Occurrence and Importance of Foliar Diseases on Maize (*Zea mays* L.) in Central Europe

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Dedicated to

Hubert and Christa

-Estudia, que un día te alegrarás-

José Luis Ramos

Contents

Abbreviations

Abbreviations

BBCH	phenological development stages scale
bp	base pair
°C	degree celsius
cm	centimetre
dNTP	deoxynucleotide triphosphate
e.g.	for example (*exempli gratia*)
fg	fentogram
g/l	gram/ litre
h	hour
ha	hectare
i.e	that is (*id est*)
kg	kilogram
klx	kilolux
km	kilometre
l	liter
m	metre
m^2	square metre
mbp	mega base pair
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	milimolar
pg	picogram
pmol	picomol
rpm	revolutions per minute
s	second
U	units
UV	ultraviolet Light
%	percentage
xg	times gravity
µg	microgram
µM	micromolar
µmol	micromol

1. Introduction

1.1. *Zea mays* (L.): Origin, domestication and actual cultivation in Central Europe

Zea mays (family Poaceae) is the only species in the Genus *Zea* (Paliwal 2001). It was domesticated from its wild ancestors teosinte (*Zea mays L.* ssp. *parviglumis* Illtis & Doebley and spp. *mexicana*) and tripsacum between the basin of the Balsas River and the highlands of Mexico´s Meseta Central approximately 9,000 years ago according to the phylogenetic analysis of Matsuoka et al. (2002). This theory is supported by the diversity of maize in these regions, as well as the prevalence of ssp. *mexicana* growing as a weed in the corn fields of the Mexican highlands. Furthermore, fossil pollen and maize cobs in caves of the Mexican region of Puebla have been discovered along with various ceramic remains from the period in question (Paliwal 2001; Segovia & Alfaro 2009). However, uncertainties surrounding single and multiple domestication are still extensively discussed in the literature (Paliwal 2001; Matsuoka et al. 2002). Although some authors maintain that maize was being cultivated in the central Caribbean Archipelago much earlier, the consensus is that by the 11th century maize was distributed across a large number of areas situated in the region (Sanoja 1989; Higuera-Gundy 1991 cited by Newsom 2009; Newson & Deagan 1994; Pagán Jímenez et al. 2005; Lane et al. 2008). Here, the name “mahiz” (from the Arawak language) was given to the plant by the Taíno people, who inhabited the Bahamian Archipelago and the Greater Antilles (Rouse 1992). With the first voyage of Christopher Columbus (1492-1493), maize kernels were transported back to Europe, consequently being cultivated in the 16th Century in southern European regions (Anghiera 1907 (1st ed. 1530) cited by Dubreuil et al. 2006; Staller 2010).

Thus, initially maize was most likely cultivated in warm European areas such as southern Spain (Andalusia) and the surrounding Mediterranean regions (Dubreuil et al. 2006). Later, the introduction of further Northern American maize populations was decisive for the adaptation and distribution of maize in other European regions as molecular data from Rebourg et al. (2002) and Dubreuil et al. (2006) reveal.

The genetic variability of maize has permitted its presence in diverse conditions, reflected by the current large-scale cultivation of the plant. This adaptation has allowed for the development of varieties that can grow at lower temperatures and mature in a shorter time span, hence the crop can grow from temperate to tropical regions (Krishna 2013; Sood et al. 2014).

In Germany, the crop was initially cultivated in the southern region of Baden-Württemberg, where towards the end of the 19th century, the cultivated area in the region had expanded to approximately 2,600 ha. The introduction of modern silage preparation techniques in the first

half of the 20th century encouraged further production of maize, establishing its importance and increasing its cultivated area to 55,000 ha by the late 1960s (Zscheischler et al. 1990).

Additional aspects that definitively promoted the cultivation of maize in Germany and other European countries were the introduction of new and improved sowing and harvesting techniques, chemical control against weeds and the genetic enhancement of varieties that were adapted to the different climatic regions of Germany and Europe. This has allowed for an increase in yields and the adaptation of the plant to different soil types (Zscheischler et al. 1990).

At present, maize has several uses in Europe such as food, animal feed, bioenergy and industrial products. This range of uses has also led to a further increase in the production of maize across the continent, especially outside of the traditionally largest maize growing countries, namely France, Romania and Italy[1]. Taking Germany again as an example, in 2004 the government introduced feed-in-tariffs to promote increased maize production for use in biogas as alternative energy in line with the German Renewable Energy Act [German: Erneuerbare-Energien-Gesetz (EEG)]. This has turned Germany into one of the major producers of maize in Europe, augmenting its cultivated area from 1.6 million ha in 2005 to 2.56 million ha in 2011[2] (DMK 2015). With an approximate cultivated area of 2.5 million ha, maize has now become the second most cultivated crop in Germany, surpassed only by wheat with 3.2 million ha (Federal Statistical Office of Germany 2015).

By far the largest increase in cultivation has been witnessed in the Ukraine, moving from sixth to first among European producers since the turn of the century (see Figure 1). Despite maize being the fourth most cultivated crop in the Ukraine (behind wheat, barley and sunflower seeds), the size of the country and hence potential cultivation area is very large, and in recent years the production of barley has declined due to growers preferring the more profitable maize, with higher prices and demand on world markets (FAO 2014). Most of the growing regions are situated in central-northern areas of the country, which are appropriate for maize production due to their flat topography, good soils and abundant availability of water during summer (Bussay 2015).

[1] Compared to Germany, which only had a cultivated area 59 046 ha in 1968, France and Italy already had 1 023800 and 967 207 ha, respectively. Data for Romania is only available from 1999 onwards, however in 1999 it had a cultivated area of 3 071 000 ha, making it the second largest producer in Europe at the time.

[2] Note that in 2012, the tariff structure of the EEG was changed, offering less incentives for biogas. Maize production in Germany has remained relatively stable since (DMK 2015).

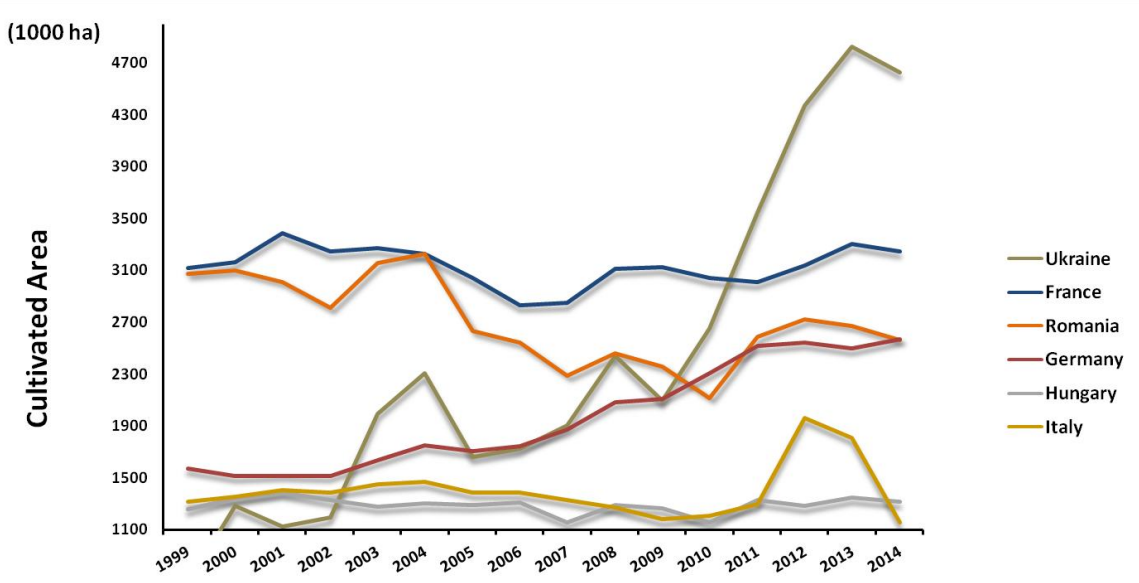

Fig. 1. Development of maize cultivation area in the main European producer countries in recent years (data compiled from ZMP 2008; FAO 2014; USDA 2015; State Statistics Service Ukraine 2015).

Meanwhile, France has maintained a stable level of cultivation in recent years, while Romania has experienced a reduction, often being affected by drought conditions (ZMP 2008, FAO 2014, DMK 2015). In 2012, for example, yield losses in Romania were estimated to be 46.1% compared to the average yield from 2009-2011 (Mateescu et al. 2013). The other traditional producer of maize mentioned above, Italy, has been overtaken in recent years by Germany and the Ukraine, with the cultivated area of maize falling from the early 60s to around 1990 and remaining relatively stable since then. Italy, like Romania, has also been affected by drought over the last few decades (Diodato & Bellocchi 2008).

Overall, the general increase in the cultivated area of maize in Europe has led to more intense maize cultivation in many regions, often accompanied by narrowed crop rotations.

1.2. Distribution and spread of maize pathogens across continents

Several diseases in the cultivation of maize are likely to have been present since the time maize was grown in its areas of origin in Mexico, where, in addition to soil exhaustion, abandoned milpas[3] and grass invasion (Lundell 1937; Willey and Shimkin 1973; Olson 1978 cited by Brewbaker 1979), pests and diseases have been proposed as limiting factors for maize cultivation in the Maya civilization (Brewbaker 1979). A number of these diseases were initially confined to the origin areas of their main host plants *Z. mays* or *Sorghum* spp.

[3] Land dedicated to the cultivation of maize, and occasionally other crops (Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua).

and as their cultivation spread across the globe, the diseases also began to appear in other growing areas.

Survival techniques based on the production of a large number of airborne spores which can be dispersed across long distances are the most viable way through which pathogens can survive across regions. Especially for biotrophic fungi, the ability to travel long distances allows for a prolongation of their life cycle and thus find plants to infect as permanent new hosts (Brown et al. 2002). This is the case for maize rusts, which are also believed to have their origin on the American continent and occur in teosinte or *Euchlanena mexicana,* making these their first hosts. For example, the origin of *Puccinia sorghi* can be traced back to the American continent (Savile 1984) and is presently endemic in North and South America – it was first described in Europe in 1838 (Holland) and later in 1858 in Germany (Neuhaus 1970 cited by Kreisel & Scholler 1994). Cammack (1959) also traces the initial appearance of the southern corn rust (American corn rust) produced by *Puccinia polysora* to the American continent, until it was carried to Africa in 1949 via air currents of viable uredospores. Contrary to this, at present *Physopella zeae* (tropical rust) is still confined to its regions of origin in Central America and has also spread somewhat in South America (Malaguti 2000; CYMMIT 2004; Da Costa 2007).

Another technique which has contributed to the long-distance spreading of several maize diseases is that of the pathogen accompanying its host plant as it has been distributed across continents. This has allowed certain pathogens to infect and survive as dormant propagules within, among other plant material, the seed tissue or by sticking to the seed coat (Neergaard 1969). One of the most important maize pathogens worldwide, *Exserohilum turcicum*, was confirmed as a maize and sorghum seedborne pathogen (Navi et al. 1999; De Rossi et al. 2012). This could explain its distribution from its traced origin in Mesoamerica or Central Africa (origin of the host plant *Sorghum* spp.) according to molecular analyses of populations by Borchardt et al. (1998). Several maize leaf pathogens such as *Bipolaris zeicola* and *Colletotrichum graminicola* have been confirmed also to be seedborne (Warren 1977; Nelson 1982; Niaz & Dawar 2009). This mechanism of transmission could also explain the arrival and distribution of the maize leaf pathogen *Kabatiella zeae*, which was first described in Japan (Narita & Hiratsuka 1959), into other continents and remote areas like New Zealand (Reifschneider & Arny 1979). Therefore, seedborne dispersion through commercial seeds could also have contributed to the introduction of certain diseases into Europe.

1.3. Main maize leaf diseases in Central Europe

1.3.1. Turcicum leaf blight

Nomenclature

The causal agent, *Exserohilum turcicum* (Pass.), Leonard et Suggs (1974), teleomorph: *Setosphaeria turcica* (Lutrell) Leonard et Suggs phylogenetically belongs to the Eumycota, phylum Ascomycota, class Dothideomycetes, order Pleorosporales and family Pleorosporaceae (Mycobank 2016). The pathogen was first described as *Helminthosporium turcicum* in Italy in 1876 by Passerini. Two years later, Cooke and Ellis described the disease in the United States (Holliday 1980). Although the sexual stage can be obtained without difficulty in the laboratory via recombination of the mating types MAT1-1 and MAT1-2 (Chang & Fan 1986), and both of these coexist in some regions (Abadi et al. 1993), it was not officially reported in the field until it was discovered in 2012 in two maize fields in Thailand (Bunkoed et al. 2014). Because the asexual stage is present in nature much more frequently than the sexual stage (Borchardt et al. 1997; Bunkoed et al. 2014), and is therefore the main causal agent of the disease, the asexual stage was used more frequently for descriptions.

The disease is commonly referred to as “Turcicum leaf blight” or “northern corn leaf blight”. The latter term was first used in the 1950s in reference to corn leaf blights in the United States. Although Turcicum leaf blight is found in most growing regions of the country and is especially prevalent in the southern part of the Corn Belt[4], it can also reach northward. Hence, in order to distinguish it from “southern corn leaf blight” produced by *Bipolaris maydis* (teleomorph *Cochliobolus heterostrophus*), which is especially prevalent in southern states and does not spread as far north, the name “northern” was assigned to the disease (Robert 1953; Holliday 1980).

Distribution

Turcicum leaf blight is one of the most important diseases affecting maize growing areas in both the northern and southern hemisphere. Prevalence and epidemics have been reported in the majority of maize growing regions in the United States and Canada (Lipps 1997; Tenuta and Zhu 2012; Bergstrom 2014; Wise 2015), Mexico, Ecuador, southwest Brazil, Argentina (Casela et al. 1998; Formento 2010; De Rossi *et al*. 2010; Couretot 2011; Culqui-Recalde 2015), India (Harlapur 2005), north-eastern and northern China (Wang et al. 2014), Uganda, Kenya and Tanzania (Adipala et al. 1993; Muiru 2008; Nwanosike et al. 2015), New Zealand and Australia (Fowler 1985; Watson & Napier 2006). In Europe, the disease is spread across the continent, from northern Spain and the United Kingdom (González &

[4] Note that while geographic depictions can differ slightly, the Corn Belt generally refers to the major corn-growing states in the US. These are located in the Midwest and include Illinois, Iowa, Nebraska, Minnesota, Wisconsin and Indiana, among others.

González Varela 2007; Mainka et al. 2012), to Latvia, Romania and the Caucasus (Jinjikhadze 2001; Borcean et al. 2012; Treikale et al. 2014). Focusing on Central European regions, a higher disease severity has been observed in the southern German regions of Bavaria and the Upper Rhein Valley, Austria (LfL 2015) and the French region of West-Bretagne (Cassini 1973; Mainka et al. 2012).

Epidemiology

E. turcicum first shows a hemibiotrophic phase, germinating to penetrate the living tissue, invading it in the first phase of the infection and subsequently behaving as a necrotroph, obtaining nutrients from the necrotic tissue (Walsh et al. 2008). Optimum temperatures between 20 and 25°C and prolonged high humidity with dew periods lasting over seven hours favour fungal sporulation, which can be distinguished by dark zones on the lesions (Berger 1970, White 2010).

The fungus overwinters as conidia, mycelia or chlamydospores (formed from the cells of the spores or from mycelial hyphae) in infected plant debris in several regions worldwide (Cassini 1973; Shang 1980; Levy 1984) or in host plants such as *Sorghum halepense* (Levy 1984) giving rise to larger epidemics in fields where tillage is not a common practice. In some maize regions, e.g. Argentina, spontaneous maize plants (resistant to herbicides) that grow across rotations can also be an important source of inoculum (personal observation). In the first few months of the maize season, high humidity and adequate temperatures favour the initial sporulation in debris in the field. These spores then give rise to first infections. Thereafter, further sporulation will occur in the lesions and wind currents are the predominant force through which the spores are transported across fields, subsequently serving as inoculum for new infections (Berger 1970). The pathogen can also attack grasses such as *Sorghum* spp. *(S. halepense*, *S. bicolour* L.), Echinochloa *(Echinochloa-cruss-galli*) and teosinte (*Zea mays* spp. mexicana) (Bunker and Mathur 2006).

1.3.2. Kabatiella eyespot

Revision in the categorisation of the fungus

The causal organism of eyespot disease, *Kabatiella zeae,* belongs to the genus *Kabatiella* described by Bubák and Kabát in 1907. With the first morphological observations of the pathogen in 1956 by Narita and Hiratsuka, it was classified within the Kabatiella Bubák species. Later, due to the similar *in vitro* behaviour of *Kabatiella* and *Aureobasidium* species and until new studies provided more information, Dingley (1973) considered including all the species in a single genus, namely *Aureobasidium*, belonging to the “black yeasts” fungi group. Fungi in this group have common morphological characteristics such as melanised cell walls, conidiophore-like structures and conidia enclosed in a polymeric matrix. However,

they may differ in terms of specialisation level, taxonomy and genetic relations (Cooke 1962; Sterflinger 2006). Further morphological studies by Hermanides-Nijhof (1977) also considered *Kabatiella* species to belong to the anamorph genus *Aureobasidium* after morphological comparisons with the ubiquitous fungus *A. pullulans.* Recently, several phylogenetic studies have shown the affinity of some *Kabatiella* species to *A. pullulans,* whereas various *K. zeae* strains are considerably different genetically from other *Aureobasidium* species (De Hoog et al. 1999, Bills et al. 2012). Furthermore, Zalar et al. (2008) emphasised that, in addition to genetic differences in some species, morphological distinctions such as the conidia shape (most *Kabatiella* species have sickle-shaped conidia, whereas in *Aureobasidium* they are normally ellipsoidal) could be observed between *Kabatiella* and *Aureobasidium* species. Nevertheless, the authors could not definitively exclude the possibility of a common grouping of both species. The reasoning behind this is that the studies were carried out with the saprophytic phyllosphere fungus *A. pullulans,* as opposed to the pathogen itself. Consequently, the classification of both genera is not clear to date and further studies need to be carried out (Bamadhaj et al. 2016).

Although the teleomorph has yet to be observed to date, all these studies have contributed to the classification of the pathogen. As with other *Kabatiella* and related *Aureobasidium* species, it is thought to be connected to teleomorph species of Discosphaerina (De Hoog et al. 1999). Thus, according to Mycobank (2016), *K. zeae* is classified as follows: Kingdom Mycota, phylum Ascomycota, class Dothideomycetes, order Dothidiales and family Dothioraceae.

Distribution

Since its first description (Narita & Hiratsuka 1959), *K. zeae* has been reported in several temperate regions worldwide, including the northern Corn Belt of the United States and the Canadian regions of Ontario and Quebec (Arny et al. 1970; Gates & Mortimore 1969; Munkvold & Martison 2001; Wise 2015), China (Xu et al. 2000), Argentina (Linares & Martínez 1971; Formento 2010), and New Zealand (Dingley 1973), but also in tropical and subtropical humid regions in Brazil (Esteves 1984; Dos Santos et al. 2007). In European regions, despite the disease being known since the 1970s (Smiljakovic & Pencic 1971; Schneider & Krüger 1972), in recent years it has appeared or increased in fields in Wales, south and southwest England (Finch et al. 2014), Denmark (Jørgensen 2012), northern Germany and the Netherlands (Kropf & Schlüter 2013, Mainka et al. 2012).

Epidemiology

Following germination of the spores and penetration of maize leaf tissue, the mycelium grows within epidermal cells on the leaf and in intercellular spaces. In the tissue, pigmented globose cells with thick walls will be formed in clusters or stroma-like structures. From here,

conidiophores emerge through the stomata and produce conidia simultaneously (Narita & Hiratsuka 1959; Dingley 1973; Schneider and Krüger 1972). The disease is characterized by a short latency period ranging from seven to 10 days (Reifschneider & Arny 1980a; Munkvold & Martinson 2001).

The pathogen survives in infected residues - mainly leaves (Arny et al. 1970; Chinchilla 1987) - but the conidia may not be able to germinate in the long-term, thus playing a minimal role in pathogen survival (Cassini 1973; Chinchilla 1987). The formation of stromatic hyphae, which form a thick melanised mycelium, has been shown to be the principle means through which the pathogen survives (Chinchilla 1987). The conidia can be disseminated through air currents (Arny et al. 1970) and splashing rain (Rapilly et al. 1975), the latter probably being most important for secondary disease cycles in the field (Chinchilla 1987). The pathogen can also be transmitted via seeds (Reifschneider & Arny 1979). Generally, the disease is favoured by mild (14-17°C) and humid weather (Narita & Hiratsuka 1959; Arny et al. 1970).

Besides maize, other alternative hosts have not been reported in nature but instead via artificial inoculations. Reifschneider and Arny (1980b) showed that other *Zea* spp. and *Zea mays* ssp. (*Zea perennis*, *Zea mays* ssp. *mexicana,* among others) can also be considered as host plants. However, the same authors could not confirm the role these plants play in the epidemiology in the field due to the absence of *K. zeae* in the areas in Mexico in which they are endemic.

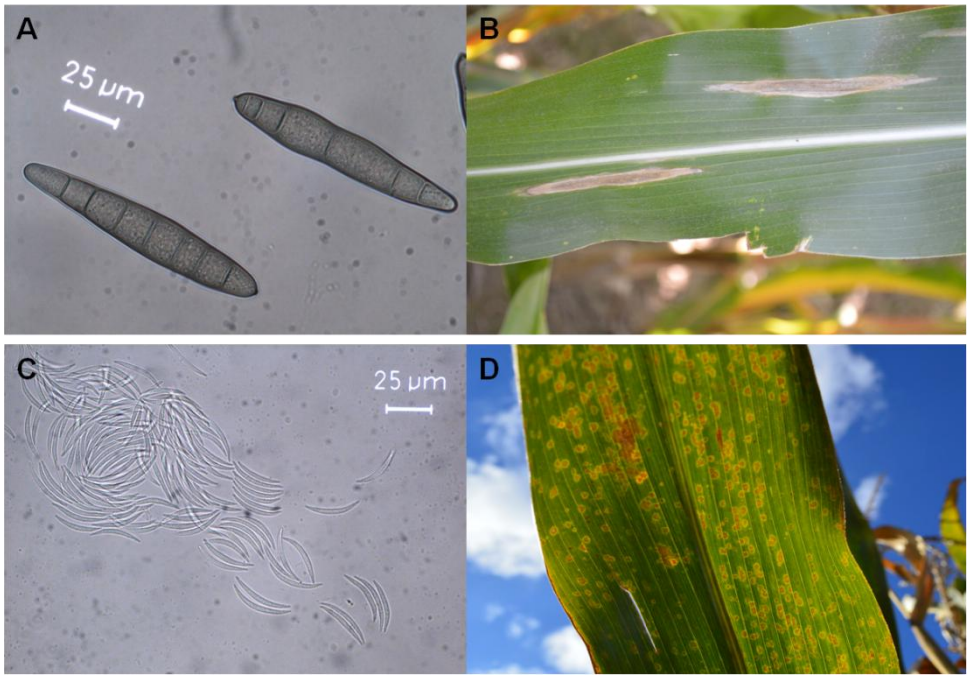

Fig. 2. The two main leaf diseases and their respective causal pathogens on maize in Central Europe. *E. turcicum* conidia (A) and typical symptoms on maize leaves (B). *K. zeae* conidia (C) and typical symptoms on maize leaves (D). Scale bars =25 µm (40x).

1.4. Maize leaf diseases of secondary importance in Central Europe

1.4.1. Common rust

Common maize rust is caused by the fungus *Puccinia sorghi*, which is classified in the Phylum Basidiomycota, class Pucciniomycetes, order Pucciniales (Mycobank 2016). It was first described by Schweinitz in 1832 (Hooker 1985), who believed it to infect sorghum. Evidently there was some confusion as sorghum is not a host plant, i.e. in reality the susceptible host is maize (Arthur & Bisby 1918). The disease has been reported in several regions of the world, including a wide distribution in fields throughout North America (Hooker 1985; Pataky & Eastburn 1993; Wise 2015), Central and South America (Casela et al. 1998; Darino et al. 2016), Asia (Dey et al. 2015), Africa (Dunhin et al. 2004) and Europe (Mercer & Gilliland 1999; Pataky et al. 2001; Arvalis 2012).

Like other rust diseases, the obligate biotrophic pathogen produces infectious uredospores, teliospores and basidiospores. When the plants are infected, pustules are developed on maize leaf surfaces. In the pustules approximately 5,000 orange to brown uredospores will be produced. These show a characteristic spherical to oval form (Hooker 1985; Jackson 2008). Due to the noticeable form and shape of the pustules, their presence is the first indicator of the disease in maize fields. Uredinia sporulate on the upper and lower surfaces of the leaf. *P. sorghi* is characterised by the production of explosive epidemics with short latency periods of about five to ten days at temperatures of 15-25°C (Hooker 1985; Vitti et al. 1995; Pataky & Tracy 1999) and is more common at a relative humidity of at least 98% (Hooker 1985).

Late in the season, the pustules become brown to black, indicating that the uredospores have been replaced by teliospores, the next and non-infectious stage in the fungus cycle. The teliospores can survive the winter (Mahindapala 1978). In spring, the two haploid teliospores first fuse their nuclei, and the germination occurs thereafter. A basidium is formed through meiosis, which produces basidiospores. These can infect several *Oxalis* spp. - in Europe mainly *O. corniculata* and *O. stricta* – which serve as alternate hosts for the pathogen (Zogg & Scherrer 1945; Gäumann 1959; Mahindapala 1978). From basidiospores, spermagonia with spermatia will be formed. After fusion of spermatia with hyphae of the opposite mating type, aecia will be formed. These produce aecidiospores which may land on maize leaves, subsequently infecting them and completing the life cycle by producing uredospores once again (Dunhin et al. 2004). The occurrence of the aecial infection has also been reported in Europe (Zogg 1949). This life cycle could be absent in areas where maize is grown continuously. Here, infections merely occur through the uredial stage, which will then gradually be transferred from old to more recently planted maize during the year as in the tropics (Hooker 1985). The pathogen may also survive from year to year as uredospores in

maize debris (Kellermann 1906) or emanate from southern regions and be dispersed through wind or storm currents to northern regions during the season (Hooker 1985).

1.4.2. Northern corn leaf spot

Bipolaris zeicola was first described in 1930 by Stout as *Helminthosporium zeicola*. Its teleomorph, *Cochliobolus carbonum* (in reference to the burnt appearance of an infected ear), was discovered by Nelson in 1959. As the anamorph stage is more frequently observed in nature as being the main causal agent of the disease, the name *Bipolaris zeicola* is used more commonly than *Cochliobolus carbonum*. *B. zeicola* belongs to the Ascomycota, Dothideomycetes, order Pleosporales, family Pleosporaceae (Mycobank 2016).

The pathogen is the causal agent of northern corn leaf spot but can also infect other Poaceae grasses including *Sorghum* spp. (Sivanesan 1987), *Oryza sativa* (Xiao et al. 1991), and other plant families (Mendes et al. 1998). *B. zeicola* infects maize worldwide (Sivanesan 1987; Mendes et al. 1998; Canhua et al. 2014; Wise 2015). In Europe, the disease has been reported to occur in Germany, Austria, France, Serbia and Hungary (EPPO 2016; Welz & Geiger 1995; Stankovic et al. 2007).

The pathogen can survive as mycelium and through formation of chlamydospores in maize debris in the field during winter (Nelson 1982). The pathogen invades the tissue intracellulary and induces lesions, which can vary from small and round-oval to lineal in shape, and grayish to brown in appearance, depending on the described pathogenic “races” (0 through 4) in question. In this case, the term “race” is mainly applied based on the leaf symptoms produced, with race 1 being the only *B. zeicola* race which shows a different reaction when inoculated onto a set of different maize lines (Multani et al. 1998). Epidemics caused by race 0 in the 70s can be avoided nowadays because resistance gene *Hm* is present in all cultivars (White 2010). Seed transmission is also considered to be an important infection source (Warham et al. 1997). Plant infection is favoured by moderate temperatures (18-26°C) and dew periods (Lipps & Mills 2001).

1.4.3. Maize anthracnose

Maize anthracnose is caused by the ascomycete *Colletotrichum graminicola* (teleomorph *Glomerella graminicola* D.J. Politis 1975), classified in the class Sordariomycetes, order Glomerellales, family Glomerellaceae (Mycobank 2016). The disease has been reported in various temperate, tropical and subtropical areas, but is particularly established and severe in warm, humid conditions (Wheeler et al. 1973; Gatch & Munkvold 2002; Jackson-Ziems et al. 2014; da Costa et al. 2014; Zhang et al. 2014; Wise 2015). It has also been observed in Europe (Sukno et al. 2014; personal observation) but in most cases with a restricted distribution limited to individual plants in the field.

C. graminicola produces a complex disease on maize plants which can involve leaf blight, die back, stalk rot, root rot, kernel and ear infections. The pathogen forms acervuli, which are composed of spores embedded in a polysaccharide protein mucilaginous matrix with black setae (appendages). The matrix protects against desiccation and supports germination and penetration of the tissue (Nicholson & Moraes 1980). The pathogen can penetrate either directly or indirectly through wounds. The indirect method is more efficient and in the stalk, for example, the initial wounds are caused by insects such as the European corn borer or other stalk rot diseases (Gatch & Munkvold 2002). On the plant surface, the spore germinates into hyphae and develops an appresorium, which allows the pathogen to penetrate the tissue. From the appresorium, a secondary hypha will colonise the cells, first through a biotrophic interaction with the host, followed by a necrotrophic stage causing infection (Wheeler et al. 1973; Venard & Vaillancourt 2007; Sukno et al. 2008; Behr et al. 2010). *C. graminicola* can overwinter in infected maize debris as a parasite (Vizvary & Warren 1982, Lipps 1983) and, as recent reports have suggested, probably as microsclerotia in the soil as well (Sukno et al. 2008).

C. graminicola is also known to infect other important crops such as wheat and oat (Dickson 1956). This could lead to the assumption that crop rotation or intercropping of these crops with maize could lead to an increase in infections. Nevertheless, while Wheeler et al. (1973) reported the ability of *C. graminicola* maize isolates to infect members of the genus *Sorghum* such as *Sorghum bicolor* and *Sorghum halepense,* these same maize isolates did not infect other cereals like wheat, oat, barley or millet. The authors also reported that isolates from these four cereals were non-pathogenic on maize, indicating different host specificity.

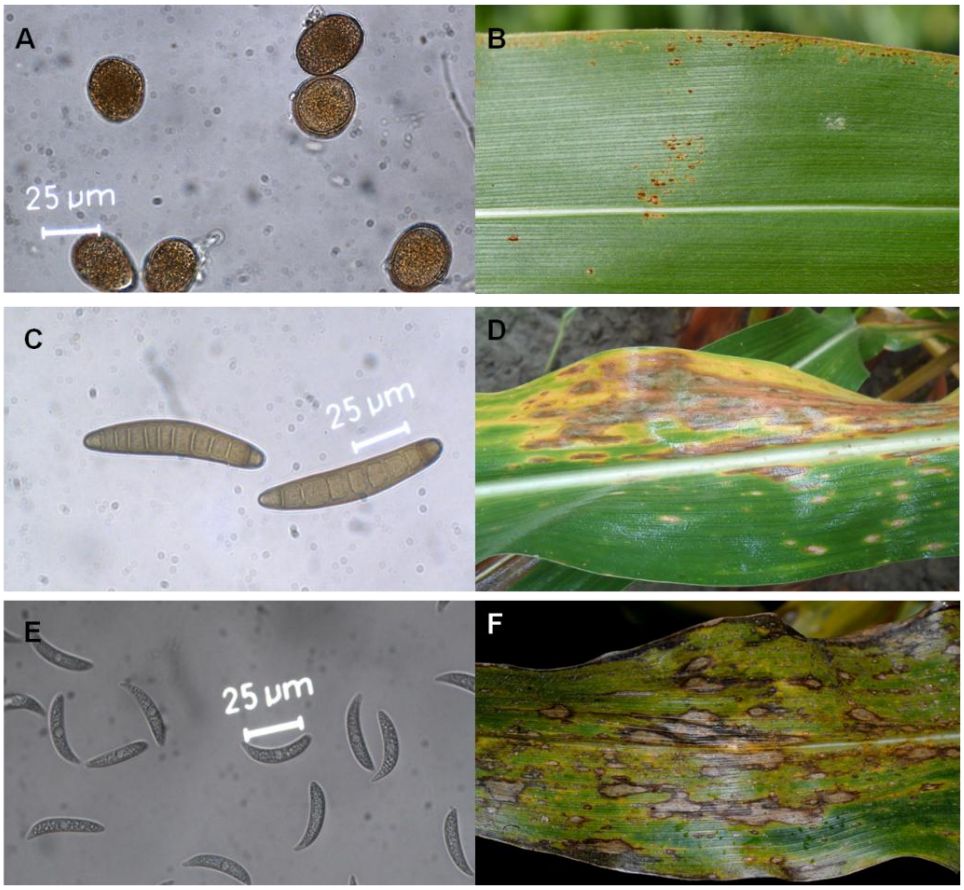

Fig. 3. Other causal agents of maize leaf diseases present in Central Europe. *P. sorghi* uredospores (A) and initial typical brown pustules formed on maize leaves (B). *B. zeicola*: conidia (C) and leaf symptoms (D). *C. gramicola*: conidia (E) and leaf symptoms (F) Scale bars =25 µm (40x).

1.4.4. *Phoma* spp. complex

In the literature, several *Phoma* spp., or other pathogens related to this genus, are associated with maize, whether it be in pathogenic or saprophytic form. Here, fungal taxonomists are still dealing with the morphological and molecular identification, and consequent classification (Boerema et al. 2004; Aveskamp et al. 2008; Aveskamp et al. 2010). There are several reasons behind this complication. Firstly, *Phoma* comprises a large number of species (more than 3,000), which are classified based on their respective host plant and extended morphological characteristics. Secondly, these characteristics vary greatly with regard to *in vitro* culture (Aveskamp et al. 2010).

A review on the most frequently found Phoma leaf pathogen in temperate regions, namely *Phoma zeae-maydis,* is provided below. Comparisons with other *Phoma* spp. present on maize are also summarised.

Yellow leaf blight

Yellow leaf blight, produced by *Phoma zeae-maydis* (syn. *Didymella zeae-maydis*), is the most important Phoma leaf disease described in temperate regions (Frezzi 1972; Cassini 1973; Jimenez-Diaz & Boothroyd 1979). The disease has been reported since the mid-late sixties in Ohio and Pennsylvannia, USA (Scheifele & Nelson 1969; McFeeley 1971) and Ontario, Canada (Gates & Martimore 1969), making it one of the most recently discovered maize leaf diseases. Initially, the causal agent was depicted as a *Phyllosticta* species. A further description of the pathogen was carried out by Arny and Nelson (1971), resulting in its classification as *Phyllosticta maydis*.

In the past, *Phyllosticta* was designated for those species inhabiting leaves, while *Phoma* was designated for those inhabiting stalks and roots until new criteria for their classification were recently introduced (Van der Aa & Vanev 2002; Boerema et al. 2004). Through these criteria, some *Phyllosticta* or *Ascochyta* species were reclassified in the *Phoma* genus (Aveskamp et al. 2008). In accordance with this concept and as the epithet was occupied by other *Phoma* spp. (*Phoma zeae*, *Phoma zeicola, Ph. maydis*), Punithalingam (1990) reclassified the species as *Phoma zeae-maydis.*

De Gruyter (2002) included *P. zeae-maydis* in the section *Macrospora,* having been introduced by Boerema (1997). Nevertheless, Aveskamp et al. (2010) excluded the importance of the spore size as an informative and reliable characteristic, instead accommodating this species in the *Phoma* section *Peyronellaea* (anamorphic genus) based on its production of multicellular chlamydospores (dyctiochlamydospores) and DNA phylogeny. The teleomorph stage was first described as *Mycosphaerella zeae-maydis* in 1973 by Mukunya & Boothroyd. Further molecular analyses carried out by Chen et al. (2015) establish the teleomorphic genus of the fungus as *Didimella zeae maydis* in an effort to resolve “the Phoma enigma”.

Distribution and epidemiology

Following overwintering in leaf debris, pseudothecia are considered to be the first source of inoculum at the beginning of the season, favoured by low temperatures and darkness (Jimenez-Diaz & Boothroyd 1979). After infection of lower leaves, the necrotic tissue becomes a suitable substrate for the development of further pseudothecia (Jimenez-Diaz & Boothroyd 1979). After formation of pycnidia and conidia, secondary infections develop, with these asexual stages being favoured by light and higher temperatures (optimal temperature 24°C) (Arny & Nelson 1971; Jimenez-Diaz & Boothroyd 1979). Ascospores are carried by wind over long distances, infecting other fields, and conidia are disseminated through water splash in the local field (Munkunya & Boothroyd 1973; Jimenez-Diaz & Boothroyd 1979).

Other *Phoma* spp. on maize

Many other *Phoma* spp. species related to *Phoma zeae-maydis* that produce maize leaf diseases or live saprophytically on leaves can be found in the literature. A summary is given in Table 1.

Tab. 1. Some *Phoma* spp. reported to be isolated from maize leaves or seeds

***Phoma* spp.**	**Sexual stage**	**Disease**	**Interaction**	**Reported from (Source)**
P. maydis *P. sorghina*	*Phaeosphaeria maydis*	Phaeosphaeria leaf spot complex	Pathogenic	Brazil (Casela et al. 1998); (Do Amaral et al. 2004) Argentina (Díaz 2011), United States (Carson 2005)
P. zeae Stout	*Mycosphaerella zeae*	--	Pathogenic	Czechoslovakia* (Cejp 1967 cited by Arny & Nelson 1971) Rumania (Scheifele & Nelson 1969) North America (Stout 1930; Scheifele & Nelson 1969; McFeeley 1971; Arny & Nelson 1971) Review: (Punithalingam 1990)
P. maydis Fautrey	*Mycosphaerella maydis*	--	Not specified	Lambotte & Fautrey (1894) North America (Stout 1930) Review: (Punithalingam 1990)
P. pomorum	--	--	Not specified	Denmark (Sørensen et al. 2010)
P. subherbarum	--	--	Saprophytic seeds	Canada (De Gruyter et al. 1993)

1.5. Epidemics and potential yield losses

Foliar diseases can have a direct influence on the amount of dry matter stored in the grain or in the final biomass of the plant (Shah & Dillard 2006; Couretot et al. 2012). In this context, different foliar maize fungal species have been reported to produce yield losses worldwide when high rates of severity are reached. “Helminthosporium leaf blights” (named after previous nomenclature) including Turcicum leaf blight, southern corn leaf blight (causal agent *Bipolaris maydis*), northern corn leaf spot and gray leaf spot (causal agent *Cercospora zeae-maydis*) have been reported to cause serious problems in various maize producing areas worldwide (Lipps 1998; De Rossi et al. 2010; Señerez Arcibal 2013; Wise 2014; Mubeen et al. 2015). Nevertheless, the southern corn leaf blight seems to be less important in northern European regions (Jørgensen 2012), while grey leaf spot does not have a noticeable

relevance in Europe, presumably due to scarce information about the disease. Conversely, in recent years, Turcicum leaf blight has been frequently reported in many European regions. For example, severe epidemics were noted in 2002, 2008 and 2010 in the southern German region of Bavaria (Mainka et al. 2012; Urban 2012; Zellner 2012). Worldwide, the disease has been reported to cause severe damage in recent years and also in several following seasons (Wise 2013; Wise 2014; De Rossi 2015).

Regarding northern corn leaf spot, its importance in Europe is restricted to certain regions, e.g. Lower Saxony (Bornemann 2015), and is not seen as a major concern for yield losses at present. Worldwide, while in some maize producing countries the disease is considered to cause significant damage only on inbreds used in hybrid seed production, with outbreaks like in the Corn Belt in the United States (White 2010) being an exception, in other regions, e.g. hilly and mountainous areas of China, it is considered an important limiting factor to corn production (Liu et al. 2015).

Common rust, together with Turcicum leaf blight, is considered to be the most damaging foliar disease in the maize fields of several regions. It produces severe epidemics which, in some cases, can generate 100% yield losses (Jeffers & Chapman 1994).

Compared to the aforementioned diseases, severe outbreaks produced by *K. zeae* have so far not been widely reported. In Europe, a severe epidemic was observed in 2011 in some regions of Denmark and Germany (Schleswig-Holstein and Lower Saxony), where yield losses varying from 10% to 30% were recorded, respectively (Hanhart 2012; Jørgensen 2012; Kropf & Schlüter 2013). The disease has been suggested to have a similarly destructive potential to that of northern corn leaf spot if optimal conditions are present (Reifschneider 1983).

A summary of the significant yield losses caused worldwide in recent years by key foliar diseases (by natural infection) of maize present in Europe is shown in Table 2.

Tab. 2. Recorded disease severities and correlated yield losses reported in recent years under natural infection.

Maize leaf disease (Causal pathogen)	Recorded disease severity and/or yield losses	Source
Turcicum leaf blight ***(E. turcicum)***	Disease severity 10-50% depending on the variety 2008/2010 Yield losses of about 40% recorded in Argentina.	De Rossi et al. 2010
	15-30% yield losses in South Africa	Cramptom 2015
	33% to 62% in the humid highlands & between 4% to 35% in the dry, coastal lowlands in Tanzania	Nwanosike et al. 2015
	2002 – 10-30% yield losses in Germany	Zellner 2012
	2013 - approx. 3.36 million tonnes (1%); 2014 - approx. 8.9 million tonnes (2.5%) in the United States and Ontario (Canada).	Wise 2013; 2014
Common rust ***(P. sorghi)***	Up to 60% yield losses in India	Dey et al. 2012
	2000 - high severity in Indiana	Wise 2010
	100% yield losses in some Mexican field trials 7.8 million hectares (34%) affected in subtropical-through-highland maize ecologies worldwide	Jeffers & Chapman 1994.
	2013 approx. 1.34 million tonnes (<1%); 2014 aprox. 2.79 million tonnes (<1%) in the United States and Ontario (Canada).	Wise 2013; 2014
Northern corn leaf spot ***(B. zeicola)***	2013- approx. 0.10 million tonnes (<1%); 2014 approx. 0.21 million tonnes (<1%) yield losses in the United States and Ontario (Canada).	Wise 2013; 2014
	Major cause of great losses in Yunnan Province (China)	Zhang et al. 2013
Eyespot ***(K. zeae)***	10% at trials in Ostenfeld (Schleswig-Holstein, Germany) (2013)	Kropf & Schlüter 2013
	21% at trials in Ostenfeld with monoculture and non-tillage (2011)	Schlüter 2012
	30% in Denmark (2011)	Hanhart 2012
	2013 - approx. 0.33 million tonnes (<1%); 2014 - approx. 0.36 million tonnes (<1%) in the United States and Ontario (Canada).	Wise 2013; 2014

1.6. Fungicides as control measure

In the effort to avoid yield losses, maintaining some maize leaf diseases under a certain level, i.e. a threshold through which economical damage is avoided, can be achieved via the use of resistant varieties (Ogliari et al. 2005; Kumar et al. 2011; Sillón 2012; Formento et al. 2014) and phytosanitary measures such as tillage practices and crop rotation (Arny et al. 1970; Levy 1984; Chinchilla 1987; Nyvall & Martison 1997; Dill-Mackey & Jones 2000; Formento et al. 2012; Draper et al. 2009). Where these measures are not sufficient or cannot be included as a suitable method for the management of the crop, the use of fungicides becomes a feasible option as part of modern and efficient maize production in order to control epidemics (Jurca Grigolli 2009; Couretot et al. 2012; Díaz et al. 2012). With regard to the maize crop, the application is based on systemic leaf compounds, which include triazoles as active substances from the demethylation inhibitors fungicide group (DMI) and strobilurines from the quinone outside inhibitors group (Q_oI-fungicides) (FRAC 2016). These compounds are summarised in Table 3.

