) Coverage 11%	MADTKAKLTLNGDTAVELDVLKGTLGQDVIDIRTLGSKGVFTFDPGFTST ASCESKITFIDGDEGILLHRGFPIDQLATDSNYLEVCYILLNGEKPTQEQ YDEFKTTVTRHTMIHEQITRLFHAFRRDSHPMAVMCGITGALAAFYHDSL DVNNPRHREIAAFRLLSKMPTMAAMCYKYSIGQPFVYPRNDLSYAGNFLN MMFSTPCEPYEVNPILERAMDRILILHADHEQNASTSTVRTAGSSGANPF ACIAAGIASLWGPAHGGANEAALKMLEEISSVKHIPEFFRRAKDKNDSFR LMGFGHRVYKNYDPRATVMRETCHEVLKELGTKDDLLEVAMELENIALND PYFIEKKLYPNVDFYSGIILKAMGIPSSMFTVIFAMARTVGWIAHWSEMH SDGMKIARPRQLYTGYEKRDFKSDIKR
4	Succinyl-CoA ligase	Score 49 Mass 41652 Matches 2 (1) Sequences 2 (1) Coverage 7.5 %	MNLHEYQAKQLFARYGLPAPVGYACTTPREAEEAASKIGAGPWVVKCQVH AGGRGKAGGVKVVNSKEDIRAFAENWLGKRLVTYQTDANGQPVNQILVEA ATDIAKELYLGAVVDRSSRRVVFMASTEGGVEIEKVAEETPHLIHKVALD PLTGPMPYQGRELAFKLGLEGKLVQQFTKIFMGLATIFLERDLALIEINP LVITKQGDLICLDGKLGADGNALFRQPDLREMRDQSQEDPREAQAAQWEL NYVALDGNIGCMVNGAGLAMGTMDIVKLHGGEPANFLDVGGGATKERVTE AFKIILSDDKVKAVLVNIFGGIVRCDLIADGIIGAVAEVGVNVPVVVRLE GNNAELGAKKLADSGLNIIAAKGLTDAAQQVVAAVEGK
55 kb band from non-induced pET-16*cfs1* transformed cells			
1	Trypto-phanase	Score 121 Mass 53139 Matches 13 (8) Sequences 7 (6) Coverage 17 %	MENFKHLPEPFRIRVIEPVKRTTRAYREEAIIKSGMNPFLLDSEDVFIDL LTDSGTGAVTQSMQAAMMRGDEAYSGSRSYYALAESVKNIFGYQYTIPTH QGRGAEQIYIPVLIKKREQEKGLDRSKMVAFSNYFFDTTQGHSQINGCTV RNVYIKEAFDTGVRYDFKGNFDLEGLERGIEEVGPNNVPYIVATITSNSA GGQPVSLANLKAMYSIAKKYDIPVVMDSAR*FAENAYFIK*QREAEYKDWTI EQITRETYKYADMLAMSAKKDAMVPMGGLLCMKDDSFFDVYTECRTLCVV QEGFPTYGGLEGGAMERLAVGLYDGMNLDWLAYRIAQVQYLVDGLEEIGV VCQQAGGHAAFVDAGKLLPHIPADQFPAQALACELYKVAGIRAVEIGSFL LGRDPKTGKQLPCPAELLRLTIPRATYTQTHMDFIIEAFKHVKENAANIK GLTFTYEPKVLRHFTAKLKEV
2	ATP synthase sununit beta	Score 113 Mass 50308 Matches 10 (5) Sequences 5 (3) Coverage 17 %	MATGKIVQVIGAVVDVEFPQDAVPRVYDALEVQNGNERLVLEVQQQLGGG IVRTIAMGSSDGLRRGLDVKDLEHPIEVPVGKATLGRIMNVLGEPVDMKG EIGEEERWAIHRAAPSYEELSNSQELLETGIKVIDLMCPFAKGGKVGLFG GAGVGKTVNMMELIRNIAIEHSGYSVFAGVGERTREGNDFYHEMTDSNVI DKVSLVYGQMNEPPGNRLRVALTGLTMAEKFRDEGRDVLLFVDNIYRYTL AGTEVSALLGRMPSAVGYQPTLAEEMGVLQERITSTKTGSITSVQAVYVP ADDLTDPSPATTFAHLDATVVLSRQIASLGIYPAVDPLDSTSRQLDPLVV GQEHYDTARGVQSILQRYQELKDIIAILGMDELSEEDKLVVARARKIQRF LSQPFFVAEVFTGSPGKYVSLKDTIRGFKGIMEGEYDHLPEQAFYMVGSI EEAVEKAKKL

* Consecutive peptides for clear distinction are alternately shown in normal and in italic letters, respectively. Overlapping peptides of different lemgth are underlined.

Table 2 continued

Hit	Protein	Parameter	Sequence with peptide hits (marked in yellow)*
3	Cfs1	Score 113 Mass 54787 Matches 5 (5) Sequences 4 (4) Coverage 11 %	MPAHHHPSSSAPCVSFPSSSKALQSSSLLSALSPRSWTISFGKVNPTASY TDVALTYLPLARNSILAVLEDAITVGRLTISDSEGDHQYGERQPGCNDVR LRIVNDNFWMRILLSGDVGFSEAYMIGDCEVQTGLKGAMDLWLDNQSGME MTLSSTVARISSAMTALYNSFLGQTKSQARLNAIASYDQSNELFKAFLSK EMMYSCALWGENEGGVRGDLELGPTPGDLEAAQLRKLHHVLRAARVKPGD RILEFGSGWGGLAIEAARTFGCEVDTLTLSIEQKTLAEERIAEAGLEGVI RVHLMDYREIPAEWEHAFDAFISIEMIEHVGPKYYNTYFKLVDFALKPQK AAAVITSSTFPESRYSSYQAEDFMRKYMWPNSSLPSATALITAAHTASQG RFTLQGVENHAAHYPRTLREWGRRLERNLTQELVARDYPSLKDNADYESF KRKWQYLFAYAGAGFSKGYITCHMLTFIRENDIPERCD
4	Glycerol kinase	Score 109 Mass 56480 Matches 15 (8) Sequences 8 (6) Coverage 18 %	MTEKKYIVALDQGTTSSR*AVVMDHDANIISVSQR*EFEQIYPKPGWVEHDP MEIWATQSSTLVEVLAKADISSDQIAAIGITNQRETTIVWEKETGKPIYN AIVWQCRRTAEICEHLKRDGLEDYIRSNTGLVIDPYFSGTKVKWILDHVE GSRERAHRGELLFGTVDTWLIWKMTQGRVHVTDYTNASRTMLFNIHTLDW DDKMLEVLDIPREMLPEVRRSSEVYGQTNIGGKGGTRIPISGIAGDQQAA LFGQLCVKEGMAKNTYGTGCFMLMNTGEKAVKSENGLLTTIACGPTGEVN YALEGAVFMAGASIQWLRDEMKLINDAYDSEYFATKVQNTNGVYVVPAFT GLGAPYWDPYARGAIFGLTRGVNANHIIRATLESIAYQTRDVLEAMQADS GIRLHALRVDGGAVANNFLMQFQSDILGTRVERPEVREVTALGAAYLAGL AVGFWQNLDELQEKAVIEREFRPGIETTERNYRYAGWKKAVKRAMAWEEH DE
5	Outer membrane protein TolC	Score 94 Mass 53708 Matches 8 (5) Sequences 5 (4) Coverage 14 %	MKKLLPILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAF EKINEARSPLLPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDM SKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQA QKEAIYRQLDQTTQRFNVGLVAITDVQNAR*AQYDTVLANEVTAR*NNLDNA VEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARL SQDLAREQIRQAQDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN MGQNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVR SSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTT LYNAKQELANARYNYLINQLNIKSALGTLNEQDLLALNNALSKPVSTNPE NVAPQTPEQNAIADGYAPDSRHQSFSKHPHALPPVTVITLSVTDDDDGAS APSERKAT
	45 kb band from IPTG-induced pET-16*cfa* transformed cells		
1	Cfa	Score 296 Mass 44337 Matches 33 (21) Sequences 14 (1) Coverage 37 %	MSSSCIEEVSVPDDNWYRIANELLSRAGIAINGSAPADIRVKNPDFFKRV LQEGSLGLGESYMDGWWECDRLDMFFSKVLRAGLENQLPHHFKDTLRIAS ARLFNLQSKKRAWIVGKEHYDLGNDLFSRMLDPFMQYSCAYWKDADNLES AQQAKLKMICEKLQLKPGMRVLDIGCGWGGLAHYMASNYDVSVVGVTISA EQQKMAQERCEGLDVTILLQDYRDLNDQFDRIVSVGMFEHVGPK*NYDTYF* *AVVDR*NLKPEGIFLLHTIGSK*KTDLNVDPWINK*YIFPNGCLPSVRQIAQS SEPHFVMEDWHNFGADYDTTLMAWYERFLAAWPEIADNYSER*FKR*MFTYY LNACAGAFR*ARDIQLWQVVFSR*GVENGLRVAR
2	Elongation factor Tu	Score 115 Mass 43427 Matches 12 (5) Sequences 6 (3) Coverage 17 %	MSKEKFERTKPHVNVGTIGHVDHGKTTLTAAITTVLAKTYGGAARAFDQI DNAPEEKARGITINTSHVEYDTPTRHYAHVDCPGHADYVKNMITGAAQMD GAILVVAATDGPMPQTREHILLGR*QVGVPYIIVFLNK*CDMVDDEELLELV EMEVRELLSQYDFPGDDTPIVRGSALKALEGDAEWEAKILELAGFLDSYI PEPERAIDKPFLLPIEDVFSISGRGTVVTGRVERGIIKVGEEVEIVGIKE TQKSTCTGVEMFRKLLDEGRAGENVGVLLRGIKREEIERGQVLAKPGTIK PHTKFESEVYILSKDEGGRHTPFFKGYRPQFYFRTTDVTGTIELPEGVEM VMPGDNIKMVVTLIHPIAMDDGLRFAIREGGRTVGAGVVAKVLG
	55 kb band from IPTG-induced pET-16*cfs1* transformed cells		
1	Cfs1	Score 768 Mass 54787 Matches 48 (35) Sequences 19 (15) Coverage 47 %	MPAHHHPSSSAPCVSFPSSSK*ALQSSSLLSALSPR*SWTISFGKVNPTASY TDVALTYLPLARNSILAVLEDAITVGRLTISDSEGDHQYGERQPGCNDVR LRIVNDNFWMRILLSGDVGFSEAYMIGDCEVQTGLKGAMDLWLDNQSGME MTLSSTVARISSAMTALYNSFLGQTKSQARLNAIASYDQSNELFKAFLSK EMMYSCALWGENEGGVRGDLELGPTPGDLEAAQLRKLHHVLRAARVKPGD RILEFGSGWGGLAIEAARTFGCEVDTLTLSIEQKTLAEERIAEAGLEGVI RVHLMDYREIPAEWEHAFDAFISIEMIEHVGPKYYNTYFKLVDFALKPQK *AAAVITSSTFPESR*YSSYQAEDFMR*KYMWPNSSLPSATALITAAHTASQG* *RFTLQGVENHAAHYPRTLREWGRRLERNLTQELVARDYPSLKDNADYESF* KRKWQYLFAYAGAGFSKG*YITCHMLTFIR*ENDIPERCD

also found in the bands from non-induced cells but with lower score among other similar-sized *E. coli* proteins (Table 2). This indicated production of Cfa and Cfs1 in lower amounts in absence of IPTG and confirmed a leaky gene expression in strain BL21(DE3) from the T7*lac* promoter in each of the two vector constructs.