Tab. 3. Classification of the main active ingredients in fungicides applied to the maize crop, according to their mechanism of action (FRAC 2016).

Target effect on fungal pathogen	Target site and code	Group name	Chemical group	Common Name
Sterol biosynthesis in membranes	C14-demethylase in sterol biosynthesis (erg11/cyp51)	DMI fungicides (Demethylation inhibitors)	Triazoles	Cyproconazole Epoxiconazole Flusilazole Propiconazole Prothioconazole
Respiration	Complex III Cytochrome bc1 (ubiquinol oxidase) at Qo site (cy b gene)	QoI-fungicides (Quinone outside inhibitors)	Methoxy-acrylates	Azoxystrobin
			Methoxy-carbamates	Pyraclostrobin
			Dihydro-dioxazines	Fluoxastrobin

Triazoles belong to the DMI fungicides group and thus the biochemical action mechanism is based on the inhibition of the enzyme C14-demethylase. This enzyme is responsible for the production of ergosterol, which is a part of the fungal cell membrane (Mauler-Machnik et al. 2002). Although the germ tube will be formed, its elongation and hyphal growth will be

impeded through disorders in the division and further development of the cell walls (Heitefuss 2000; Häuser-Hahn et al. 2004).

In strobilurin-based fungicides (Q_oI-fungicides), the mechanism of action implies the inhibition of the electron transport at the Q_o-center of the cytochrome bc_1 of the complex III in the mitochondrial membrane, interrupting the respiratory chain. Thus, several developmental stages of the pathogen essential for the colonization of the plant such as spore germination, germ-tube growth, penetration and mycelia growth will be either directly or indirectly interrupted (Becker et al. 1981; Bartlett et al. 2002; Fernández-Ortuño et al. 2010). Strobilurines show a high level of effectiveness against sporulation and spore development, an effect which azoles do not show (Häuser-Hahn et al. 2004). Therefore, strobilurines will usually be applied prior to infection or in early stages of the fungal life cycle (as a protective and early curative application). Strobilurines are never applied as an individual treatment but rather in combination with other active ingredients from other fungicide groups (Bartlett et al. 2002).

Triazoles and strobilurines are effective against a broad fungal spectrum, which includes important species from the Ascomycetes and Basidiomycetes (Bartlett et al. 2002) such as *Helminthosporium* species, *K. zeae and P. sorghi* in maize (Pinto 2004; Couretot et al. 2012; Wise 2015).

In line with this, fungicide treatments for the management of maize leaf diseases are registered as a control option in maize fields of key maize producing countries like the United States, Argentina, Brazil and India, among others (Bradley et al. 2010; Formento 2010; Juliatti et al. 2013; UNL Extension 2014; Kumar et al. 2014). In Europe, they are permitted in some countries such as France, Hungary, Poland, United Kingdom, Austria, Germany and Denmark (French Ministry of Agriculture 2015; Agrinex 2015; Minrol 2016; HSE 2016; AGES 2016; BVL 2016; SEGES 2016). In Germany, the farmer assesses the risk of disease and makes an independent decision on the adequacy of a fungicide treatment. If the farmer requires further advice, he can consult the responsible local extension service (Bornemann 2015; Agravis 2015). The application of fungicides in maize in Germany is only permitted once per season (Bornemann 2015).

1.7. Aim of the thesis

The primary aim of this thesis was to review the phytosanitary state of maize crops in Central European regions, focusing on fungal leaf diseases and their epidemiology, particularly addressing less-known or novel pathogenic species. To this end, a qualitative monitoring was carried out in different Central European regions through the collection and subsequent analysis of leaf samples. In regions with a higher disease pressure, the correlation between favourable weather conditions, development of the disease and seasonal fluctuations in inoculum was analysed for the main pathogens in order to gain more in-depth knowledge on the dynamic of these diseases in the field. This was to be achieved by using a Burkard spore trap in three locations. With the aim of accelerating the assessment of spore trap samples, the possibility of a molecular biological detection of propagules of the main leaf pathogens was investigated. In addition, the latent period of the disease was determined. Finally, studies were conducted to provide insight into whether fungicide application is necessary and how to determine the optimal timing of application. The suitability of fungicides for the optimal control of the disease was the final goal of the thesis. In this regard, the effect of different timing of application on infected leaf area and yield were analysed. Based on the relationship between infected leaf area and yield, the possibility of establishing an economic action threshold was investigated.

2. Materials and Methods

2.1. Materials

Chemicals

Trisiloxan/ Emulgator (Silwet® Gold)	Spiess-Urania Chemicals
Hexane	Carl Roth GmbH + Co. KG Karlsruhe
Vaseline Ratiomed	Megro GmbH & Co. KG, Wesel
Taq-Buffer A with $MgCl_2$ (10x)	Nippon Genetics, Dueren
dNTPs Mix (10 mM)	Bioline, Luckenwalde
Proteinase K (20 mg/ml)	AppliChem, Darmstadt
Ribonuclease (RNAse) (100 x 4U/mg)	AppliChem, Darmstadt
Taq polymerase (5U/µl) FastGene	Nippon Genetics, Dueren
Glycoblue coprecipitant (15mg/ml)	Thermofisher Scientific, Dreieich
DNA Gel Loading Dye (6 X)	Thermo Fisher Scientific, Dreieich
Midori Green Advance	Nippon Genetics, Dueren
Thrichloromethane/Chloroform	Carl Roth GmbH + Co. KG Karlsruhe
Isoamyl alcohol	AppliChem, Darmstadt
Polyethilene glycol (PEG) 6000	AppliChem, Darmstadt
β-Mercaptoethanol	Sigma-Aldrich Chemie, Steinheim
Phenol	Carl Roth GmbH + Co. KG Karlsruhe
Glycerol	Carl Roth GmbH + Co. KG Karlsruhe

Buffer compounds

CTAB buffer
(Brandfass & Karlovsky 2008)

N-Cetyl-N,N,N-trimethyl-ammoniumbromid (CTAB) (0.02 M)	Merck, Darmstadt
Ethylenediaminetetraacetic acid disodium (Na-EDTA) (20 mM)	AppliChem, Darmstadt
Sorbitol (0.13 M)	Merck, Darmstadt
N-Lauroylsarcosin-Sodiumsalt (0.03 M)	Merck, Darmstadt
TRIS-HCl pH 8.0 (10 mM)	Applichem, Darmstadt
NaCl (0.8 M)	Applichem, Darmstadt
Polyvinylpyrroidon K30 1% (w/v)	AppliChem, Darmstadt

DNA gel loading dye	Thermofisher, Dreieich
TE buffer (Brandfass & Karlovsky 2008)	
Ethylenediaminetetraacetic acid disodium (Na-EDTA) (100 mM, pH 8.0)	AppliChem GmbH, Darmstadt
Tris (1M, pH 8.0)	AppliChem GmbH, Darmstadt
TBE buffer (TRIS-Borat-EDTA)	AppliChem GmbH, Darmstadt

2.1.1. Media

The different solid agar media were compiled as follows (for 750ml H_20_{dest}):

Malt agar:

10 g maltose monohydrate
1.5 g peptone
15 g agar

Oat agar (OA)

15 g oat flour
11.25 g agar

Potato dextrose agar (PDA)

22 g potato dextrose agar — Sigma-Aldrich Chemie, Steinheim

Synthetic nutrient-poor agar (SNA)

0.75 g KH2PO4
0.75 g KNO3
0.375 g MgSO4
0.375 g KCl
0.15 g glucose
0.15 g saccharose
11.25 g agar

Vegetable 8 (V8)

1.5 g Ca CO3
11.25 g agar
75 ml V8 vegetable juice

Yeast malt extract agar (YE+ME)

25.2 g yeast malt extract agar Sigma-Aldrich Chemie, Steinheim

Water agar 0.8 % (WA)

6 g agar

***K. zeae*-liquid medium**

7.5 g carboxymethylcellulose (low viscosity) Sigma-Aldrich Chemie, Steinheim
3.7 g maltose,
1.1 g peptone,
0.75 g monobasic potassium phosphate

For all the media:
Agar: Carl Roth GmbH + Co. KG, Karlsruhe.

The necessary amount of nutrient medium was autoclaved with steam at 121ºC under 2 bar pressure for 20 min. After autoclaving, streptomycin (200 ppm) (Duchefa Biochemie, Haarlem) was added.

2.1.2. Maize seeds

Variety Ricardinio, certified	KWS GmbH, Einbeck
Variety Barros, certified	KWS GmbH, Einbeck
Variety Fernandez, certified	KWS GmbH, Einbeck
Variety Kalvin, certified	Syngenta GmbH, Maintal
Variety Ronaldinio, certified	KWS GmbH, Einbeck
Variety NK Silotop, certified	Syngenta GmbH, Maintal

2.2. Inventory and validation of fungal pathogens on maize leaves

In order to reflect the actual situation of maize leaf diseases in Central Europe and due to the scarce information about their occurence in Central European maize fields, a qualitative monitoring of the occurrence of potential leaf infecting species was carried out within regions of Germany, the Netherlands, Czech Republic, Austria, France and Poland during the years 2012 and 2013. The inventory and validation is based on the prevalence of the respective diseases and the completion of Koch´s postulates for the less known diseases to verify that the obtained fungal organism produces the disease. To achieve this, symptomatic leaf samples were collected and fungal organisms were isolated and analysed morphologically. For a number of isolates, pathogenicity tests were conducted by inoculating healthy plants in the greenhouse with spore suspensions prepared from single-spore cultures.

Koch's postulates were followed in order to subsequently establish the relationship between a disease and the organism it infects and comprise the following *in vitro* and *in vivo* methods. All four of the following steps must be completed for their fulfilment:

1. **The organism has to be detected in the diseased tissue.**
2. **It has to be isolated and grown in pure culture.**
3. **With the pure culture, the disease must be reproduced on the host plant.**
4. **The pathogen has to be recovered from the inoculated plant.**

These steps were carried out one after another using the experimental setup described in the next sections.

2.2.1. Sampling locations

Samples of infected maize leaves were collected in 2012 and 2013 from selected fields in Germany (27 locations), the Netherlands (six locations), Czech Republic (six locations), Austria (three locations), France (two locations) and Poland (two locations) in order to perform a qualitative monitoring (Table 4 and Figure 4). These locations are comprised of breeding stations, trial locations and conventional agricultural fields. Fields belong to conventional farms, maize trial locations focused on fungicide application (Syngenta, Landwirtschaftskammern) and breeding locations (Syngenta).

Tab. 4. Sampled locations for the monitoring in 2012 and 2013.

Code	Location	Country	Year	Variety	Purpose	Organisation
			North and Centre			
12.14/13.14	Ommen	Netherlands	2012/2013	--	Breeding	Syngenta
12.15	Princepeel	Netherlands	2012	--	Breeding	Syngenta
12.28	Groningen	Netherlands	2012	--	Breeding	Syngenta
12.30	Biddinghausen	Netherlands	2012	--	Breeding	Syngenta
12.21	Ottersum	Netherlands	2012	--	Breeding	Syngenta
13.35	centre	Netherlands	2013	SY Milkitop	Breeding	Syngenta
12.2/13.2	Ostenfeld	Germany	2012/2013	Ronaldinio	Trials	FH Kiel
12.20/13.20	Nordholz	Germany	2012/2013	--	Breeding	Syngenta
12.19	Cloppenburg	Germany	2012	--	Breeding	Syngenta
12.23	Rade	Germany	2012	Ricardinio	Trial	LWK SH
12.24	Beesten	Germany	2012	--	Breeding	Syngenta
13.40	Wessin	Germany	2013	Ronaldinio	Trial	Syngenta
13.42	Klein Marzehns	Germany	2013	Diverse	Conventional	--
13.43	Bodenwerder	Germany	2013	Ronaldinio	Conventional	--
13.44	Taaken	Germany	2013	Ricardinio	Conventional	--
12.27	Bad Oldesloe	Germany	2012	Fernandes	Trial	Syngenta
12.11	Köhn (Plön)	Germany	2012	Kalvin	Trial	Syngenta
12.31	Giekau	Germany	2012	--	Trial	Syngenta
12.13	Stapel	Germany	2012	Ricardinio	Trial	LWK NS
12.12	Schleswig	Germany	2012	Agro Yoko	Conventional	--
13.45	Linum	Germany	2013	Ricardinio	Conventional	--
13.47	Waake	Germany	2013	--	Conventional	LWK Hessen
13.48	Göttingen	Germany	2013	--	Trial	Uni Göttingen
13.49	Dewitz	Germany	2013	Ronaldinio	Trial	BioChem agrar
12.3	Liesborn	Germany	2012	--	Breeding	Syngenta
12.25	Milte	Germany	2012	Zidane/Logo	Trial	LWK NRW
			South			
13.39	Windsbach-Untereschenbach	Germany	2013	--	Trial	Syngenta
12.32	Marburg	Germany	2012	--	Trial	Syngenta
12.16/13.16	Mariaporsching	Germany	2012/2013	--	Trial	Syngenta
12.17	Mintraching	Germany	2012	--	Breeding	Syngenta
13.36	Mittich	Germany	2013	Zidane	Trial	Syngenta
13.37	Hartkirchen	Germany	2013	DKC4590	Trial	Hetterich
13.38	Ruhstorf (Rott)	Germany	2013	Ricardinio	Conventional	Syngenta
12.10/13.10	S. Peter am Hart	Austria	2012/2013	--	Breeding	Syngenta
12.18	Schönering	Austria	2012	--	Breeding	Syngenta
13.46	Steiermark	Austria	2013	--	Trial	Syngenta
			East			
12.26	Galowo	Poland	2012	--	Breeding	Syngenta
12.29	Zybiszów	Poland	2012	--	Breeding	Syngenta
12.4	Nechanice	C. Republic	2012	--	Breeding	Syngenta
12.5	Lysice	C. Republic	2012	--	Breeding	Syngenta
12.6	Lesany	C. Republic	2012	--	Breeding	Syngenta
12.7	Bylany	C. Republic	2012	--	Breeding	Syngenta
12.8	Caslav	C. Republic	2012	--	Breeding	Syngenta
12.9	Plana	C. Republic	2012	--	Breeding	Syngenta
			West			
12.22	Moorlas	France	2012	--	Breeding	Syngenta
12.34	Garlin	France	2012	--	Breeding	Syngenta

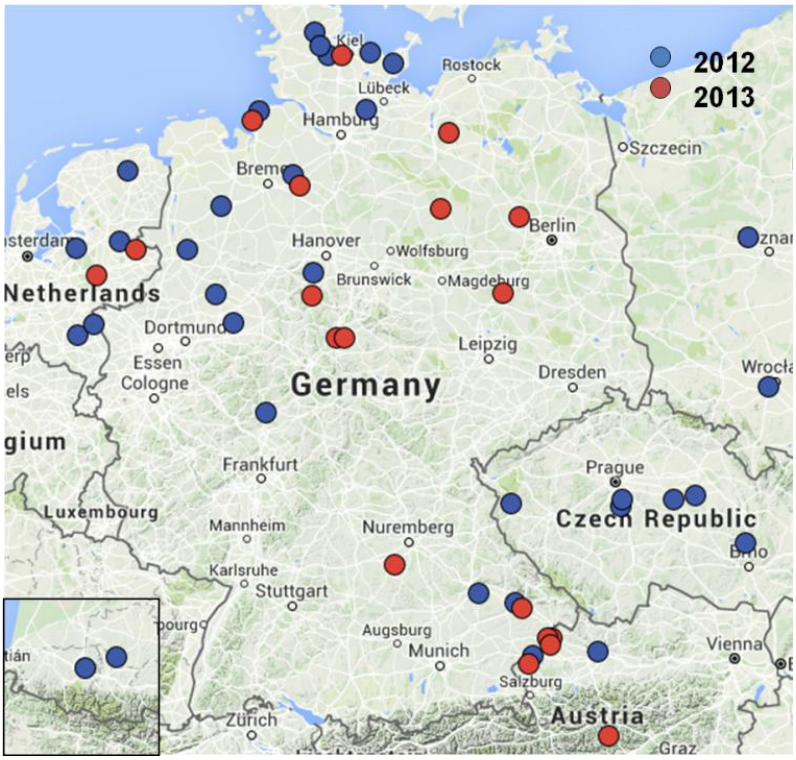

Fig. 4. Distribution of the monitoring locations in 2012 and 2013 in Germany (27 locations), the Netherlands (six locations), Czech Republic (six locations), Austria (three locations), France (two locations) and Poland (two locations).

2.2.2. Isolation of fungal organisms

Two different methods were used for the recovery. This was dependent on the capacity of the target pathogen to sporulate under artificial conditions and, consequently, the difficulty of isolating it from the infected sample in the presence of strongly sporulating saprophytes.

a) Placement of the infected tissue on sterile filter paper or SNA.

Samples of infected maize leaves (approx. 1-2 cm in length) exhibiting lesions were segmented and transferred into a glass beaker, where they were disinfected in a 2% sodium chloride (NaOCl) solution for 45 s and then rinsed in sterile water for another 45 s. This last step was repeated and finally the leaf samples were dried with absorbent sterile paper. Following this, sterile filter paper was moistened with sterile water. This surface was used for *E. turcicum* as it facilitates fungal sporulation and can be isolated directly from the leaf. An alternative was the placement of the infected tissue on SNA medium as it provides a higher level of humidity than filter paper and for a longer period of time. It also allows the organism to not only sporulate on the leaf, but also facilitate its growth and sporulation on the agar surface. This procedure was regularly used for *K. zeae, B. zeicola, C. graminicola, Phoma* spp. and *Fusarium* spp., among others.

b) Waring blendor technique (Arny et al. 1970, adapted from Stover & Waite 1953).

Using this method, the samples containing lesions were also segmented into small pieces (approx. 1 cm in length), but in this case were merely washed in running water, dried with absorbent paper, transferred to sterile water in a beaker, and blended for five min. Three successive dilutions were performed with sterile water (1:10) and the suspensions were plated on to SNA Petri dishes. After settling, the excess suspension layer was discarded, leaving the dish in a slanted position for one hour to remove the excess liquid. The fungus was isolated from the developed colonies on the agar after approximately one to two days. This method was used in difficult cases as concerns the isolation of *K. zeae, Phoma* spp. or *Fusarium* spp., caused by poor sporulation and growth or a high abundance of saprophytes.

c) Isolation from seeds

A seed lot sample of the commercial variety Kalvin was received in 2012 from Ostenfeld for examination of seedborne fungal pathogens. From the seed lot, 200 seeds were washed in running water, disinfected in a 2% sodium chloride (NaOCl) solution for 45 s and then rinsed in sterile water for another 45 s. Under sterile conditions on the clean bench, the seeds were dissected into two parts and placed on water agar (1%) plates. *In vitro* sporulation of fungal organisms was observed during the next nine days.

2.2.3. Preparation of single spore cultures

Single cultures were obtained for a number of isolates. This was done for several purposes, including inoculation in the greenhouse, storage, or, as in the case of *Phoma* spp., further DNA analysis. To obtain single spore cultures, two different methods were used depending on the feasibility of separating single spores:

a) Smearing out a spore

This method consists of placing a spore on transparent and selective poor nutrient agar, SNA or WA, using an inoculation needle. With the help of a stereo microscope, spores were selected, laid separately and marked. After germination of the spores, these were then transferred to a second dish on a suitable solid medium and grown under optimal conditions (see Table 5). This method was used for organisms with relatively large and pigmented spores such as *E. turcicum*, *Bipolaris* spp., *Alternaria* spp. and *Epiccocum nigrum*, among others.

b) Dilution plating

In this method, spores were transferred directly from plant material or obtained colonies through the Waring Blendor technique onto a solid medium, where, after *in vitro* growth of the

colony (up to Ø 0.5 mm), 1-2 ml sterile water was added to the colony. The colony was rinsed with water and the resulting spore suspension was plated on transparent agar (SNA) by streaking out with a Drigalski spatula to distribute single spores. After two to three days, the Petri dish was checked for the formation of monosporic colonies, which, if present, were removed and allocated to an adequate medium for the respective fungi. This method was used for relative small or hyaline spores of *K. zeae*, *C. graminicola, Phoma* spp. and *Fusarium* spp.

2.2.4. *In vitro* cultivation

To achieve *in vitro* sporulation, the selected organisms for the study were cultured on different agar media in accordance with the requirements of each fungus (Table 5).

Tab. 5. Media and conditions used for cultivating different fungi for the inventory.

Fungus	Medium	Temperature	Light/dark h regime
E. turcicum	V8	24°C	Dark
K. zeae	PDA/MA	24°C	12h/12 h.
B. zeicola	V8	24°C	UV-L or 12h/12 h.
C. graminicola	PDA	18°C-20°C	UV-L
***Phoma* spp.**	OA	20°C	UV-L and dark*
***Fusarium* spp.**	PDA/SNA	18°C-20°C	UV-L/24°C
***Alternaria* spp.**	PDA	24°C	12h/12h
Epiccocum nigrum.	WA	24°C	12h/12h

*For morphological analyses, both were necessary for comparisons according to Boerema et al. (2004).

2.2.5. Morphological identification of causal agents

The morphological parameters were analysed through macro and microscopy. Images were taken using a DFC 240® microscope camera (Leica, Ernst Leitz Wetzlar GmbH). The identification of the disease was based on leaf symptoms and on the morphological features of the causal agent following the respective keys in Table 6.

Tab. 6. Literature keys for the identification of frequently found fungi on maize leaves.

Target pathogen	Key
E. turcicum	Alcorn 1988; White 2010
B. zeicola	Alcorn 1988; Stankovic 2007; White 2010
K. zeae	Narita et Hirasuka 1959; Arny et al. 1970; Schneider & Krüger 1972
***Puccinia* spp.**	Savile 1984; White 2010
C. graminicola	White 2010
***Phoma* spp.**	Stout 1930; Boerema & Dorenbosch 1973; Punithalingam 1990; Aveskamp et al. 2010; Boerema et al. 2004; qBank Database 2012-2015
***Fusarium* spp.**	Leslie and Summerell 2006; White 2010
Other and saprophytes *(Alternaria* spp.*, Epiccocum* spp.*, Cladosporium* spp. ...)	Ellis 1971; Ellis 1976; White 2010; Watanabe 2010

In contrast to other maize leaf pathogens, a precise identification of *Phoma* species, which implies the recognition of several micromorphological features, was carried out. Based on data from Boerema et al. (2004), Aveskamp et al. (2010), and related summaries from the Q-Bank database, the following macroscopic and microscopic parameters were analysed to obtain more accurate descriptions:

Description of the colony

- *In vitro* growth rate (after seven days)
- Colour (according to the scale of Rayner 1970)
- Production and characterisation of mycelium

Microscopic observations

- Shape and size of the pycnidia (by averaging the measurements of 5-10 samples)
- Shape and size of the conidia (by averaging the measurements of 30 samples)
- Presence of chlamydospores/ multicellular chlamydospores (dyctiochlamydospores)

Unless otherwise indicated, the analysis was carried out after incubation of the plates for two weeks in complete darkness at 20-22°C. To guard against cases where these conditions did not produce pycnidia, a second plate was also incubated for each isolate with a UV light regime of 12/12 h darkness to stimulate the formation of pycnidia.

Furthermore, six *Phoma* isolates from the CBS culture collection (CBS-KNAW Central Biodiversity Center, Utrecht, the Netherlands) were compared morphologically with the isolated specimens (see Table 7). CBS freeze-dried isolates were obtained in lyophilized form and revived under incubation in OA medium. The selected isolates were: *Ph. glomerata*, *Ph. pomorum, Ph. sorghina*, *Ph. subherbarum* (two isolates) and *Ph. zeae-maydis.* The selection of these CBS isolates was based on their relation to *Phoma* species colonising maize in the literature.

Tab. 7. Isolates of *Phoma* species obtained from the CBS (Central Biodiversity Center, Utrecht, the Netherlands) for morphological comparisons with the isolated *Phoma* specimens in the monitoring.

***Phoma* spp. (syn.)**	**CBS Number**	**Origin**	**Selected after**
Ph. glomerata *(Didymella glomerata)*	528.66	Netherlands	Payak et al. 1987
Ph. pomorum *(Didymella pomorum)*	838.84	Germany	Sørensen et al. 2010
Ph. sorghina *(Epiccocum sorghinum)*	180.80	South Africa	Do Amaral et al. 2004
Ph. subherbarum *(Didymella subherbarum)*	250.49	Peru	De Gruyter et al. 1993
Ph. subherbarum *(Didymella subherbarum)*	249.49	Peru	De Gruyter et al. 1993
Ph. zeae-maydis *(Didymella maydis)*	588.69	USA, Wisconsin	Arny & Nelson 1971

2.2.6. Molecular identification of *Phoma* spp.

Due to the limitations of identification based on morphological characters, further molecular analysis were carried out for nine isolates (12.18; 12.20; 13.2P, 13.2C, 13.2B; 12.13; 12.19; 12.36; 12.37).

2.2.6.1. Obtaining of DNA from pure cultures

Pure cultures of the different fungi were grown on suitable solid medium at 24°C for 2 weeks. Liquid cultures were inoculated with a single mycelial plug (diameter approx. 0.5 cm) in 30 ml of Czapek-DOX (34 g/l) in 100 ml Erlenmeyer flasks. Cultures were incubated on a shaker at 20-22°C and 100 rpm for 14 to 21 days. After this period, the culture was harvested by filtration through a Büchner funnel with filter paper and using a water-jet pump. To prevent cross contamination, the funnel was disinfected between samples with 70% ethanol. The mycelium was washed with autoclaved water, freeze-dried for 48 h and stored in Falcon tubes with Parafilm at -20°C until DNA extraction was carried out.

For the DNA extraction of fungal species, the method described by Brandfass and Karlovsky (2008) was followed. Lyophilized dry material was ground into a fine powder with liquid nitrogen in a mortar. From the powdered mycelium, 30-40 mg were transferred to a 2 ml Eppendorf tube and kept cold in nitrogen. A 1 ml of CTAB buffer, 2 µl mercatoethanol and 1 µl proteinase K (1:10) were added. The tubes were placed in an ultrasonic cleaner for 5 s followed by incubation in a heated bath for 10 min at 42°C, and then another 10 min at 65°C. The suspensions were emulsified with 800 µl of chloroform:isoamylalcohol mixture (24:1), vortexed, and then cooled for 10 min on ice. Next, the tubes were centrifuged for 15 min at 13,000 rpm (9,500 xg) and 600 µl of the supernatant were collected and mixed with 194 µl PEG (30%) and 100 µl 5M NaCl. The solution was centrifuged for 15 min at 13,000 rpm (9,500 xg) and the supernatant was carefully discarded to avoid destroying the pellet. It was then washed with 800 µl ethanol (70% v/v). Thereafter, it was centrifuged for 2 min, while the last drops were discarded through pipetting and the sample was dried for 10 min at 30°C in a speed vacuum. The pellets were dissolved in 100 µl of TE buffer (1x), incubated for 30 min at room temperature and stored at -20°C. To remove possible RNA contamination from DNA templates, RNAse (1:10) was added to the samples and incubated at 37°C for 30 min.

2.2.6.2. Assessment of the obtained DNA yield and quality from cultures

In order to check the concentration of the obtained DNA, 7 µl of each sample were taken and pipetted in a microtest plate with 2 µl DNA Gel Loading Dye (6 X). The samples were loaded onto a 1.0 % TBE agarose gel. Electrophoresis was conducted at 3V/cm for 90 min in TBE buffer (0.5x), to which Midori Green was added. Finally, the samples were analysed using Phage Lambda DNA (Sigma-Aldrich Chemie, Steinheim) to estimate the DNA concentration under UV light.

2.2.6.3. Conditions for PCR assay

The 5.8S nrRNA gene with the two flanking internal transcribed spacers 1 and 2 were amplified with the generic primers ITS4 and ITS5 (White et al., 1990). Primer sets were obtained from Invitrogen™ Life Technologies GmbH (Darmstadt, Germany), resuspended with nuclease-free water and stored at -20°C.

Tab. 8. Primers used for the amplification of the internal transcribed spacers 1 & 2.

Primer	Primer Name	Primer Sequence	Amplified fragment size	Source
5’ primer	ITS 5	TCCTCCGCTTATTGATATGC	630 bp	White et al. 1990
3’ primer	ITS 4	GGAAGTAAAAGTCGTAACAAG		

Standard PCR amplification reactions (final volume of 25 µm) were performed containing nuclease-free water, 1 X Taq Buffer (contains a final $MgCl_2$ concentration of 1.5 mM), 0.2 mM of dNTPs, 1 µM of each primer, 2.5 units of Taq DNA Polymerase and 10 ng of template DNA. Gradient PCR reactions were performed on TProfessional basic Gradient Thermocycler (Biometra, Germany). To establish the melting temperature (T_m) of the specific primers, a range of temperatures above and below the calculated Tm (55-65°C) were tested simultaneously in a preliminary analysis for two isolates (12.10 & 12.18). The final selected assay conditions are shown in Table 9.

Tab. 9. Universal PCR cycling assay for generic primer set ITS4 and ITS5.

Step	Temperature	Time	Repetitions
Initialisation	94°C	5 min	
Denaturation	94°C	1 min	x 36
Annealing	61°C	1 min	
Elongation	72°C	1 min	
Final extension	72°C	10 min	

To avoid undesired proteins and inhibitors, PCR products were purified with the "QIAquick PCR Purification Kit" (Qiagen GmbH, Hilden, Germany) according to the instructions provided. Finally, the purified DNA was quantified through electrophoresis as described above.

2.2.6.4. DNA sequencing and analysis

DNA samples were sent for sequencing (Eurofins MWG operon, Ebersberg, Germany). The forward and reverse strands were sequenced with the same primers used in the PCR amplification (ITS 4 & ITS 5). The sequences obtained were analysed using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) Database[5]. Comparisons were carried out with those sequences registered with accession numbers of the Central Biodiversity Center (CBS-KNAW, Utrecht, the Netherlands).

2.2.6.5. Identification by the Fungal Biodiversity Center (CBS-KNAW)

For most of the *Phoma* isolates, DNA sequencing of the 5.8S nrRNA gene with the two flanking internal transcribed spacers 1 and 2 resulted in several species. In these cases, a concrete determination of the species was not possible for all isolates. Therefore, three representative isolates collected in 2012 and 2013 were sent to the CBS-KNAW for further identification. The procedure was based on the generation of the internal transcribed spacer 1 and 2 regions and partial sequences of the actin and translation elongation factor 1α gene, and a further comparison with CBS sequence databases.

[5] Database can be found here: http://blast.ncbi.nlm.nih.gov/Blast.cgi

2.2.7. Preservation techniques for fungal organisms

The conservation of the isolated fungal species should not only guarantee survival, but also virulence and capacity to produce a large amount of spores. These are necessary parameters for further *in vitro* and *in vivo* studies. Freshly grown fungal colonies (1-2 weeks old) were used. Different methods from the literature were selected, adapted for each different genus, and are explained in this section. In some cases, more than one method had to be used when the inefficacy of certain methods was discovered as work progressed, obligating a change of preservation method (section 4.1.3, Discussion). Parallel to the methods described here, representative isolates were dispensed in storage vials, prepared through freeze-drying (lyophilisation) and deposited for long-term preservation at 4°C at the Division of Plant Pathology and Plant Protection of the University of Göttingen.

a) Preservation with water and glycerol (25%)

Autoclaved water and glycerol (75:25) was added to the colony of the fungus grown on solid agar and the colony was crushed with a sterile glass rod. In order to remove remains of mycelia, the suspension was poured onto a sterile cheese cloth inserted into a funnel, which was inside a 50 ml tube. 1 ml of the spore suspension was pipetted in 1.5 µl Eppendorf tubes and stored at -20°C. This method was used for *Phoma* spp.

b) Preservation in silica gel granules with skim milk solution (in accordance with Perkins 1962; Vaillancourt Laboratory 1995 modified from Tuite & Lutrell 1969)

Due to non-survival or a low survival rate of *K. zeae*, *B. zeicola* and *C. graminicola* spores after storage with autoclaved water and glycerol (25%), this second method was used. Given the absence of moisture, the use of a solid medium like silica gel granules prevents all fungal growth and metabolism. Silica gel granules (1-2.3 mm; 10-18 mesh without indicator) were sterilised by heating for two hours in a drying oven at 160°C and cooled to 4°C thereafter. Screw cap tubes of 10 ml were filled with approx. 4 g of the sterile silica gel granules. A 7.5% (v:v) solution of dry powdered skim milk was prepared with autoclaved water, autoclaved and cooled to 4°C. 30 min before use, as well as during usage, the tubes with silica granules were placed in an ice bath. Freshly produced spores were collected by following the same procedure used for the prior method (water + glycerol), but on this occasion the culture was washed with the prepared 7.5% skim milk suspension. From the obtained skim milk spore suspension, 250 µl were pipetted into the silica granules and the mixture was placed immediately onto a vortex mixer adding another 250 µl of skim milk. The caps were sealed with Parafilm, left in an ice bath for approx. 30 min, and stored at 4°C. To check viability, a few silica granules from selected isolates were sprinkled onto a suitable agar medium after

two weeks. This sprinkling process was repeated regularly thereafter to check for viability of the spores.

c) Storage on sterile filter paper (adapted from Formento 2015, pers. comm., 23 February)

Parallel to the silica gel method, an alternative method was used because some *K. zeae* and *B. zeicola* isolates could not be obtained from the silica gel granules after several months and sprinkles. Following the method of Formento (2015) for some fungal pathogens on maize leaves, initially autoclaved filter papers were placed directly on medium next to a growing fungal colony until they were overgrown by the mycelium of the respective fungi. Due to the fact that pieces of mycelium are also stored through this procedure, a second variant was also used. In this case, the filter papers were placed into 1.5 ml Eppendorf tubes (6 filter papers/tube) and autoclaved. Freshly produced spores were collected through suspension of the colony in autoclaved water and crushing it with a glass rod. In order to remove remains of mycelia, the suspension was poured onto a sterile cheese cloth inserted into a funnel, which was inside a 50 ml tube. From the obtained spore suspension in the tube, 50 µl were pipetted into the filter papers, vortexed, dried and stored at -20°C. Due to the capability of storing spores for a long period of time, enhanced accuracy, low material costs and a less time-consuming procedure, this method was also practical for common saprophytes like *Alternaria* spp. and *E. nigrum*.

2.2.8. Production of inoculum

Spores of the various fungal species served as inoculum for greenhouse experiments. *Bipolaris* spp., *C. graminicola, Phoma* spp., *Fusarium* spp., *Alternaria* spp. and *E. nigrum* isolates were grown on suitable solid medium and under optimal conditions (Table 5). The mycelium was suspended in a water solution with the surfactant agent Silwet® Gold (0.0125%), which facilitated moistening and spreading of the spore solution onto the leaf by reducing the surface tension. After crushing the mycelium with a glass rod, freshly produced spore suspensions were collected and in order to remove remains of mycelia, the suspension was poured onto a sterile cheese cloth inserted into a funnel, which was inside a 50 ml tube.

Spores were counted under the microscope (20x- 40x magnification) with a Thoma-counting hemacytometer for *Phoma* spp., and a Fuchs-Rosenthal hemacytometer for all other fungal organisms. Spore suspensions were adjusted to the final spore concentration shown in Tables 10-11 (section 2.2.9) depending on the organism and isolate.

Production of inoculum for *K. zeae*

Due to the slow growth of some *K. zeae* isolates and its limited ability to produce spores on agar, a liquid medium known as *K. zeae* medium (Reifschneider and Arny 1979) was used first. Flasks (1 L) were filled with 500 ml of the *K. zeae* liquid medium and each flask was inoculated with four-day-old colony plugs and placed on the shaker at 100 rpm for seven to 10 days at room temperature (approx. 18-20°C). Spore suspensions were then calculated and adjusted by adding water to achieve the required densities (Reifschneider and Arny 1979).

Due to the dissimilarity between isolates to produce spores, a modification of this method with an improved step provided by Algermissen (2014, pers. comm., 24 March) was used for the production of large and uniform amounts of *K. zeae* spores. Here, one agar colony plug was inoculated into 30 ml of *Kabatiella zeae* liquid medium described by Reifschneider and Arny (1979) and incubated for four days. From here, 400 µl of the liquid medium were pipetted onto malt agar plates, evenly distributed with a Drigalski spatula, and incubated at 24°C. After four days, a high amount of *K. zeae* colonies with masses of spores had already formed in the plates. The Petri dish was suspended in a water solution with the surfactant agent Silwet Gold (0.0125%). After crushing the mycelium with a glass rod, freshly produced spore suspensions were collected and, in order to remove remains of mycelia, the suspension was poured onto a sterile cheese cloth inserted into a funnel, which was inside a 50 ml tube.

2.2.9. Evaluation of pathogenicity in the greenhouse

Pathogenicity tests were carried out in the greenhouses of the Division of Plant Pathology and Plant Protection of the Georg-August-University Göttingen. The orientation of the greenhouses is north-south and the benches inside have an east-west orientation. The greenhouses consist of side and roof ventilation systems and can be ventilated and heated automatically. The day/night light regime (14/10h) was automatically controlled in the greenhouse in winter through sodium vapour lamps (Philips SON-T Plus 400 watt 20 klx).

For a number of isolates of less known diseases in Central Europe, namely *K. zeae*, *B. zeicola*, *C. graminicola*, *Phoma* species, *Fusarium* spp., *Alternaria* spp., and *E. nigrum* (see Tables 10-11), pathogenicity tests were conducted by inoculating healthy plants with spore suspensions prepared from single-spore cultures in order to fulfil Koch's postulates.

Maize varieties Ricardinio and Barros were selected because they show a reasonable level of susceptibility to some maize leaf diseases. This is based on personal observations from field visits made during the monitoring. In the greenhouse, plants were sown (one seed/pot)

in pots (11x11 cm) containing a mixture made up of one part of sand, one part of compost and one part of standard substrate with pea and clay (Einheitserdewerke Werkverband e.V., Sinntal-Altengronau). The mixture was then covered with a small layer of sand. As nutrient fertiliser, Hakaphos® (15% N, 15% K_2O, 11% P_2O, 1% MgO) was applied at a concentration of 4 g/l.

Maize seedlings were inoculated with spore solutions using a pump sprayer once the fifth or sixth leaf had unfolded (5^{th}-6^{th} leaf stage). This was usually four to five weeks after sowing. The control was inoculated with water and the surfactant agent (Silwet Gold 0.0125%). Each fungal isolate treatment consisted of three replications for every maize variety. The replications were fully randomised and placed in a humidity chamber, maintaining a high level of humidity and moisture by spraying the chamber and the leaves with water. After three days, the plants were removed and placed on greenhouse benches.

Due to the extended range of *Phoma* species and current lack of knowledge about them, a second pathogenicity test was conducted. Plants were incubated in a climate chamber at a controlled temperature and relative humidity of 20°C and 90%, respectively.

Tab. 10. Isolates of different fungi tested for pathogenicity in the greenhouse and concentrations (spores/ml) used.

Organism	Isolate	Location	Spores/ml
K. zeae	12.10	Braunau am Inn	$5x10^5$
	12.11	Köhn (Plön)	$5x10^5$
	12.13	Stapel	$1x10^5$
	12.14	Ommen	$50x10^3$
	12.17	Mintraching	$25x10^3$
	12.24	Beesten	$5x10^5$
	12.28	Groningen	$2x10^5$-$5x10^5$
	12.30	Biddinghausen	$3x10^5$
	12.31	Giekau	$3x10^5$
***Bipolaris* spp.**	12.2S	Ostenfeld	$1x10^6$
	12.7	Bylany	$1x10^6$
	12.8	Caslav	$1x10^6$
	12.9	Plana	$1x10^6$
	12.18	Schönering	$1x10^6$
	12.20	Nordholz	$1x10^6$
	12.27	Bad Oldesloe	$1x10^6$
C. graminicola	12.15	Princepeel	$1x10^6$
	12.22	Moorlas	$1x10^6$
***Phoma* spp.**	12.2	Ostenfeld	$3.8x10^6$-$1x10^7$
	13.2d	Ostenfeld	$1x10^7$
	13.2b	Ostenfeld	$1x10^7$
	13.2.1	Ostenfeld	$1x10^7$
	13.2P	Ostenfeld	$1x10^7$
	13.2B	Ostenfeld	$1.1x10^6$-$4.5x10^6$
	13.2C	Ostenfeld	$1x10^7$
	13.2.1	Ostenfeld	$1x10^7$
	12.10	Braunau	$1x10^7$
	12.12	Schleswig	$1x10^7$
	12.13	Stapel	$1x10^7$
	12.18	Schönering	$5x10^6$-$1x10^7$
	12.19	Cloppenburg	$1x10^7$
	12.20	Nordholz	$1x10^7$
	12.23	Rade	$1x10^7$
	12.23.2	Rade	$2x10^6$-$4x10^6$
	12.27	Bad Oldesloe	$1x10^7$
	12.28	Groningen	$1x10^7$
	12.31	Giekau	$1x10^7$
	13.20	Nordholz	$1x10^7$
	13.36	Mittich	$1x10^7$
	13.37	Hartkirchen	$1x10^7$
	13.47	Kassel	$1x10^7$
	13.48	Kassel	$1x10^7$
	14.51	Offenburg*	$65x10^4$-$2.5x10^6$
***Phoma* spp. CBS**	*Ph. Glomerata*	528.66	$1x10^7$
	Ph. Pomorum	838.84	$1x10^7$
	Ph. Sorghina	180.80	$6x10^4$-$7x10^4$
	Ph. Subherbarum	249.92	$1x10^7$
	Ph. Subherbarum	250.92	$1x10^7$
	Ph. zeae-maydis	588.69	$17x10^4$-$75x10^4$
***Alternaria* spp.**	14.50*	Inzing	$5x10^4$
	14.51*	Offenburg*	$1x10^4$
***Epiccocum* spp.**	14.51*	Offenburg*	$2x10^4$-$6x10^4$

* Received sample from 2014 not belonging to the monitoring of 2012/2013

Tab. 11. Isolates of *Fusarium* spp. tested for pathogenicity in the greenhouse and concentrations (spores/ml) used.