Surprisingly then in this proteomics analysis, an unexpected Cfs1 peptide was found in the BL21(DE3) protein sample with the pET-16*cfs1* plasmid that started with the normal amino acid M. Such peptide would not be expected to be present in the sample, unless the native start codon was in error recognized for start of translation. The results from proteomics suggest thus that the sequence at the pET-16b*cfs1* fusion point CTCGA*GGATCC*T**ATG** (the *Bam*HI site used in cloning is shown in italic; the native *cfs1* ATG startcodon is shown in bold) contains an erroneous RBS (ribosome binding site). Within this sequence, there is a the GAGG-stretch with a 7-bases-aligned spacing to the *cfs1* startcodon which resembles a partial Shine-Delgarno (SD) sequence with the *E. coli* consensus AGGAGGU and the motif GAGG in *E. coli* virus T4 early genes (Chen et al. 1994; Ma et al. 2002; Malys 2012). Because the distance of such motif to an ATG-startcodon is decisive for a potential function in initiation of translation through binding ribosomes (Chen et al. 1994; Ma et al. 2002; Malys 2012), it is this sequence in pET-16b*cfs1* that likely serves as a minor RBS for start of mRNA translation into protein at the ribosomes.

Proteomic analysis of transformed* E. coli *ZK126 and its mutant YYC1272. All sense and anti-sense pET-constructs (Table 1) were also transformed into *E. coli* ZK126 and YYC1272, respectively. Cultures (non-induced by IPTG) were grown for further 1 h in LB medium at 37 °C, acid-challenged for 1 h and then, intracellular proteins were isolated from harvested cells. Protein samples were separated on 12 % SDS gels. Banding patterns in all samples were very similar to each other, regardless of the *E. coli* strain or the transformed vector used or of the pH applied to the cultures (Fig. 2A and B).

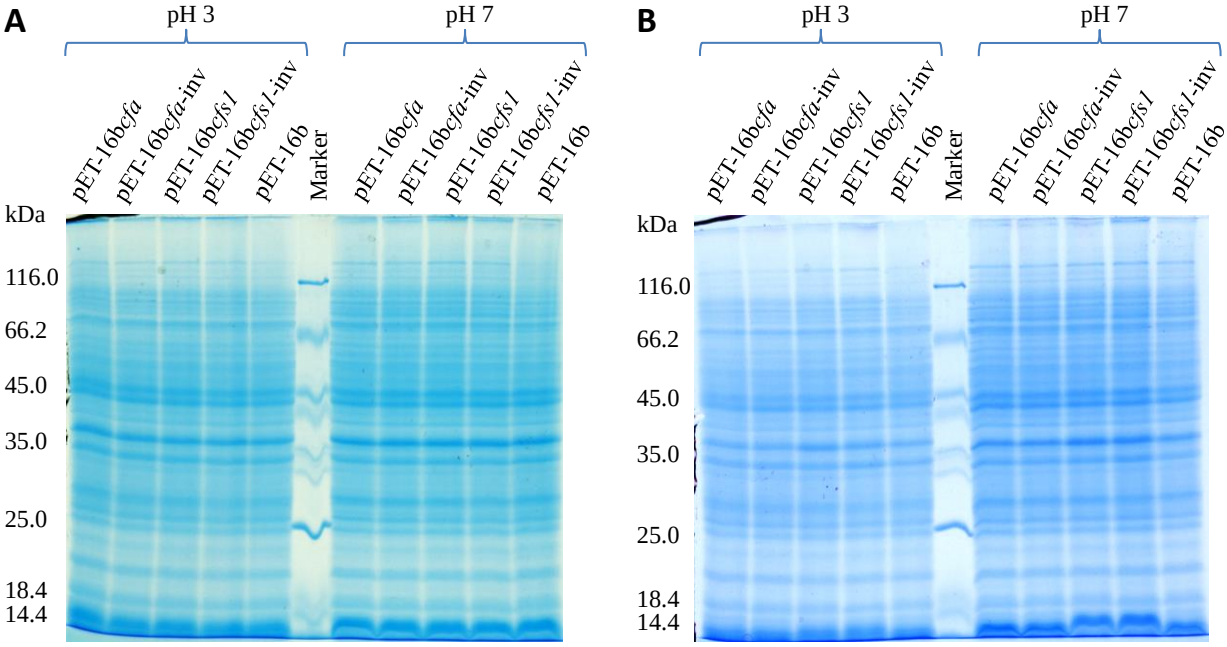

Fig. 2 12 % SDS gels with total protein extracts from *E. coli* ZK126 (A.) and YYC1272 cells (B.) transformed with pET-16b expression plasmids as indicated above the lanes after the acid test.

Marker: Protein size marker #SM0431 (12.5 µl) from Fermentas. Left: Protein extracts (25 µl per lane) from cultures kept at pH 3 (left) and from cultures kept at pH 7 (right).

Bands of sizes of about 45 to 50 and 55 to 60 kDa were excised from the gels, trypsin-digested and resulting peptides separated in mass-spec analysis for protein identification. Mascot searches identified then variably in good scores and with at least two peptides per protein for the samples from mutant strain YYC1272 in the 42 to 50 kDa size range from the host *E. coli* the elongation factor Tu [found before also in BL21(DE3); Table 2], isocitrate lyase [found before also in BL21(DE3); Table 2], glutamate decarboxylase, 6-phosphogluconate dehydratase, phosphoglycerate kinase, enolase, long-chain fatty acid transporter FadL, and trigger factor involved in protein export (data not further shown). Mascot searches identified in the 55 to 60 kDa size range periplasmic oligopeptide-binding protein OppA, chaperonin GroEL, AidA-I family adhesin (probably processed), nitrate reductase subunit beta, PTS *N*-acetyl glucosamine transporter subunit IIABC, malate synthase, and glucose-6-phosphate isomerase (data not further shown). Several of these proteins were also detected in the samples taken from the wild type strain ZK126 (not further shown). However, neither any one of the peptides from *E. coli* Cfa nor from *C. cinerea* Cfs1 were found in the bands excised from the gels in Fig. 2.

Loos (2002) formerly analyzed protein extracts from IPTG-induced YYC1272 pET-16b*cfs1*-transformed cells in western blots with tetra-His-antibodies that recognize the His-tag of pET-vector-encoded fusion proteins. By the sensitive western analysis, she detected also no band of the expected size of ca 55 kDa for the whole-length Cfs1-fusion protein in SDS-PAGE separated protein extracts. However, in dependency of IPTG-induction, she found in western analysis ladders of smaller bands which indicated fast degradation processes of recombinantly expressed His-tag-fused Cfs1 protein (Loos 2002). Accordingly in the approach taken here with only cutting out bands of expected full-length protein sizes, any size-reduced degradation products of the 10x-His-tagged Cfs1 protein would not have been possible to detect. In the literature, it is reported that the *E. coli* Cfa protein is very unstable and shortlived (Chang et al. 2000). The same could be true for the *C. cinerea* Cfs1 protein in *E. coli* as it is indicated by the work of Loos (2002). Under only low level expression in *E. coli* in a protease-proficient strain, full length proteins would then by low concentrations with short life-times be difficult to find.

As another point, suggested by the range of highly expressed *E. coli* proteins that were found in the proteomics analysis of gel-excised bands in this study (see above), the sensitivity in detecting tryptic peptides from gel-excised protein bands by mass-spec might likely not have been sufficient enough to discover any lowly expressed instable proteins. In this work, one could have expected at least for the native Cfa protein to find it in the samples from the acid-stressed *E. coli* ZK126 clones which harbor a functional *cfa* wildtype gene (Chang and Cronan 1999). Because this was not the case, more advanced whole-shot-gun proteomic techniques with higher sensitivities in total peptide detection could be applied in the future for identification of the proteins isolated of the transformed strains. With higher sensitivity, peptides from any full-length proteins as well as from possible degradation products might be detected, possibly even in quantitative comparative analyses in acid resistance tests between parallel IPTG-induced and IPTG-non-induced cultures.

Acid resistance tests with pET-vectors in* E. coli *ZK126 and its mutant YYC1272. As already described above, strains ZK126 and YYC1272 both do not contain a T7 RNA polymerase gene (Chang and Cronan 1999). Differentially as demonstrated above to happen in *E. coli* BL21(DE3) with an inserted T7 RNA polymerase gene, leaky expression at the T7 promoter by some non-induced background production of T7 RNA polymerase in the absence of IPTG (Fig. 1; Table 2) thus cannot occur in the strains ZK126 and YYC1272 (see above). ZK126 and YYC1272 cultures in the proteomics experiment presented with the gels in Fig. 2 were not IPTG-treated. Under such conditions, any repression of expression the cloned genes *cfa*

and *cfs1* mediated at the phage T7 promoter by binding of LacI to the *lacO* operator fragment would not have been expected to be relieved.

Whether any leaky expression of the two cloned genes from the T7*lac* promoter could nevertheless be possible in strains ZK126 and YYC1272 was further addressed by series of acid tests with the transformed *E. coli* clones, with and without IPTG-induction using MME (Protocol B, Table 3) and LB (Protocol A, Table 4) in cultivations, respectively. First, experiments were perfomed with using Protocol B with minimal medium MME, because Chang and Cronan (1999) stated that this test was less delicate than Protocol A and less likely to obtain erratic results based on when cultures were grown longer into the stationary phase. At this time of experimental work, the antisense plasmid pET-16b*cfs1*inv was not yet available why only four clones each of the wildtype strain ZK126 and of its *cfa*-mutant YYC1272 were compared with each other in behavior, that was from each strain transformants with either control vector pET-16b, the sense constructs pET-16b*cfa* and pET-16b*cfs1*, or the antisense construct pET-16b*cfa*inv.

Table 3 Cell survials of transformed ZK126 and YYC1272 clones in 1 h-long acid resistance tests – cultivation in MME medium according to Protocol B