Organism	Isolate	Location	Spores/ml
***Fusarium* spp.**	12.2S	Ostenfeld	$1x10^6$
	13.2	Ostenfeld	$1x10^6$
	12.8	Caslav	$1x10^7$
	12.10	Braunau	$1x10^6$-$1x10^7$
	12.12	Schleswig	$1x10^6$
	12.13	Stapel	10^5
	12.14	Ommen	$8.5x10^5$
	12.19	Cloppenburg	$8.5x10^5$
	12.23	Rade	$1x10^6$
	12.27	Bad Oldesloe	$1x10^6$
	12.30	Biddinghuizen	$1x10^6$
	12.31	Giekau	$1x10^6$

The evaluation of pathogenicity of the isolates on maize was carried out 14 days after inoculation, on the fourth, fifth and sixth unfolded leaves (BBCH 15-16). The visual scale used was adapted for each pathogen depending on the development of the lesions (Tables 12 & 13).

Tab. 12. Disease rating scale adapted for maize seedling plants inoculated with *B. zeicola, C. graminicola* and *K. zeae*.

Grade	Pathogen	Symptoms	Reaction
0	*B. zeicola*, *C. graminicola, K. zeae*	No symptoms were observed	--
1	*B. zeicola*, *C. graminicola, K. zeae*	Slight chlorosis <5% of the leaf area	Slight
2	*B. zeicola*, *C. graminicola, K. zeae*	Chlorosis spreading >5% leaf area	Moderate
3	*B. zeicola*, *C. graminicola K. zeae*	Chlorosis develops to dark spots/ necrosis Chlorosis develops a central brown ring	Moderate
4	*B. zeicola*, *C. graminicola K. zeae*	Chlorosis expansion with dark spots/necrosis Chlorosis/ brown rings develop in patches	Moderate
5-6	*B. zeicola, C. graminicola K.zeae*	Necrosis/ dark spots prevail Necrotic lesions coalesce Leaf begins with senescence	Moderate-Severe
7-8	*B. zeicola, C. graminicola K.zeae*	Leaf begins with senescence Leaf senescence	Severe
8-9	*B. zeicola, C. graminicola K.zeae*	Leaf has died	Severe

Tab. 13. Disease rating scale for seedling plants inoculated with *Phoma* spp.

Grade	Symptoms	Reaction
0	No symptoms were observed	--
1	Slight chlorosis < 5% leaf area	Slight
2	Chlorosis spreading >5% leaf area	Moderate
3	Chlorosis develops to brown spots	Moderate
4	Spreading of the brown spots: - Typical spots red-brownish, dispersed; (Typical early disease symptoms) >5% leaf area	Moderate-severe
5	Spot expansion	Moderate-severe
6	Lesions coalesce	Severe
7	Beginning of leaf senescence	Severe
8	Leaf senescence	Severe
9	Leaf has died	Severe

2.2.10. Re-isolation of the pathogen from the infected tissue

To confirm the fungal organism as the causal agent of the disease produced, the same fungal organism has to be recovered from the infected leaf tissue of the inoculated maize plant. Therefore, infected leaf samples were collected from the inoculated plant in the greenhouse, and disinfected and incubated in SNA agar plates at room temperature for sporulation. The leaf samples were examined after one to two days by using the microscope to confirm the presence of the pathogen.

2.3. Field locations for spore trapping and fungicide application studies

2.3.1. Locations for fungicide trials

Fungicide trials for the main pathogen *E. turcicum* were carried out in 2013 in the municipality of Mittich (48°4485’ N 13°394013’ E) and in 2014 in the municipality of Inzing (48°410126’ N, 13°407447’ E), which are separated by a geodesic distance of approximately four kilometres. The reason for the change in the location is that after two to three years of designing experiments in a particular field, the intermediate pathways created for access to the plots (and rows) can still be distinguished, which can have a negative impact on subsequent trials. Both municipalities are situated in the community of Pocking, an area in the southeast of Bavaria (Passau district) through which the river Inn flows. This alpine foreland region is characterised by a humid continental climate, with cold winters and hot, rainy summers. The average annual precipitation in the region is 769 mm, while the average annual temperature is 9.2°C (1991-2015). The hottest and at the same time rainiest months

are June, July and August (average over 1981-2010 period). These conditions facilitate a natural pressure of *E. turcicum* in these locations.

For the second main pathogen *K. zeae,* fungicide trials were conducted in 2013 and 2014 at the Experimental Station Lindenhof (54°318843'N, 9°801423'E), which belongs to the Department of Agricultural Sciences of the University of Applied Sciencies of Kiel. The station is located in the municipality of Ostenfeld (district of Regensburg–Eckernförde) in the northern region of Schleswig-Holstein. In this location, a high natural pressure of *K. zeae* has been observed in recent years. The region is characterised by mild winters and temperate summers. The average annual precipitation is 826 mm, while the average annual temperature is 8.8°C. The hottest and rainiest month is July. The area is also prone to strong winds. Further information about the characteristics of the locations is provided in Table 14.

Tab. 14. Soil and geographical parameters of the trials in Bavaria and Schleswig-Holstein.

Parameter	Location		
	Mittich	**Inzing**	**Ostenfeld**
Soil order	Clay-loam	Sandy-loam	Loamy-sand
Soil quality index	65	65	50
Soil type	Luvisol	Luvisol	Luvisol
Altitude above mean sea level	319 m	309 m	14 m
Nearest geodesic distance to a river	2.4 km (Inn River) 374 m (Rott: Inn's secondary river)	1 km (Inn River) 4.5 km (Rott: Inn's secondary river)	- 7.8 km (North Sea Canal) 30.5 km (Baltic Sea)

Sowing and Field Management

The agronomical measures for field preparation and sowing were carried out according to the practical standards relevant to each region by the personnel of the cooperating institutions (FH Kiel in Ostenfeld (Lindenhof), and Syngenta GmbH and Hetterich field trials in Inzing and Mittich). For each location and season, these standards are provided in detail in Table 15 and Appendix Tables A1 and A2. In order to achieve a higher pressure of the disease, conservation tillage or maize cultivation in the previous season was preferred where possible; if, for example, other agricultural measures were not required to reduce other damaging factors such as the overwintering survival of the larvae of *Ostrinia nubilalis* in the southern German locations of Mittich and Inzing.

The maize hybrids selected for the studies are varieties which are commonly used in the locations and surrounding region. In the southern locations, the early-maturing grain variety Zidane (K240) and the late-maturing variety NK Silotop (S270) were used in 2013 (Mittich)

and 2014 (Inzing), respectively. Regarding resistance to *E. turcicum*, Zidane and NK Silotop are classified as “susceptible” and “moderately resistant”, respectively (LWK NW 2012; Hiltbrunner et al. 2015). In Ostenfeld, the silage middle early-maturing varieties Ronaldinio (S240) and Kalvin (S200) were sown. These varieties have been observed as being moderately susceptible to the disease in some northern fields (Schlüter 2012; personal observation).

Tab. 15. Sowing and management in test fields.

Location	Mittich (2013)	Inzing (2014)	Ostenfeld (2013)	Ostenfeld (2014)
Maize variety	Zidane®	NK Silotop®	Ronaldinio®	SY Kalvin®
Sowing date	25.04.2013	22.04.2014	22.04.2013	16.05.2014
Seeding rate	9 seeds/m^2	9 seeds/m^2	10 seeds/m^2	10 seeds/m^2
Soil preparation	Conventional tillage/ plough	Conventional tillage/plough	Conventional tillage/plough	Minimal tillage
Soil preparation Mechanical method	Rotary harrow	Rotary harrow	Rotary harrow	Rotary harrow
Debris management	debris worked with flail mower and grubber	debris worked with flail mower and grubber	Rototiller in autumn Grubber	Rototiller in autumn Grubber
Crop rotation	Maize (2010) Maize (2011) Winter wheat-mustard (2012) Maize (2013)	Maize (2010, 2011) Winter wheat- under sown crops (2012) Maize (2013) Maize (2014)	Maize (since 2007)	Maize (since 2007)

2.3.2. Locations for spore trapping

Spore traps were placed in the fields of the fungicide trials in Inzing in 2014 for the detection of *E. turcicum,* as well as in Lindenhof in 2013 and 2014 for *K. zeae* (1 spore trap/location). In 2015, further epidemiological studies through spore traps were suspended due to the absence of the fungal disease in Ostenfeld, while in Inzing the trial was damaged by drought.

Therefore, a third location, which forms part of the "Göttinger Miniplot Soil Warming Experiment" (51°5578'N, 9° 9519'E) at the Division of Plant Pathology and Plant Protection at the Georg-August-University Göttingen was used to analyse the development of both diseases. This was done through artificial inoculation of maize plants as part of a related bachelor project[6]. In this experiment, plants were inoculated at the end of tasseling (BBCH 59-63) and plots were covered with plastic, providing a saturated atmosphere (100% RH) to favour the initial establishment of the pathogen in the plant.

This third location is characterised by moderately cold winters and temperate summers with rainy periods. In the summer period, both the hottest (July - average temperature of 17°C) and the rainiest month coincide (June - average precipitation of 81 mm)[7].

Tab. 16. Soil and geographical parameters in Göttingen ("Miniplot" experimental site).

Parameter	Göttingen
Soil order	Loamy- sand
Soil quality index	80
Altitude above mean sea level	150
Nearest geodesic distance to a river	2.6 km (Leine)

[6] "Relationship between Infection and Yield Losses for *E. turcicum* and *K. zeae".* Bachelor thesis project Jakob Schnackenberg. Division of Plant Pathology and Plant Protection. Georg-August-University Göttingen. Experimental trials from July to September 2015.
[7] Information from Wetterstation Göttingen: http://www.wetterstation-goettingen.de/klimabericht.html

2.4. Epidemiological studies based on spore trapping in the field

To investigate the dynamic of the main fungal foliar pathogens, epidemiological studies of *E. turcicum* and *K. zeae* were carried out based on their air dispersal pattern. Therefore, a Burkard 7-day spore-recording volumetric trap (Burkard Manufacturing Co. Rickmansworth, UK) was used to monitor the airborne inoculum.

2.4.1. Trapping season

Spore samples were collected during the maize crop season in summer and at the beginning of autumn when epidemics of *K. zeae* and *E. turcicum* occur and spores of both pathogens are released. For *K. zeae* in Ostenfeld, the period under investigation lasted from 16 July to 1 October in 2013, and from 30 June to 5 October in 2014. For the examination of *E. turcicum* in Inzing, samples were collected from 8 July until 6 October 2014, with the exception of 23 September due to the malfunction of the trap. In Göttingen, samples from both pathogens were collected from 15 July until 24 September 2015, except on 29 July and 20 August due to malfunction of the trap.

2.4.2. Air sampling and analysis via microscopy

Spore traps were placed approx. 120 m from the studied field in Inzing and approx. 10 m from the nearest maize field in Ostenfeld. In Göttingen, due to the space restrictions in the “Miniplot” site, the spore trap had to be situated at a distance of 5 m from the trial. The spore trap was oriented in the direction of the prevailing winds in each location. Once set up, the spore trap absorbs air particles (10 l min^{-1}) at 1 m above ground level through a small orifice with the assistance of a motor attached to the bottom of the trap. The energy for the motor was provided by weekly battery changes. The absorbed spores are captured on a cellophane tape, the surface of which is coated with a mixture of vaseline and hexan (40:40). The tape is secured in a metal drum with a fixed circumference, which rotates in a 7-day cycle using a timer. This allows for specific measurement of the day the spores were captured on the tape, which was changed upon the completion of each 7-day cycle. The upper part of the spore trap consists of a vane, which permits the trap, and simultaneously the orifice absorbing the spores, to move with the wind.

The cellophane tapes were carefully removed from the drum of the spore trap each week and divided into seven daily segments. Tapes for microscopy examination were stored at 4°C to preserve spore morphology and avoid germination or degradation. Each slide (representing 24 hours) was mounted on microscope slides (76 x 26 mm) and the daily

number of spores captured was examined microscopically at 20x to 40x magnification. In order to obtain the most accurate results, the total number of conidia on the tape surface was counted.

2.4.3. Spore release, development of the disease and weather conditions

For the correlation between Kabatiella eyespot and Turcicum leaf blight disease development and the obtained data from the spore trap, the progression of the percentage of infected leaf area from the non-treated control of the fungicide trials in Ostenfeld and Inzing was compared. Rating scales and number of assessments for Inzing and Ostenfeld are described in detail in section 2.6.2. In Göttingen, the progression of the percentage of infected leaf area was obtained as part of the aforementioned bachelor project (section 2.3.2). In this location, due to the fast development of the diseases, four assessments were carried out: the vegetative stage (BBCH 39), end of flowering development of the grain (69-71), end of development of the fruit (75-79), and ripening (BBCH 87-89). Spore release data were correlated with climatic parameters (section 2.6.4).

2.5. Coupling spore trapping with PCR and qPCR assays

After a visual count of the number of spores via microscope, the samples were extracted from the vaseline following the procedure for air samples published by Kaczmarek et al. (2009) for *Leptosphaeria maculans* (pathogen in oilseed rape) with some modifications. The detection through real time qPCR was first adapted, optimised and finally validated for the detection of *K. zeae* and *E. turcicum* DNA. The PCR method was used as part of the specificity test and to check for a high amplified quantity of organic material (see section 2.5.6. and 2.5.8., respectively).

2.5.1. DNA extractions from the spore trap tape

Each daily section of the tape was cut into two parts and introduced into a 2 ml screw-capped tube filled with lysing glass beads (300-400 mg). 500 µl of CTAB buffer, 2 µl mercaptoethanol and 1µl proteinase K (1:10) were added to the sample tubes with the beads and sample tapes. The mixture was prepared using the FastPrep-24 Instrument®, which breaks the cells down through a multidirectional, continuous beating in order to obtain their cellular content. The samples were set to six cycles of 30 s at a velocity of 6 m s^{-1} until most of the spore cells lyse. This step was carried out after checking for spore remains with the microscope. To avoid overheating of the biological material, the samples were incubated in

ice for 30 s between repetitions. The suspension was incubated for 30 min at 70°C (mixed for 10 min each), emulsified with an equal volume of a chloroform:isomylalcohol mixture (24:1), vortexed, and chilled for 10 min on ice. Next it was centrifuged for 15 min at 12,000 rpm (8,050 xg) and the supernatant was collected and mixed with a double amount of absolute ethanol (96-99% v/v) and 0.1 volume of sodium acetate (3 M, pH 5). The solution was incubated for 1 h at -20°C centrifuged for 15 min at 13,000 rpm (9,500 xg) thereafter. The supernatant was carefully discarded to avoid destroying the pellet, which was washed with ice-cold ethanol (70% v/v) and 3 ul of the coprecipitant Glycoblue (1:3). It was then centrifuged for 2 min to separate the DNA, and the last drops were discarded by pipetting and the sample was dried for 10 min at 30°C in a speed vacuum. The pellets were dissolved in 100 ul of 1 x TE buffer, incubated for 30 min at room temperature and stored at -20°C.

2.5.2. Specific primer sets for amplification

PCR and qPCR assays were carried out with specific primers which are derived from the Internal Transcribed Spacer (ITS). The primer set JB 585/JB 586 for *E. turcicum* and JB 616/ JB 618 for *K. zeae* (Table 17) amplify a specific DNA target region within the target pathogen (Beck 1997). Primer sets were obtained from Invitrogen™ Life Technologies GmbH (Darmstadt, Germany), resuspended with nuclease-free water and stored at -20°C.

Tab. 17. Specific primer set information used for the amplification of *E. turcicum* and *K. zeae* extracts.

Fungal Organism	Primer Name	Primer Sequence	Amplified fragment size	Source
E. turcicum 5' primer	JB 586	5'TGGCAATCAGTGCTCTGCTG3'	485bp	Beck 1997
E. turcicum 3' primer	JB 595	5'TCCGAGGTCAAAATGTGAGAG3'		
K. zeae 5' primer	JB 616	5'TGTTGTTAAAACTACCTTGTTGC3'	455bp	Beck 1997
K.zeae 3' primer	JB 618	5' GTTTCTGTCGGCAGAAGTC3'		

Additionally, due to the low number of positive amplified samples for the analysis of *K. zeae* from Ostenfeld, DNA samples from this location (2013 and 2014) were checked for DNA quality via PCR with the generic primers ITS4 and ITS5 described in section 2.2.6.3. Therefore, unsuccessful DNA extraction could be excluded.

2.5.3. Conditions for PCR assay

Standard PCR amplification reactions were performed as described in section 2.2.6.3. using thermal cycling conditions shown in Table 18. These conditions were selected according to the instructions provided. To establish the melting temperature (T_m) of the specific primers, a range of temperatures above and below the calculated Tm (55-65°C) were tested simultaneously in a preliminary analysis for *E. turcicum* and *K. zeae* primer sets. The annealing temperature was finally set after testing non-target fungal organisms. This was done in a way which reduced possible non-specific amplifications. The final selected assay conditions are shown in Table 18. To check DNA quality with the generic primers ITS4 and ITS5, a universal fungal PCR cycling assay established in the laboratory was used (Table 19).

Tab. 18. PCR conditions used for the amplification of DNA with the specific primer sets JB 585/JB 586 for *E. turcicum* and JB 616/JB 618 for *K. zeae*.

Step	Temperature	Time	Repetitions
Initialisation	94°C	5 min	
Denaturation	94°C	30 s	x 35
Annealing *E.turcicum*/ *K. zeae*	64.°C/65.2°C	45 s	
Elongation	72°C	45 s	
Final extension	72°C	10 min	

Tab. 19. Universal PCR cycling assay for generic primer set ITS4 and ITS5 used to check DNA quality of the spore trap tape samples.

Step	Temperature	Time	Repetitions
Initialisation	95°C	5 min	
Denaturation	95°C	1 min	x 36
Annealing	57°C	1 min	
Elongation	72°C	1 min	
Final extension	72°C	10 min	

2.5.4. Assessment of the obtained DNA yield from tapes via PCR

In order to check the amplified DNA size through PCR, 5 µl of each sample were taken and pipetted in a microtest plate with 2 µl DNA Gel Loading Dye (6 X). The samples were loaded onto a 1% TBE agarose gel, to which Midori Green (6 %) was added. Electrophoresis was conducted at 3V/cm for 300 min in TBE buffer (0.5x). Finally, the samples were analysed using a 100 bp molecular weight marker (Nippon Genetics GmbH, Dueren) to estimate the concentration, and the DNA concentration was evaluated under UV light.

2.5.5. Conditions for qPCR assay

Standard 10 µl qPCR amplification reactions were performed with the 2x SensiFAST SYBR® Lo-ROX Kit (5 µl) (Bioline, Luckenwalde), nuclease free water (3.2 µl), 0.4 µM of each primer (0.40 µl), and 10 ng of template DNA (1 µl). RT-qPCR reactions were performed on the RT-PCR System CFX 384 Touch™ C1000 Detection System (Bio-Rad, Hercules, CA, USA) using thermal cycling conditions, shown in Table 20.

Tab. 20. qPCR cycling protocol for specific primers JB 585/JB 586 for *E. turcicum* and JB 616/JB 618 for *K. zeae*

Step	Temperature	Time	Repetitions
Initialisation	95°C	2 min	
Denaturation	95°C	5 sec	x 34
Annealing	68°C	25 sec	
Elongation	72°C	30 sec	

If the amplifications were above the established baseline threshold and the melting temperature curve coincided with the pure *E. turcicum* melting temperature curve, samples were considered as positive.

2.5.6. Primer specificity evaluation

The specificity of the selected primers was checked during the development of this method. This is important in order to guarantee the exclusive amplification of the targeted sequence of DNA from the respective pathogens *E. turcicum* and *K. zeae.* Thus, to evaluate the specificity of the primers used for *E. turcicum* and *K. zeae*, pure cultures of 13 other fungi (Table 21) were tested. These organisms were selected due to their airborne dispersal and presence in European maize fields, as well as in surrounding field crops, or because they are genetically related to the target species being investigated. The organisms were obtained from the samples collected during the monitoring carried out as part of this project (section 2.2), as well as from collections of the Division of Plant Pathology and Plant Protection of the University of Göttingen and the CBS-KNAW in Utrecht.

Tab. 21. Target and non-target fungal organisms used for PCR specificity tests with JB 585/JB 586 (*E. turcicum*) and JB 616/JB 618 (*K. zeae*) primer set.

Fungal organism	Obtained from	Id. code	Medium
E. turcicum (target organism)	Monitoring	13.10	V8
K. zeae (target organism)	Monitoring	12.31	MA
Alternaria spp.	Monitoring	13.10	PDA
Aspergillus spp.	APP	0006	PDA
Bipolaris sorokiniana	APP	3198	V8
Bipolaris zeicola	Monitoring	13.2	V8
Botrytis cinerea	APP	--	--*
Cladosporium spp.	Monitoring	13.10	PDA
Epicoccum nigrum	Monitoring	13.10	SNA
Fusarium graminearum	Monitoring	12.12	PDA
*Kabatiella caulivora****	CBS-KNAW	242.64	MA
Phoma lingam	APP	C40	--**
Penicillium spp.	APP	0282	PDA
Septoria tritici	APP	0606	YE+ME
Trichoderma spp.	APP	0320	OA

*Culture was obtained in frozen form from the Division of Plant Pathology and Plant Protection of the University of Göttingen (APP). **DNA was obtained directly from the Division of Plant Pathology and Plant Protection of the University of Göttingen (APP). ***only tested for *K. zeae.*

The obtention of DNA from pure cultures of the different fungi was carried out through the method described in section 2.2.6.1. Further assessment through electrophoresis of the obtained DNA yield and quality is described in section 2.2.6.2.

The 13 fungi species were tested by PCR under the same conditions described in section 2.5.3, but with annealing temperatures ranging from 60 to 68°C. With the selected melting temperature (T_m) from the PCR assay, a range of temperatures above it were tested through qPCR in order to avoid possible non-target amplifications. Parallel to the check for possible amplifications with specific primer sets JB 585/JB 586 (*E. turcicum*) and JB 616/JB 618 (*K. zeae*), DNA samples of the 13 fungi were checked for DNA quality via PCR with the generic primers ITS4 and ITS5 mentioned above.

The qPCR assay was performed as described in section 2.5.5 with one of the non-target fungal organisms, namely *Alternaria* spp. for *E.turcicum* primers, and *K. caulivora* for *K. zeae* primers. These non-target fungal organisms were selected because they both resulted in more complications in terms of non-specific amplifications during the PCR test for specificity at lower temperatures or at the same selected annealing temperature. Through qPCR, with the selected primers for *E. turcicum* (JB 585/JB 586), dilutions of *Alternaria* spp. of 1, 10 and

100 pg μl^{-1} were tested, while for the selected primer of *K. zeae* (JB 616/JB 618) the dilutions were 10 fg, 1 and 10 pg μl^{-1}. The final selected assay conditions are shown in Table 20 above. Unexpected signals were investigated through DNA electrophoresis.

2.5.7. Primer sensitivity evaluation

To obtain quantification standard curves and determine the limit of detection through qPCR, a series of diluted genomic DNA standards (100 pg μl^{-1},10 pg μl^{-1}, 1 pg μl^{-1}, 100 fg μl^{-1}, 10 fg μl^{-1},) was generated from pure mycelia of *E. turcicum* and *K. zeae* using the software Multi-Analyst (Bio-Rad, Hercules, CA, USA).

Furthermore, the qPCR assay was tested for detection of spore trap samples with artificially inoculated spore tapes. A suspension from pure *E. turcicum* agar cultures and autoclaved water was prepared. Suspension drops were pipetted onto fresh vaseline plastic tapes equal in size to those used in the spore trap in the field. The final number of spores per tape was then counted via microscope (1 to 330 spores per slide). DNA extraction from tape samples and evaluation through PCR and qPCR assay were carried out as described above (sections 2.5.1 to 2.5.5).

Due to the difficulty to detect *K. zeae* spores on the vaseline tape via microscope, a series of spore suspensions from pure *K. zeae* agar cultures was prepared. The dilution range consisted of 1,000, 500, 250, 125, 62, 31 to 15 spores in a total pipetted volume of 10 µl per tape. This total volume was inoculated onto the freshly prepared vaseline tapes, divided into two drops of 5 µl. Three repetitions were done for each sample. DNA from the vaseline tape was extracted from all samples following the procedure for DNA extraction described in section 2.5.1.

2.5.8. Dilutions of DNA yield as template

A high quantity of organic matrix material can inhibit the amplification of DNA through qPCR (McDewitt et al. 2007; Kaczmarek et al. 2009). Therefore, samples were first amplified through PCR. When amplified samples with the specific primer sets for *E. turcicum* or *K. zeae* showed prominent bands of DNA on agarose gel, the samples were diluted (1:4).

2.6. Control of the main diseases through fungicides in the field

To evaluate fungicides as an effective method against the main leaf diseases, i.e. Turcicum leaf blight, common rust and Kabatiella eyespot, the development of the infected leaf area was analysed under the effect of different fungicide applications at different stages. Kabatiella eyespot was evaluated in Ostenfeld and Turcicum leaf blight in Mittich and Inzing in 2013 and 2014, respectively. Due to the higher pressure of *P. sorghi* (common rust) in Mittich in 2013, this disease was also evaluated.

2.6.1. Treatment design and fungicide application

The experimental units were rectangular plots with four repetitions for each treatment and fully randomised (Table 22). Due to the placement of the experiment and in order to complete the randomised block, both the number of repetitions and blocks could vary between four and five in Ostenfeld depending on the treatment. The individual plot measurements were 7 m (length) x 3 m (width) for Ostenfeld, 12 m x 3 m for Mittich, and 10 m x 3 m for Inzing. Row spacing was 0.75 m in all locations.

Tab. 22. Design of experiments for the trials in 2013 and 2014

Location	Year	Treatments + Control	Repetitions	Blocks
Mittich	2013	5	4	4
Inzing	2014	6	4	4
Ostenfeld	2013	6	5	5
Ostenfeld	2014	5	3-5	3

In 2014, three further trials were planned in the location of Ostenfeld using the variety Ronaldinio sown in April. These trials were excluded from further analysis because the leaf area infected by *K. zeae* was less than 1 % at the end of the season in both the treated plots and the untreated control. Results for the trial sown in May using the variety SY Kalvin will, however, be presented.

As a representative example for the fungicide treatment trials in each location, the design of the experiments in Ostenfeld and Mittich in 2013 is presented in Figure 5.

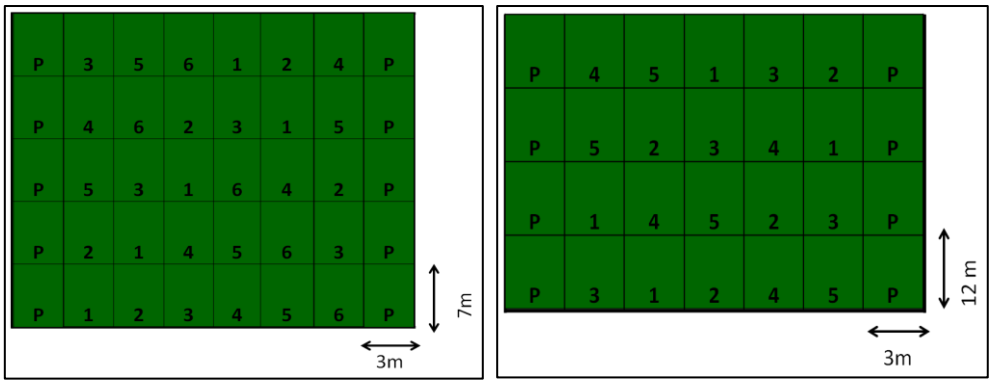

Fig. 5. Design of the experiment in Ostenfeld (left) and Mittich (right) in 2013. Different numbers indicate different treatments. Each row represents a single block. P= Periphery border.

Different treatments were applied at various growth stages of the plant in order to obtain different levels of the disease, which could then be correlated with diverse yield losses. Thus, this should provide more information about an adequate control schedule between early and late treatments.

Parallel to the untreated control, treatments consisted of:

- Propiconazole and azoxystrobin (Quilt Excel®), which is registered on the German market for foliar application on maize against Turcicum leaf blight;
- Epoxiconazole and pyraclostrobin (Opera®), which is used in Denmark to control maize leaf diseases such as Kabatiella eyespot. In Germany, it is only applied to cereals.
- Fluopyram and prothioconazole (Propulse®) and carbendazim and flusilazole (Harvesan®), which are registered for application to other crops like cereals (against *Septoria* spp., *Blumeria graminis* and *Puccinia* spp.) and oilseed rape (against leaf and stalk diseases such as *Alternaria brassicae* and *Sclerotinia sclerotiorum*).

In Mittich, applications were carried out at two different growth stages, BBCH 33 (vegetative) and BBCH 65 (flowering). In 2014 in Inzing, the fungicides were applied at BBCH 51 (tasseling) and BBCH 65 (flowering). A summary of the different fungicide treatments and timing of application in 2013 and 2014 is shown in Tables 23 and 24. Because the seeds were additionally treated with the fungicide Aatiram® in Inzing in 2014, two controls, one without seed treatment and another with seed treatment, were arranged to detect possible effects of the seed treatment on the different foliar treated variants.

Tab. 23. Fungicide foliar treatments tested on variety Zidane in Mittich in 2013

No.	Active ingredients	Form	g/l	l/ha	Stage	Product	Producer
1	**Control**	-	-	-	-	-	-
2	**Propiconazole Azoxystrobin**	SE*	11.7 13.5	1	32	Quilt Excel®	Syngenta
3	**Propiconazole Azoxystrobin**	SE	11.7 13.5	1	63	Quilt Excel®	Syngenta
4	**Epoxiconazole Pyraclostrobin**	SE	50 133	1.5	32	Opera®	BASF
5	**Epoxiconazole Pyraclostrobin**	SE	50 133	1.5	63	Opera®	BASF

*SE = Suspo-emulsion.

Tab. 24. Fungicide foliar treatments tested on variety NK Silotop in Inzing in 2014.

No.	Active ingredients	Form	g/l	l/ha	Stage	Product	Producer
1	**Control**	-	-	-	-	-	-
2	**Control seed treatment**[1]	-	-	-	-	-	-
3	**Propiconazole Azoxystrobin**	SE*	11.7 13.5	1	51	Quilt Excel®	Syngenta
4	**Propiconazole Azoxystrobin**	SE	11.7 13.5	1	65	Quilt Excel®	Syngenta
5	**Fluopyram Prothioconazole**	SE	125 125	1	32	Propulse®	BASF
6	**Epoxiconazole Pyraclostrobin**	SE	50 133	1.5	63	Opera®	BASF

[1] For more details, see Appendix Table A1. *SE = Suspo-emulsion.

In Ostenfeld, treatments were applied using a wheel sprayer with a hand-held spray boom with flat fan nozzles (model IDK-120-025, 50 cm nozzle spacing). The application was calibrated to a volume of 250 l/ha at 3 bars and sprayed at about 4.5 km/h (6 s/7 m long plot).

In both Mittich and Inzing, treatments were applied using a backpack sprayer with a hand-held spray boom with flat fan nozzles (model Airmix 110-02, 50 cm nozzle spacing). The application was calibrated to a volume of 260 l/ha at 2.8 bars for Mittich in 2013, and 300 l/ha at 2.5 bars for Inzing in 2014. In both cases, the spraying rate was about 4 km/h (9-11 s/10-12 m long plot).

In Ostenfeld in 2013 and 2014, the mixture of propiconazole and azoxystrobin (Quilt Excel®) was applied at three different crop growth stages, BBCH 33 (vegetative), BBCH 55 (appearance of the first symptoms or tasseling) and BBCH 63 (flowering). In 2013, other treatments were applied at BBCH 55. A summary of the different fungicide treatments and timing of application is shown in Tables 25 and 26.

Tab. 25. Fungicide foliar treatments applied to the variety Ronaldinio in Ostenfeld in 2013.

Nr	Active ingredients	Form	g/l	l/ha	Stage	Product	Producer
1	**Control**	-	-	-	-	-	-
2	**Propiconazole** **Azoxystrobin**	SE*	11.7 13.5	1	33	Quilt Excel®	Syngenta
3	**Propiconazole** **Azoxystrobin**	SE	11.7 13.5	1	55	Quilt Excel®	Syngenta
4	**Propiconazole** **Azoxystrobin**	SE	11.7 13.5	1	63	Quilt Excel®	Syngenta
5	**Fluopyram** **Prothioconazole**	SE	125 125	1	55	Propulse®	Bayer
6	**Carbendazim** **Flusilazole**	SE	125 250	0.8	55	Harvesan®	DuPont

*SE = Suspo-emulsion.

Tab. 26. Fungicide foliar treatments applied to variety Kalvin in Ostenfeld in 2014.

Nr	Active ingredients	Form	g/l	l/ha	Stage	Product	Producer
1	**Control**	-	-	-	-	-	-
2	**Propiconazole Azoxystrobin**	SE*	11.7 13.5	1	**33**	**Quilt Excel®**	Syngenta
3	**Propiconazole Azoxystrobin**	SE	11.7 13.5	1	**55**	**Quilt Excel®**	Syngenta
4	**Propiconazole Azoxystrobin**	SE	11.7 13.5	1	**63**	**Quilt Excel®**	Syngenta

*SE = Suspo-emulsion.

2.6.2. Disease assessment

Disease severity assessments began when the infected leaf area was at least sufficient to carry out a visual rating of the untreated control (normally at flowering, i.e. BBCH 63-65). Further assessments were carried out when a noticeable increase in the disease severity was observed in the plots, normally during the development of the grain (BBCH 71-73) and at ripening (BBCH 83-87). A schematic graph showing the development of the plant in relation to fungicide application and time of rating is exhibited in Figure 6.

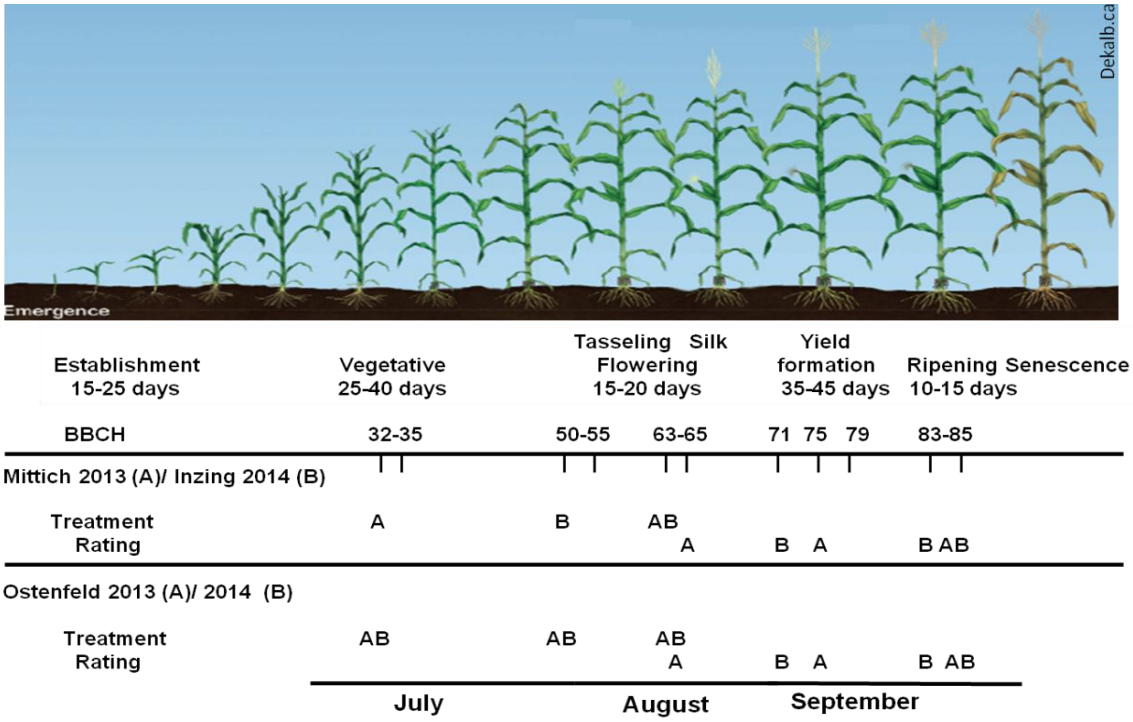

Fig. 6. Schedule of fungicide application and disease ratings related to maize stages in 2013 (Mittich and Ostenfeld) and 2014 (Inzing and Ostenfeld) trials (maize development data obtained from Hack et al. 1992 and Dekalb 2015).

In accordance with the EPPO Guidelines (2011), disease assessments were made on five successive plants in the centre of each of the two central rows in each plot. Five leaves, consisting of the main ear leaf (L0), two leaves above the main ear (L+1 & L+2), and two below (L-1 & L-2), were given a score based on a visual rating of the percentage of infected leaf area (0-50%). The visual rating was done following the keys from the EPPO Guidelines (2011) for the development of Kabatiella eyespot and common rust, and from the University of Wisconsin Extension (2011) for the development of Turcicum leaf blight (Figures 7 & 8).

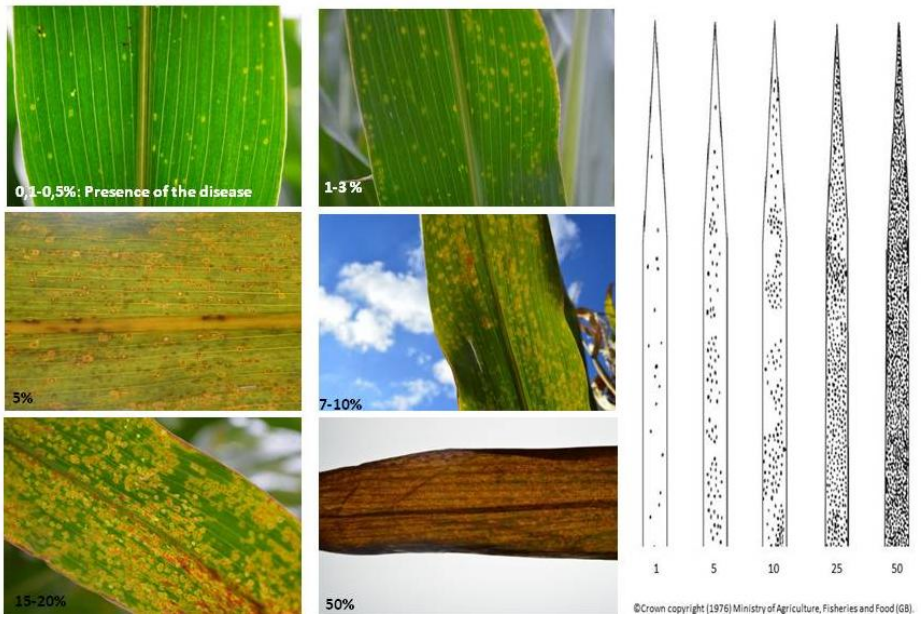

Fig. 7. Diagrammatic representation for rating of percentage of infected leaf area used to evaluate disease severity of Kabatiella eyespot and common rust based on the Ministry of Agriculture, Fisheries and Food (GB) (1976) and the EPPO Guidelines (2011).

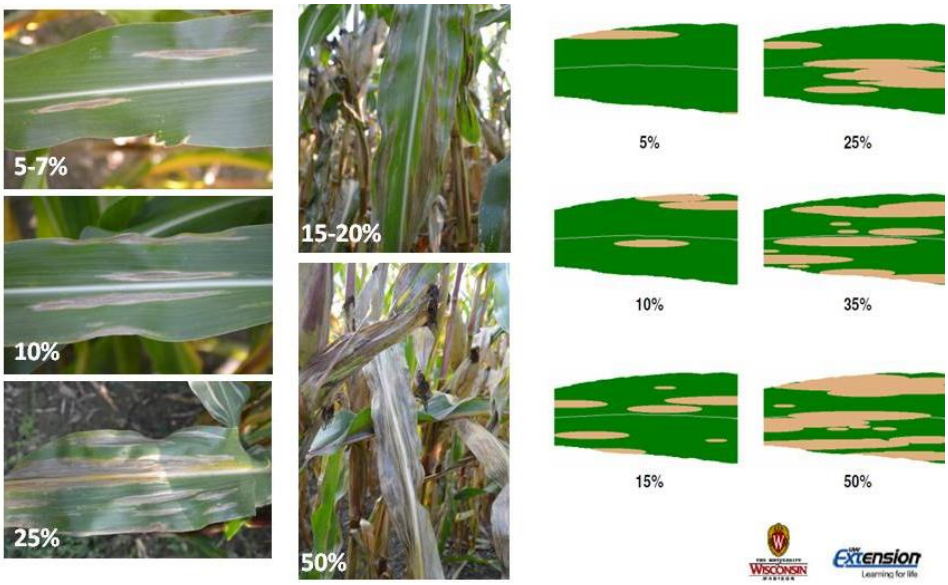

Fig. 8. Diagrammatic representation for rating of the percentage of infected leaf area used to evaluate disease severity for Turcicum leaf blight based on the University of Wisconsin Extension (Esker 2010).

2.6.3. Biomass and grain yield

As the main purpose of use of maize varieties in the region is silage and biogas production, the amount of biomass was recorded in Ostenfeld. Harvest and preparation of the samples were carried out by the personnel of the cooperating institution, FH Kiel. Plants from the central row of each plot were harvested for quantitative recording of yield once a wet matter level of 33% had been reached in the plant (specified German national standard). Plants were dried in an oven at 105°C for two days. The total dry weight (kg/ha) of the harvested plants (including the ears) was then recorded.

In the southern locations of Inzing and Mittich, grain yields were measured as maize used for livestock feeding is predominant in the region. The central rows of each plot were harvested and weighed. The harvest and preparation of the samples was completed at the facilities of the participating companies (Syngenta GmbH and Hetterich Field Trials).

2.6.4. Correlation of disease development with weather factors

Because the development of the foliar maize diseases is reliant on specific weather factors, the correlation of both is necessary for the characterisation of the development pattern of the disease in the field. Therefore, in order to describe these epidemics, the daily means of temperature (°C), relative humidity (%), precipitation (mm) and wind (m s^{-1}) were recorded. For Ostenfeld (Schleswig-Holstein), the weather station was situated directly in the experimental fields. For Mittich and Inzing (southern Bavaria), the climatic parameters were obtained from the weather station of the Bavarian State Research Centre for Agriculture (LfL). This is situated in Bärnau (Pocking), at a geodesic distance of approx. 4 km from Inzing and 8 km from Mittich.

2.7. Data management and statistical analysis

2.7.1. Epidemiological studies based on spore trapping in the field

The number of spores was counted through microscopy and their corresponding DNA yield was assessed via qPCR. Given one categorical variable, the log model y= exp (a + ax), assuming a negative binomial distribution, was used to determine the relation between number of spores and DNA yield, as well as to make an inference about the model parameters. To facilitate reading and interpretation, the x and y axes were flipped. Analyses were carried out with the *E. turcicum* samples from sensitivity tests and samples from Mittich 2013 and Inzing 2014 because the number of spores could be assessed via microscope. For

K. zeae, only the artificial spore trap samples were used as it was not possible to detect spores through visual assessment (microscopy).

For the statistical analysis, the GENMOD procedure by SAS was used. Model parameters were tested by the corresponding Pearson's chi-squared test. In all analyses, the general level of significance was set to $p<0.05$.

2.7.2. Control of the main diseases through fungicides in the field

From the disease rating in the field, values were obtained for the main ear leaf (L0) and the two leaves above (L+1 & L+2) and below the main ear (L-1 & L-2).

During leaf ripening, a distinction between senescence and pathogen damage became more and more difficult. Consequently, the number of observed data per treatment was different. This led to unequal sample sizes which require the use of weighted means [(least square means (LS means)] to prevent confounding of treatment effects. For every treatment and treatment combination the LS means were calculated by using PROC GLM or MIXED and MINITAB.

The experiments were arranged in a completely randomised split-split block design. For the estimation of treatment effects and the comparison of means for each experiment, an analysis of variance (ANOVA) was conducted using GLM, MIXED and MINITAB. The same software was used for the regression analysis. In all statistical procedures, the residual analysis and appropriateness was examined using SAS. The sample means out of ten observations formed the ANOVA database. For these means, the Central Limit Theorem applies, which means no data transformation is required. To study the relation between infected leaf area and yield adjusted for block effects, the GLM co-variance procedure was employed. Treatment effects and the regression coefficients were tested by the corresponding F-test and the Bonferroni multiple mean comparisons method was applied to estimate mean differences. In all analyses, the general significance level was set to $p<0.05$. For the results obtained, the general linear model used with the following components was the most adequate assumption:

$$Y_{ijkl} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \gamma_k + (\tau\gamma)_{ik} + (\beta\gamma)_{jk} + (\tau\beta\gamma)_{ijk} + \delta_l + (\tau\delta)_{il} + (\gamma\delta)_{jl} + (\tau\gamma\delta)_{ikl} + \varepsilon_{ijkl}$$

Y_{ijkl} = Observed values are composed of the following effects:

μ = General mean

Main effects

τ = 6 Treatments, β = 4 Blocks (random), γ = 5 Position, δ = 3 Stages (random)

First order joint effects

$(\tau\gamma)_{ik}$ Treatment* position, $(\tau\delta)_{il}$ Treatment* stage and $(\gamma\delta)_{kl}$ Position* stage

Second order joint effects

$(\tau\gamma\delta)_{ikl}$ = Treatment* position* stage

Error terms

$(\tau\beta)_{ij}$ = Error1
$(\beta\gamma)_{jk} + (\tau\beta\gamma)_{ijk}$ = Error2
ε_{ijkl} = Error3

From all factors used in the above model, only the blocks which can be seen as randomly chosen environments are random. Treatment, leaf position and stage are fix factors since their levels are all chosen according to the specific research questions. In a mixed model, the GLM as MIXED procedure in SAS allows the hypothesis test about LS means and their differences for main and joint factor effects.

Considering the fact that the treatments and severity of the diseases differed from one year to the other, a combination of the 2013 and 2014 data was not considered reasonable for a common analysis of variance.

3. Results

3.1. Inventory and validation of fungal pathogens on maize leaves in Central Europe

Knowledge about the appearance and regional distribution of maize leaf diseases and their respective pathogens is an indispensable prerequisite to achieve effective control.

During 2012 and 2013, potential leaf infecting pathogens isolated from symptomatic leaves were identified according to different literature keys (see section 2.2.5). In the case of *P. sorghi,* the characteristic symptoms or pathogenic structures on the leaf were a clear signal of the presence of the pathogen in the field. This meant that a microscopic examination was only required in single cases. Similarly, *E. turcicum* was recognisable in all cases by the symptoms described in the literature. Typical development of the lesion was favoured due to the absence of resistance in the observed plant varieties.

The determination of *K. zeae, B. zeicola*, *C. graminicola* and *Phoma* spp., in contrast, was more difficult during the monitoring as they occasionally exhibited similar symptoms in the initial stages of the formation of lesions on the leaf. This led to further isolation on solid media and *in vitro* observation for several isolates.

Pathogenicity tests served to confirm the correlation between the isolated organisms and the disease, and were therefore conducted for less known pathogens which induce lesions such as *K. zeae*, *B. zeicola*, *C. graminicola*, *Phoma* spp. and *Fusarium* spp. Here, a series of isolates was tested, also serving for subsequent diagnosis on the leaf. The only exception was *Phoma* spp., for which all of the isolates were tested because it was necessary to carry out pathogenicity tests in order to reveal and distinguish between pathogenic and saprophytic *Phoma* species. The resulting symptoms of the disease are described in detail below (based on in-depth visual assessment) and are compared with the symptoms observed in the field.

3.1.1. Symptoms and morphological characterisation of *E. turcicum* and *P. sorghi*

E. turcicum

The initial symptoms are green or yellow lesions, which develop into elliptical or cigar-shaped lesions of approximately 3-20 cm in length and fuse with time. After periods of high humidity or moisture, dark zones of fungal sporulation can be distinguished in the lesions. However, after long dry periods, the lesions expand without sporulation. Lower and middle leaves generally exhibited a higher rate of infection than the upper leaves.

Conidia are olive green to brown, 2-9 septa, spindle-shaped with a strongly protruding hylum. Their size ranged within the parameters given by De Rossi et al. (2015) (10-20 to 30-147 µm). The conidia show mono and bipolar germination. Formation of chlamydospores and shorter, stroma-like mycelium cells could be observed *in vitro* when the plates were incubated at 4°C for several months.

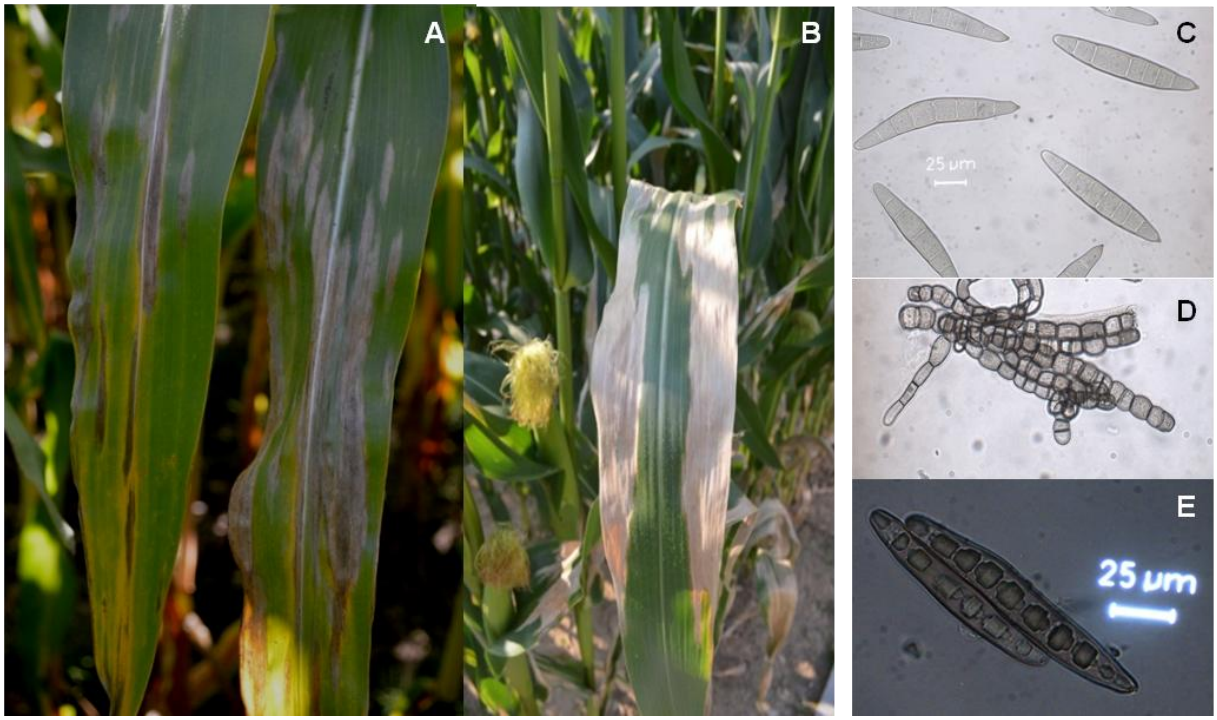

Fig. 9. *E. turcicum*. A. Typical symptoms on maize leaves B. Extreme leaf blight without sporulation. C. Conidia. D-E. Compacted mycelium and production of chlamydospore-like structures in mycelium and conidia. Scale bars =25 µm (40x).

P. sorghi

Brown pustules containing orange to brown uredospores develop on the surface of maize leaves. Due to the noticeable form and shape of the pustules, their presence was the first indicator of the disease in the maize field. Uredinia of *P. sorghi* sporulate on the upper and lower surfaces of the leaf. In the late season, the pustules become brown to black, indicating that the uredospores have been replaced by teliospores. Uredospores are orange to brown, with a spherical to oval form. Teliospores are smooth, oblong to ellipsoid, with two cells detached at the septa and with a long pedicel. The disease tends to develop on the middle and upper leaves of the plant.

Fig. 10. *P. sorghi*. A. Pustules on maize leaves. B. Pustules with teliospores. C. Uredospores. D. Teliospores (40x).

3.1.2. *K. zeae*, *B. zeicola* and *C. graminicola*

K. zeae

In the field, the pathogen produces circular to oval lesions (up to 4 mm), which develop a narrow, yellow halo. Later, the halo will be surrounded by a brown, black or purple ring inducing the form of an eye, hence the name “eyespot”. The lesions can be delimited in patches and later fuse to form large necrotic areas. In some locations, the disease was restricted to the upper leaves of the plant. The symptoms can also be observed in ear husks and leaf sheaths.

After incubation of the lesions in a humidity chamber, slimy masses of spores (similar to sporodochia) cover the lesions. In the plant tissue, dark chains of stromatic mycelium are formed. In some cases, these colonies can be confused with the colonies of *Fusarium* spp. on the leaf. In this case, an analysis via microscope is necessary to confirm the isolate in question. Conidia are hyaline, curved to falcate with pointed ends, which later can suffer deformations, probably due to germination of the spore. Conidia are normally non-septate, but one or two septa could be formed. Their size varies between 1-4 and 24-33 µm (average 3 x 27 µm).

According to Reifschneider & Arny (1980a), one of the most reliable parameters for the identification of *K. zeae* is its *in vitro* growth. Colonies show a slow-growing mycelium on malt

agar and PDA. It is initially characterised by a light pink or white colour and wrinkled mycelium, which later turns dark and coriaceous (Figure 11). A hairy mycelium develops in some isolates. Formation of sections with both dark and light colours frequently occurs, but subculturing of these results in normal colonies.

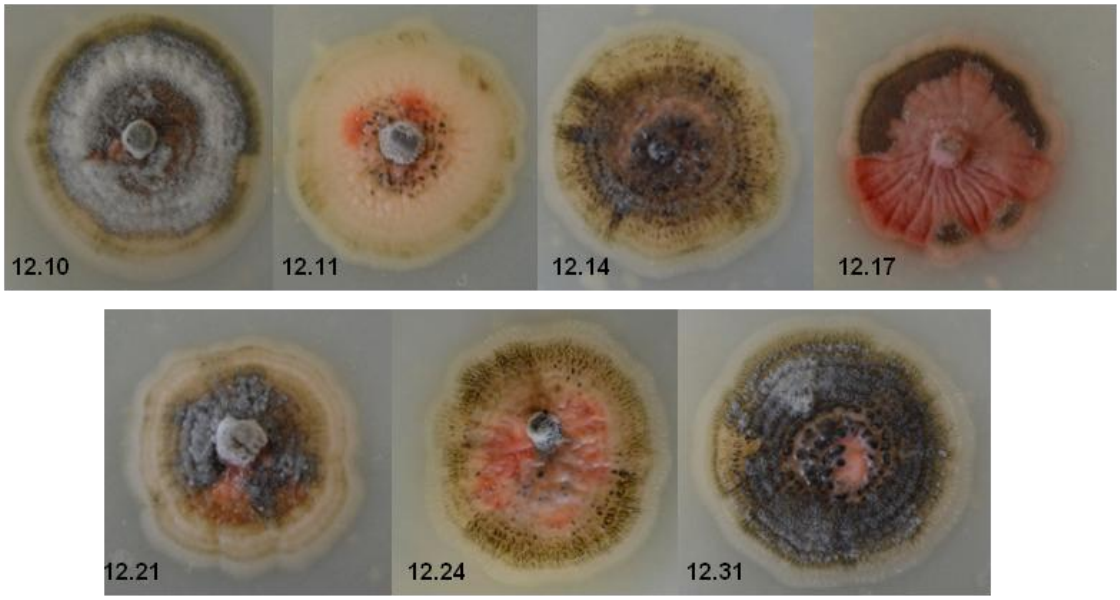

Fig. 11. Ten-day-old colonies of *K. zeae* isolates on PDA.

Pathogenicity tests in the greenhouse

After inoculation in the greenhouse, both tested maize varieties presented circular spots with a tan centre, brown border and yellow halo. The symptoms appeared 7-10 days after inoculation, coalescing and developing into necrosis. In general, these symptoms coincided with those which developed in the field (Figure 12 A-D). Nevertheless, some atypical symptoms were also produced where the typical circular spots had developed in an irregular form. All isolates which were recovered from the lesions produced in the inoculated plants have the same characteristics as the initial inoculated organism and therefore fulfil Koch's postulates. A summary of the pathogenicity test for all isolates is given below.

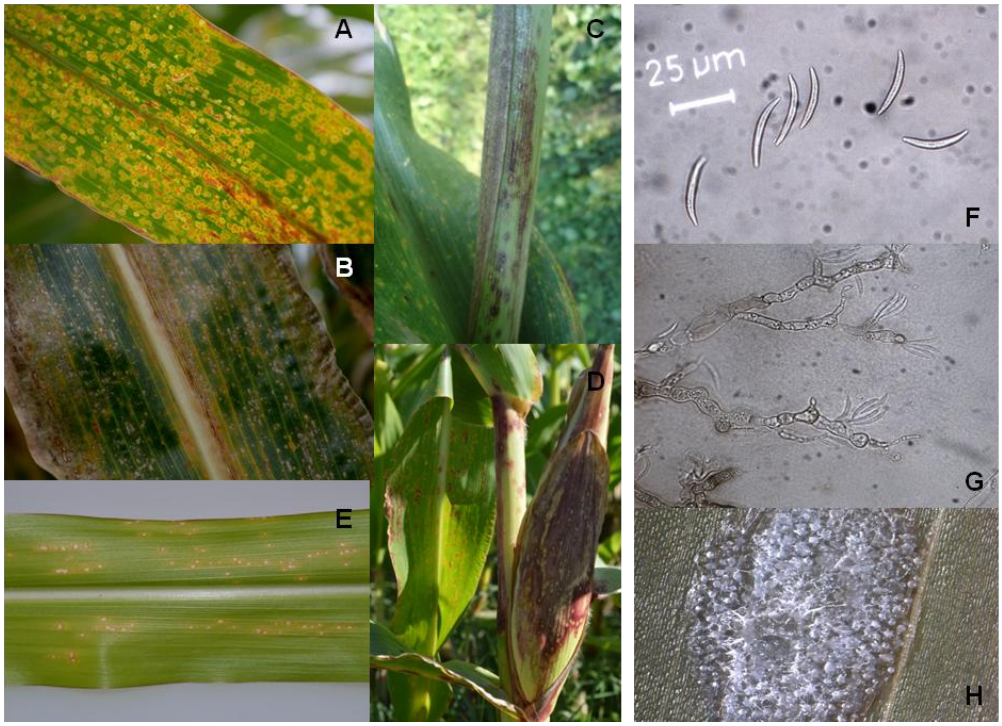

Fig. 12. Eyespot disease and its causal organism *K. zeae*. A-D. Initial and late symptoms in the field. E. Symptoms after artificial inoculation in the greenhouse. F-G. Conidia and disposition of the conidia in the mycelium. H. Sporulating colonies on the lesions.

***Bipolaris zeicola* and *Bipolaris* spp.**

Different types of symptoms were observed for *B. zeicola* in the field. The most common consisted of brown elongated and irregular spots, which resemble the description for *B. zeicola* race 2 in the literature (Figure 13 A-B). Characteristic symptoms for this race were observed in leaves originating from Schönering (sample 12.18) and Nordholz (sample 12.20). Other observed symptoms were the production of linear, greyish lesions along the leaf vein (Figure 13 C), indicating the presence of race 3 (sample 12.7, Bylany). *B. zeicola* could also be diagnosed in commercial seeds which were planted at the Ostenfeld location (sample 12.2S). Small necrotic lesions were observed in samples from location Bad Oldesloe (sample 12.27), sharing the leaves with *K. zeae*. Two isolates of *Bipolaris* spp. (sample 12.8, Caslav and sample 12.9, Planá) were isolated from oval to irregular-shaped lesions, tan in colour and a clearly defined dark border.

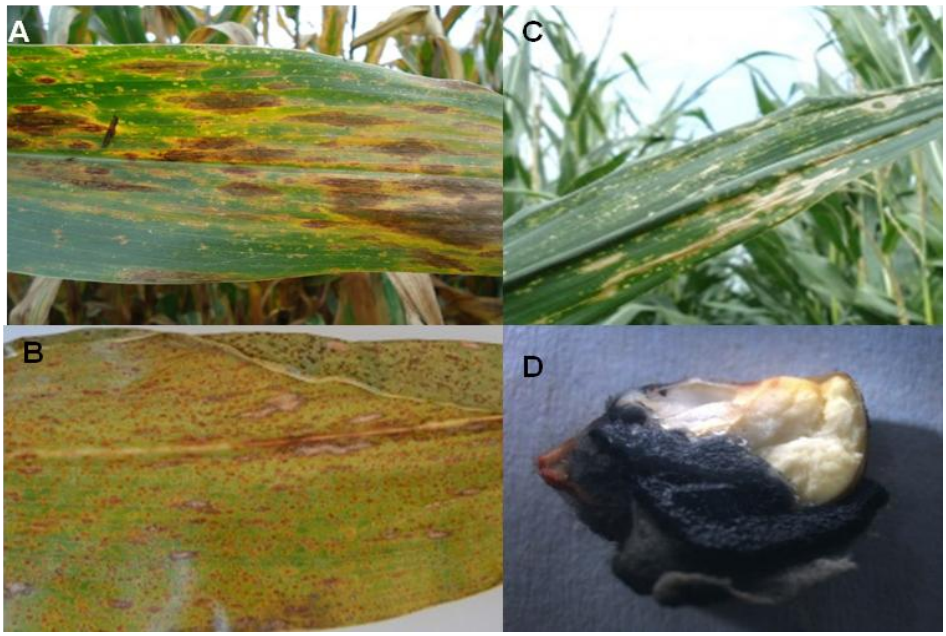

Fig. 13. *B. zeicola* symptoms in the field. A-B. Symptoms produced by race 2: observed in sample 12.18, Schönering (A) and initial symptoms observed in sample 12.20, Nordholz (B). C. Symptoms produced by race 3 in sample 12.7, Bylany. D. Commercial seed infected with *B. zeicola*.

In vitro sporulation of young cultures was favoured by UV light. Mycelium growth and pattern varied considerably among isolates after two weeks (Figure 14). Ascending white masses of compacted mycelium, either isolated or in groups, were also developed (Figure 15).

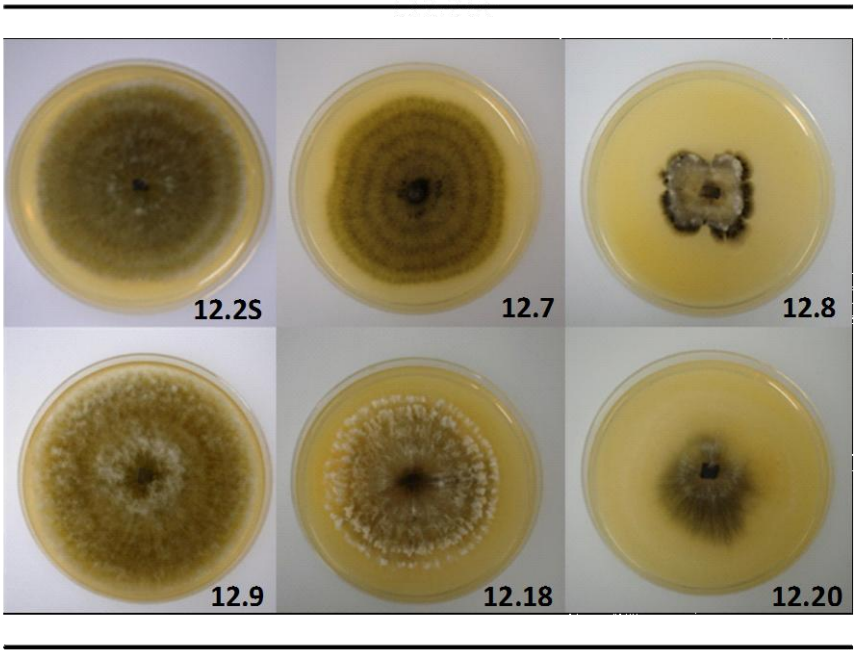

Fig. 14. Fourteen-day old colonies of *B. zeicola* and *Bipolaris* spp. isolates on V8.

Fig. 15. Ascending white isolated masses of compacted mycelium typical from *Bipolaris* spp.

Conidia of *B. zeicola* are olive to dark brown, slightly curved, with rounded ends and septate. The number of septa and size of the conidia oscillates within the range given by White (2010), i.e. 7-18 x 25-100µm. Other identified *Bipolaris* spp. spores differ from the *B. zeicola* species described, with a darker appearance when observed directly on the sample leaf and also when cultivated *in vitro*. The majority of the spores of these isolates exhibit less or, in some cases, no curvature compared to the spores of *B. zeicola* (Figure 16, E-F). Some spores also exhibited flat, irregular borders. Growth in agar differs greatly between the two isolates; isolate 12.8 shows slow growth after 14 days (2-3 cm), is dark in colour and exhibits formation of white buffs, as is characteristic for other *Bipolaris* spp. Isolate 12.9, on the other hand, exhibits fast growth on the medium, with a grey mycelium and formation of buffs, closely mirroring colonies of *B. zeicola*.

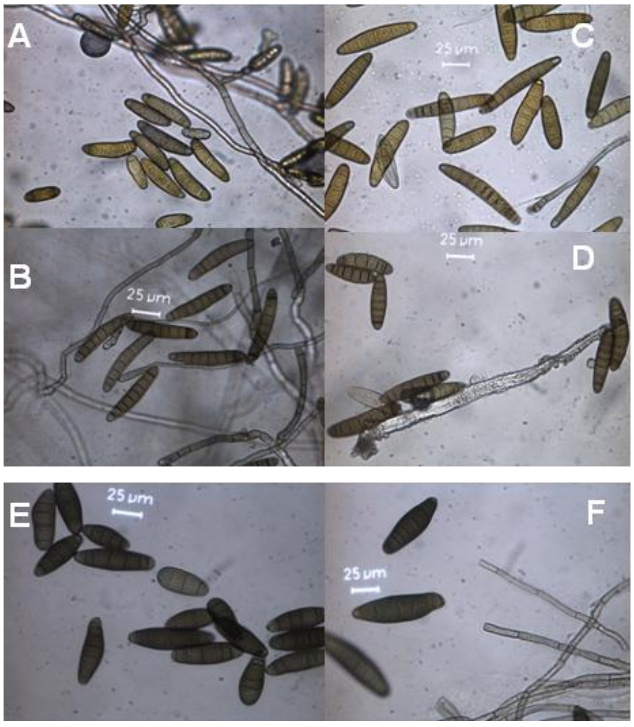

Fig. 16. Conidia of *B. zeicola* (A-D). Isolate 12.2S (A) Isolate 12.7 (B) Isolate 12.18 (C) Isolate 12.27 (D). Conidia of *Bipolaris* spp. (E-F). Isolate 12.8 (E) Isolate 12.9 (F).Scale bars= 25 µm

Pathogenicity tests in the greenhouse

Two days after inoculation, the two maize varieties tested showed symptoms for all five *B.zeicola* isolates. The appearance and severity of these symptoms differed among isolates (Figure 17). Isolate 12.7 exhibited linear and yellow-brown to greyish lesions, which are the typical symptoms described in the literature for race 3 of *B. zeicola.* The symptoms were also similar to those found in the field. Although the two varieties exhibited chlorotic symptoms around the lesions, which are usually regarded as being characteristic of plant resistance, this did not impede the progression of the disease, which induced premature senescence and ultimately the death of the leaf. In fact, isolate 12.7 reached a higher level of severity than any of the other isolates (grade 9 for the variety Ricardinio/grade 8 for Barros). Isolate 12.2S, originating from commercial seeds, produced very small lesions (0.5-2 cm) with a tan centre and marked brown border. Its development was rather slow compared to isolate 12.7, with the maturing of the plant occurring before further development of the disease could be observed. Despite this, the organism was able to produce necrosis (grade 6 for the variety Ricardinio/5 variety Barros).

The symptoms observed for isolate 12.18 were similar to those of isolate 12.2S. In both varieties, small necrotic lesions were produced, with a subsequent fusing of the lesions similar to that found in the field in Schönering. This even induced the beginning of a premature senescence in the Ricardinio variety (grade 7). Isolate 12.20 produced tan spots similar to those of the isolates above, but with the formation of lesions with pronounced borders and spread out across the leaf. Isolate 12.27 produced necrosis with less-defined borders than those of isolate 12.2S, 12.18 and 12.20. In contrast to the other isolates, chlorotic halos were not produced.

Of the two varieties tested, the variety Barros exhibited a higher level of resistance than Ricardinio for all *B. zeicola* isolates. For all *B. zeicola* isolates, the successful recovery of the same fungal organism from the artificially inoculated and subsequently infected tissue was achieved, completing Koch´s postulates. This confirms that the isolated fungal organism is the causal agent of the disease.

The unidentified *Bipolaris* spp. isolate 12.8 produced similar initial spots (necrotic centre and chlorotic halo) as *B. zeicola* isolates 12.2S, 12.18, 12.20. For isolate 12.9, small chloroses were produced, which started to develop into necrosis after two weeks. It was not possible to observe a further development of the disease on the plant for both isolates due to the natural maturing of the leaf. However, the pathogen was successfully re-isolated in both cases from the low number of lesions that had already developed, fulfilling Koch's postulates and confirming the pathogenicity of the isolates. Between the two varieties, Barros again exhibited a higher level of resistance than Ricardinio to the two *Bipolaris* spp. isolates.

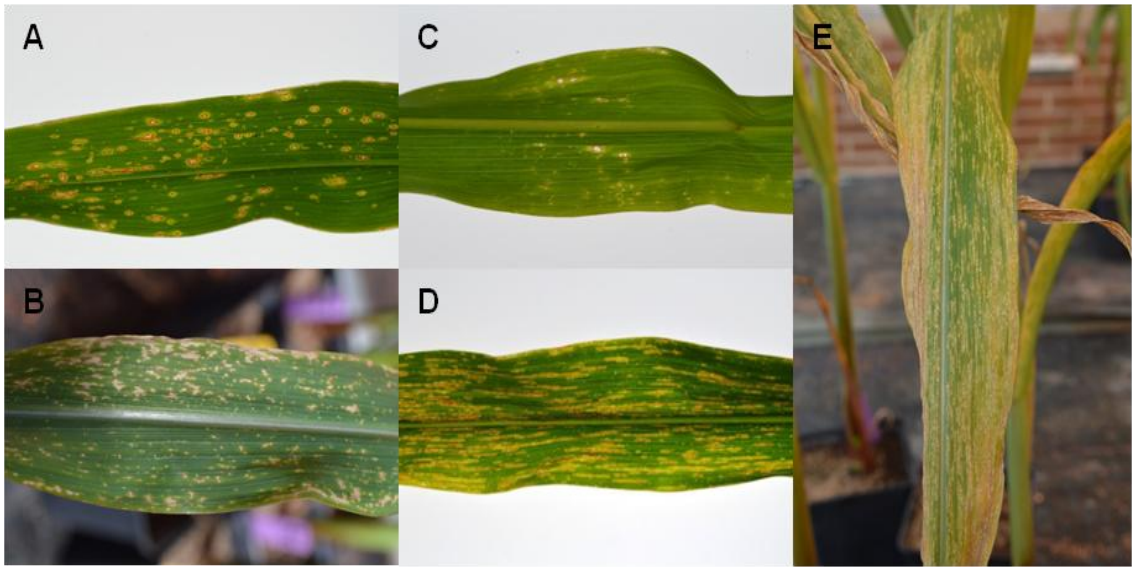

Fig. 17. Symptoms produced by *B. zeicola* and *Bipolaris* spp. after artificial inoculation in the greenhouse. A. Isolate 12.20 B. Isolate 12.27 C. Isolate 12.8 D-E. Isolate 12.7 clearly reflecting the typical linear lesions produced by race 3.

Colletotrichum graminicola

Symptoms on maize leaves are oblong to rectangular lesions with a tan centre and red to reddish-brown borders (Figure 19, A-F). These develop through the deformations produced via the penetration of the leaf tissue by the lesions. The acervuli can be clearly observed within the lesions, making it one of the unequivocal parameters for the diagnosis of the disease in the field. After incubation of the leaves in a humidity chamber for two days, substantial appresorium formation could be observed.

In vitro, the colony grows quickly under UV light conditions, completely covering the agar with orange masses of spores which are embedded in a mucilaginous matrix with black setae (acervuli) and a dark, flat mycelium. The colony therefore exhibits a dark colour with slimy, dotted orange groupings at the surface (Figure 18). Spores are hyaline and show a distinctive drop in the centre. Conidial size was within the range described by White et al. (1987) (5 x 30 µm).

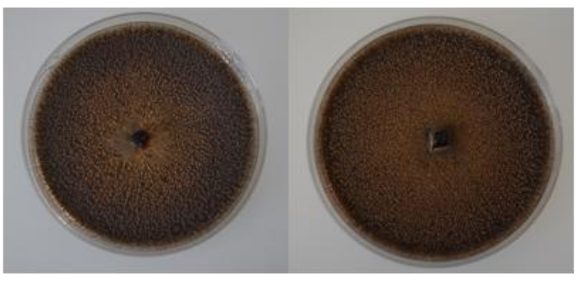

Fig. 18. Fourteen-day-old colonies of *C. graminicola* isolates (12.15 and 12.22) on PDA.

Pathogenicity tests in the greenhouse

Both isolates, 12.22 and 12.15, produced a similar pattern of development on the two maize varieties tested. Chlorotic spots (1 mm) were produced two days after inoculation, developing into long brown and necrotic areas along the leaf, coalescing with time. In some lesions, acervuli could be observed. Necrotic areas were also developed in the midrib and progressed further from here into the surrounding middle areas of the leaf, giving the appearance of strangulating the leaf. Dead leaves turned a reddish-brown colour. Stalk discolouration and necrosis were also produced by both isolates. Both fungi could be recovered from the symptoms on both varieties and the corresponding colonies have the same features as the initially inoculated fungal pathogens, fulfilling Koch's postulates.

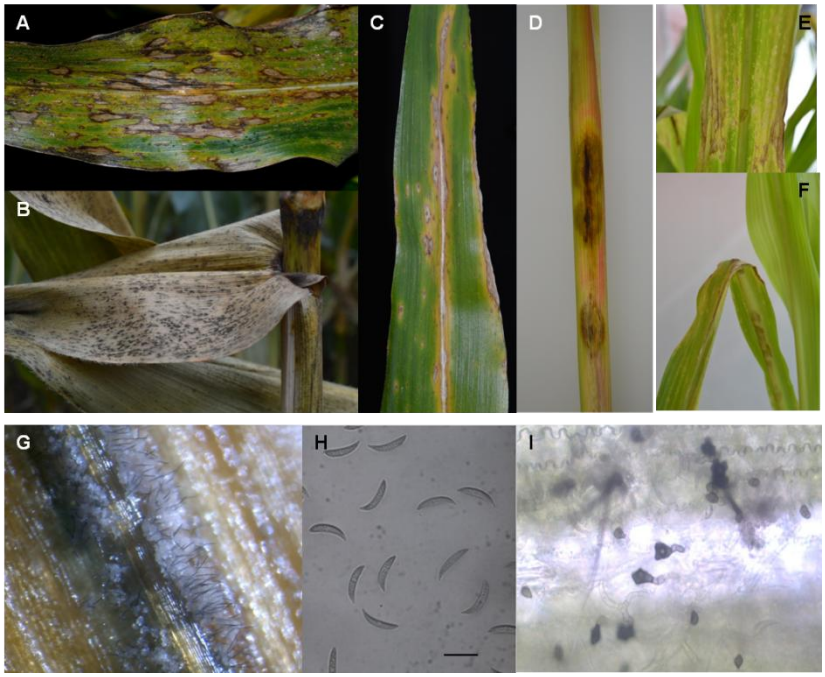

Fig. 19. *C. graminicola*. A-B. Symptoms in the field and presence of acervuli in the lesions. C-F. Symptoms after artificial inoculation in the greenhouse. G-H. *in vitro* observations: G. acervuli H. Conidia. Scale bar =25 µm. I. Formation of appressoria.

The results of all isolates tested are summarised in Table 27 below. The score data refer to the maximum score obtained from all leaves in each variety. The respective controls (non-inoculated) did not exhibit symptoms in any of the experiments.

Tab. 27. Summary of the isolates tested for Koch's Postulates from the monitoring in 2012 and 2013.

					Inoculation into a healthy plant Grade rating variety Ricardinio/Barros			
Organism	**Isolate**	**Location**	**Presence of the pathogen in symptomatic leaves**	**Isolated and *in vitro* pure culture**	**Leaf#4**	**Leaf#5**	**Leaf#6**	**Re-isolation**
K.zeae	12.11	Köhn (Plön)	√	√	0/0	4/3	4/0	√
	12.13	Stapel	√	√	0/0	3/2	3/1	√
	12.17	Mintraching	√	√	1/1	4/3	3/3	√
	12.24	Beesten	√	√	4/0	5/4	5/5	√
	12.28	Groningen	√	√	0/0	3/4	5/5	√
	12.30	Biddinghausen	√	√	3/0	5/2	5/3	√
	12.31	Giekau	√	√	4/0	5/4	5/5	√
B. zeicola	12.2S*	Ostenfeld	√	√	4/4	5/4	6/5	√
	12.7	Bylany	√	√	9/8	8/8	7/7	√
	12.8	Caslav	√	√	1/2	4/1	2/1	√
	12.9	Plana	√	√	3/2	2/1	2/1	√
	12.18	Schönering	√	√	7/2	3/1	4/6	√
	12.20	Nordholz	√	√	4/3	5/3	6/4	√
	12.27	Bad Oldesloe	√	√	7/5	5/3	5/2	√
C. graminicola	12.15	Princepeel	√	√	9/9	8/8	7/8	√
	12.22	Moorlas	√	√	9/9	8/8	7/7	√

*isolated from commercial seeds.

3.1.3. Symptoms and pathogenicity tests for *Phoma* spp.

In order to avoid confusion in the *Phoma* analysis of this work, the anamorph synonym *Phoma*, e.g. *Phoma zeae-maydis* (current synonym *Didymella maydis*) will also be used.

Lesions observed in the field resemble the descriptions provided in the literature for the pathogenic species on maize, *Ph. zeae-maydis*. These are oval to elliptical spots with a brown border and grey leaf centre, with a ring in the centre of the lesion. The leaf blade turned yellow as the lesions developed (Figure 20, A1-A3). After incubation of the lesions in a humidity chamber, *Phoma* pycnidia emerged from the lesions (Figure 20 B). In the samples with lesions from Ostenfeld (13.2C), in addition to pycnidia, pseudothecia resembling those from *Mycosphaerella zeae-maydis* (*Didymella maydis*) were observed (Figure 20, C-D). After cultivation on agar, instead of further pseudothecia, pycnidia related to *Phoma* spp. were produced. Nevertheless, the conidia produced *in vitro* are significantly smaller than those described for *Ph. zeae-maydis*.

In some samples, more than one *Phoma* species was found in *Phoma* lesions. On some occasions it was also sharing the leaf sample with other fungal pathogens, as was frequently the case for *K. zeae.* A total of 21 isolates were obtained and tested for pathogenicity. For a better comprehension of the importance of the different isolates, a description of the obtained pathogenicity results is given first, followed by the respective morphological characterisation.

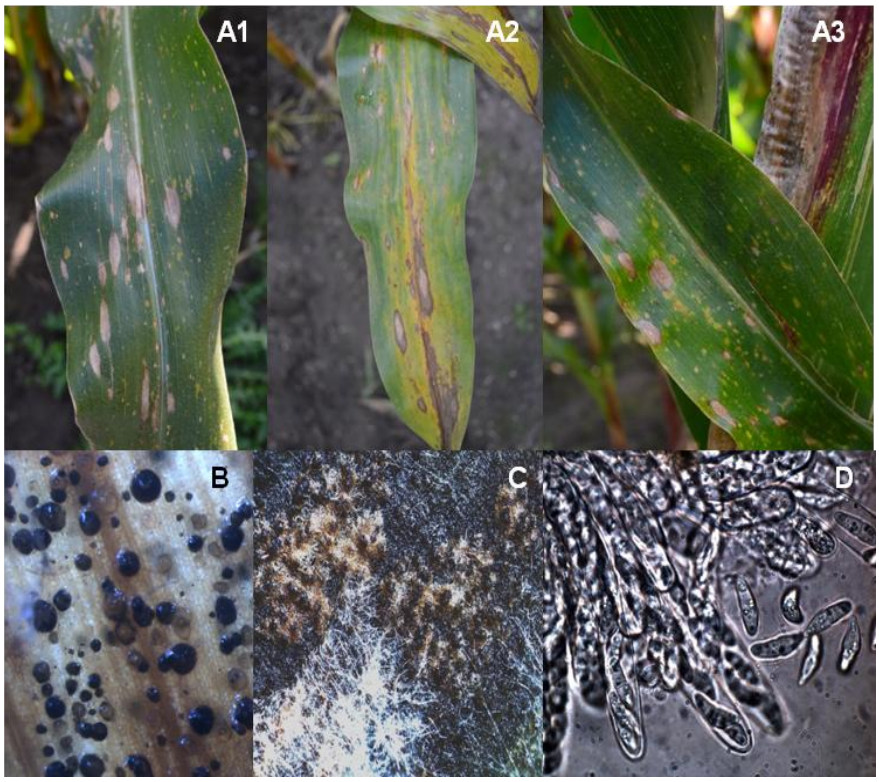

Fig. 20. *Phoma* spp. A1-3. Field symptoms B. Pycnidia on/in the leaf C. Pseudothecia D. Ascospores.

Pathogenicity tests in the greenhouse

Chlorotic lesions (1-2 mm) were observed in the varieties Ricardinio and Barros two to three days after inoculation for six isolates (12.18, 12.20, 12.23.2, 13.2P, 13.2C, 13.20). The chlorosis developed into round to oval lesions with an orange centre and yellow halo, turning into a necrotic centre, resembling the initial symptoms of the specific disease in the field (Figure 21). The lesions expanded along the leaf veins, coalescing in large areas which provide space for chlorotic zones that surrounded the lesions. From the pathogenic isolates, 12.18 and 12.20 induced a premature death of the leaves, thus achieving the highest grade of virulence of all tested isolates (grade 9).

Of the *Phoma* spp. isolates obtained from the CBS, only isolate 588.69, *Ph. zeae-maydis (syn. Didymella maydis*), produced lesions which tend to expand (grade 5). Isolate 180.80 (*Phoma sorghina*) produced only very slight chlorosis.

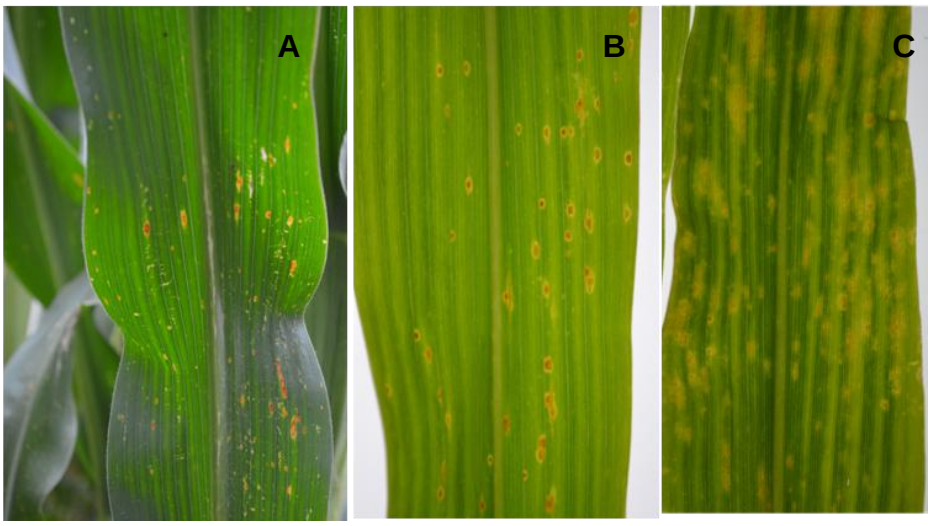

Fig. 21. Initial *Phoma* spp. lesions. A. Field symptoms B-C. Symptoms after artificial inoculation on healthy plants in the greenhouse with isolates 12.20 (B) and *Phoma zeae-maydis* (CBS 588.69) (C).

A seventh isolate (12.3B) developed chlorosis on both maize varieties but these did not turn into necrosis. Isolates 13.36 and 13.37 produced very limited slight chlorosis to the upper leaf in the variety Ricardinio only. Based on these results, none of these isolates (13.2B, 13.36; 13.37) could be considered as causal agents of the disease produced in the field. The rest of the isolates obtained during the monitoring did not exhibit any symptoms. A summary of the results is presented in Table 28.

From the lesions produced by isolates 12.18, 12.20, 12.23.2, 13.2P, 13.2C, 13.20 and CBS 588.69, the fungal organism could be recovered from the infected tissue, resembling the initially inoculated conidia and therefore fulfilling Koch's postulates.

Tab. 28. Summary of the *Phoma* isolates obtained during the monitoring in 2012 and 2013 and tested for Koch's postulates.

				Inoculation on healthy plants Score rating variety Ricardinio/Barros			
Isolate	**Location**	**Presence of the pathogen in symptomatic leaves**	**Isolated and *in vitro* pure culture**	**Leaf#4**	**Leaf#5**	**Leaf#6**	**Re-Isolation**
12.2	Ostenfeld	√	√	0/0	0/0	0/0	-
13.2d	Ostenfeld	√	√	0/0	0/0	0/0	-
13.2P	Ostenfeld	√	√	4/3	4/4	6/4	√
13.2B	Ostenfeld	√	√	0/0	2/0	2/2	(*)
13.2C	Ostenfeld	√	√	5/5	5/5	5/5	√
12.10	Braunau	√	√	0/0	0/0	0/0	-
12.13	Stapel	√	√	0/0	0/0	0/0	-
12.18	Schönering	√	√	9/9	8/8	6/6	√
12.19	Cloppenburg	√	√	0/0	0/0	0/0	-
12.20	Nordholz	√	√	9/8	7/6	6/6	√
12.23.1	Rade	√	√	5/5	5/5	5/5	√
12.23.2	Rade	√	√	0/0	0/0	0/0	-
12.27	Bad Oldesloe	√	√	0/0	0/0	0/0	-
12.28	Groningen	√	√	0/0	0/0	0/0	-
12.31	Giekau	√	√	0/0	0/0	0/0	-

* only chlorosis

Tab. 28. (continued) Summary of the *Phoma* isolates obtained during the monitoring in 2012 and 2013 and tested for Koch's postulates.