E. coli strain			Wildtype ZK126 x 10^7 cells					*cfa* mutant YYC1272 x 10^7 cells				
Gene in pET-16b			-	*cfa*$^+$	*cfa*^{inv}	*cfs1*$^+$	*cfs1*^{inv}	-	*cfa*$^+$	*cfa*^{inv}	*cfs1*$^+$	*cfs1*^{inv}
Exp.	IPTG	pH										
1	-	3	1.1	0.6	0.3	0.7	-	0.1	0.1	0.3	0.7	-
		7	1.5	1.7	1.6	2.0	-	0.8	0.6	1.5	2.0	-
		% 3/7	77.3	35.3	18.8	35.0	-	12.5	16.7	20.0	35.0	-
	+	3	1.3	0.9	0.6	0.4	-	0.1	0.2	0.1	1.3	-
		7	1.4	1.6	2.0	2.1	-	0.5	0.6	1.7	2.1	-
		% 3/7	92.9	56.3	30.0	19.1	-	20.0	33.3	5.9	61.9	-
2	-	3	1.8	1.5	1.6	1.3	-	1.4	1.8	1.8	1.9	-
		7	5.0	4.6	3.2	4.7	-	4.1	4.6	4.4	5.1	-
		% 3/7	36.0	32.6	50.0	27.7	-	34.2	39.1	40.9	37.3	-
	+	3	1.7	2.3	1.3	2.3	-	1.6	2.0	1.7	2.2	-
		7	6.7	4.8	4.8	5.8	-	5.5	5.0	4.5	6.7	-
		% 3/7	25.4	47.9	27.1	39.7	-	29.1	40.0	37.8	32.8	-
3	-	3	2.4 ± 0.2	2.1 ± 01	2.2 ± 0.0	1.9 ± 0.0	-	2.2 ± 0.1	3.1 ± 0.1	2.3 ± 0.0	2.5 ± 0.0	-
		7	7.6 ± 0.4	7.5 ± 0.9	8.6 ± 0.0	7.2 ± 1.1	-	7.3 ± 2.3	7.5 ± 0.9	8.3 ± 0.2	7.8 ± 0.1	-
		% 3/7	31.6	28.0	25.6	26.4	-	30.1	41.3	27.7	32.1	-
	+	3	2.0 ± 0.2	2.2 ± 0.0	1.8 ± 0.1	1.4 ± 0.1	-	2.2 ± 0.1	2.7 ± 0.2	2.1 ± 0.3	1.5 ± 0.1	-
		7	9.1 ± 0.3	8.4 ± 0.4	8.3 ± 0.6	7.8 ± 0.3	-	9.0 ± 0.1	7.8 ± 0.3	8.7 ± 0.6	9.1 ± 1.2	-
		% 3/7	22.0	26.2	21.7	18.0	-	24.4	34.6	24.1	16.5	-
4	-	3	5.3	4.1	4.1	5.6	-	2.8	3.1	2.8	4.1	-
		7	22.4	28.8	19.9	17.8	-	17.4	28.7	29.5	12.1	-
		% 3/7	23.7	14.2	20.6	31.5	-	16.1	10.8	9.5	33.9	-
	+	3	4.4	4.3	4.2	3.8	-	2.4	4.6	1.9	4.1	-
		7	17.4	20.9	16.4	12.8	-	18.8	11.3	11.0	14.7	-
		% 3/7	25.3	20.6	25.6	29.7	-	12.8	40.7	17.3	27.9	-
5	-	3	2.8	3.4	2.7	3.8	-	2.5	2.2	3.4	3.6	-
		7	3.5	3.6	3.4	4.4	-	3.8	3.0	4.6	4.3	-
		% 3/7	80.0	94.4	79.4	86.4	-	65.8	73.3	73.9	83.7	-
	+	3	1.9	2.4	3.7	3.8	-	2.5	2.0	3.3	4.5	-
		7	3.2	5.0	4.1	4.1	-	4.3	3.3	4.5	4.9	-
		% 3/7	59.4	48.0	90.2	92.7	-	58.1	60.6	73.3	91.8	-
6	-	3	3.5	5.0	6.0	3.7	-	3.9	4.2	4.1	3.7	-
		7	3.7	6.3	7.3	4.1	-	7.0	6.6	7.4	5.7	-
		% 3/7	94.6	79.4	82.2	90.2	-	55.7	64.7	55.4	64.9	-
	+	3	3.7	4.6	5.9	3.7	-	3.4	4.9	4.2	3.1	-
		7	3.9	5.2	6.9	4.0	-	5.7	5.8	5.6	6.1	-
		% 3/7	94.9	88.5	85.5	92.5	-	59.7	84.5	75.0	50.8	-
7	-	3	2.1	1.2	2.6	1.9	-	1.2	1.4	2.3	1.7	-
		7	3.2	1.9	3.4	2.8	-	2.1	1.9	3.9	2.7	-
		% 3/7	65.6	63.2	76.5	67.9	-	57.1	73.7	59.0	63.0	-
	+	3	1.4	1.1	1.1	1.5	-	1.4	1.1	1.5	1.1	-
		7	2.8	1.6	2.2	2.9	-	1.8	1.9	2.8	2.6	-
		% 3/7	50.0	68.8	50.0	51.7	-	77.8	57.9	53.6	42.3	-

Table 4 Cell survials of transformed ZK126 and YYC1272 clones in 1 h-long acid resistance tests – cultivation in LB medium according to Protocol A*