				Inoculation on healthy plants Score rating variety Ricardinio/Barros			
Isolate (syn.)	**Location**	**Presence of the pathogen in symptomatic leaves**	**Isolated and *in vitro* pure culture**	**Leaf#4**	**Leaf#5**	**Leaf#6**	**Re-Isolation**
13.20	Nordholz	√	√	5/3	5/4	5/4	√
13.36	Mittich	√	√	0/0	0/0	0/1	-
13.37	Hartkirchen	√	√	0/0	0/0	0/1	-
13.47	Kassel	√	√	0/0	0/0	0/0	-
13.48	Kassel	√	√	0/0	0/0	0/0	-
Ph. glomerata *(D. glomerata)*	CBS 528.66	-	-	0/0	0/0	0/0	-
Ph. pomorum *(D. pomorum)*	CBS 838.84	-	-	0/0	0/0	1/0	-
Ph. sorghina *(E. sorghinum)*	CBS 180.80	-	-	0/0	0/0	0/1	-
Ph. subherbarum *(D. subherbarum)*	249.92	-	-	0/0	0/0	0/0	-
Ph. subherbarum *(D. subherbarum)*	250.92	-	-	0/0	0/0	0/0	-
Ph. zeae-maydis *(D. maydis)*	CBS 588.69	-	-	0/1	4/3	5/2	√

For all *Phoma* isolates tested, the respective controls did not exhibit any symptoms in any of the experiments.

3.1.4. Characterisation of *Phoma* spp.

The different *Phoma* spp. isolates are characterised by high variability in their respective *in vitro* growth, as well as morphological differentiations indicating that several *Phoma* species or strains exist. The exhaustive morphological characterisation of each isolate and the CBS isolates for comparison is exhibited below.

Colony size measurements were taken after the first incubation week (dark, 20-22°C). Further characterisation of the colony was described after 14 days. In addition, plates were also cultivated under UV light. If any changes were observed in the colony growth or morphological features of the pycnidia and conidia, these were also noted.

After searching for similarities using BLAST, 100% similarity was found for only two (non-pathogenic) isolates, 12.36 and 12.37, which matched with *Ph. pomorum* CBS 838.84. As described below, morphological characters of this isolate also coincide with *in vitro* descriptions of isolates 12.36 and 12.37. For the rest of the isolates (13.2C, 13.2P, 12.18, 12.20, 13.2B, 12.13 and 12.19), molecular analysis resulted in several *Phoma* species for each isolate (Apendix Table A3). All of these *Phoma* species had a similarity level of 98%-99% with our isolates and no definitive conclusions could be drawn. Data from the obtained forward and reverse strands can be found for all species in the appendix.

Further analysis by the Central Biodiversity Center (CBS-KNAW, Utrecht, the Netherlands) confirmed the isolates 13.2B (considered saprophytic), 12.20 (pathogenic) and 12.13 (considered saprophytic) as three putative new species within the genus *Peyronellaea* (syn. *Didymella*; anamorph related to the genus *Phoma*).

The *Phoma* spp. isolates described below are grouped according to similar *in vitro* characteristics and pathogenicity. First, six pathogenic isolates (classified as pathogenic after fulfilment of Koch's postulates) are presented and described in detail, followed by those considered non-pathogenic.

Pathogenic *Phoma* spp. isolates

In general, pathogenic isolates present a slow growth rate between 24 and 38 mm after seven days. Moderate to abundant production of aerial mycelium floccose to woolly, green to olivaceous, brown or white with regular border. Chlamydospores or dictyochlamydospores are present in some isolates. Spore size varies within the range 4.1-7.9 x 1.5-3.2 µm.

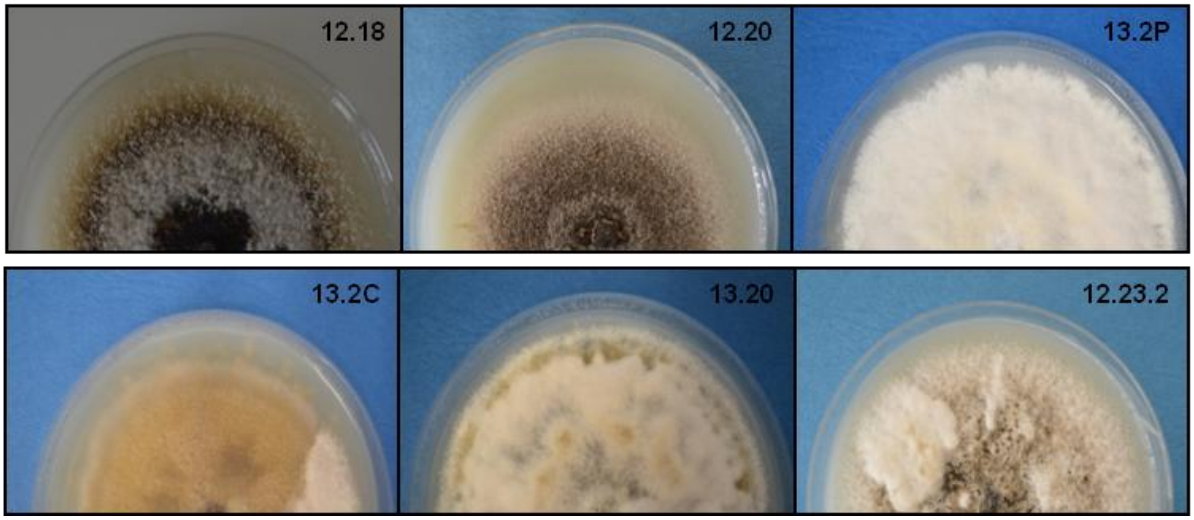

Fig. 22. *Phoma* spp. pathogenic isolates. Fourteen-day-old colonies on OA incubated in a dark regime.

Isolate 13.2C

Growth rates between 35 and 39 mm after seven days. Moderate to abundant production of aerial mycelium floccose to woolly, green to olivaceous, brown or white with regular border.

Size of the conidia: 4.7- 7.5 x 1.8-2.6 µm (average 6.2 x 2.2 µm). Conidia are ellipsoidal, single-celled, hyaline, presenting one to four polar, small to medium size guttules. Pycnidia are produced at a moderate rate under the mycelium layer and frequently found in the centre of the colony. Pycnidia are also produced in agar and more frequently in the centre of the colony when agar plates are incubated in UV light conditions. Pycnidia are dark brown to black, subglobose on agar and mostly irregular in agar, glabrous, with pseudoparenchymatous thick-walled cell structure. The size of pycnidia range between 50-200 x 50-150 µm and are solitary or confluent with other pycnidia. The number (0-4) and form of ostioles can vary considerably, presenting slightly to conspicuously papillate forms. In some cases, the formation of numerous small cavities (possibly ostioles) was also observed when the isolate was subcultured. The pycnidia present a white matrix (in which conidia are embedded) with moderate production of conidia. Chlamydospores present.

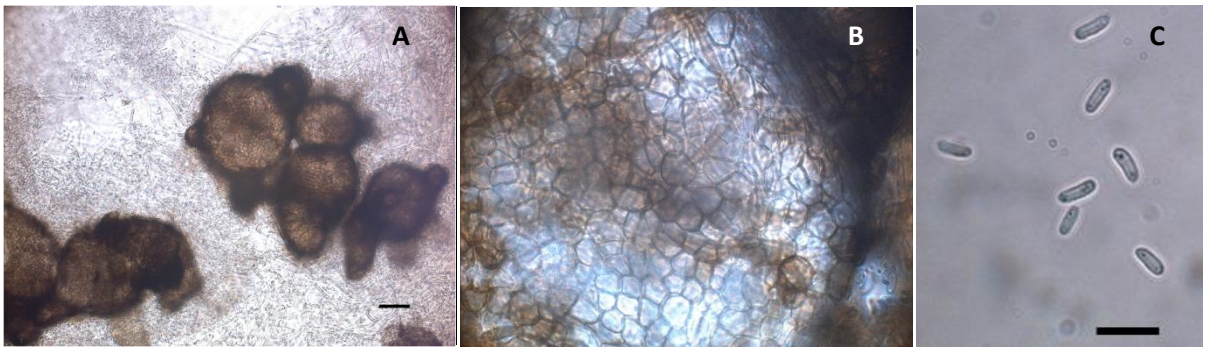

Fig. 23. Phoma isolate 13.2C. A. Pycnidia. B. Textura angularis (pseudoparenchymateous cell wall). D.Conidia. Scale bars A=50 µm; C=10 µm.

Isolate 13.2P

Growth rates of the colony between 36 and 38 mm after seven days. The production of aerial mycelium is moderate to abundant, usually white and wooly in appearance, developing some greenish/olivaceous to brown areas when cultivated in UV light conditions. The colony presents regular borders with scarce to moderate pycnidia production. Pycnidia under the mycelium layer and most commonly in the centre of the colony. Size of the conidia: 4.1-7.6 x 2-3.2 µm (average 6 x 2.6 µm). Conidia are ellipsoidal to oblong, single-celled, hyaline, presenting one to five mostly polar, medium-sized guttules. Pycnidia are brown to black, globose to subglobose, glabrous, normally with a pseudoparenchymatous thick wall. In every pycnidium, a unique, slightly papillate ostiole was observed. In some cases, however, it was either difficult to recognize or absent entirely. The size of the pycnidia varies within the range of 125-300 x 125-300 µm. The pycnidia present a white-yellowish colour matrix with moderate production of conidia, or an absence thereof. Formation of light and dark brown chlamydospores and dictyochlamydospores.

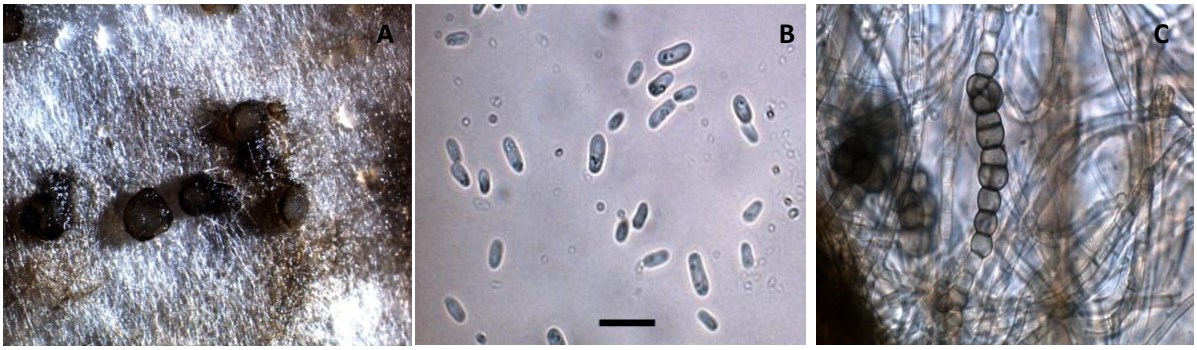

Fig. 24. Phoma isolate 13.2P. A. Pycnidia on the agar medium surface. C. Conidia. D. Dictyochlamydospores. Scale bars B=10 µm.

Isolate 12.18

The diameter of the colony ranged from 29 to 30 mm after seven days with regular border. Moderate production of concentrical zones of floccose to wooly, white, brown, green to olivaceous aerial mycelium which can develop felty areas near the colony centre with an abundant presence of pycnidia. Under UV light conditions, the wooly white and brown mycelium could be produced in sectors. Size of the conidia: 3.7-6.1 x 1.5- 2.3 µm (average: 5 x 2 µm). Conidia are ellipsoidal, single-celled, hyaline, presenting one to four polar, small-medium size guttules. Pycnidia are produced at a moderate rate and are brown to black in colour, globose and subglobose in the agar, taking an irregular form once situated on the agar. The pycnidia surface is glabrous, with pseudoparenchymatous thick-walled cell structure. Solitary and confluent pycnidia fall within the range of 50-100 x 50-100 µm and

present one slightly papillate ostiole. The pycnidia present a white matrix with moderate production of conidia.

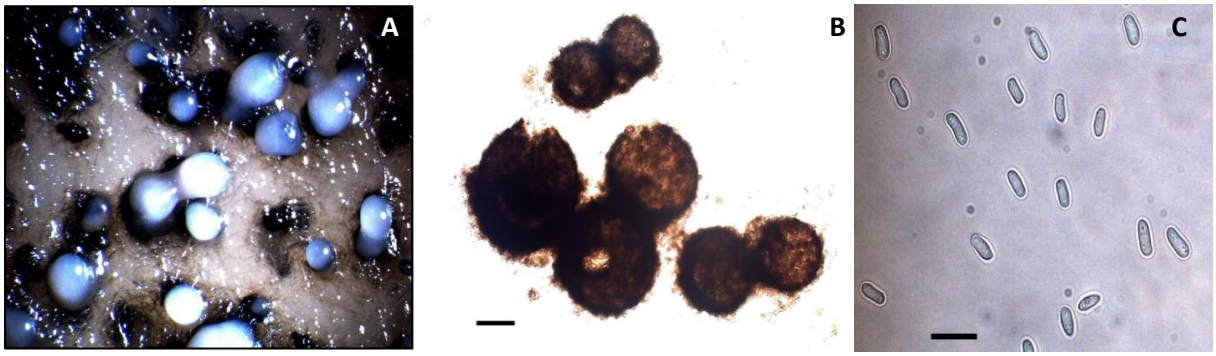

Fig. 25. Phoma isolate 12.18. A. Pycnidia in/on the agar medium surface. B. Pycnidia. C. Spores. Scale bars B=50 µm C=10 µm.

Isolate 12.20

According to the CBS analysis: *Peyronellaea* new species. (syn. *Didymella* spp.). The colony showed regular growth, varying between 24 and 28 mm after seven days Moderate production of concentrical zones of flat/effuse to floccose white and brown aerial mycelium. Size of the conidia: 4.5-7.9 x 1.6-3.9 µm (average 6.1 x 2.7 µm). Conidia are ellipsoidal, single-celled, hyaline, presenting one to four polar, medium-large size guttules. Under UV light conditions, the concentric zones remain predominantly different tones of dark to pale brown colours. Pycnidia production varies from scarce to moderate and is situated under the mycelium layer. The pycnidia are dark brown to black, globose on agar, subglobose in agar, with pseudoparenchymatous cell structure. Pycnidia are either solitary or confluent and their size ranges between 75-200 x 50-150 µm. Pycnidia mostly present one ostiole, which varies in form from slightly to conspicuously papillate. White matrix present with moderate production of conidia.

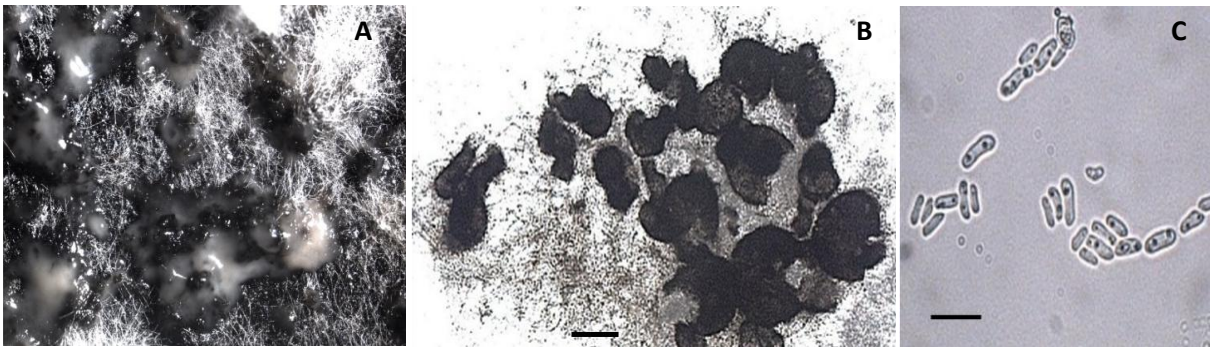

Fig. 26. Phoma isolate 12.20. A. Pycnidia in/on the agar medium surface. B. Pycnidia. C. Spores. Scale bars B=100 µm; C=10 µm.

Isolate 13.20

Growth rates of the colony ranged between 37 and 38 mm after seven days with a regular border. Abundant production of whitish wooly mycelium with some green olivaceous to grey areas scattered in the mycelium or in the margin zone. Size of the conidia: 4.3-7.7 x 1.8-2.9 µm (average 5.7 x 2.4 µm). Conidia are ellipsoidal to oblong, single-celled, hyaline, presenting none to three polar, small-medium size guttules. Pycnidia are scarcely to moderately produced under the mycelium layer, mostly on the agar surface. Pycnidia are brown, globose to subglobose, solitary and confluent, glabrous with a pseudoparenchymatous thick wall. A single, slightly papillate ostiole was observed for each pycnidia. The size of the pycnidia varies within the range of 100-250 x 100-300 µm. The pycnidia present a white matrix, with moderate production of conidia.

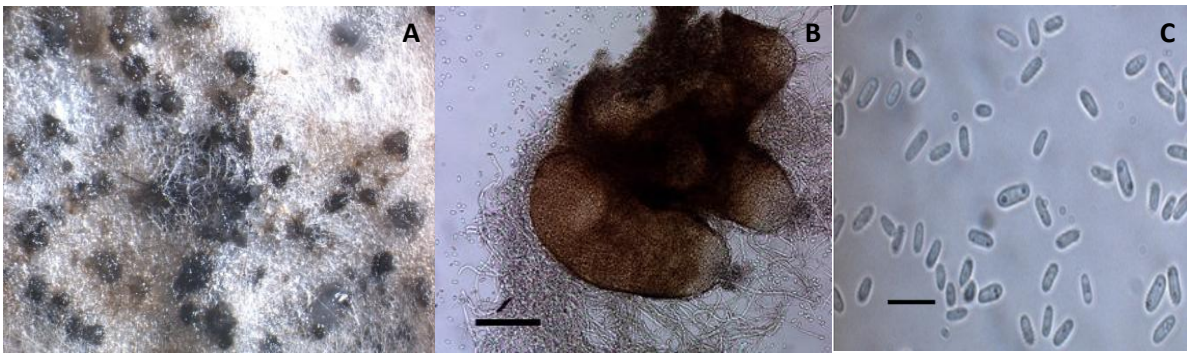

Fig. 27. Phoma isolate 13.20. A. Pycnidia on the agar medium surface. B. Pycnidia. C. Conidia. Scale bars B=100 µm; C=10 µm.

Non-pathogenic *Phoma* spp. isolates

Here, only a few representative isolates are presented. Descriptions of further non-pathogenic isolates can be found in the appendix.

In general, non-pathogenic *Phoma spp.* isolates present a faster growth than the pathogenic *Phoma* spp. isolates, which, with the exception of isolate 13.2B and 12.10 (range 35-43) oscillate between 45-75 mm after seven days. With the exception of isolate 13.2B (11.9 x 3.9 µm), spore size varies within the range 2.9-6.3 x 1.3-3.1 µm.

Isolate 13.2B

According to the CBS analysis: *Peyronellaea* new putative species.

The colony showed regular growth, varying between 35 and 38 mm after seven days. There was scarce production of brown, flat to effuse aerial mycelium, which can develop some white floccose areas in the centre of the colony. Under UV light conditions, mycelium production is inhibited, producing only pycnidia. Size of the conidia: 9.9-11.9 x 3.3-4.3 µm

(average 11.9 x 3.9 µm). Conidia are variable, ellipsoidal to allantoid, single-celled, hyaline, with one to five polar and apolar, medium-size guttules. Pycnidia are dark brown, produced on the agar surface, moderately and mostly solitary. Globose, subglobose and irregular pycnidial forms were observed. The pycnidia surface is pilose with a pseudoparenchymatous thick wall. The pycnidia size falls within the range of 200-500 x 100-400 µm. Although in some cases several ostioles per pycnidia could be observed, the pycnidia regularly present only one, slightly papillate ostiole, from which a white colour matrix emerges with moderate production of conidia. Chlamydospores were present.

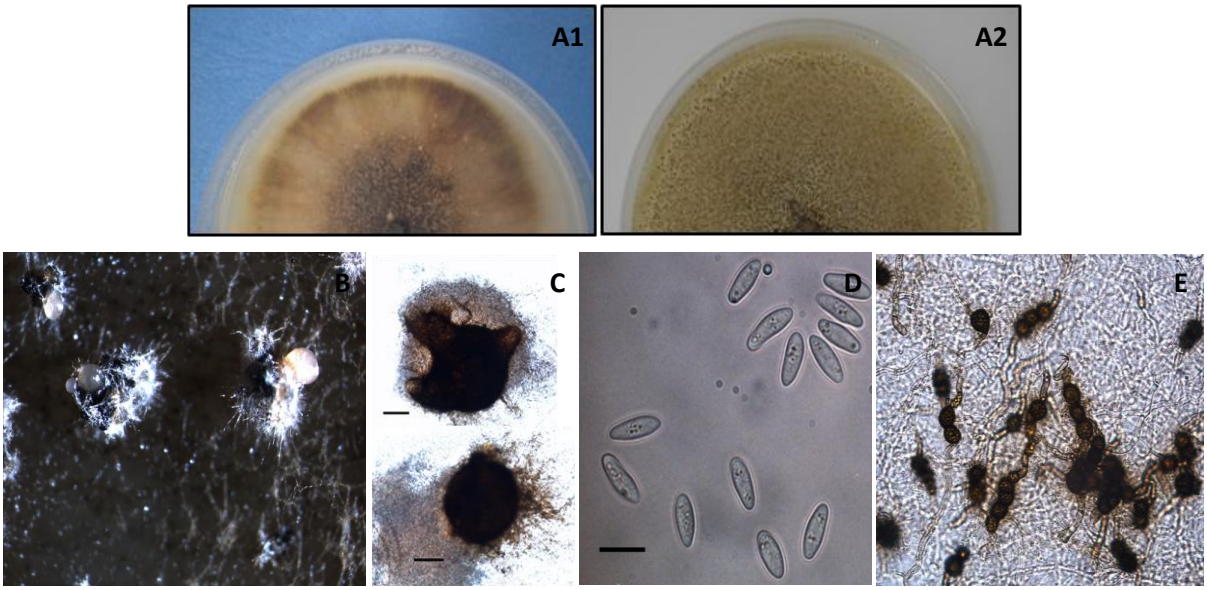

Fig. 28. Phoma isolate 13.2B, origin: Ostenfeld. A. *In vitro* growth on OA after fourteen days. Dark conditions (A1). UV light regime (A2). B. Pycnidia on the agar medium surface. C1-2. Pycnidia. D. Conidia. E. Chlamydospores. Scale bars C1-2=100 µm; D=10 µm.

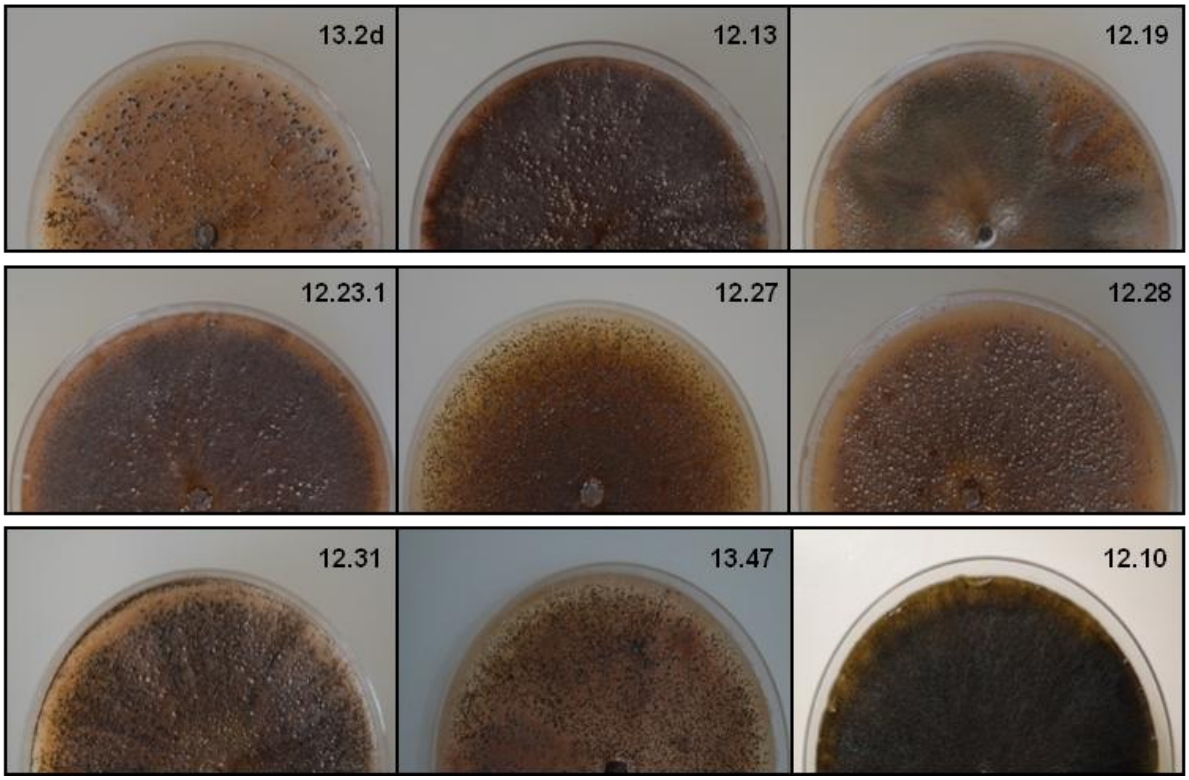

Fig. 29. Non-pathogenic *Phoma* spp. isolates. Fourteen-day-old colonies on OA.

Isolate 12.13

According to the CBS analysis: *Peyronellaea* new putative species. (syn. *Didymella* spp.). Colony size 70-75 mm in diameter after seven days, with a regular border. Scarce production of aerial mycelium, which is salmon and flat/effuse to scattered. Mycelium is immersed, dark brown or red/vinaceous. Size of the conidia: 2.9-5.2 x 1.2- 2.0 µm (average 3.4 x 1.6 µm). Conidia are ellipsoidal, single-celled, hyaline, presenting either one or no guttule, which is polar and small in size. Large spores could also be observed but were unusual. Pycnidia are produced in abundance and homogeneously distributed on the plate, situated on the agar surface and also in the agar. Pycnidia are brown, globose to irregular, glabrous or with hyphal outgrowths, with pseudoparenchymatous cell wall structure. The size of the pycnidia ranges between 75-300 x 75-200 µm and are either solitary or confluent with other pycnidia. Pycnidia mostly present a single ostiole, while in some cases it is absent. If present, it is conspicuously papillated and so-called ‘necks” are also formed. Pycnidia present a rosy buff, white conidial matrix. Conidia are produced in abundance and extruded in cirri.

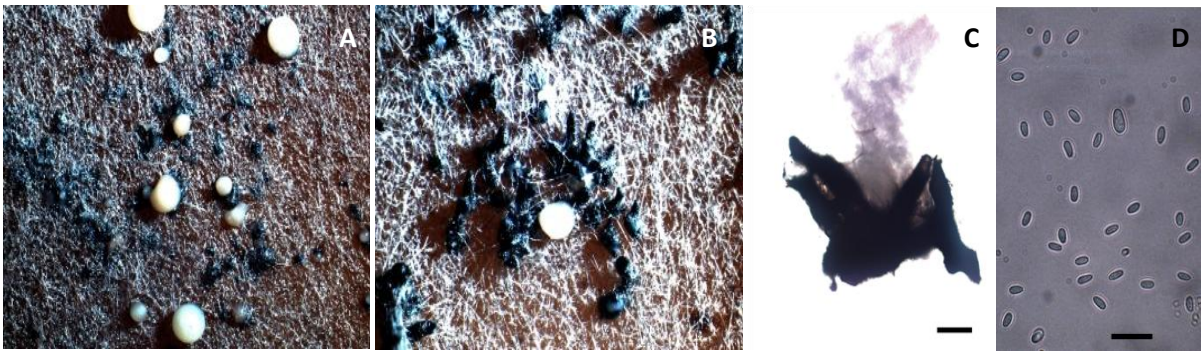

Fig. 30. Phoma isolate 12.13. A1-2. Pycnidia on the agar medium surface with white conidial matrix. B-C. Irregular pycnidia. D. Conidia. Scale bars C=100 µm; D=10 µm.

Isolate 12.19

Colony diameters after seven days: 65 and 72 mm with a regular border. Immersed mycelia are flat, red vinaceous to brown. Aerial mycelium brown, flat to effuse with some scattered areas of effuse mycelium. Size of the conidia: 3.3-4.7 x 1.5-1.9 µm (average 3.9 x 1.7 µm). Large spores could also be observed but were unusual. Conidia are ellipsoidal, single-celled, hyaline, presenting one to three polar and small guttules. Pycnidia are produced in abundance and mostly at the margin of the colony situated on the agar surface and also in the agar. Pycnidia are brown to black, globose, subglobose and flask-shaped, glabrous or with hyphal outgrowths, with a pseudoparenchymatous cell wall structure. The size of the pycnidia ranges between 75-150 x 75-200 µm and are located solitarily or in confluence with other pycnidia. Pycnidia mostly present, with or without ostiole, predominantly slightly or non-papillate. Conidial matrix whitish to yellow, containing conidia in abundance.

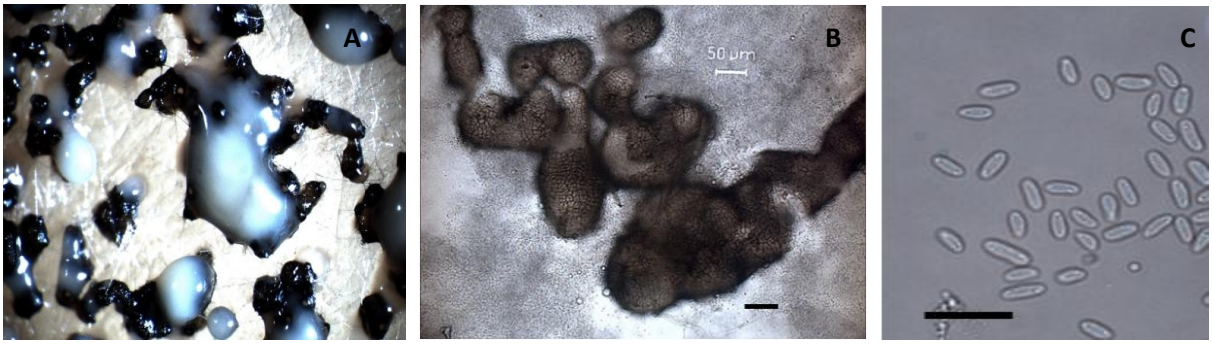

Fig. 31. Phoma isolate 12.19. A. Pycnidia on the agar medium surface. B. Pycnidia. C. Conidia. Scale bars B=50 µm; C=10 µm.

Isolate 13.36

According to data from the DNA sequencing and molecular analysis: *Phoma pomorum* (syn. *Didymella pomorum*). Growth rates of the colony between 54 and 58 mm after seven days with regular border. Moderate to abundant production of green/olivaceous buff, floccose to wooly aerial mycelium with some floccose white tufts. Under UV light, the colony develops a felty black centre. Size of the conidia: 3.8-6.5 x 1.6-2.3 µm (average 4.6 x 2 µm). Conidia are ellipsoidal single-celled, hyaline, presenting two to three polar, small-sized guttules. Pycnidia are produced in abundance both on and in medium. Pycnidia are brown to black, globose to subglobose, solitary and confluent, glabrous with pseudoparenchymatous wall. Size of the pycnidia varies within the range of 50-200 x 50-200 µm. Absent or single, slightly to conspicuously papillate ostiole. The pycnidia present a hyaline-pinkish matrix with abundant production of conidia.

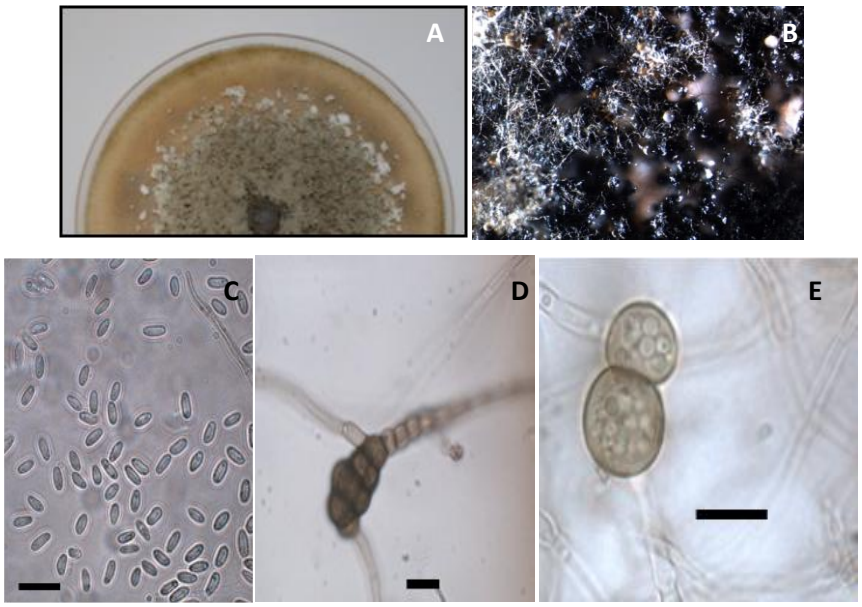

Fig. 32. Phoma isolate 13.36. A. *In vitro* growth on OA after fourteen days (dark conditions). B. Pycnidia on the agar medium surface. C. Conidia. D. Dyctiochlamydospores. E. Chlamydospores. Scale bars C-D=10 µm.

***Phoma* spp. obtained from the CBS**

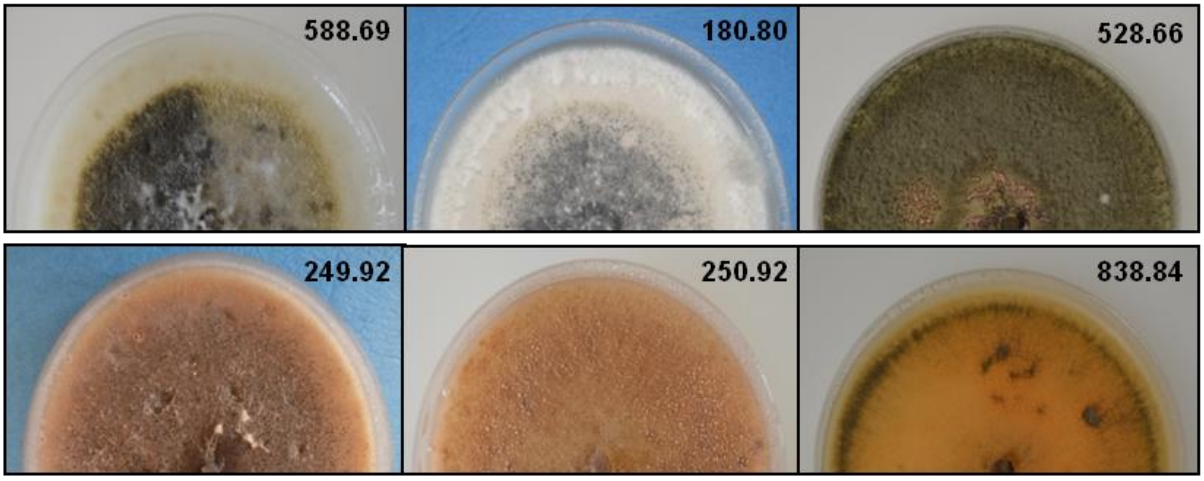

Fig. 33: Fourteen-day-old *Phoma* spp. isolates obtained from the CBS on OA.
588.89 *Ph. zeae-maydis* 180.80 *Ph. sorghina* 528.*66 Ph. glomerata* 249.92-250.92 *Ph. subherbarum*. 838.84 *Ph. pomorum.*

Phoma zeae-maydis CBS 588.69 (syn. *Didymella maydis*)

Growth rates of the colony between 32 and 34 mm after seven days with regular border. Scarce to moderate production of whitish, green/olivaceous flocosse mycelium with black felty centre and yellow border. Size of the conidia: 10.7-16.5 x 3.7-4.8 µm (average 13.5 x 4.1 µm). Conidia are distinctively larger than those of all the other *Phoma* isolates presented. Conidia are ellipsoidal, mostly single-celled but single septa can be developed. Conidia are hyaline, presenting one to five polar and apolar, large guttules. Pycnidia are produced moderately under the mycelium layer, mostly on the agar surface. Pycnidia are brown, globose to subglobose and distribution is predominantly solitary. Pycnidium surface glabrous or with hyphal outgrowths with pseudoparenchymatous wall. Pycnidia do not have ostioles or if present, it is single and non-papillate. Size of the pycnidia varies within the range of 180-250 x 150-250 µm. The pycnidia present a white matrix with scarce to abundant production of conidia.

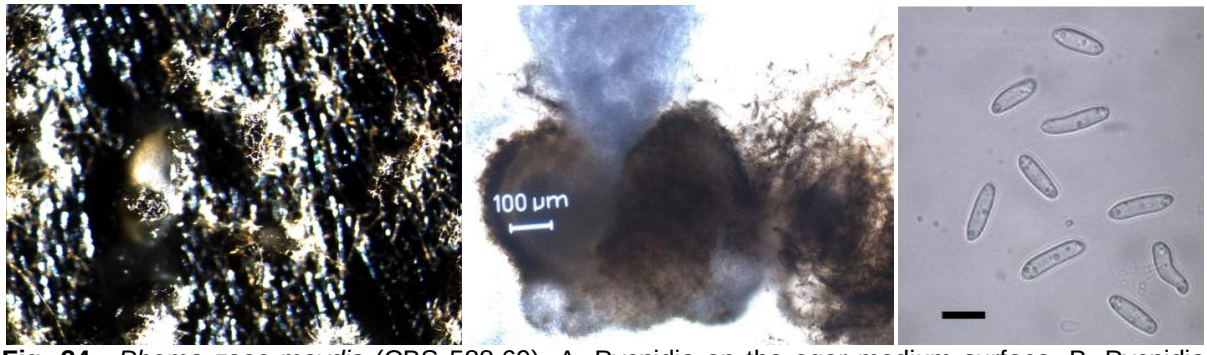

Fig. 34. *Phoma zeae-maydis* (CBS 588.69). A. Pycnidia on the agar medium surface. B. Pycnidia. C. Conidia. Scale bars B= 100 µm; C= 10 µm.

Phoma glomerata CBS 528.66 (syn. *Didymella glomerata*)

Growth rates of the colony between 50 and 54 mm after seven days with regular border. Moderate to abundant production of white, green/olivaceous flocosse and wooly aerial mycelium. Under UV light, the mycelium tends to be more floccose. The pattern of *in vitro* growth varies greatly, but green/olivaceous flocosse mycelium remains permanent. Size of the conidia: 4.3-6.6 x 2-2.9 µm (average 5.4 x 2.6 µm). Conidia are ellipsoidal to clavate, single-celled, hyaline, presenting one to two polar, medium-sized guttules. Brown to black pycnidia are produced in abundance on the medium, subglobose, both solitary and confluent, glabrous or with hyphal outgrowths and with pseudoparenchymatous wall. A single, slightly to conspicuously papillate ostiole was observed most of the time for every pycnidium. The pycnidia present a white to rosy buff matrix, with abundant production of conidia.

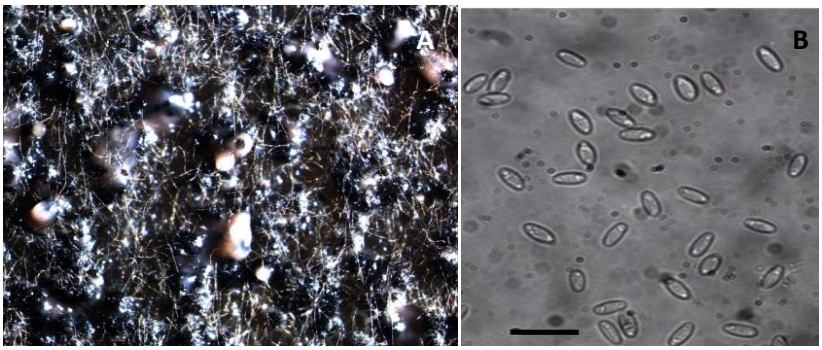

Fig. 35. *Phoma glomerata* (CBS 528.66) A. Pycnidia on the agar medium surface. B. Conidia. Scale bar=10 µm.

Phoma pomorum CBS 838.84 (syn. *Didymella pomorum*)

Growth rates of the colony between 50 and 54 mm after seven days with regular border. Moderate to abundant production of white, brown, yellow buff flat/effuse aerial mycelium. Size of the conidia: 4.1-5.9 x 1.9-3.1 µm (average 5 x 2.5 µm). Large conidia were also observed. Conidia are ellipsoidal to oblong, single-celled, hyaline, presenting one to two polar, small-medium sized guttules. Pycnidia are produced in masses and are submerged in the medium where only their pinkish orange matrix emerges from the agar. Pycnidia are pale brown, subglobose, solitary and confluent, glabrous or with brown hyphal outgrowths, with pseudoparenchymatous wall. It seems to be that pycnidia only have a single, slightly papillate ostiollum (or this is absent).

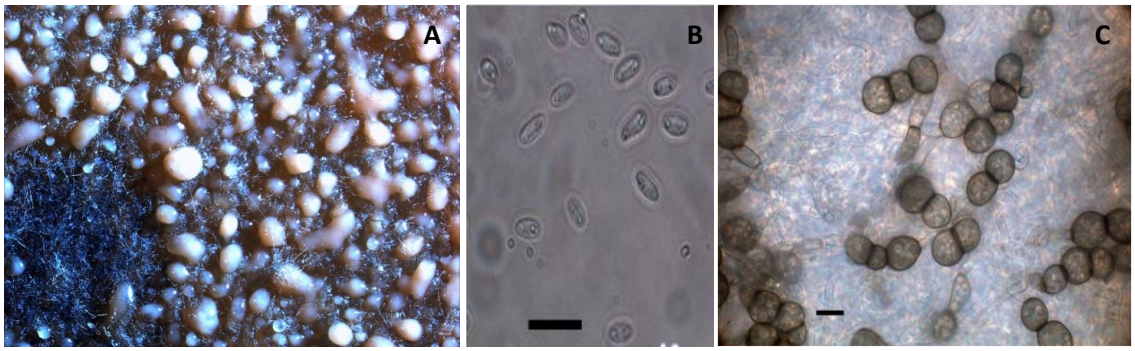

Fig. 36. *Phoma pomorum* (CBS 838.84). A. Conidial matrix (pycnidia submerged in the agar medium surface). B. Conidia. C. Chlamydospores. Scale bars B-C=10 µm.

Phoma sorghina CBS 180.80 (syn. *Epiccocum sorghinum*)

Growth rates of the colony between 45 and 49 mm after seven days with regular border. Scarce to moderate production of white, grey, blue cyan floccose or wooly aerial mycelium under dark conditions, whereas a UV light regime inhibits its production and favours the development of salmon-orange pigments in the medium. Size of the conidia: 4.5-7.6 x 2.7-3.9 µm (average 6 x 3.2 µm). Conidia are ellipsoidal to ovoid, single-celled, hyaline, presenting none to two polar, small-sized guttules. Pycnidia are mostly produced on the medium and distinctly scattered in the mycelium. Conidial matrix frequently absent, but if present, hyaline to white, while in mature pycnidia orange-brown and normally only when wall is fractured. Pycnidia are black and vary from globose to subglobose. Pycnidia are mostly solitary but also confluent in some cases. Pycnidia size ranges from 50-100 x 50-125 µm. The surface is normally glabrous with pseudoparenchymatous wall. Pycnidia have one to three ostioles and these are slightly to conspicuously papillated. Conidia extruded in cirri can frequently be observed.

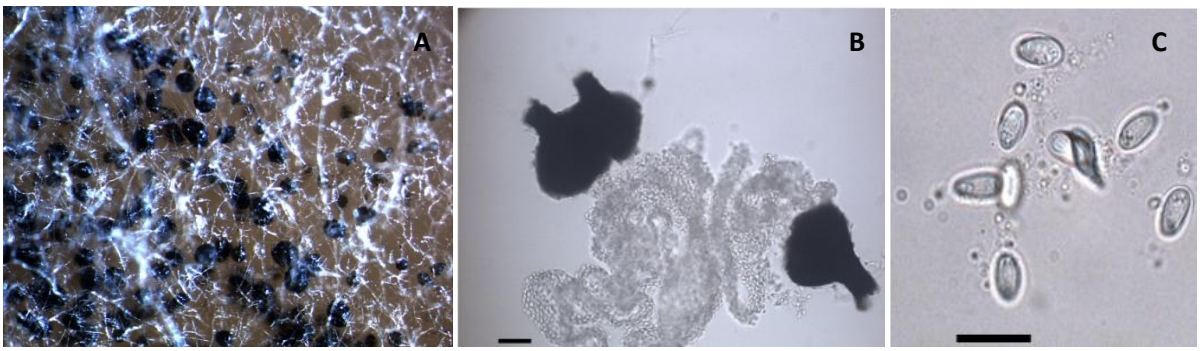

Fig. 37. *Phoma sorghina* (CBS 180.80). A. Pycnidia on the agar medium surface and scattered on the mycelium. B. Pycnidia C. Conidia. Scale bars B=50 µm; C=10 µm.

Phoma subherbarum 249.49 (syn. *Didymella subherbarum*)

Colony diameter after seven days about 50 and 55 mm with regular border. Aerial mycelium is white to salmon, flat to effuse and scattered. Immersed mycelium brown. Brown to red/vinaceous pigments are produced in the medium. Size of the conidia: 2.7-4.9 x 1.4-2.4 µm (average 4 x 2 µm). Conidia are ellipsoidal, single-celled, hyaline, either with or without one or two polar, small guttules. Pycnidia are produced in abundance and evenly distributed on the plate, on the surface and in the medium. Pycnidia are either solitary or fuse with other pycnidia. The characteristical shape is mostly subglobose and irregular, with glabrous surface and pseudoparenchymatous wall. Size of the pycnidia falls within the range of 50-250 x 50-250 µm. Pycnidia present with one to four, slightly to conspicuously papillate ostioles with possible development of 'necks'. Micropycnidia also present. Conidial matrix initially hyaline and later becomes white to pinkish, containing conidia in abundance.

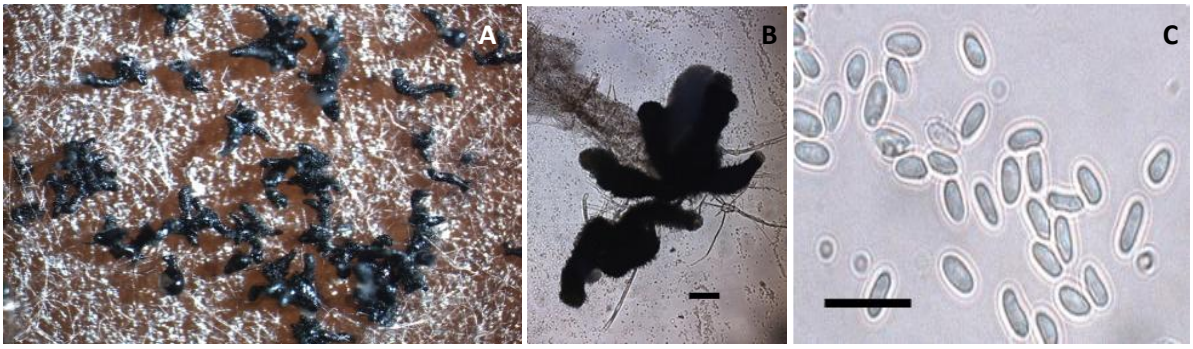

Fig. 38. *Phoma subherbarum* (CBS 249.49). A. Pycnidia on the agar medium surface. B. Pycnidia C. Conidia Scale bars B=100 µm; C=10 µm.

Phoma subherbarum 250.92 (Syn. *Didymella subherbarum*)

Colony diameter after seven days 61 and 65 mm with regular border. *In vitro* growth resembles isolate CBS 249.92 (also *Ph. subherbarum*), but the colour spectrum is lighter with white, flat, scattered aerial mycelium, with immersed light brown and salmon pigments in the agar. Size of the conidia: 2.9-4.8 x 1.2-2.0 µm (average 3.9 x 1.8 µm). Conidia are ellipsoidal, single-celled, hyaline, either without or with one or two small guttules, normally polar. Pycnidia are produced in abundance, both on the surface and in the agar. Pycnidia are brown to black, mostly solitary and globose to subglobose. Pycnidia have a glabrous surface or with hyphal outgrowths and pseudoparenchymatous cell wall. Size of the pycnidia falls within the range of 100-150 x 100-150 µm. Pycnidia present one to two non- papillate ostioles. Conidial matrix white, yellow or pinkish containing conidia in abundance.

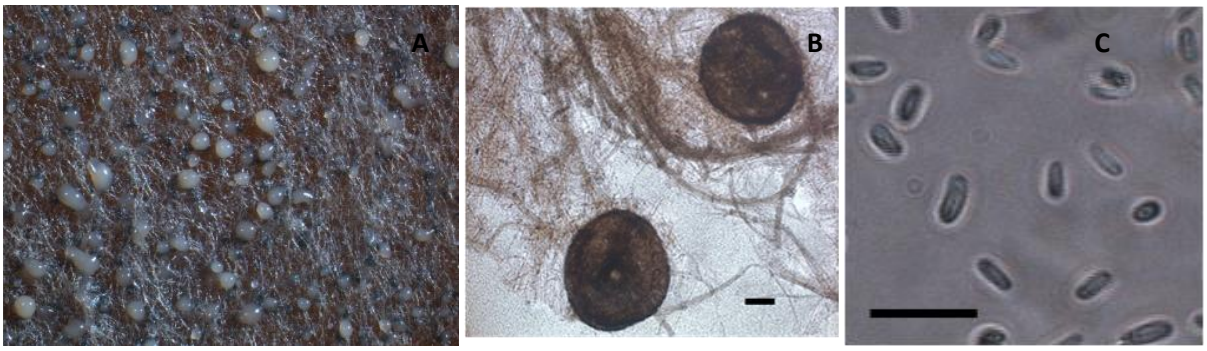

Fig. 39. *Phoma subherbarum* (CBS 250.92). A. Conidial matrix and pycnidia on/in agar medium surface. B. Pycnidia. C. Conidia. Scale bars B=50 µm; C=10 µm.

3.1.5. Characterisation of *Fusarium* spp. and pathogenicity tests

Fusarium spp. were frequently found in the infected leaf samples, together with other fungal organisms. Testing for the Koch's postulates becomes necessary for two reasons: first, to confirm or reject *Fusarium* species as symptomatic pathogens producing leaf spots, and second, to accurately associate less known pathogenic fungal organisms (such as those belonging to the *Phoma* spp.) when *Fusarium* was also present in the same lesions.

Fusarium symptoms were observed in the field in corn husks, leaf sheaths, stalks and ears (Figure 40). On the leaf, the pathogen was normally sharing lesions with another pathogen. In total, 21 *Fusarium* isolates were obtained (Table 29 and Figure 41).

Due to the secondary role of the pathogen in producing foliar maize spot diseases, the identification in this work, if possible, was only carried out through morphological observations. From the 21 isolates, 12 could be identified. These belong to four species: *F. graminearum*, *F. avenaceum*, *F. cerealis* and *F. proliferatum*.

In the greenhouse, inoculations of healthy plants were carried out for 12 *Fusarium* spp. isolates (see Table 29). None of the isolates could produce any symptoms after spraying spore suspensions onto healthy leaves of the plant. Thus, *Fusarium* spp. could not be confirmed as main leaf pathogens which produce symptomatic spots on maize leaves.

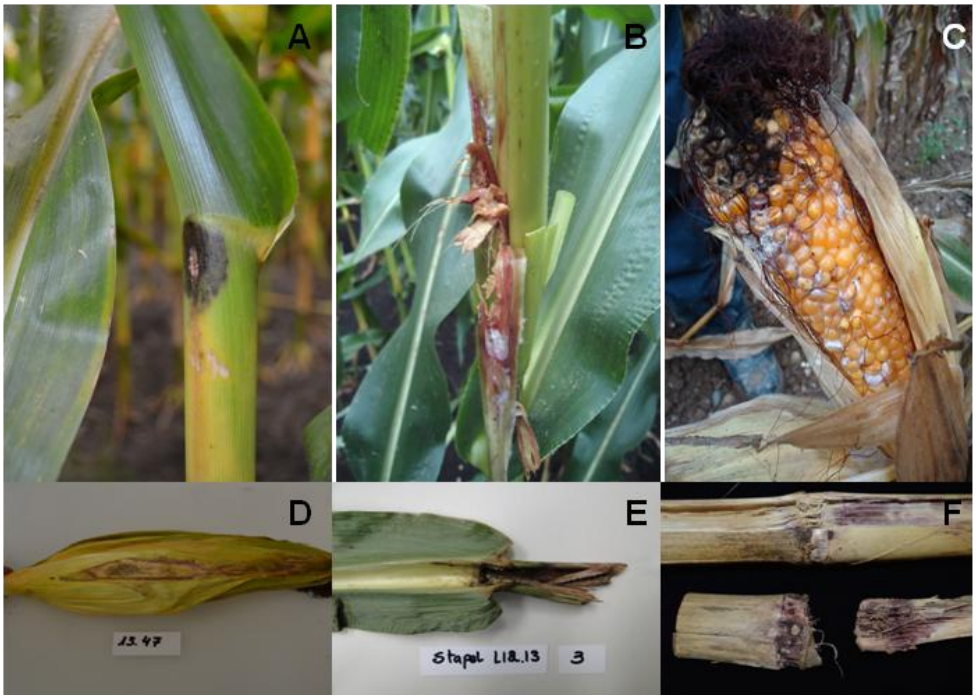

Fig. 40. *Fusarium* spp. symptoms observed in the field on: A-B. Stalk. C. Ear kernels. D. Corn husks. E. Leaf sheaths. F. Presence in maize debris (*Giberella zeae*).

Tab. 29. *Fusarium* spp. isolates obtained from the monitoring in 2012 and 2013.

Location	*Fusarium* spp.	Substrate
12.2*	***F. graminearum***	**Seeds**
13.2	***F. graminearum***	**Stalk debris**
12.8*	***Fusarium* spp.**	**Leaf**
12.10*	***Fusarium* spp.**	**Leaf**
12.12*	***F. graminearum***	**Leaf**
12.13*	***F. graminearum***	**Leaf shead**
12.14*	***Fusarium* spp.**	**Leaf shead**
12.19*	***F. avenaceum***	**Leaf shead**
12.23*	***Fusarium* spp.**	**Leaf shead**
12.23b	***Fusarium* spp.**	**Leaf**
12.27*	***F. avenaceum***	**Leaf**
12.30*	***Fusarium* spp.**	**Leaf**
12.31*	***F. avenaceum***	**Leaf**
13.18	***Fusarium* spp.**	**Leaf**
13.20	***F. avenaceum***	**Leaf**
13.42	***Fusarium* spp.**	**Leaf**
13.43	***Fusarium* spp.**	**Leaf**
13.47	***Fusarium* spp.**	**Corn husk**
13.47b	***Fusarium* spp.**	**Corn husk**
13.47c	***Fusarium* spp.**	**Leaf**
13.47d	***F. graminearum***	**Leaf**

*isolate tested for pathogenicity

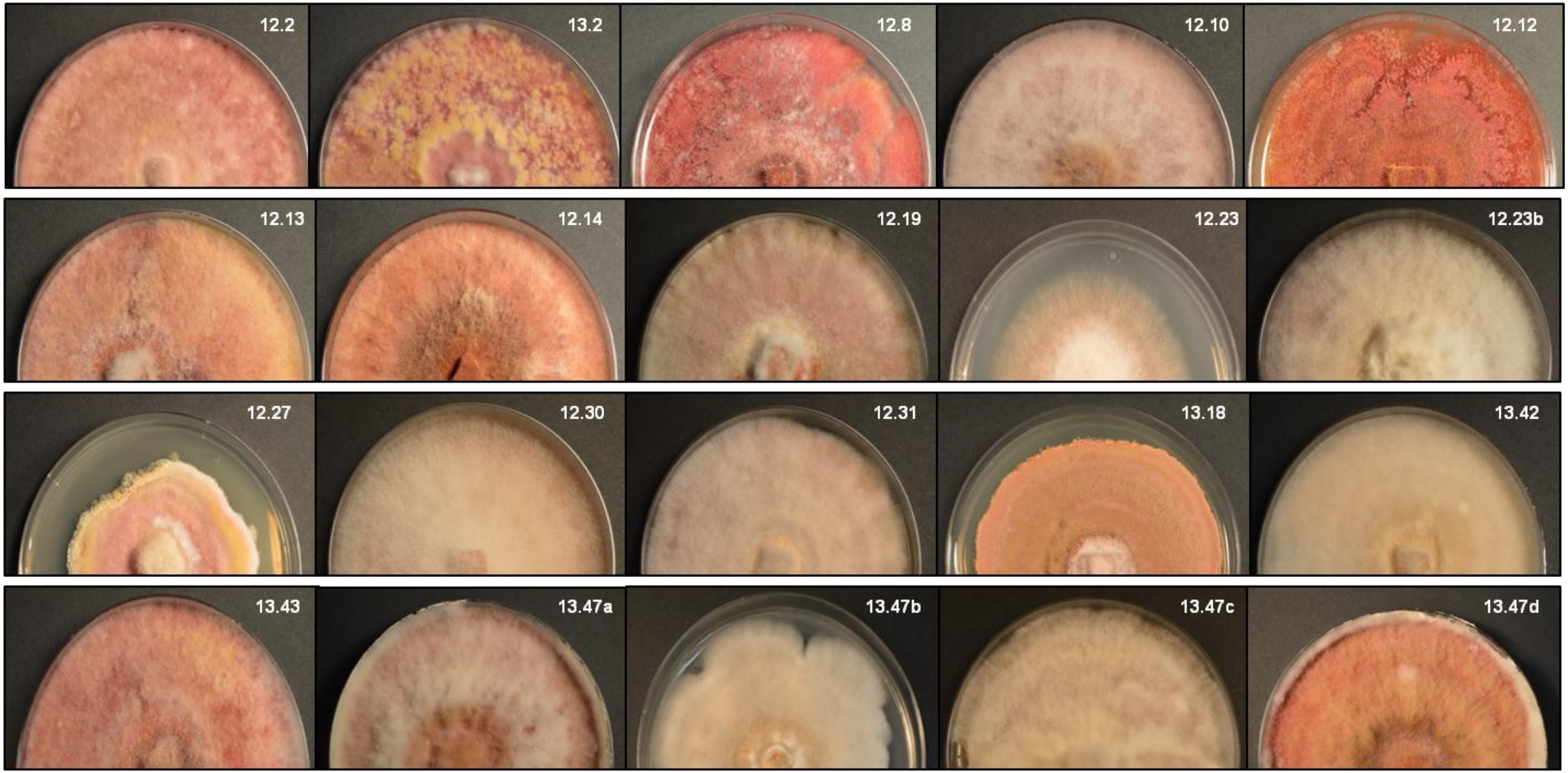

Fig. 41. Fourteen-day-old colonies of *Fusarium* spp. isolated from maize samples during the monitoring 2012 and 2013.

3.1.6. Testing of further organisms

Following the same procedure used for all fungi species tested, two isolates of *Alternaria* spp. and one of *Epiccocum nigrum* were evaluated. None of the isolates produced symptoms on the inoculated plants. Consequently, these fungal species could not be classified as causal agents of maize leaf spots.

3.1.7. Summary of the inventory

This summary is based on observations both in the field and the laboratory, as well as the results obtained after Koch's postulates. Therefore, tested and non-tested isolates are included in this section. Table 30 below provides a regional overview of the final results of the monitoring carried out in 2012 and 2013 for numerous fungal leaf diseases. Other pathogens observed like *Ustilago maydis* and *Sclerophthora macrospora* or *Septoria* spp. are also mentioned.

Tab. 30. Summary of the monitoring of maize leaf diseases in Central Europe during the 2012 and 2013 seasons.

Code location	Location	Season	*E. turcicum*	*K. zeae*	*P. sorghi*	*B. zeicola*	*C. graminicola*	*Phoma spp**	*Fusarium spp.**	Others
North										
12.2	**Ostenfeld**	2012		X		X		Xs	Xs	
12.11	**Köhn (Plön)**	2012		X						
12.12	**Schleswig**	2012						Xs	Xs	
12.13	**Stapel**	2012		X				Xs	Xs	
12.20	**Nordholz**	2012		X		X	X	X		
12.23	**Rade**	2012		X				X/Xs		
12.27	**Bad Oldesloe**	2012				X		Xs	Xs	
12.31	**Giekau**	2012		X				X/Xs		
13.2	**Ostenfeld**	2013		X		X		X		
13.20	**Nordholz**	2013		X			X	X		*U.maydis*
13.40	**Wessin**	2013		X		X		Xs		
13.44	**Taaken**	2013								
13.49	**Dewitz**	2013								
North-West										
12.19	**Cloppenburg**	2012	X	X				Xs	Xs	
12.24	**Beesten**	2012	X	X						
12.14	**Ommen**	2012	X	X	X				Xs	*S. macrospora*
12.15	**Princepeel**	2012	X		X	X	X			
12.21	**Ottersum**	2012	X	X	X					
12.28	**Groningen**	2012	X	X				Xs		*Septoria* spp.
12.30	**Biddinghuizen**	2012	X	X		X				*Septoria* spp.
13.14	**Ommen**	2013	X	X						

* X: pathogenic isolates producing symptoms. Xs: isolates considered saprophytes or not producing symptoms on maize leaves after Koch's postulates.

Tab. 30. (continued) Summary of the monitoring of maize leaf diseases in Central Europe during the 2012 and 2013 seasons.

Code location	Location	Season	*E. turcicum*	*K. zeae*	*P. sorghi*	*B. zeicola*	*C. graminicola*	*Phoma spp**	*Fusarium spp.*	Others
Central										
12.3	**Liesborn**	2012	X					Xs		*U. maydis*
12.25	**Milte**	2012	X	X						
12.33	**Springe**	2012			X					
13.42	**K. Marzehns**	2013								
13.43	**Bodenwerder**	2013								
13.45	**Linum**	2013				X				
13.47	**Waake**	2013						X		
13.48	**Göttingen**	2013		X				Xs		*U. maydis*
East										
12.4	**Nechanice**	2012			X					
12.5	**Lysice**	2012	X		X				Xs	*U. maydis*
12.6	**Lesany**	2012	X						Xs	
12.7	**Bylany**	2012				X			Xs	*Bipolaris* spp.
12.8	**Caslav**	2012							Xs	*Bipolaris* spp.
12.9	**Plana**	2012								
12.26	**Galowo**	2012				X				
12.29	**Zybiszów**	2012			X					*P. polysora*

* X: pathogenic isolates producing symptoms. Xs: isolates considered saprophytes or not producing symptoms on maize leaves after Koch's postulates.

Tab. 30. (continued) Summary of the monitoring of maize leaf diseases in Central Europe during the 2012 and 2013 seasons.

Code location	Location	Season	*E. turcicum*	*K. zeae*	*P. sorghi*	*B. zeicola*	*C. graminicola*	*Phoma spp**	*Fusarium spp.*	Others
					South					
12.10	**Braunau**	2012	X	X	X			Xs	X	
12.16	**Mariaporsching**	2012	X							
12.17	**Mintraching**	2012	X	X						
12.18	**Schönering**	2012	X	X		X		X		
12.32	**Marburg**	2013	X		X					
13.10	**Braunau**	2013	X			X				
13.39	**Windsbach**	2012								
13.36	**Mittich**	2013	X	X	X			Xs		*S. macrospora*
13.37	**Hartkirchen**	2013	X			X		Xs		
13.38	**Ruhstorf (Rott)**	2013	X							
13.41	**Mariaporsching**	2013								
13.46	**Steiermark**	2013								
					South-West					
12.22	**Moorlas**	2012	X				X			
12.34	**Garlin**	2012	X							

* X: pathogenic isolates producing symptoms. Xs: isolates considered saprophytes or not producing symptoms on maize leaves after Koch's postulates..

3.1.8. Distribution and prevalence of fungal pathogens occurring on maize leaves

In this section, the prevalence and distribution (Table 31 & Figure 42) of the pathogens which are considered the most common causal agents of symptoms on maize leaves, i.e. *E. turcicum*, *K. zeae*, *P. sorghi, C. graminicola*, *Phoma* spp., are presented according to the data from the monitoring. Note that only the pathogenic *Phoma* isolates are included on the map.

E. turcicum

The pathogen was diagnosed in 18 locations in 2012 and only six in 2013, representing a prevalence of 54% and 32%, respectively. It was frequently observed in the central and eastern regions of the Czech Republic and also in the Netherlands. Although the pathogen is also frequently found in the north-western part of Lower Saxony, it is endemic in southern parts of Germany and northern Austria – in these regions, a higher disease pressure was observed for both years.

P. sorghi

Although the pathogen was diagnosed in various areas of the Netherlands and central Germany, southern Germany is the main region in which the pathogen is widespread across fields. In total, the pathogen was diagnosed in nine locations in 2012 and one location in 2013, which corresponds to a prevalence of 27% of all studied locations in 2012, falling to 5% in 2013. After *E. turcicum*, *P. sorghi* can be considered the second most important disease in southern Germany.

K. zeae

The presence of the pathogen was confirmed in a total of 16 locations in 2012 (48%) and six locations in 2013 (32%). While it was found on just a few plants in the southern German and northern Austrian locations, it was particularly widespread in fields of the Netherlands and northern Germany (northern and western Lower Saxony and Schleswig-Holstein).

B. zeicola

The pathogen was distributed across various regions. It was observed in both the southern and northern regions, as well in the west (north-western Germany) and east (Czech Republic). There was no dominant region for the disease, but a higher prevalence was observed in the northern German location of Nordholz. Prevalence ranged from 30% in 2012 to 26% in 2013.

C. graminicola

The pathogen was only identified on isolated plants in north-western Germany, the Netherlands, and France. It was diagnosed in three locations in 2012, representing 9% prevalence, falling to 5% in 2013 when it was only diagnosed in a single location.

Phoma spp.

Pathogenic *Phoma* species were identified in the northern German locations of Ostenfeld (2012 and 2013), Rade (2012), Nordholz (2012 and 2013), as well as in Waake in central Germany (2013) and in Schönering (northern Austria). In total, it was identified in three locations in 2012 and two locations in 2013. Non-pathogenic *Phoma* species are generally distributed across all the studied regions. In 2012, non-pathogenic *Phoma* species were isolated from 13 locations in 2012 and four locations in 2013, which represents a prevalence of 30% and 21%, respectively.

Tab. 31. Prevalence of maize leaf diseases according to the 2012 and 2013 monitoring.

Disease	**Turcicum leaf blight**	**Kabatiella eyespot**	**Brown spot**	**Common rust**	**Anthracnose**	***Phoma* spp.***	
Causal Agent	***E. turcicum***	***K. zeae***	***B. zeicola***	***P. sorghi***	***C. graminicola***	**NP**	**P**
2012							
Locations	18	16	10	9	3	10	3
Prevalence (100%=33)	54%	48%	30%	27%	9%	30%	9%
2013							
Locations	6	6	5	1	1	4	2
Prevalence (100%=19)	32%	32%	26%	5%	5%	21%	16%

***NP: non pathogenic. P: pathogenic.**

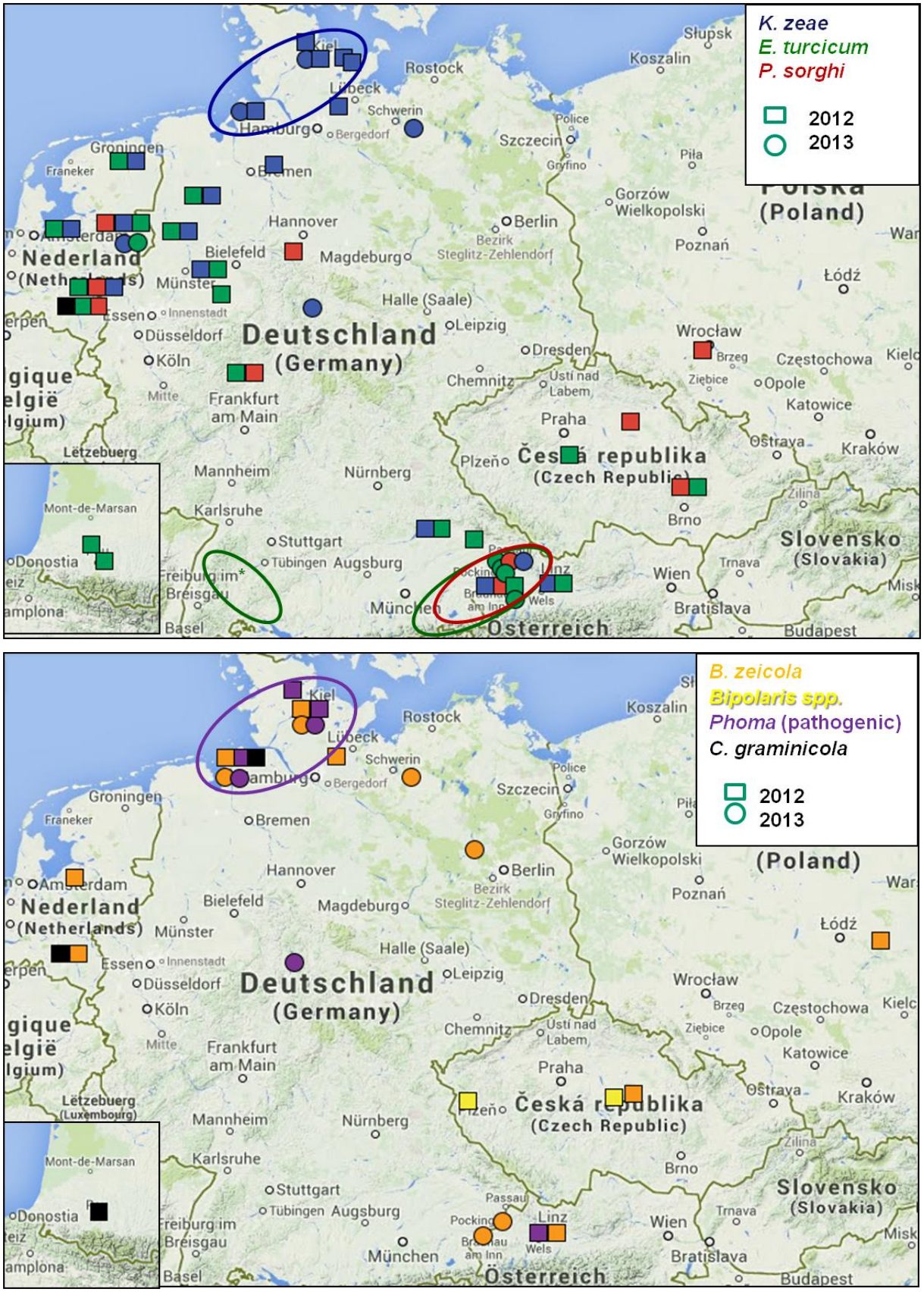

Fig. 42. Distribution of fungal pathogens on maize leaves in Central Europe from the samples collected in 2012 and 2013. Main foliar diseases, *K. zeae*, *E. turcicum* and *P. sorghi*, are shown above and of secondary importance, *B. zeicola*, *Bipolaris* spp. *Phoma* spp. and *C. graminicola*, are shown below. Principle regions of distribution are circled.

3.2. Meteorological conditions

In order to compare the development of the epidemic in the field, it is important to analyse the weather conditions which prevailed from the first appearance of the symptoms in the middle of the season to the late growing season stages. Weather conditions varied in both locations markedly from one year to another.

3.2.1. Mittich and Inzing

Figure 43 depicts the relationship between temperature (daily average), precipitation and relative humidity for the years in which trials were conducted, 2013 and 2014.

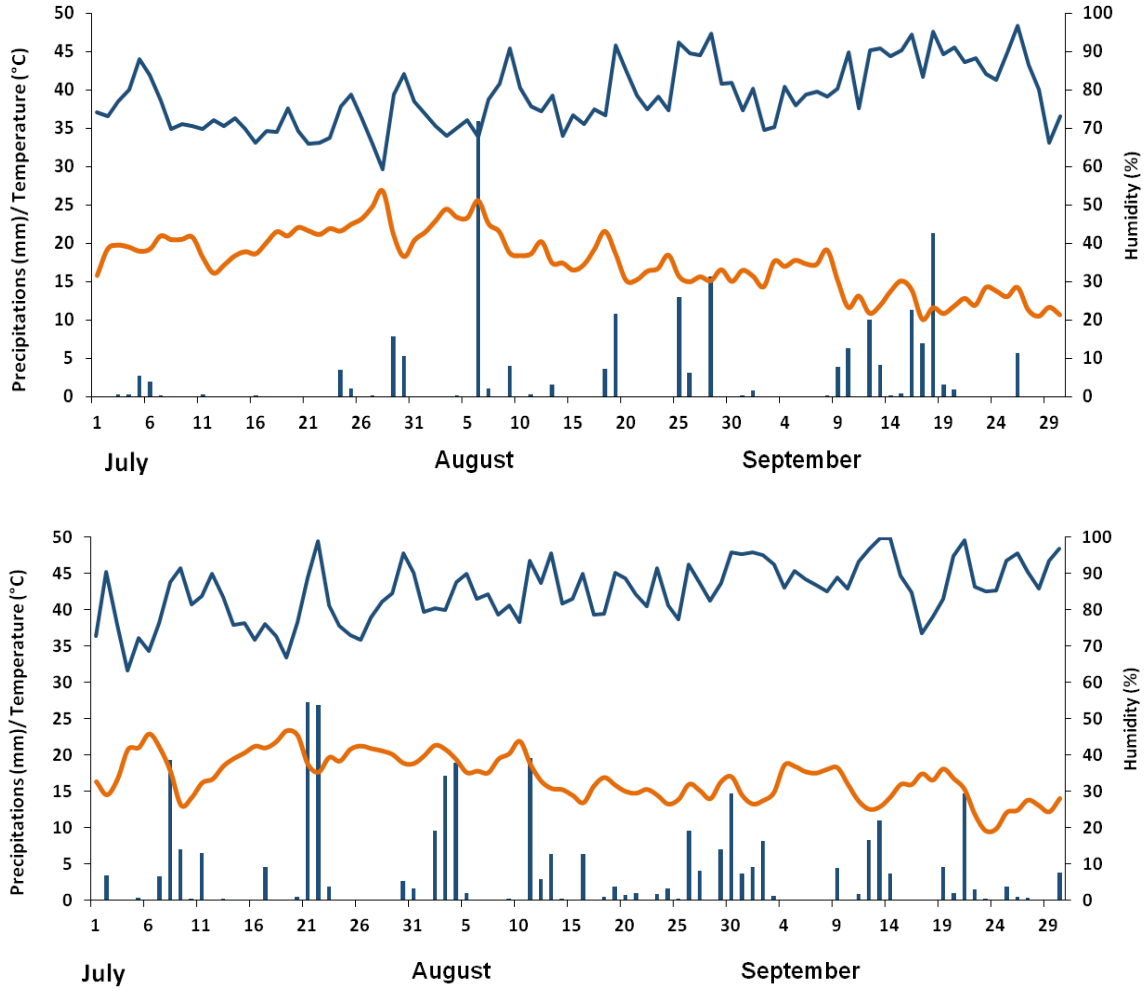

Fig. 43. Weather conditions in the region of Mittich in 2013 (above) and Inzing in 2014 (below). Precipitation — Temperature (daily average) — Relative humidity.

In 2013 (Figure 43, above), it is clear that there was very little precipitation in July, with some more frequent rain falling towards the end of the month. In August, there was a day of very heavy rain in the first week and some accumulation of precipitation towards the end of the month. Although a slightly higher quantity of precipitation was recorded in August than in September, it rained more frequently in September, i.e. on more days. In comparison, precipitation in 2014 was higher but still with frequent dry intercalary periods. This was especially noticeable in July and August, where several events of precipitation are evident.

Regarding temperatures in 2013, in July they initially rose slowly (from 15.8°C to 20.8°C), with a slight decrease around the middle of the month (20.5°C to 16.1°C) and a constant rise (peak temperature 26.8°C) until a few days before the end of the month. At the beginning of August, the temperatures rose again (21.4°C to 25.5°C), slowly falling from the second week onwards (lowest temperature 15.0°C), while in September the temperature was stable until a notable decrease in the second week (19.1°C to 10.9°C). In 2014, there was some fluctuation in July, with a clear increase in the initial days of the month (14.5°C to 22.9°C) and a sharp fall at the end of the first week (22.9°C to 13.0°C). Thereafter, there was a constant increase in the temperature until another decrease occurred (22.7°C to 17.6°C), coinciding with two days of very heavy rain on 21 and 22 July. The temperatures in August were more constant, in particular after they dropped in the second week (21.9°C to 13.4°C). September was less consistent, with higher temperatures initially, a decrease in the second week (18.2°C to 12.5°C), followed by an increase in the middle of the month and a second drop in temperature (18.1°C to 9.5°C). Overall, the average temperature in September 2014 was slightly higher than in 2013, while for July and August it was lower than 2013 (see Table 32).

To gain a greater overview of the weather patterns either side of 2013 and 2014 and make subsequent comparisons with past years, Table 32 displays the average temperatures and levels of precipitation for July, August and September from 2010 to 2015. The starting year is 2010 due to the epidemic of Turcicum leaf blight which occurred in this season (Urban 2012).

Tab. 32. Overview of weather conditions for the region of the locations of Mittich and Inzing from 2010 to 2015.

Mittich/ Inzing region	July (vegetative to reproductive phase)	August (reproductive phase to development of the fruit)	September (fruit development to ripening)
Temperature			
2010	20.6	17.8	12.9
2011	16.8	18.7	14.7
2012	19.0	18.9	14.2
2013	20.3	18.7	13.7
2014	19.2	16.7	14.8
2015	21.5	21.1	13.7
Long-term average (1981-2010)	18.7	18.1	13.9
Precipitation			
2010	190.8	146.7	36.1
2011	172.3	66.8	71.3
2012	78.9	88.9	66.4
2013	23.7	89.3	73.6
2014	105.3	127.5	69.6
2015	42.0	20.6	47.4
Long-term average (1981-2010)	112.0	109.0	72.0

For the southern German locations of Mittich and Inzing, it is clear that the temperatures varied from 2010 to 2015, especially in July and August. Here, the average monthly temperatures ranged from 16.8°C (2011) to 21.5°C (2015) and 16.7°C (2014) to 21.1°C (2015) in July and August, respectively. The levels of precipitation also varied greatly between 2010 and 2015. This was noticeable in July and August, ranging from a low of 23.7 mm (2013) to a high of 190.8 mm (2010) in July, and 20.6 mm (2015) and 146.7 (2010) in August. In September, while the average temperature and precipitation was more stable from 2010-2015, there was some variation (especially in 2010). Despite this variation, clear patterns still emerge. It is noteworthy that 2010-2015 were relatively warm and dry seasons compared to the long-term average (1981 to 2010). In fact, only six (July 2011, August 2010, August 2014, September 2010, September 2013 and September 2015) of the 18 months fell below the corresponding long-term average temperature, while only five (July 2010, July

2011, August 2010, August 2014, September 2013) of the 18 months had a higher level of precipitation than the corresponding long-term average. 2010 stands out here, however, with very high levels of precipitation.

3.2.2. Ostenfeld

Figure 44 depicts the relationship between temperature (daily average), precipitation and relative humidity for the years in which trials were conducted, 2013 and 2014.

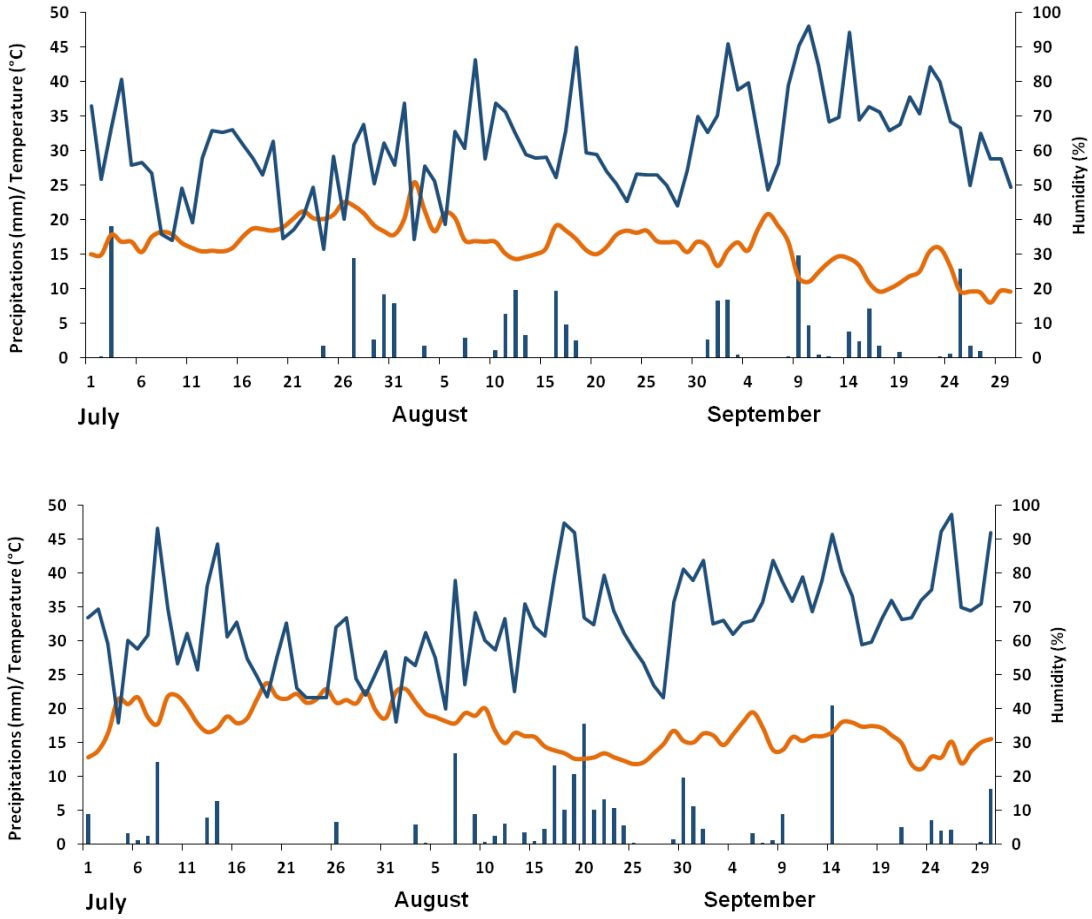

Fig. 44. Weather conditions in Ostenfeld in 2013 (above) and 2014 (below). ▌Precipitation — Temperature (daily average) — Relative humidity.

In Ostenfeld, there was minimal precipitation in both July 2013 and 2014. While some rainfall occurred around the middle of the month in August 2013, a dry period thereafter meant that the level of precipitation was much lower than in August 2014. In contrast, precipitation was more frequent in September 2013 compared to September 2014 (with a clear, isolated peak on 14 September).

Regarding the temperature in 2013, in July the temperatures were relatively constant, slowly rising (peak 22.5°C) until a slight drop occurred at the end of the month (22.5°C to 17.8°C). At the beginning of August there was a sharp rise (25.4°C) and subsequent fall (18.3°C) in the temperature, with a relatively constant trend thereafter. The temperatures in September, on the other hand, fluctuated frequently, with several notable increases and decreases within a few days (high 20.8°C; low 9.6°C), and a slight downward trend overall. In 2014, there was a sharp increase in the temperature at the start of July (12.9°C to 21.7°C) and overall it was a warmer month compared to 2013. August 2014, on the other hand, was cooler than in 2013, with a relatively constant downward trend (high 22.9°C; low 11.9°C) until an increase in the temperature at the end of the month (11.9°C to 16.8°C). As in 2013, temperatures in September 2014 fluctuated but were more consistently warm.

To gain a greater overview of the weather patterns either side of 2013 and 2014 and make subsequent comparisons, Table 33 displays the average temperatures and level of precipitation for August, July and August from 2011-2015. The starting year is 2011 due to the epidemic of Kabatiella eyespot which occurred in this season in Schleswig-Holstein (Urban 2012; Schlüter 2011).

Tab. 33. Overview of weather conditions in Ostenfeld from 2011 to 2015.

Ostenfeld region	July (vegetative to reproductive phase)	August (reproductive phase to development of the fruit)	September (development of the fruit to ripening)
Temperature (°C)			
2011	16.4	16.5	14.5
2012	16.4	17.2	13.4
2013	18.1	17.5	13.1
2014	19.8	16.1	15.5
2015	16.8	18.2	13.0
Long-term average (1981-2010)	17.3	16.9	13.5
Precipitation (mm)			
2011	110.7	244.8	107.2
2012	145.5	60.2	63
2013	55.4	44.9	70.1
2014	33.9	111.8	48.8
2015	186.6	56.5	76.2
Long-term average (1981-2010)	84.0	82.0	76.0

There is notable variation in the temperature and level of precipitation. Regarding the temperature, there were noticeable differences for all months, with lows and highs ranging from 16.4°C to 19.8°C (July), 16.5°C and 18.2°C (August), and 13.0°C and 15.5°C (September). Precipitation levels also exhibited noticeable differences, with distinctly dry months in July (2013 and 2014), as well as very wet months (2012 and 2015). August 2011 was an unusually wet month compared to August 2012-2015, while September was relatively stable in comparison. In contrast to Mittich and Inzing, no clear patterns emerge with respect to the long-term average. Eight (seven) of the 15 months analysed were below (above) the long-term average for temperature, while this was also the case for precipitation (eight below average / seven above average).