E. coli strain			Wildtype ZK126 x 10^8 cells					*cfa* mutant YYC1272 x 10^8 cells				
Gene in pET-16b			-	*cfa*$^+$	*cfa*^{inv}	*cfs1*$^+$	*cfs1*^{inv}	-	*cfa*$^+$	*cfa*^{inv}	*cfs1*$^+$	*cfs1*^{inv}
Exp.	IPTG	pH										
1	-	3	3.9	4.7	4.8	4.9	3.9	0.1	2.8	2.2	3.7	2.9
		7	5.0	4.9	5.0	5.8	4.7	5.7	4.5	4.4	5.1	5.1
		% 3/7	78.0	95.9	96.0	84.5	83.0	1.8	52.2	50.0	72.6	56.9
	+	3	3.8	3.6	3.9	4.1	4.3	0.2	2.4	2.9	3.9	2.4
		7	4.9	4.5	4.7	5.5	4.5	5.4	4.4	4.3	4.9	4.9
		% 3/7	77.6	80.0	83.0	75.6	95.6	3.7	54.6	67.4	79.6	49.0
2	-	3	7.2	5.3	6.1	6.9	7.1	0.2	2.9	2.8	3.2	2.4
		7	7.8	6.7	6.8	7.6	7.7	7.4	6.5	6.6	7.2	6.7
		% 3/7	92.3	79.1	89.7	90.8	92.2	2.7	44.6	42.2	44.4	35.8
	+	3	7.2	4.4	5.1	6.1	7.3	0.2	4.3	2.2	4.2	1.6
		7	7.5	6.2	6.6	7.3	7.9	7.6	6.0	6.1	6.8	6.4
		% 3/7	96.0	71.0	72.3	83.6	92.4	2.6	**71.7**	36.1	**61.8**	25.0
3	-	3	3.2	3.0	3.0	3.2	3.5	1.4	2.6	1.2	2.7	1.3
		7	3.4	3.2	3.3	3.4	3.6	3.1	2.8	2.9	2.9	2.9
		% 3/7	94.1	93.8	90.9	94.12	97.2	45.2	**92.9**	41.4	**93.1**	44.8
	+	3	3.0	2.9	2.7	3.1	3.4	1.3	2.4	1.2	2.8	1.4
		7	3.0	3.2	3.2	3.3	3.7	2.9	2.7	2.9	2.8	2.7
		% 3/7	100	90.6	84.4	93.9	91.9	44.8	**88.9**	41.4	**100**	51.9
4	-	3	5.1 ± 0.9	4.7 ± 0.3	4.8 ± 0.1	5.3 ± 0.1	4.0 ± 0.0	3.5 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	3.7 ± 0.3	2.4 ± 0.2
		7	12.3 ± 0.6	13.0 ± 0.9	12.3 ± 0.7	12.6 ± 1.0	12.5 ± 0.8	12.4 ± 0.1	12.3 ± 0.4	12.6 ± 0.1	12.5 ± 0.0	12.2 ± 0.8
		% 3/7	41.46	36.2	39.0	42.1	32.0	28.2	22.8	23.0	29.6	19.7
	+	3	6.0 ± 0.8	7.3 ± 0.6	5.5 ± 0.4	5.4 ± 0.1	4.5 ± 0.2	3.6 ± 0.0	4.5 ± 0.2	4.3 ± 0.2	5.0 ± 0.2	2.6 ± 0.4
		7	12.2 ± 0.6	13.4 ± 0.3	12.2 ± 0.9	12.6 ± 0.7	12.2 ± 0.9	12.4 ± 0.3	12.0 ± 0.6	12.6 ± 0.2	12.1 ± 0.4	12.8 ± 0.4
		% 3/7	49.2	54.5	45.1	42.9	36.9	29.0	**37.5**	34.1	**41.3**	20.3

* Underlined in yellow are experimental series with data that would fit the idea that Cfa and Cfs1 are both expressed in uninduced conditions from the sense constructs pET-16b*cfa* and pET-16b*cfs1*, respectively and that the proteins mediate acid resistance.

Own experiences with Protocol B. In total, seven different experimental series were performed in MME according to Protocol B (Table 3), with variable acid incubation times for either only 1 h (experiments 1, 2 and 7), or for 1 h, 2 h and 3 h (experiments 3, 4, 5, and 6). Further in the experiments 2, 4, 5 and 6, samples of cells as freshly grown main cultures for the application of the acid resistance test were also plated for better overview (to control/confirm no loss of viability during further incubation in the pH 7.0 growth control samples). For the test cultures then, the actual length of acid treatment time played clearly a role in the overall survival of cells. Incubation for 1 h resulted variably in relative pH 3.0/pH 7.0 survival rates of ca. 10 to 60 % of empty-vector control samples of the YYC1272 mutant (Table 3) whereas longer incubation times killed much more of the acid-treated cells of the YYC1272 mutant, to survival frequencies below 10 or even 1 % with much higher variability between samples, irrespectively of absence or potential presence of proteins Cfa or Cfs1 (not further shown). To conclude this, acid treatment of 1 h appeared to be best for the performance of the experiments.

In line with the expectations, in all instances of 1 h-incubation experiments presented in Table 3, regardless of the strain or the plasmid transformed into a strain or whether cells were IPTG induced or not, cells survived the pH 7 treatments better than the pH 3.0 treatments. In experiments 1, 4, 5, and 6, the survival of clones of strain ZK126 was overall better than survival of clones of mutant YYC1272. In contrast, clear differences in overall behaviour between clones of the two strains were not observed in experiments 2, 3 and 7. Survival rates between different series of experiments varied a lot. Also within individual experiments and against expectations for a stable experimental approach, survival rates between the four clones of the wildtype control strain ZK126 varied in most of the experiments, regardless of whether IPTG was applied or not. Because the results on the

wildtype control strain ZK126 indicated potential experimental irregularities, data from mutant YYC1272 could also not be considered reliable. In accordance, also between the four clones tested of mutant YYC1272 within individual experiments, survival rates varied a lot. Between experiments, the best surviving clone(s) of mutant YYC1272 also varied. A clone of generally better acid resistance performance could thus not be identified. In summary therefore, in none of the experiments as summarized in Table 3, expectations were convincingly fulfilled that the sense constructs pET-16b*cfa* and pET-16b*cfs1* would mediate acid resistance.