3.2.3. Göttingen

For this location, only the weather records for 2015 are presented (Table 34 and Figure 45) as the experiment consisted of a single artifical inoculation in that year. The region is not characterised by the presence of maize leaf diseases, thus reducing the likelihood of possible inoculum. Comparisons with previous years are therefore not justified here.

Tab. 34. Summary of the weather data for the Göttingen "Miniplot" site.

Göttingen "Miniplot"	July* (vegetative to reproductive phase)	August* (reproductive phase to development of the fruit)	September* (development of the fruit to ripening)
Temperature 2015	18.7°C	20.2°C	14.0 °C
Precipitation 2015	50	69	58

*Data registered during the experiment from 13.July (artificial inoculation) until 29 September (harvest).

The average temperature during the vegetative to reproductive phase of the plant was 18.7°C. The end of July and beginning of August was characterised by a dry and warm period with isolated rain showers. In the middle of August, there was a decrease in the temperatures and some heavy rain was recorded. Although a higher quantity of precipitation was recorded in August than in September, it rained more frequently in September, i.e. on more days.

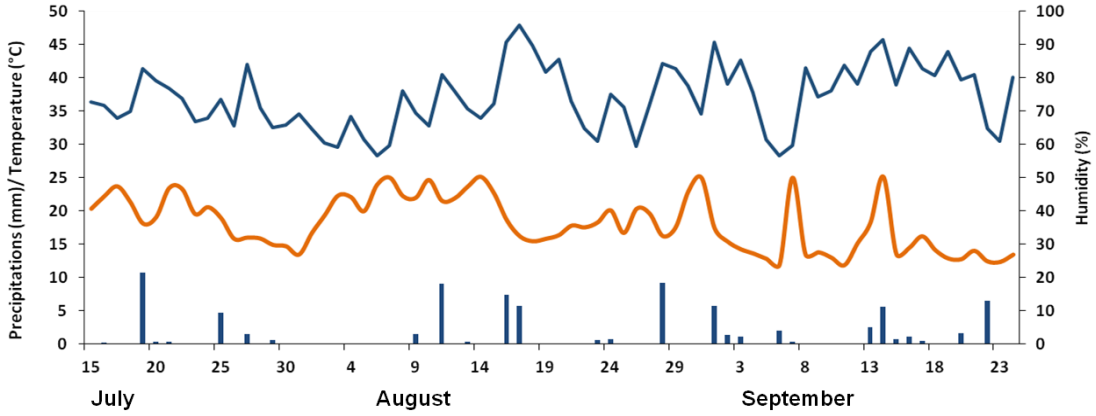

Fig. 45. Weather conditions in the "Miniplot" in Göttingen in 2015. ▌ Precipitation ▬ Temperature ▬ Relative humidity.

3.3. Epidemiological studies based on spore trapping in the field

3.3.1. Seasonal incidence of airborne conidia of *E. turcicum*

The seasonal incidence of conidia of *E. turcicum* was investigated through the use of Burkard spore traps during the 2014 season in Inzing (natural inoculum) and 2015 season in Göttingen (artificial inoculum). The evaluation of the samples, initially through microscopy, is presented in this section. Different patterns of *E. turcicum* conidial release and dispersal were considered according to previous reports for *E. turcicum* (Casselman & Berger 1970; Leach 1975; Leach et al. 1977; Bleicher & Balmer 1993; Rapilly 1991), i.e. those influenced by wind, rain, variations in relative humidity (henceforth RH) and temperature. Further estimations of latent periods based on the data obtained from the spore trap and the correlation with weather conditions are discussed in detail in section 4.3.1.

3.3.1.1. Inzing 2014

A total of 90 tapes were collected in 2014 for Inzing. From the collected samples, 69% presented *E. turcicum* conidia through microscopic assessment.

First conidia were detected on 8 July (first day of monitoring), when the plants were at the beginning of tassel emergence (BBCH 51). The following two months (middle of July until end of August) did not present any noticeable conidia release (mostly 0-2 conidia; max. 7 conidia) and therefore are not presented here in detail. These low amounts of conidia coincided with low precipitation and low humidity during July and August. However, at the end of July (30-31) and beginning of August (1-5), a combination of moderate temperatures (18-21°C) and six rainy days with three days (30, 31 July, 5 August) of high RH were

recorded (>90% average RH per day). For instance, on 30 July, 18 h of 100% RH were registered. Shortly thereafter, the first lesions (minimal) in the location were detected on 7 August (<1% infected leaf area). Trapped conidia remained at low levels.

Two periods of notable (10 and 17-18 September) and two of abundant (24-26 September and 3-4 October) conidia release were considered for detailed analysis and are therefore shown in detail in Figure 46.

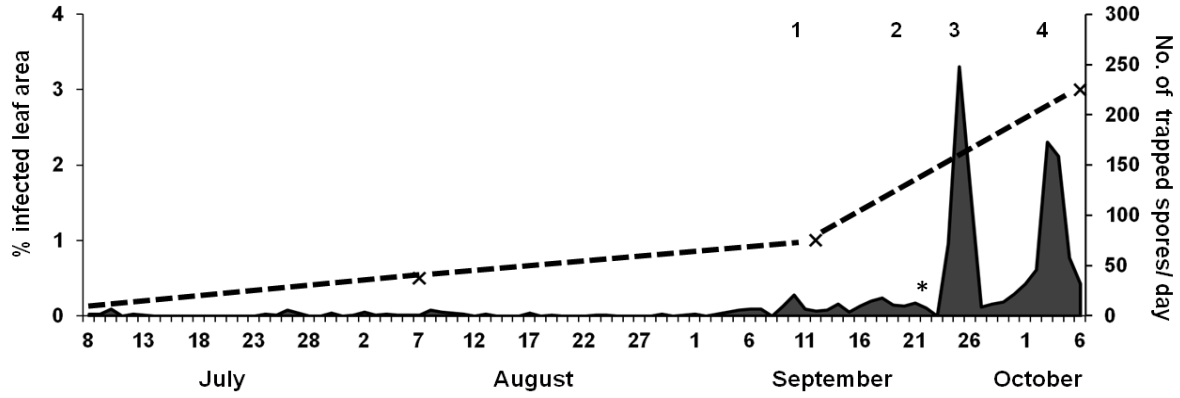

Fig. 46. Fluctuation of conidia release (▬) for the sampling period in Inzing in 2014 with development of the percentage of infected leaf area (**----**) and periods of notable (points 1 & 2) and abundant conidia release (points 3 & 4).).*= missing data

Weather data for Inzing in 2014 and correlation with the trapped number of spores are shown in Figure 47. At the end of August and beginning of September, a period of frequent rain (about 8 days) including five days of high humidity (>93%) was recorded. On several days of this period (26, 30 August and 2 September), at least 14 h of 100% RH occurred. Thereafter, a first notable peak of conidia release was registered on 10 September, albeit with a relatively low quantity of spores (21 spores). Strong winds with accompanying rain and an increase in the RH coincided on the preceding day of conidial release. On the following days (11-14 September), a decrease in the temperatures was observed (range 12-16°C), coinciding with a four-day period of rainfall and high humidity (16-23 h of 100% RH were recorded daily). On these days, only a few spores were trapped despite high wind velocities also being recorded (3.1 to 3.7 m s^{-1} on 12-13 September). A slight increase in the infected leaf area was noted on these days (Figure 46). On 17-18 September, a noticeable (low) conidia release was detected (15 and 18 conidia per day, respectively). This coincided with a strong decrease in the RH on 17 September (avg. 73%), followed by an increase in wind on 18 September, where the maximum peak was registered. In the late season, a relatively warm (17-18°C) period spanning three days (17-19 September) occurred. The end of this three-day period coincided with a noticeable amount of precipitation, which continued until 23 September and was accompanied by two days of high humidity (15 and 19 h of 100% RH, respectively).

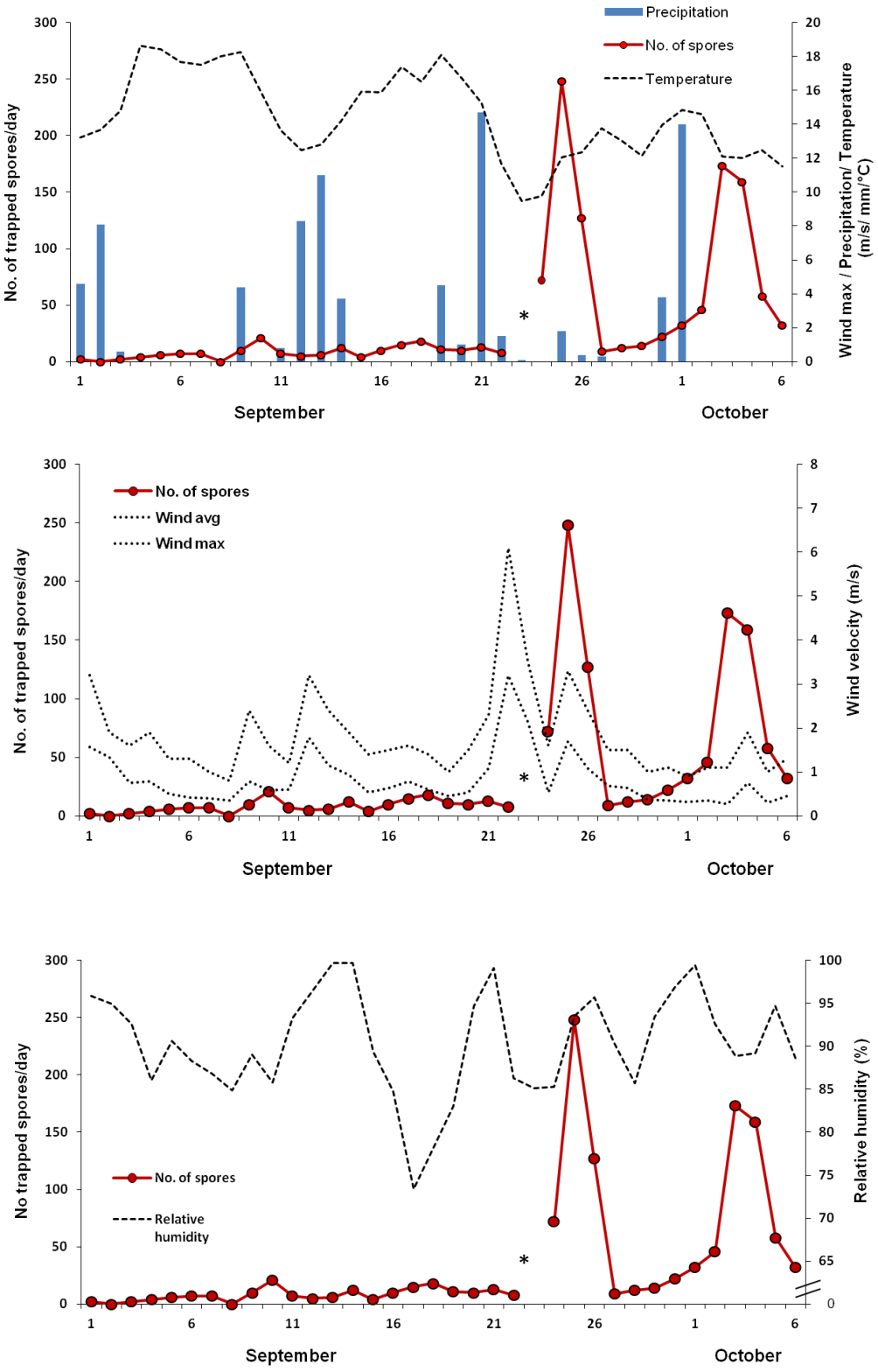

Fig. 47. Fluctuation of conidia release and weather conditions in Inzing in summer 2014: Average temperature and precipitation (above), wind velocity (centre) and average relative humidity (below). *=missing data.

First peaks of abundant conidia release were recorded during a three-day period on 24, 25 and 26 September, presenting 72, 248 and 127 conidia per tape, respectively. On the day of the maximum registered peak of the season, i.e. 25 September, a violent increase in the RH (100% for 14 h) occurred in combination with rain and wind. These enhancing factors (high humidity and rain) for conidia release and further infection were prolonged until 26 September. An increase in the temperature (up to 15°C), coinciding with a four-day period of high humidity (>93-99% RH), occurred from 30 September to 3 October - 14-21 h of 100% RH were recorded daily. A slight increase in the number of trapped spores was observed, with a clear increase on 3 and 4 October (153 and 159 spores, respectively). On 3 and 4 October, a violent decrease in the relative humidity accompanied by wind coincided with these peaks of conidial release. An increase in the infected leaf area (approx. 3% on average) was recorded after these periods (Figure 46).

3.3.1.2. Göttingen 2015

Seasonal fluctuations of airborne conidia were studied in Göttingen in 2015 after artificial inoculation of the plants through spraying with spore suspensions (13 and 24 July). *E. turcicum* conidia were recorded on 68% of the samples through microscopy.

With the exception of the first two days after inoculation - when plants were covered with plastic for 48 h and a saturated atmosphere (100% RH) is therefore assumed - the structure of the data for Göttingen did not allow for an accurate calculation of the number of hours with 100% RH. Hence, a day characterised by high RH is considered to have an average of at least 90%.

First chlorotic lesions were observed 24 hours after artificial inoculation. On 16 July, three days after artificial inoculation, first conidia were detected when the plants were, on average, at the beginning of the flowering stage (BBCH 59-63). These initial and inconsiderable quantities of conidia (1-4 per day) on the days thereafter were directly related to the sprayed inoculum (artificial inoculation) and not as a result of their own sporulation on the leaf tissue after an established process of infection. Two periods of moderate conidia release and four main periods of abundant conidia release were considered for detailed analysis. These are depicted in detail in Figure 48.

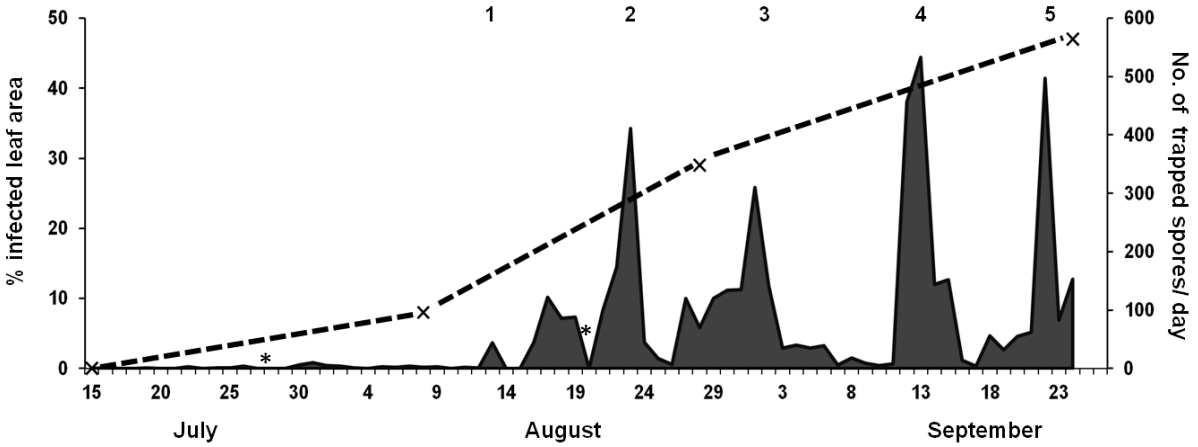

Fig. 48. Fluctuation of conidia release ( ) for the sampling period in Göttingen in 2015 with development of the percentage of infected leaf area and periods of notable (point 1) and abundant conidia release (points 2, 3, 4, 5).*= missing data

First typical cigar-shaped lesions on the leaves were recorded, on average, 12 days after the formation of the first chlorotic symptoms. Extended lesions on the leaves were observed during the dry, warm period at the end of July and beginning of August. At this stage, an infected leaf area of approximately 8% was recorded. No sporulation was observed on the lesions. On 11 August, a high amount of rain was recorded (18 mm). The first peak of notable conidia release was detected on 13 August (44 conidia) and coincided with rain and strong wind gusts (max. 4.1 m s^{-1}). A three-day period (16, 17 and 18 August) of high humidity (≥90-95%) and rain (26 mm) was recorded, with a decrease in the temperature to around 15-18°C. On these days, sporulation could be observed on the lesions.

During this period and the succeeding days (16-24 August), abundant amounts of conidia could be counted daily, with a maximum peak of 411 spores on 23 August. Rain and an increase in RH were correlated with conidia release on the preceding days (16-21 August), while strong winds coincided with the release and dissemination on 22 August. On 23 August, conidia release was accompanied by an increase in wind speed (avg 1.16 m s^{-1}; max. 4.4 m s^{-1}) and a drastic decrease in RH (average 60%).

The 23 August almost remains directly connected to the second abundant peak of conidial release, which commenced only four days afterwards and is reflected by an ascending curve of daily conidia release from 27 August to 2 September. This period of conidial release corresponds to the fluctuations in the RH, as shown by Figure 49. For example, the maximum peak (310 conidia) occurred on a day of high humidity (>90%). Despite an increase in the RH during this period, it failed to reach 90% and only a single rainy day was recorded. The constant conidial release coincided with a combination of rain and an increase in humidity during these days. Furthermore, wind (0.3 to 2.7 m s^{-1}) also strongly promoted

conidial release, particularly for the dispersal of conidia on 31 August and 1 September. In the following days, unfavourable weather conditions of frequently low humidity dominated, coinciding with a low conidial release plateau. During this period, the leaf blight lesions continued to expand with sporulation, reaching an infected leaf area of 30% (rated on 28 August). The next period of abundant conidia release occurred from 12 to 15 September, where maximum peaks of 457 and 533 conidia were registered on 12 and 13 September, respectively. On 12 September, a slight decrease in the RH coincided with spore release. On 13 and 14 September, wind, or wind combined with rain, corresponded with the peaks of spore release and dispersal. An increase in the RH (>90%) was also observed. From 13 to 17 September, frequent rain and one day of high humidity (>90%) occurred. Days of strong wind and high humidity occurred on the days thereafter, e.g. 17, 19 and 21 September, yet very few conidia (between 4 and 62) were recorded. An abundant peak of spore release (497 conidia) occurred on 22 September, attributable to the heavy rain as the wind speed was relatively low. After this period of high spore release, an infected leaf area of 46% was recorded.

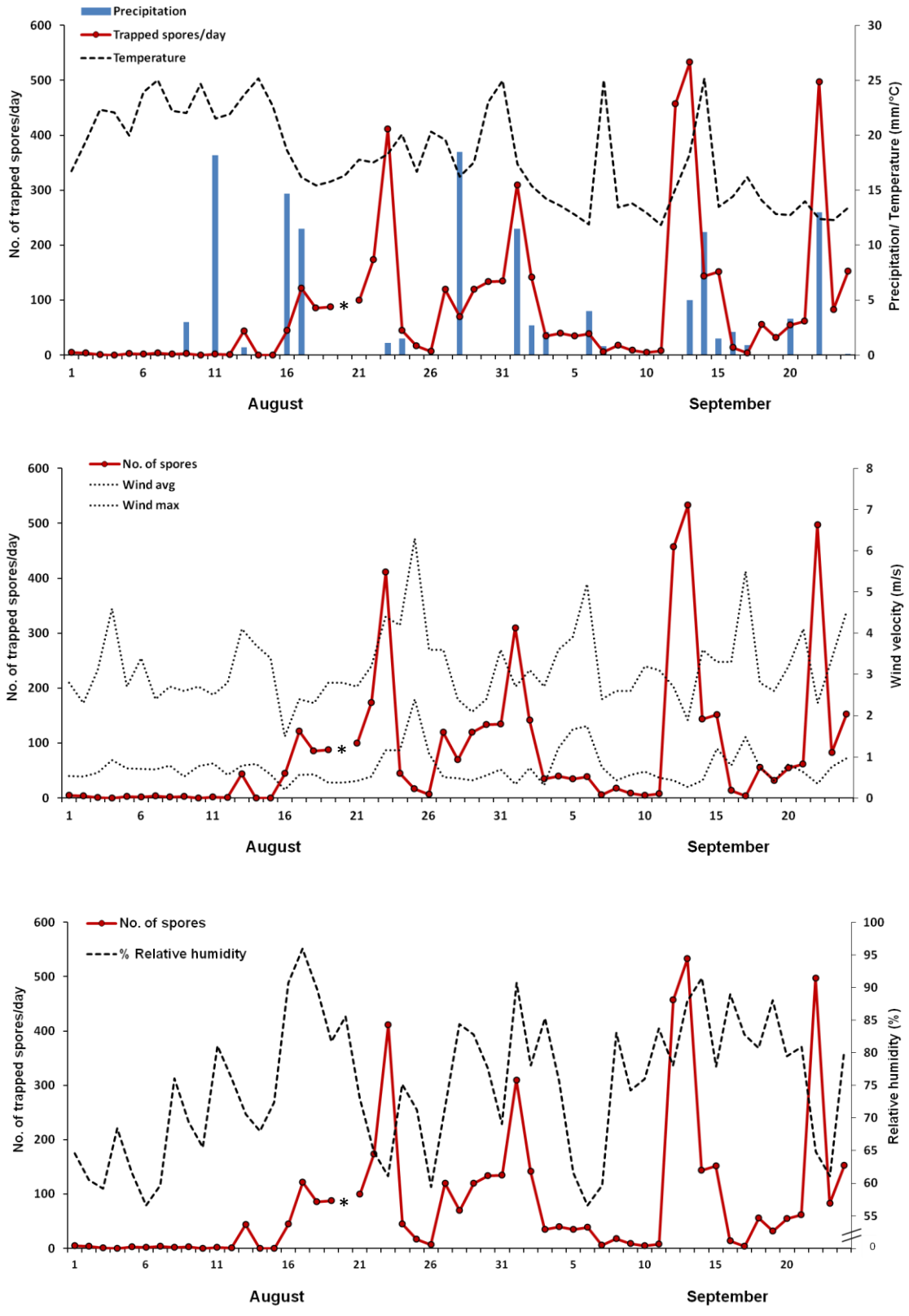

Fig. 49. Seasonal fluctuations in conidia number and weather conditions in Göttingen in summer 2015: average temperature and precipitation (above), wind velocity (centre) and average relative humidity (below).).*= missing data

3.3.1.3. Comparisons based on climatic conditions, development of Turcicum leaf blight and spore release between Inzing 2014 and Göttingen 2015

Based on the observations obtained from the spore release pattern, an outline of the different climatic conditions which enhance the development and further sporulation until the spore release described above in Inzing and Göttingen are summarised in Table 35. The latent periods described became shorter after successive infection periods when the disease was established in the field and the disease pressure was higher. Further considerations for the length of the latent period are discussed in section 4.3.1.

Tab. 35. Overview of the decisive climatic conditions enhancing the development of the disease, including sporulation and further conidial release in Inzing in 2014 compared with Göttingen in 2015.

Phenomen	**Environmental conditions enhancing the development of Turcicum leaf blight related to the spore release**	
	Inzing	Göttingen (artificial inoculation)
Infection + sporulation	**Low sporulation (early season):** • successive periods of 96-100% RH (at least 17 hours) • 18-21°C **Massive sporulation (late season):** • successive periods of 100% RH (periods ranging between 69 to 121 hours) accompanied by rainy periods (>4 days) • 9-18°C	**Infection:** • at least 24 hours of 100% RH **Sporulation:** • Low: 18 mm precipitation • High: ±3 days ≥ 90-95% RH • 26 mm precipitation • 15-18°C
Lesion expansion	• Low-moderate	• Outstanding • Favoured by a warm period of 28-30°C for lesion expansion after establishment of the infection
Latent period	• 7-14 days	• 6-14 days
Spore release coincided with	• Drastic increase and decrease in RH • Wind and/or rain	• Drastic increase and decrease in RH • Wind and/or rain
Spore spread	• Wind and/or rain	• Wind and/or rain

3.3.2. Correlation of microscope counts and DNA yield for *E. turcicum*

Samples from Inzing and Göttingen were analysed via qPCR assay to evaluate DNA yields of *E. turcicum*. Both molecular methods were evaluated and compared with the data obtained from the traditional method of visual light microscopy (section 3.3.1).

3.3.2.1. Inzing

From the 90 samples obtained in Inzing, positive amplifications were obtained through qPCR with the *E. turcicum* specific primer pair in 51% of the samples. An overview of the monitored season in Inzing in 2014, with results from the qPCR assay compared to those of the visual microscopy, is depicted in Figure 50.

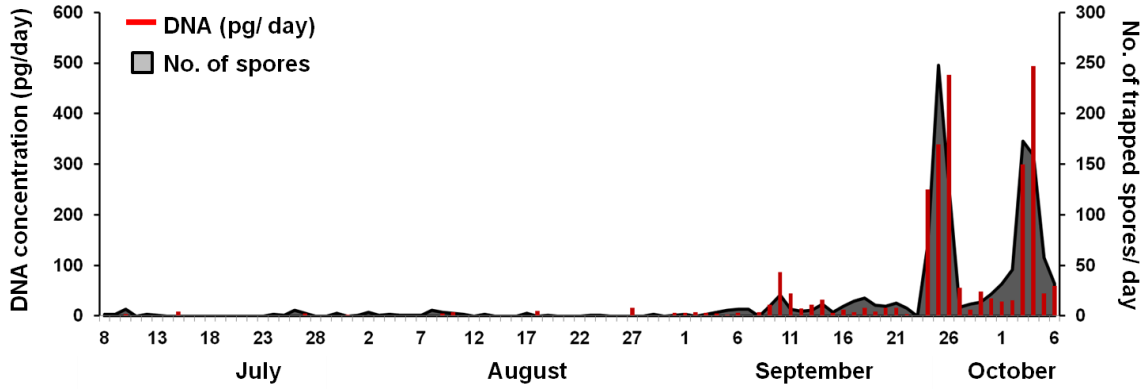

Fig. 50. Seasonal fluctuations in conidia number of *E. turcicum* assessed via microscope and DNA amount assessed via qPCR on spore trap tapes in Inzing 2014.

Through visual microscopy, the first *E. turcicum* conidia were detected on 8 July (2 conidia). First detections through qPCR assay occurred two days later on 10 July (3.04 pg), where seven conidia were counted by microscopy. Higher conidia quantities ($x>10$) were regularly amplified through both methods. A low number of conidia ($0<x\leq10$) were counted through microscopic assessment in 48% of the samples (43/90), whereas only 47% (20/43) of these samples were positive via qPCR assay. This was the case on 8 August, for example, where six conidia were counted but no amplification was achieved through qPCR.

On the contrary, there were also samples for which DNA amplifications were registered via qPCR assay despite the fact that no spores were visually detected through microscopy. An example here is 2 September, where an amplification of 7.8 pg DNA was quantified via qPCR yet no spores were visualised through microscopy. Of the 29 samples (32% of total) in which no spores were registered through microscopy, positive amplifications via qPCR assay were obtained for eight of them (28%).

The first notable peak of conidial release determined through microscopic counts on 10 September (21 spores) coincides with the first notable DNA yield determined via qPCR assay (87 pg). The highest peak achieved during the season was recorded on 25 September through microscopy (248 conidia), whereas the maximum amplification of DNA occurred on 26 September (127 conidia).

The third seasonal fluctuation in spore numbers was recorded on 3 and 4 October for both methods. While the highest number of conidia was recorded by microscopy on 3 October, the highest DNA yield was detected on 4 October (495 pg).

According to the log linear model used (see section 2.7.1), we can assume a significant ($p<0.05$) relation between DNA yield and the number of spores for the samples from Inzing 2014. At levels of up to 250 pg, it seems that the relation follows a more linear pattern, i.e. up to about 85 spores (Figure 51). The average DNA yield (28.3 pg) obtained corresponds to 24 spores, i.e. 1.15 pg/ spore.

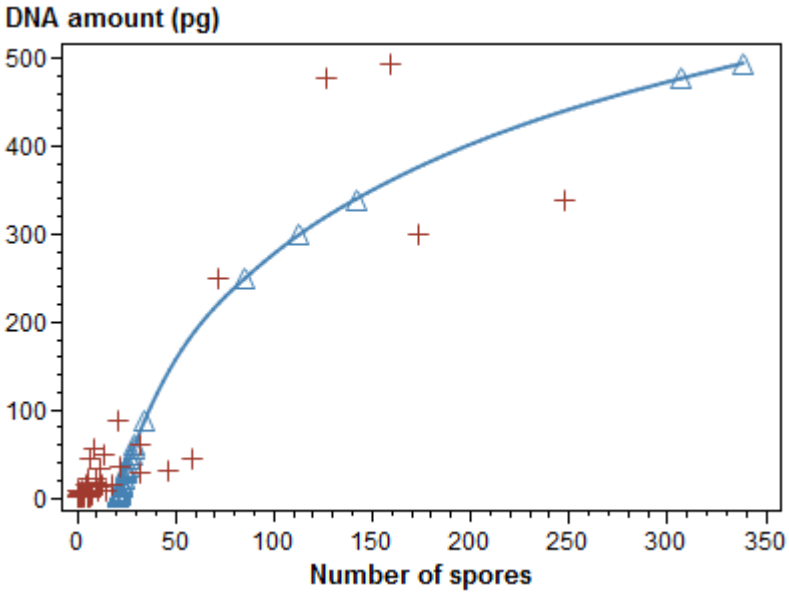

Fig. 51. Relation between number of *E. turcicum* spores estimated through microscopic counts and DNA yield assessed via qPCR samples ($p<0.05$) for Inzing 2014. Red crosses (+) are observed pairs of DNA concentration (qPCR) and the corresponding number of spores for each individual sample. Blue triangles (Δ) are the estimates of DNA concentration and number of spores according to the model equation.

3.3.2.2. Göttingen

From the 70 samples obtained in Göttingen, positive amplifications were obtained through qPCR with the *E. turcicum* specific primer pair in 67% of the samples.

Higher conidia quantities (x>10) were regularly amplified through both methods. The only exception was two days (15 and 16 September) for which no DNA amplification was achieved via qPCR. Low numbers (0<x≤10) of conidia were observed through microscopic counts in 27% of the samples (24/70), while *E. turcicum* DNA (determined via qPCR) was only detected on 42% (10/24) of these samples. Of the 12 samples (17% of total) in which no spores were registered through microscopy, positive amplifications via qPCR assay were obtained for five of them (42%). For example, on 15 August an amplification of 31 pg DNA was quantified via qPCR yet no spores were visualised through microscopy.

An overview of the complete monitored season in Göttingen in 2015 is provided in Figure 52. The first important spore release of the season occurred on 13 August with 44 spores, corresponding to 240 pg DNA obtained via qPCR. Thereafter, four periods of abundant sporulation were observed by microscopy: 17 to 23 August, 27 August to 2 September, 12 to 15 September and 22 September. These four seasonal fluctuations coincide with the periods for which DNA amplification was successful via qPCR assay.

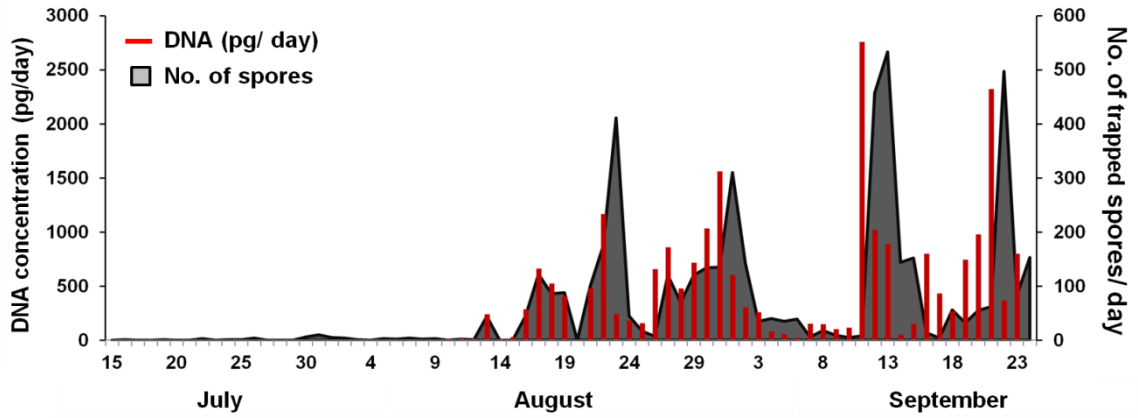

Fig. 52. Seasonal fluctuations in conidia number of *E. turcicum* assessed via microscope and DNA amount assessed via qPCR on tapes in Göttingen in 2015.

During the first and second periods of abundant conidial release, the highest DNA amplifications were detected on 17 August (662 pg) and 23 August (1768 pg), coinciding with the highest peaks of conidia (122 and 411 per day, respectively) observed on these days by microscopy.

For the third seasonal fluctuation (12-14 September), the highest peak of DNA amplification was observed on 12 September via qPCR assay, while one day later (13 September) the highest peak of spore release was recorded by microscopy. For the fourth seasonal

fluctuation, a one-day peak on 22 September was recorded via both visual assessment and qPCR.

According to the log linear model used (see section 2.7.1), we can assume a significant relation between DNA yield and the number of spores for the samples from Göttingen 2015. At levels of up to 1165 pg, it seems that the relation follows a more linear pattern, i.e. up to about 175 spores (Figure 53). The average DNA content (353.67 pg) obtained corresponds to 78 spores, i.e. 4.6 pg/ spore.

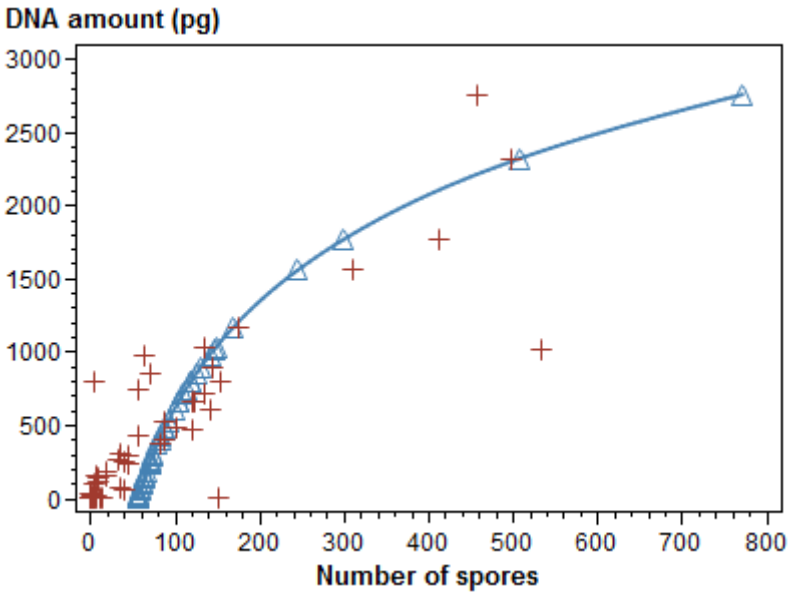

Fig. 53. Relation between number of E. *turcicum* spores estimated through microscopic counts and DNA yield assessed via qPCR samples ($p<0.05$) for Göttingen 2015. Red crosses (+) are observed pairs of DNA concentration (qPCR) and the corresponding number of spores for each individual sample. Blue triangles (Δ) are the estimates of DNA concentration and number of spores according to the model equation.

3.3.3. Seasonal incidence of inoculum of *K. zeae* analysed via qPCR

In Ostenfeld, a total of 77 tapes were collected in 2013 and 84 tapes in 2014. In Göttingen, the same samples analysed for *E. turcicum* (70 tapes) were also analysed for the presence of *K. zeae* conidia.

Tapes from Göttingen in 2015 and Ostenfeld in 2013 were analysed through light microscopy for *K. zeae* conidia but it was not possible to distinguish them from the numerous conidia of other fungi registered daily on the vaseline tape samples. Therefore, only a range of tapes from Ostenfeld in 2014 were analysed to check for *K. zeae* spores but the same difficulties as in 2013 were encountered. Consequently, it was not possible to confirm the spore release of *K. zeae* through light microscopy for any of the examined locations. Thus, only results from the molecular analysis via qPCR, if successful, can be directly correlated with the development of *K. zeae* in the field.

3.3.3.1. Ostenfeld

In 2014, it was not possible to detect DNA from the tape samples via qPCR. The infected leaf area produced by Kabatiella eyespot remained under 1% until the beginning of September (BBCH 75-79; fruit development) and a maximum infected leaf area of 3.7% was recorded for the untreated control at the end of the season.

As shown in Figure 54, in 2013, only three DNA amplifications (4% of total samples) were recorded via qPCR (16, 17, 28 September). DNA yields were lower than 1 pg day^{-1}. The three peaks of DNA amplification were detected when periods of frequent rain (at least 2-3 days) occurred on the days prior to the amplified peak. During August, while some periods of high humidity and rain could have favoured sporulation, long periods of low humidity, a lack of precipitation and moderate temperatures (17-18°C) could have simultaneously had a counteracting effect. Symptoms of Kabatiella eyespot were only observable in some plants, scattered inconsistently across the field, and DNA amplifications were not registered.

In the late season, the onset of cold weather was registered around 7 September. During this month, the average temperature decreased to 13.1°C. On 11 September, a slight increase in the infected leaf area (1.3%) compared with the first assessment at the beginning of August (0.3%) could be recorded in the untreated control. Thereafter, on 16 and 17 September, first peaks of DNA amplification were recorded, coinciding with a four-day rainy period. On 28 September, a low peak of DNA amplification was recorded after a four-day period of rain. By 2 October, when plants were at the ripening stage (BBCH 83-85), the infected leaf area in the control had developed to 5.9%.

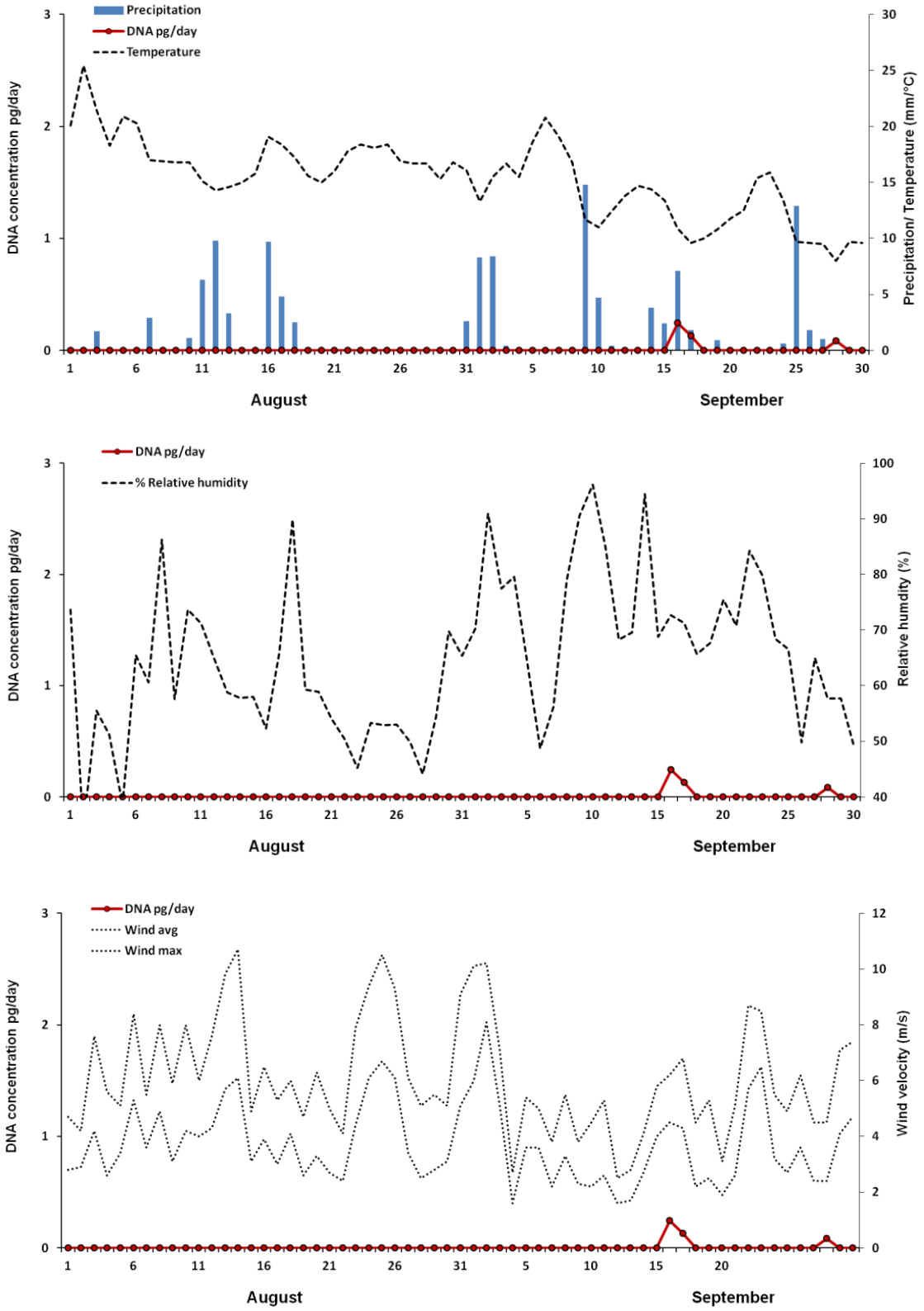

Fig. 54. Seasonal fluctuations of *K.zeae* DNA yield and weather conditions in Ostenfeld in summer 2013: average temperature and precipitation (above), average relative humidity (centre) and wind velocity (below).

3.3.3.2. Göttingen

In Göttingen in 2015, DNA amplifications were only registered on 14 September (2 pg) (Figure 55).

First lesions of Kabatiella eyespot were observed in the location seven days after inoculation (on 20 July) and by the end of July the disease was widely distributed among plants. It was not possible to observe sporulation on the leaves without incubating leaf samples in conditions of high humidity in the laboratory. After a warm and dry period (until the beginning of August), favourable weather conditions of heavy rainfall occurred in the middle and at the end of August. A slight increase in the infected leaf area was registered on 28 August (6% infection), but concentrations of *K. zeae* had yet to be amplified via qPCR. A further decrease in the average temperature, starting from 2 September until 6 September, was recorded (15 to 11°C). Although high temperatures and low levels of humidity were recorded on 7 September, cool temperatures (11-13°C) were predominant on the days immediately thereafter (8-12 September). On 14 September, the first amplification of *K. zeae* DNA yield was registered (2 pg). This occurred within a period of frequent rain. Strong rain and wind are correlated with spore release and dispersal on 14 September. A further decrease in the average temperature combined with frequent periods of rain favoured the development of the disease, which translated into an infected leaf area of 20% at the end of September. Further amplification of DNA of *K. zeae* DNA were not registered via qPCR.