What could be learned from the so far presented experiments? Performance of the experiments required throughout good excercise and technical experience. Because time is a crucial factor in killing cells at pH 3.0, fast and accurate handling of all samples in minute's time was in all steps essential, especially also after the incubation time performed for the acid shock of cells. Accordingly, there were many more possible pitfalls in the experiments, as for example:

- Some experiments in Table 3 were possibly adversely affected by a general too slow handling of the different strains in serial dilutions and plating after the acid treatment.
- After acid treatment, it had further to be made sure for statistical solid counting that dilutions with appropriate numbers of surviving cells (about 50 to 300/plate) would be plated in order to overcome non-linear effects on colony formation when too many or to few survising cells were spreaded. Best plating conditions for strain ZK126 cultivated in MME without glucose for 1 h were thus dilutions of 10^{-5} to 10^{-6} and for mutant strain YC1772 dilutions of 10^{-4} to 10^{-5}.
- By the experimental difficulty of handling so many different samples in shortest time, with exception of experiment 3, only one plate of cells per dilution (spreading each time 100 µl cells/plate) were prepared for further cultivation of surviving cells and subsequent colony counting from the eight different clones (four per strain), by the series of 1:10 sample dilutions (from 10^{-1} to 10^{-5} or 10^{-6}) made with MME medium (pH 7.0) after acid treatment for acid-shocked cells and pH 7.0-treated control cells, respectively. This gives a certain factor of insecurity for experimental control of perfect cell plating in experiments. However, data from experiment 3 with plating each time three repeats per clone and per dilution step for growth and counting of surviving colonies had reasonable standard deviations of the average of surviving cells (Table 3).
- Further of important influence for the outcome of experiments, equal starting concentrations of cells of equal physiological status were required to ensure a fair cell-to-acid relationship. Preculture (overnight culture) cell concentrations need thus to be highly similar in all strains. If some clones behaved already different in cell concentrations in overnight precultures and were then probably physiologically different in growth phase, such differences will likely reappear in all remaining steps in the experiments until their ends, even if the absolute cell numbers for the tests were adjusted between samples by adding more or less calculated volumes of the overnight precultures to the medium for the main cultures. In such cases with different initial preculture cell concentrations, the subsequent acid test results might then also vary as a result of the possible physiological differences between clones in connection with the different growth of the overnight precultures (see here also reported experiences provided by Chang and Cronan 1999). For examples in this study, cell densities as measured by ODs differed in part between overnight precultures of clones in the experiments 1, 2 and 4 presented in Table 3. In the following, survival rates of empty vector control samples for the wildtype control strain ZK126 and the *cfa* mutant strain YYC1272 were variably increased and decreased as compared to samples of their clones with genes *cfa* and *cfs1* cloned in sense direction into the pET-16b vector and

to samples of the clones with gene *cfa* cloned in anti-sense direction (Table 3). Accordingly, from this we cannot safely conclude anything solid from these experiments on different behaviours between the wild and mutant strains against low pH in the acid resistance test. Summing up, obtaining and also maintaining similar cell concentrations between samples are thus very important from the overnight preculture state onwards over the growth of the main culture up to the further acid treatment steps in the experiment.

- In the experiments in Table 3 other than 1, 2 and 4, measured cell densities of all the different clones in the precultures were in comparable ranges as well as measured cell densities in the main cultures. However, data on surviving cells were still sometimes highly variable from some series of experiments even for the transformants of the wildtype control strain ZK126 (e.g., compare data of IPTG-induced cultures in experiment 5). It suggests that there might have been other negative influences in the experiments which possibly remained unrecognized.

Own experiences with Protocol A. Similarly to the experiments following Protocol B, best experimental conditions had to be established for application of Protocol A using LB medium (Table 4). Cells were in the late logarithmic phase (OD_{600nm} of ca. 1.2) when acid-treated following Protocol A, in order to avoid negative physiological effects in the later growth phases in the LB/amp complete medium which had previously been reported to provoke erratic results in plating (Chang and Cronan (1999). Following experiences from Protocol B, a time of 1 h of acid treatment was chosen in all experiments performed after Protocol A and was found to be suitable. In total, four different experiments were performed. After the acid treatment in the first three experiments, serial dilutions of cells were plated only once per dilution step, unlike in the last experiment with three repeats of plating per serial dilution step. Standard deviations of the calculated averages of the surviving cells were reasonably low (Table 4).

Experiments performed with Protocol A included five clones per strain because at this time, the missing antisense construct pET-16b*cfs1*inv was also available (Lakkireddy and Kües 2014 – Subchapter 8.1). As in Protocol B, cell densities were photometrically measured in Protocol A. Cell densities in the series of parallel grown precultures and in the subsequent main cultures were overall comparable. According to the expectations, also when following Protocol A, survival of cells after acid treatment of control wildtype strain ZK126 was generally better than survival of cells of mutant strain YYC1772 (Table 4). Furthermore, data from the experiments performed after Protocol A were much more regular within an experiment between the five clones of control wildtype strain ZK126 and between some of the clones of the mutant YYC1772. This could hind to that with time and increasing technical experience, any problems in equal experimental performance with the various clones were better overcome. However, in experiments 1 and 2 performed after Protocol A, the YYC1272 strain transformed with the empty plasmid behaved much more sensitive than any of the other four transformants of the *cfa* mutant (Table 4). By unknown reason, strains in these two experiments might thus not have been in the same physiological stage when having been confronted with acid stress. Taking fresh clones in experiments 3 and 4 resulted then in survival rates for the YYC1272 empty-vector control clone which were comparable to those of the two clones carying the antisense constructs pET-16b*cfa*inv and pET-16b*cfs1*inv, respectively (Table 4).

Importantly from the results in Table 4, those clones of mutant YYC1772 that survived the acid treatment better than others were the transformants with the sense constructs pET-16b*cfa* and pET-16b*cfs1* for potential enzyme expression. From the four different experiments performed with Protocol A, three provided series of data (marked in Table 4 in

yellow) that followed such expectations when proteins Cfa and Cfs1 would have successfully been expressed and been active in modifying UFAs present in the cellular membrane of *E. coli* into CFAs, in order to strengthen the membranes against the acid stress applied. These positive data can however also not be considered as a final proof for functionality as long as other experimental series do not provide throughout the same tendencies in cell survival of the different clones and as long the reasons for the differences in some of the experiments presented here in Table 4 remain unknown. To possibly explain the cases of the so far positive outcomes of the experiments, working hypotheses for further study in the future are:

In three of the seemingly positive experiments shown in Table 4, the data came from the series of IPTG-treated cells where the negative control by the *lacO* sequence linked to the T7 promoter should have been relieved through the addition of IPTG (Novy and Morris 2001), similarly as previously been reported from the positive Cfs1 expression experiments by Loos (2002). Accordingly, this could possibly explain any leaky gene expression from the vectors in the case that in the absence of T7 RNA polymerase there is still in the DNA an upstream weakly recognition by the normal *E. coli* RNA polymerase for an unintended initiation of transcription (Wiggs et al. 1979).

One series of acid-treated samples (in experiment 3; Table 4) showed excellent survial of YYC1772 mutant cells transformed with either pET-16b*cfa* or pET-16b*cfs1* also under IPTG-non-induced conditions (> 90% of cells as compared to the controls with ca 40-45 % cell survival; Table 4). Even without addition of IPTG, there could be a possibility of release of repression at the *lacO* sequence at the T7 promoter by traces of lactose being present in the LB medium through the tryptone prepared from caseine from milk (84610.500; VWR Chemicals, Leuven, The Netherlands). Such derepression at *lacO* would then also open the path for the normal *E. coli* RNA polymerase if this enzyme has indeed an accidental weakly recognition site upstream of *lacO*.

Conclusions

The essential fruiting gene *cfs1* of *C. cinerea* has successfully been expressed in the Lon and OmpT protease-free *E. coli* B strain BL21(DE3). In this strain, the foreign protein Cfs1 from *C. cinerea* is apparently quite stable (Fig. 1). This may contrast the situation in the isogenic *E. coli* K12 strain ZK126 and its *cfa*-mutant YYC1272 which both carry the wiltype Lon and OmpT protease genes and in which the native Cfa protein is only very shortlived. However, deletions of genes *lon* and *groEL* in ZK126 indicated before that the Cfa protein degradation depends neither on the Lon protease nor on any special protease activity of the GroEL chaperone but is in part a response to the heat-shock regulon of a not yet identified protease activity (Chang et al. 2000). Moreover, OmpT is a protease in the outer membrane of *E. coli* and may affect the proteins only by cell lysis (Grodberg and Dunn 1988).

Degradation bands of recombinant Cfs1 detected in a western blot of proteins isolated from *cfa*-mutant YYC1272 transformed with pET-16b*cfs1* (Loos 2002) and results from some performed acid tests (Loos 2002; Lakkireddy and Kües 2014 – Subchapter 8.1; this chapter) are possible indications for IPTG-enabled expression of Cfs1 in strains ZK126 and YYC1272. Results from this study shown in Table 4 using Protocol A in acid treatment appear to support this idea. However, the final evidence for expression of *C. cinerea* Cfs1 in these strains from the recombinant vector pET-16b*cfs1* under IPTG-induction has still to be provided, such as for example by using more sensitive shut-gun proteomics to detect the proteins.

Suggested among by the good stability of the Cfs1 protein in strain BL21(DE3), the further question to proof *in vivo* the enzymatic function performed by Cfs1 might be best

addressed in the future in acid stress tests in the strain BL21(DE3) after knocking out its natural *cfa*$^+$ gene. While not conclusive in a first attempt performed in frame of this study with transformed IPTG-induced BL21(DE3) cells (data not shown), lipid analysis of cellular membranes after acid shock should in addition be possible to perform for identification of any enzymatic changes from UFAs to CFAs (Grogan and Cronan 1997). Changing with the overexpressing BL21(DE3) transformants to another possibly easier read-out system could be an additional possibility to overcome any problems in accurate experimental performance of the very difficult to perform acid resistance test. In a recent report in a newly developed functional test, growth speed and cell density in liquid culture were thus observed under osmotic stress using BL21(DE3) cells transformed by the *cfa*$^+$ gene from the bacterium *Halomonas socia* as cloned in vector pET-24ma (Choi et al. 2020). Because *C. cinerea* Cfs1 expressed in strain BL21(DE3) is apparently very stable, as a third other option of experimental enzyme testing, it is moreover possible to purify the overexpressed enzyme from the strain and use then the purified protein with unsaturated phospholipids in an *in vitro* enzyme test like formerly described and successfully performed with *E. coli* Cfa (Courtois et al. 2004).

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Curriculum Vitae

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Education

P.hD. (2009 – Present)	Georg-August-University of Göttingen, Germany Thesis Title: Factors influencing fruiting body initiation of Agaricomycetes
M.Phil. (2008 – 2017)	Bioinformatics, Acharaya Nagarjuna University, Andhra Pradesh, India Thesis Title: Homology modeling of microbial pathogenic proteins of *Haemophilus influenza*
M.Sc. (2004 – 2006)	Biotechnology, University of Madras, Tamil Nadu, India Project Title: Isolation, estimation, purification and characterization of protease from *Vibrio alginolyticus*
B.Sc. (2001 – 2004)	Microbiology, Sri Venkateswara University, Andhra Pradesh, India

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