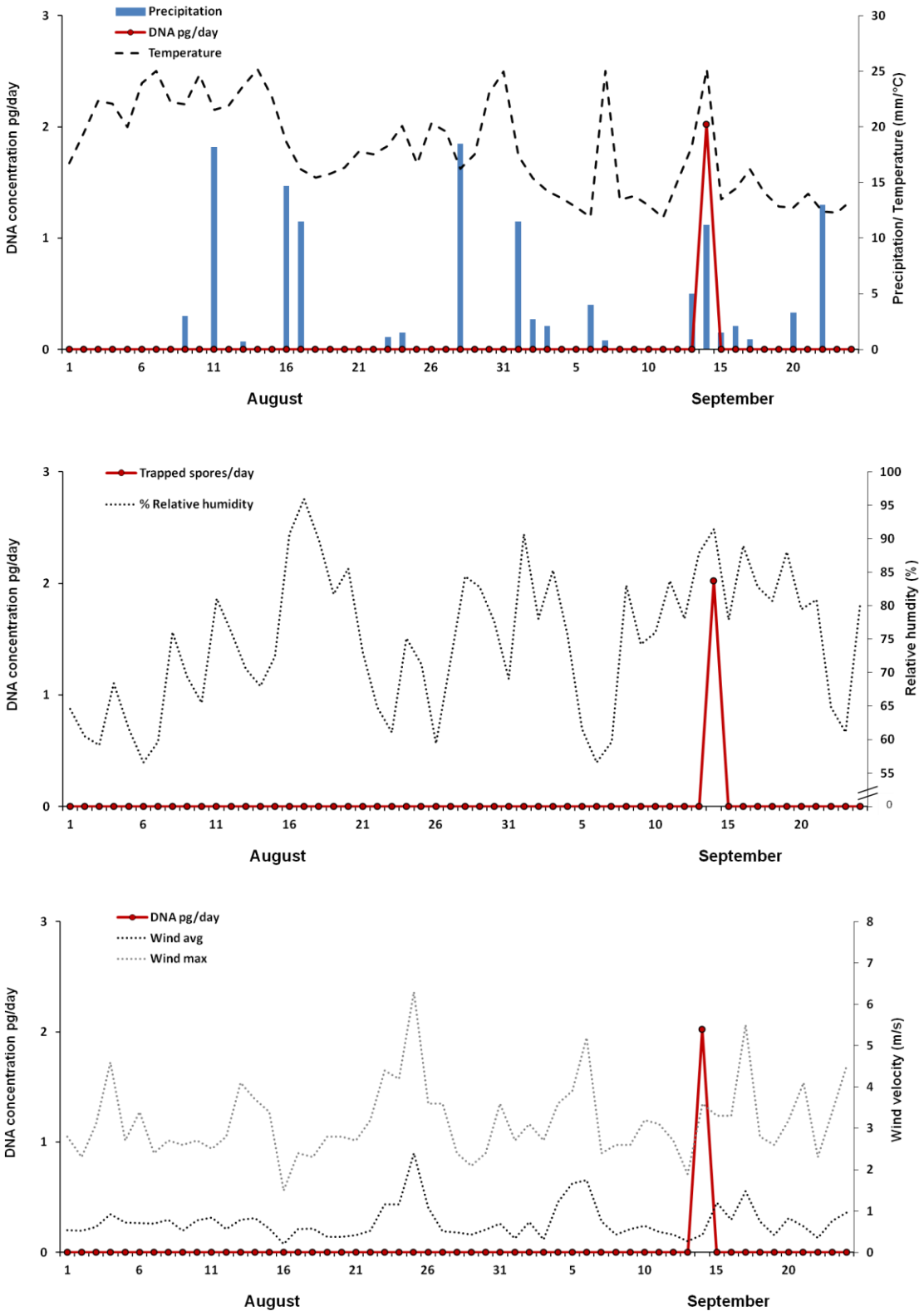

Fig. 55. Seasonal fluctuations of *K.zeae* DNA yield and weather conditions in Göttingen in summer 2015: average temperature and precipitation (above), average relative humidity (centre) and wind velocity (below).

3.3.4. Detection through qPCR and sensitivity

In order to test sensitivity of the qPCR method, a range of annealing temperatures (64-68°C) was tested, starting with the established temperature from the PCR assay (see section 2.5.7). The *E. turcicum* primer set could amplify *E. turcicum* DNA at the minimum level of 10 fg μl^{-1}, even at the highest tested annealing temperature of 68°C. Considered positive samples for *K. zeae* DNA could be detected at the minimum level of 100 fg μl^{-1} at melting temperatures ranging from 64-68°C. Ct value (cycle threshold) for the lowest detected standard concentration of *E. turcicum* (10 fg μl^{-1}) was, on average, close to 33. Ct value for the lowest detected standard concentration *K. zeae* (100 fg μl^{-1}) was close to 31.

Analysing the obtained melting curve (T_m), amplifications of *E. turcicum* resulted in a narrow symmetric single melting point curve, obtained at 86.5 °C (Figure 56, left). *K. zeae* DNA amplifications resulted in two individual melting phases, one at 89°C and another close to 85°C (Figure 56, right).

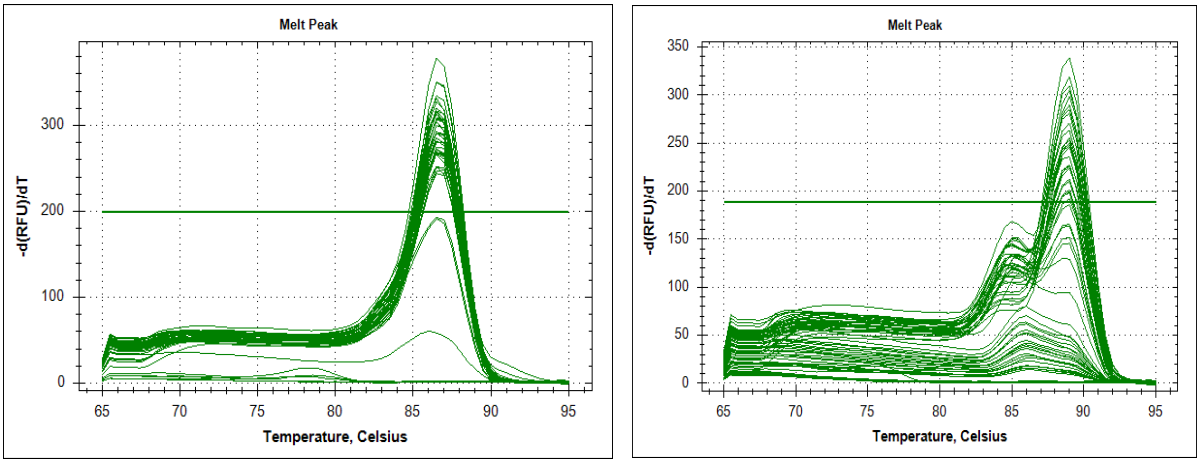

Fig. 56. Melting curves obtained via qPCR method. Narrow symmetric single melting point curve obtained at 86.5°C for *E. turcicum* (left). Double melting curve obtained for *K. zeae* at 85°C and 89°C (right).

A second experiment was conducted directly from artificial spore trap tapes, which were analysed for the lowest detectable quantities of conidia.

For *E. turcicum*, the qPCR method could detect a minimum of two to four conidia (0.06-0.1 pg μl^{-1}), which corresponds to 6-10 pg of DNA per sample (a sample corresponds to 100 µl TE). For *K. zeae*, DNA amplifications from artificial spore trap tapes amplified via qPCR were considered to be positive at a minimum of 125 conidia.

Furthermore, with the artificial spore trap tapes, the relation between the number of spores counted through microscopy and those obtained via qPCR was evaluated.

According to the log linear model used (see section 2.7.1), we can assume a significant relation ($p<0.05$) between DNA yield and the number of spores. At levels of up to 600 pg, it seems that the relation follows a more linear pattern, i.e. up to about 300 spores (Figure 57). The average DNA content (178.4 pg) obtained corresponds to 144 spores, i.e. 1.24 pg/spore.

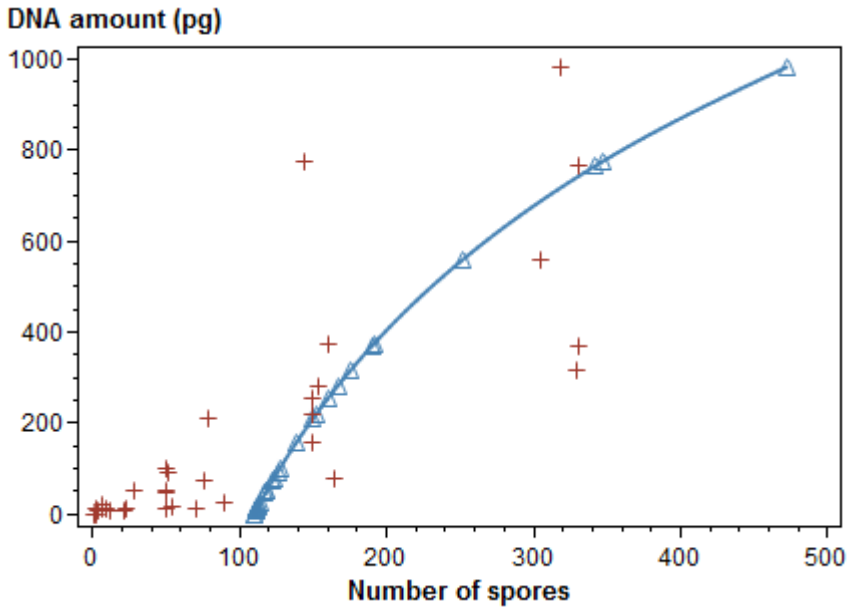

Fig. 57. Relation between number of *E. turcicum* spores estimated through microscopic counts and DNA yield assessed via qPCR samples ($p<0.05$) with artificial spore trap tapes. Red crosses (+) are observed pairs of DNA concentration (qPCR) and the corresponding number of spores for each individual sample (for range of 1-330 spores). Blue triangles (Δ) are the estimates of DNA concentration and number of spores according to the model equation.

For *K. zeae*, according to the log linear model used (see section 2.5.7), we can assume a significant relation between the number of *K. zeae* conidia in the range of 125-1000 and DNA yield. At levels of up to 1.55 pg, it seems that the relation follows a more linear pattern, i.e. up to about 719 spores (Figure 58). On average, the DNA content obtained (1.18 pg) corresponds to 606 spores, i.e. 0.002 pg/ spore.

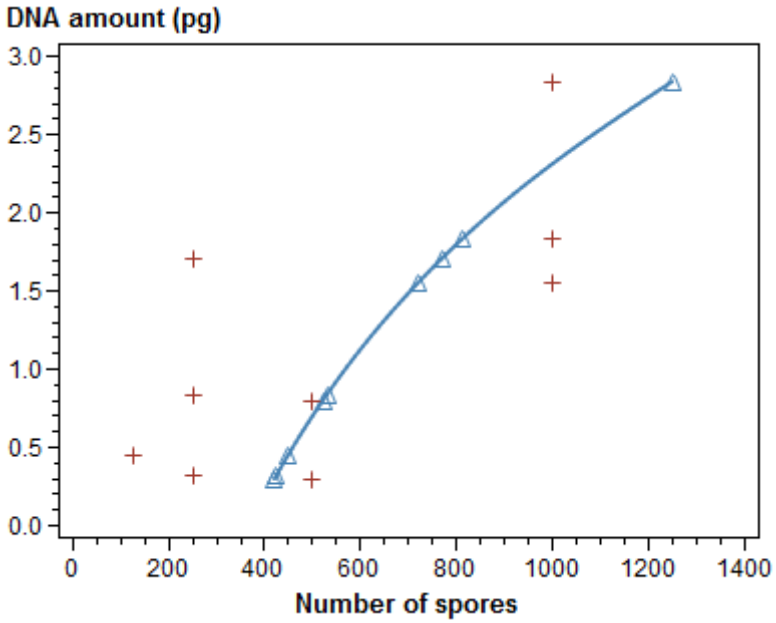

Fig. 58. Relation between number of *K. zeae* spores estimated through microscopic counts and DNA yield assessed via qPCR samples ($p<0.05$) with artificial spore trap samples. Red crosses (+) are observed pairs of DNA concentration (qPCR) and the corresponding number of spores for each individual sample (for range of 125-1000 spores; only positive samples according to the melting curve and the threshold were considered). Blue triangles (Δ) are the estimates of DNA concentration and number of spores according to the model equation.

3.3.5. Evaluation of specificity

With the purpose of testing the applicability of the protocol under natural conditions in the field, the specificity of the primers JB585/586 for *E. turcicum* and JB616/JB618 for *K. zeae* DNA was tested through PCR and qPCR.

Primer sets JB585/JB586 for *E. turcicum* and JB616/JB618 for *K. zeae* were tested against a collection of 13 other fungi through PCR (Table 36). These organisms were selected due to their airborne dispersal and presence in European maize fields, as well as in surrounding field crops, or because they are genetically related to the target species being investigated.

The specificity test through PCR for the *E. turcicum* specific primer set resulted in amplifications of the target organism *E. turcicum* and none of the 13 fungal non-target species when the annealing temperature was increased from 55°C to 64°C. Primer set JB616/JB618 selected for amplification of *K. zeae* DNA revealed additional amplifications of the genetically related species *K. caulivora* at all annealing temperatures tested (55°C-65°C), but not for the rest of the tested species. In this case, using higher annealing temperatures did not help to improve specificity through PCR. This was because the amplification of the *K. zeae* diagnostic amplicon was negatively affected.

Tab. 36. Evaluation of specificity of *E. turcicum* and *K. zeae* primer sets using different non-target fungal organisms.

Fungal organism	Amplicon with universal primers ITS4/ITS5 57°C	Amplicon with *E. turcicum* primer set JB585/JB586 64°C	Amplicon with *K. zeae* primer set JB616/JB618 65°C
E. turcicum (target organism)	+	+	-
K. zeae (target organism)	+	-	+
Alternaria spp.	+	-	-
Aspergillus spp.	+	-	-
Bipolaris sorokiniana	+	-	-
Bipolaris zeicola	+	-	-
Botrytis cinerea	+	-	-
Cladosporium spp.	+	-	-
Epicoccum nigrum	+	-	-
Fusarium graminearum	+	-	-
Kabatiella caulivora	+	n.t.	+
Phoma lingam	+	-	-
Penicillium spp.	+	-	-
Septoria tritici	+	-	-
Trichoderma spp.	+	-	-

n.t.: non tested

Based on the results of the specificity test through PCR, specificity tests were also carried out via qPCR. Through PCR, the selected primer set JB585/JB586 for amplification of *E. turcicum* DNA also amplified the non-target fungi *Alternaria* spp. at lower annealing temperatures than 64°C. Therefore, *Alternaria* spp. was selected for further specificity tests through qPCR. Positive samples were considered to be those exceeding the established threshold and coinciding with the melting temperature curve of *E. turcicum* amplifications.

Through qPCR, at 64°C (selected annealing temperature for PCR), non-specific amplification of DNA of *Alternaria* spp. was observed. In an effort to increase specificity of amplification, a progressive increase in the annealing temperature (64-68°C) was tested for three different *Alternaria* spp. concentrations (1, 10 and 100 pg μl^{-1}). When testing the highest concentrated samples (100 pg μl^{-1}), positive amplifications were obtained (corresponding to 0.06 pg of the target *E. turcicum* DNA standard curve). To confirm these non-specific amplifications as positive, *Alternaria* spp. reaction products were electrophoresed on agarose gel. These corresponded to a single band amplified at the same specific size of the target *E. turcicum* amplicon (485 bp).

Consequently, the primer set JB585/JB586 can not be considered 100% specific for *E. turcicum* amplifications.

Specificity tests through qPCR with the selected *K. zeae* JB616/JB618 primer set were carried out testing the closely related fungus *K. caulivora*. This fungus, in addition to *K. zeae*, was amplified through PCR at all tested annealing temperatures (55-65°C). Through qPCR,

three different *K. caulivora* DNA concentrations were tested (0.01, 1 and 10 pg μl^{-1}). The test resulted in non-specific DNA amplification of *K. caulivora* DNA at the previously established temperature of 65°C through PCR. A further increase in the annealing temperature (65-68°C) through qPCR resulted in non-specific amplifications, even at the highest annealing temperature of 68°C for concentrations of 1 and 10 pg (corresponding to 0.04 and 0.28 pg of the target *K. zeae* DNA standard curve, respectively). The non-specific amplification products of *K. caulivora* DNA also correspond to the two individual melting phases (at 89°C and another close to 85 °C) observed for *K. zeae*.

Consequently, the primer set JB585/JB586 can not be considered 100% specific for *K. zeae* amplifications.

3.4. Evaluation of disease control through fungicides

The development of the disease and the response of the plant to various fungicide compounds and differences in timing of application were evaluated in four field experiments in 2013 and 2014 under conditions of natural inoculum.

In 2013, Turcicum leaf blight and common rust were analysed in Mittich. In 2014 in Inzing, only Turcicum leaf blight could be assessed due to the low pressure of common rust in the location. In Ostenfeld, the development of Kabatiella eyespot could be evaluated in 2013 and 2014.

Results for the development of the diseases across the season are exhibited together with the performance of the fungicide treatments applied at different stages. The development of the disease on the leaves of the plant (L-2, L-1, L0, L+1, L+2) is also presented in order to provide detailed information about the effect that the pathogen had on each one of these leaves in the respective location. This parameter provides information on the dynamic of the pathogen. This is important in the context of fungicide management in order to know which zones of the plant are most vulnerable and consequently need to be treated.

An estimation of the destructive potential of the diseases was then carried out by comparing the final yield of the non-treated control and treated plots. Here, yield reduction was calculated for each percentage point increase in the disease.

3.4.1. Turcicum leaf blight and common rust in Mittich 2013

The first disease assessment was performed on 31 July (BBCH 65: flowering), 28 days after the first fungicide application and 8 days after the penultimate treatment. On this date, the first scattered lesions were observed in the location. Following this assessment, the dispersion of the disease in the field was inhibited, delaying the second disease assessment until an increase in the infected leaf area could be observed in the field. This ocurred on 5 September (BBCH 75-79: fruit development), 35 days after the first assessment. However, at this stage significant differences in the infected leaf area among the non-treated control and treated plots could not be observed. Twenty days after the second assessment, the final disease assessment was performed (25 September; BBCH 85: ripening of the plant). At this time, both early (at BBCH 32: stem elongation) and late fungicide applications (BBCH 63: flowering) had a significant positive effect with respect to the non-treated control (8% infected leaf area).

The mixture of epoxiconazole + pyraclostrobin applied at both stages, early (BBCH 32: stem elongation) and late (BBCH 63: flowering), showed a higher efficacy (4.6% and 4.7% reduction in infected leaf area, respectively) compared to the mixture propiconazole +

azoxystrobin (3.0% and 3.6% reduction in infected leaf area, respectively). Concerning the timing of applications using the same fungicide treatment, no significant differences were observed for either mixture.

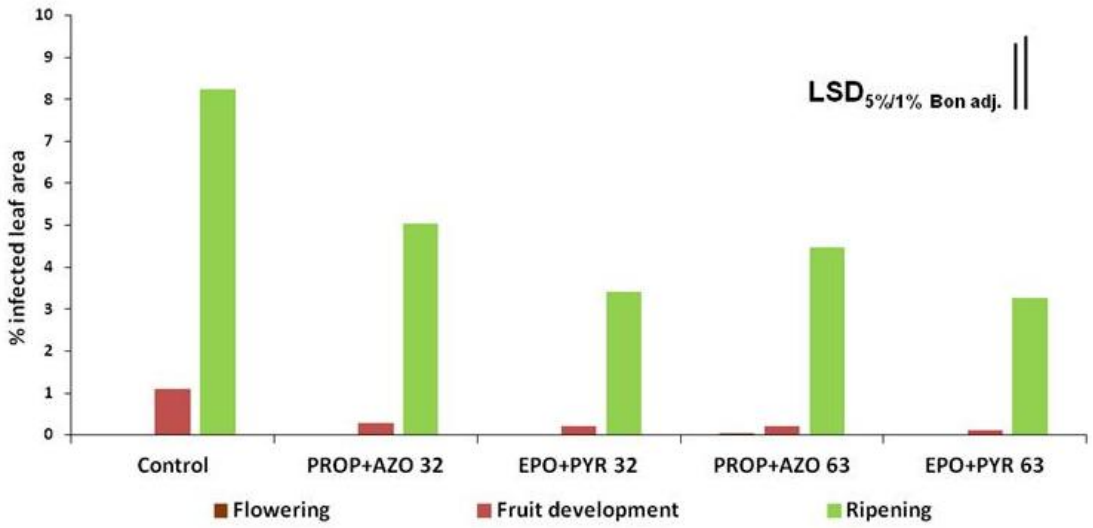

Fig. 59. Mittich 2013. *E. turcicum* infected leaf area on maize variety Zidane at three growth stages – flowering, fruit development, ripening. Comparison of control and different fungicide applications at different times. 32 (BBCH) = applied at vegetative stage (early application). 63 (BBCH) = applied at flowering (late application). PROP= propiconazole; AZO= azoxystrobin; EPO= epoxiconazole; PYR= pyraclostrobin; Least significance difference (LSD) according to Bonferroni test: LSD= 1.47 ($p<0.05$); LSD= 1.64 ($p<0.01$).

Focusing on the development of the disease in the plant (LS means of each leaf position from all treatments and the non-treated control), leaves L0, L-1 and L+1 showed a higher infected leaf area (Figure 58). The ear leaf (L0) showed the highest infected area (6.2%), followed by the leaves L-1 (5.0%) and L+1 (5.7%). Differences among these three leaves were not significant. The upper leaf L+2 (4.3%) and the lower leaf L-2 (3.1%) showed the lowest infected area. The upper (L+2) and the lower leaf (L-2) differ significantly ($p<0.05$ respectively) from L+1, L0 and L-1.

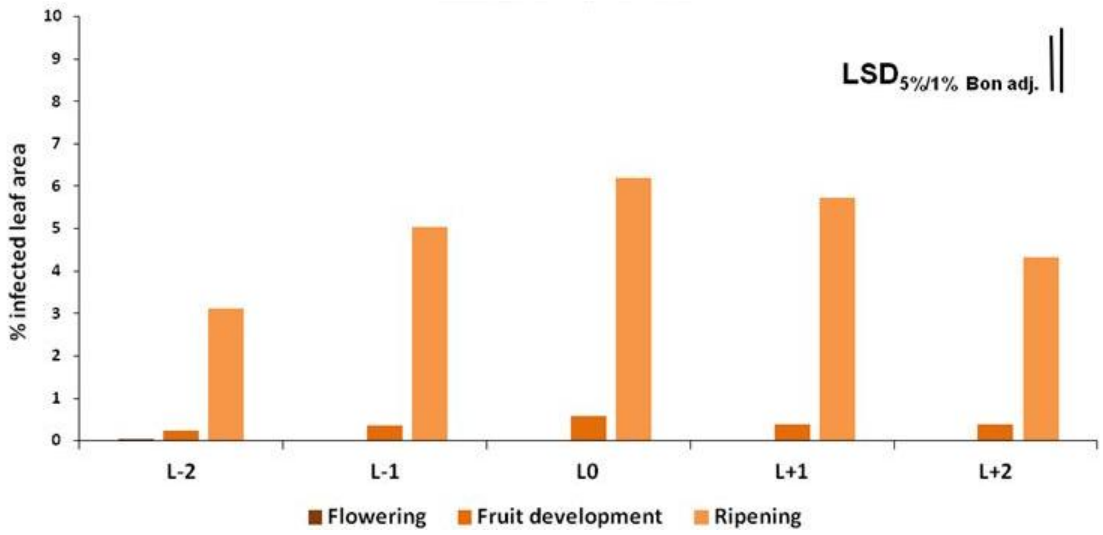

Fig. 60. Mittich 2013. *E. turcicum* infected leaf area on maize variety Zidane according to the leaf position on the plant at three growth stages - flowering, fruit development, ripening. L0= main ear leaf; L-1 and L-2= two leaves below main ear leaf; L+1 and L+2= two leaves above main ear. Data obtained from control and different fungicide applications sprayed at different times. Least significance difference (LSD) according to Bonferroni test: LSD= 1.31 ($p<0.05$); LSD= 1.47 ($p<0.01$).

Below, Figure 61 shows that although the first pustules of common rust were observed at the end of July, lesions around the pustules were not observed until the second assessment (5 September), and these represented less than 1% of the infected leaf area. In the next two weeks, the infected leaf area increased to around 5.8% in the non-treated control, whereas in the treated plots it remained below 3%. All treatments differed significantly from the non-treated control ($p<0.01$). Comparing the fungicide treatments, these had no significant effect when applied at the vegetative stage (BBCH 32). When applied at flowering (BBCH 63), however, propiconazole + azoxystrobin provided significant disease control (4.6% reduction in infected leaf area; $p<0.01$) compared to epoxiconazole + pyraclostrobin (2.9% reduction in infected leaf area). Within the same treatment, significant differences between early and late application could only be observed for the treatment propiconazole + azoxystrobin, where a late application was more effective.

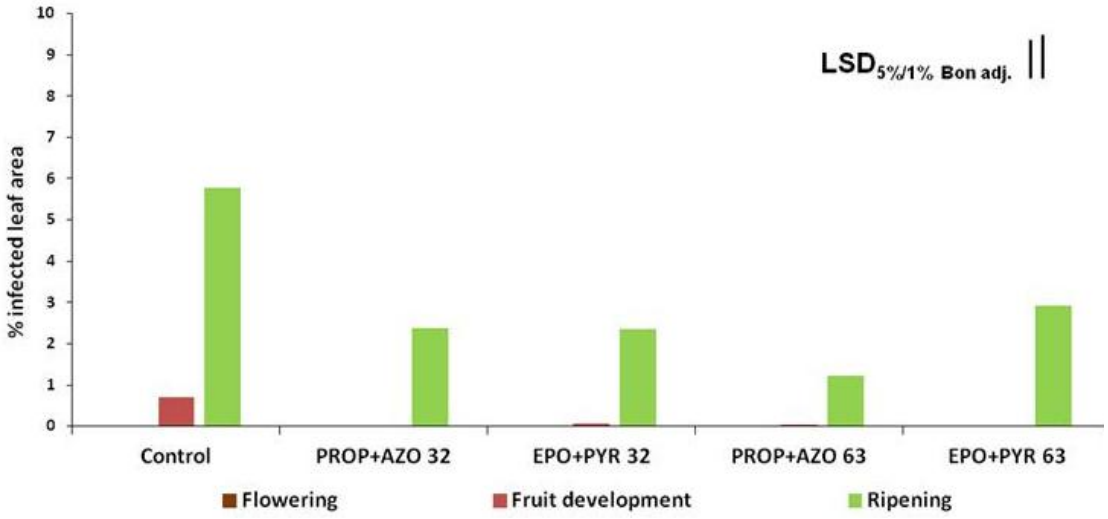

Fig. 61. Mittich 2013. *P. sorghi* infected leaf area on maize variety Zidane at three growth stages – flowering, fruit development, ripening. Comparison of control and different fungicide applications at different times. 32 (BBCH) = applied at vegetative stage (early application). 63 (BBCH) = applied at flowering (late application). PROP= propiconazole; AZO= azoxystrobin; EPO= epoxiconazole; PYR= pyraclostrobin; Least significance difference (LSD) according to Bonferroni test: LSD= 0.95 ($p<0.05$); LSD=1.06 ($p<0.01$).

Conclusions on the distribution of the infected leaf area on the plant (Figure 62) could only be drawn from observations made in the final rating on 25 September (BBCH 85). There is a slight upward trend from the lower leaves L-2 (1.3% infected leaf area) and L-1 (1.6%) to the ear leaf (2.3%) and upper leaves L+1 (4%) and L+ 2 (5.6%). A significant difference between the lower leaves (L-1 and L-2) and the upper leaves (L+1 and L+2) was observed ($p<0.01$).

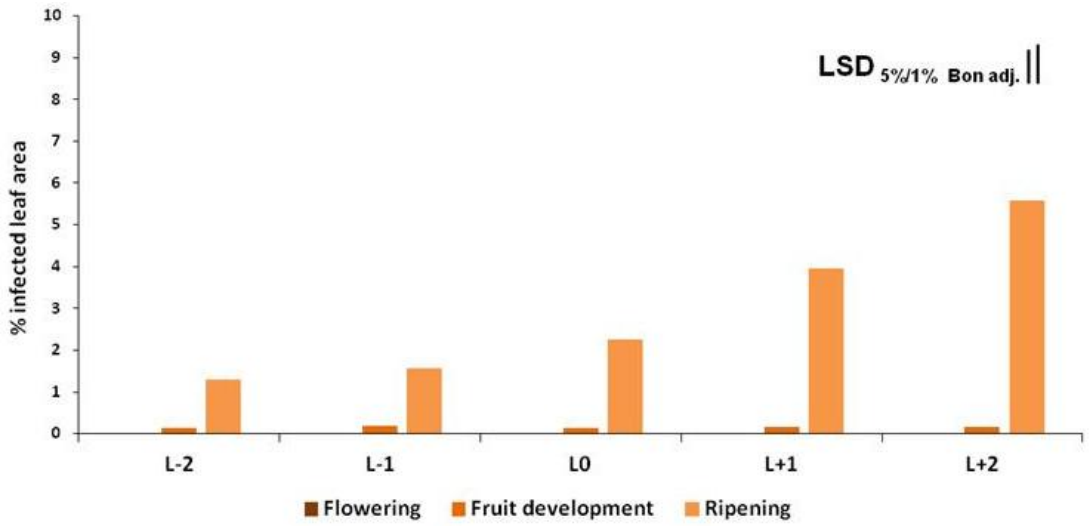

Fig. 62. Mittich 2013. *P. sorghi* infected leaf area on maize variety Zidane according to the leaf position on the plant at three growth stages - flowering, fruit development, ripening. L0= main ear leaf; L-1 and L-2= two leaves below main ear leaf; L+1 and L+2= two leaves above main ear. Data obtained from control and different fungicide applications sprayed at different times. Least significance difference (LSD) according to Bonferroni test: LSD= 0.87 ($p<0.05$); LSD= 0.98 ($p<0.01$).

3.4.2. Turcicum leaf blight in Inzing 2014

First Turcicum leaf blight symptoms were visible during the first rating on 7 August (BBCH 71: fruit development). Nevertheless, the disease did not develop further and in the second assessment on 12 September, the infected leaf area was lower than 1% in both non-treated and treated plots. At this time, the maize plants were at the end of the fruit development. On 8 October, an increase in the infected leaf area was observed, ranging from 2.4% to 2.8% in the non-treated controls (with and without seed treatment, respectively). LSD analysis did not show significant effects between the non-treated controls.

The mixture propiconazole + azoxystrobin at early application (BBCH 51: pre tassel, 10 July) showed a lower, statistically significant ($p<0.05$) infected leaf area (1.2%) in relation to the non-treated controls. This significant difference did not hold for the late application (1.4%) on 23 July (BBCH 65: flowering). Fluopyram + prothioconazole and epoxiconazole + pyraclostrobin, both applied at early stage BBCH 51, did not significantly reduce the infected leaf area with respect to the non-treated plots (1.4% & 2.4%, respectively).

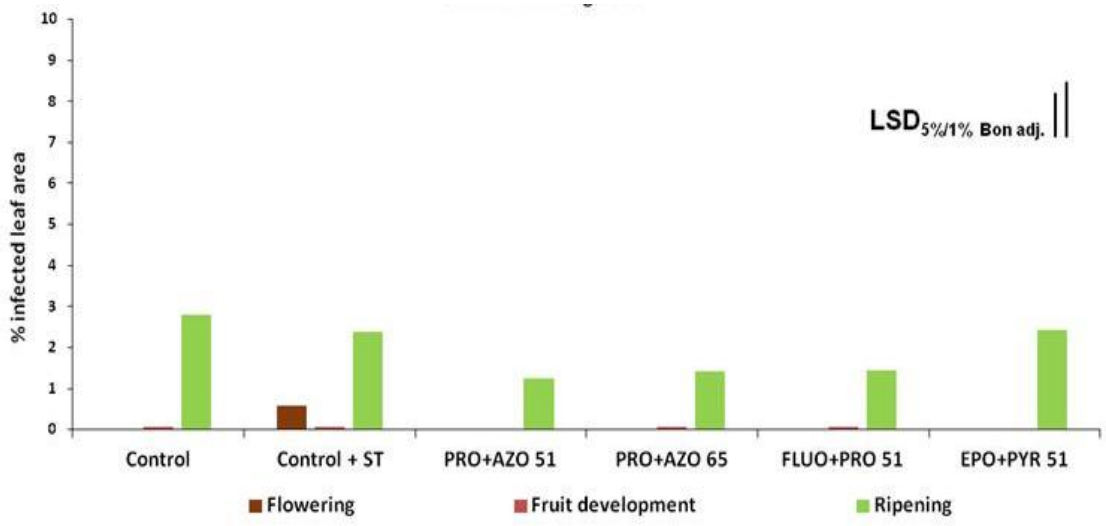

Fig. 63. Inzing 2014. *E. turcicum* infected leaf area on maize variety NK Silotop at three growth stages – flowering, fruit development, ripening. Comparison of control and different fungicide applications at different times. 51 (BBCH)= applied at tasseling (middle-late application); 65 (BBCH)= applied at flowering (late application). ST= applied seed treatment; PROP= propiconazole; AZO= azoxystrobin; FLUO= fluopyram; EPO= epoxiconazole; PYR= pyraclostrobin. Least significance difference (LSD) according to Bonferroni test: LSD= 1.1 ($p<0.05$); LSD=1.22 ($p<0.01$).

In the last assessment on 8 October, the highest levels of severity were observed on the upper leaf L+1 (3.3%), followed by leaves L0 (3.0%) and L+2 (2.9%) (Figure 64). Lower leaves, L-1 and L-2, registered an infected leaf area of less than 1%. Separating the middle (L0) and upper leaves (L+1 and L+2) from the lower leaves (L-1 and L-2) provided a statistically significant difference.

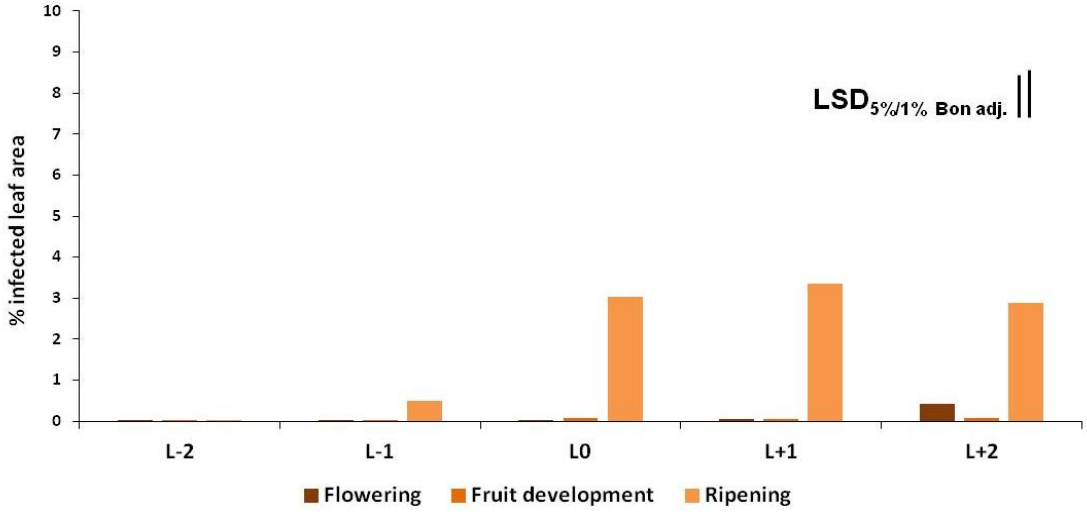

Fig. 64. Inzing 2014. *E. turcicum* infected leaf area on maize variety NK Silotop according to the leaf position on the plant at three growth stages - flowering, fruit development, ripening. L0= main ear leaf; L-1 and L-2= two leaves below main ear leaf; L+1 and L+2= two leaves above main ear. Data obtained from control and different fungicide applications sprayed at different times. Least significance difference (LSD) according to Bonferroni test: LSD= 0.96 ($p<0.05$); LSD= 1.08 ($p<0.01$).

3.4.3. Kabatiella eyespot in Ostenfeld 2013 and 2014

In 2013, the first disease assessment was performed on 7 August (BBCH 63: flowering). At this time, the first symptoms of Kabatiella eyespot were widespread in the location but without significant differences among treated and non-treated plots according to the LSD test as shown in Figure 65. In the second assessment, performed on 11 September (BBCH 79: end of the fruit development), a slightly higher infected leaf area (1.3%) was recorded in the non-treated variant, while the treated plots remained at an infected leaf area of less than 1% (Figure 63). Nevertheless, the differences here were not significant. A third assessment was carried out on 2 October (BBCH 83-85: ripening). At this point, the infected leaf area showed differences among treatments, with the control reaching the highest value of 5.9%. This was significantly different to all treatments ($p<0.01$). Among treated variants, the early season application (BBCH 33: vegetative stage) was not as effective as later applications at tasseling (BBCH 55) and flowering (BBCH 63). The early season application (BBCH 33: vegetative stage) reduced the infected leaf area by 2.5%, whereas later applications at

flowering (BBCH 55) and tasseling (BBCH 63) reduced the infected leaf area by 4.3% and 5.3%, respectively.

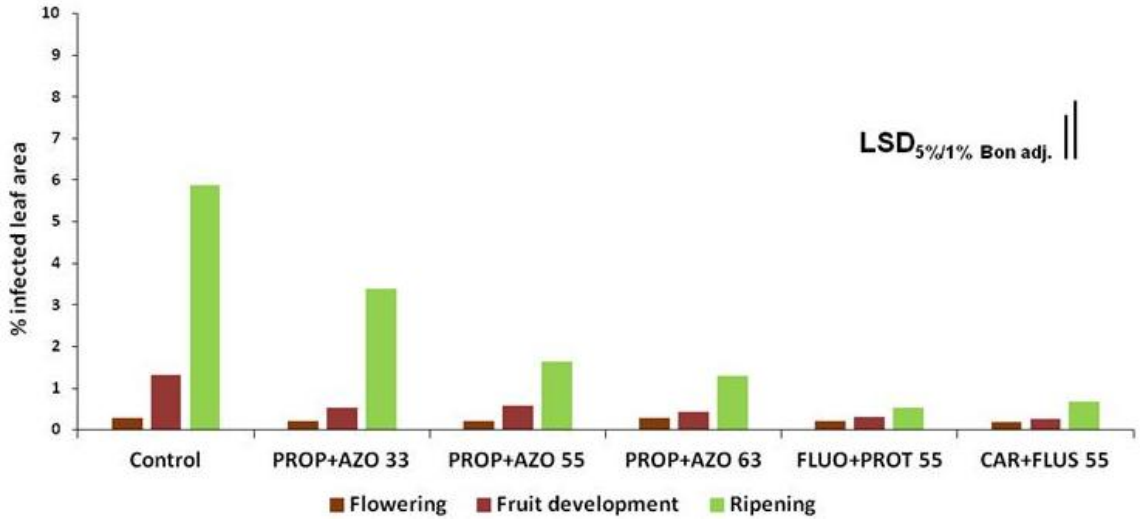

Fig. 65. Ostenfeld 2013. *K. zeae* infected leaf area on maize variety Ronaldinio at three growth stages – flowering, fruit development, ripening. Comparison of control and different fungicide applications at different times. 33 (BBCH)= applied at vegetative stage (early application); 55 (BBCH)= applied at tasseling (middle-late application); 63 (BBCH)= applied at flowering (late application). PROP= propiconazole; AZO= azoxystrobin; FLUO= fluopyram; PROT= prothioconazole CAR= carbendazim FLUS= flusilazole. Least significance difference (LSD) according to Bonferroni test: LSD= 1.1 ($p<0.05$); LSD= 1.22 ($p<0.01$).

As shown in Figure 66, in the first and second assessments on 7 August (BBCH 63: flowering) and 11 September (BBCH 79: end of the fruit development), respectively, the infected leaf area remained under 1% and did not differ significantly among leaf positions. The infected leaf area was slightly higher in the lower leaves (L-2 & L-1). By the last assessment on 2 October, the disease had spread to the upper leaves L+1 and L+2. These leaves exhibited a slightly higher infected leaf area (2.4-3.2%). The infected leaf area on the upper leaf L+2 was significantly different from leaf L-2, L-1, L0 ($p<0.01$) and L+1 ($p<0.05$).

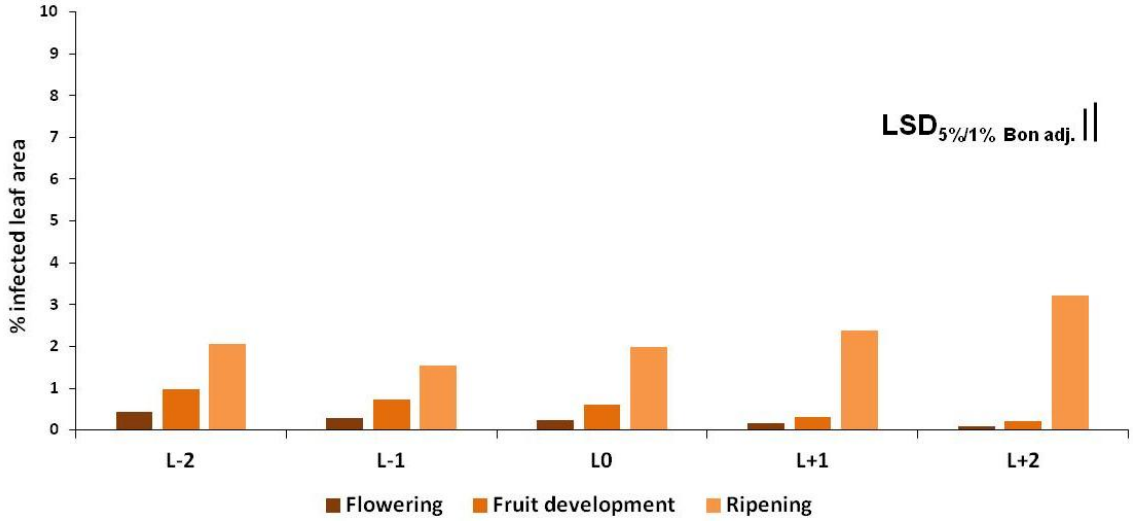

Fig. 66. Ostenfeld 2013. *K. zeae* infected leaf area on maize variety Ronaldinio according to the leaf position on the plant at three growth stages - flowering, fruit development, ripening. L0= main ear leaf; L-1 and L-2= two leaves below main ear leaf; L+1 and L+2= two leaves above main ear. Data obtained from control and different fungicide applications sprayed at different times. Least significance difference (LSD) according to Bonferroni test: LSD= 0.79 ($p<0.05$); LSD= 0.89 ($p<0.01$).

In 2014, in the first and second disease assessments on 12 August (BBCH 65; flowering) and 4 September (BBCH 75, fruit development), respectively, the infected leaf area produced by Kabatiella eyespot remained under 1% for all treatments and significant differences among treated variants and the non-treated control were still not observable, as shown in Figure 67. After two weeks, on 17 September (BBCH 85: beginning of the ripening), an infected leaf area of 3.7% was achieved in the non-treated control. Compared to the non-treated control, all the applied treatments significantly reduced the infected leaf area but no significant differences were obtained among different treatments. Large differences between repetitions (blocks) were recorded, reflected by the large LSD range.

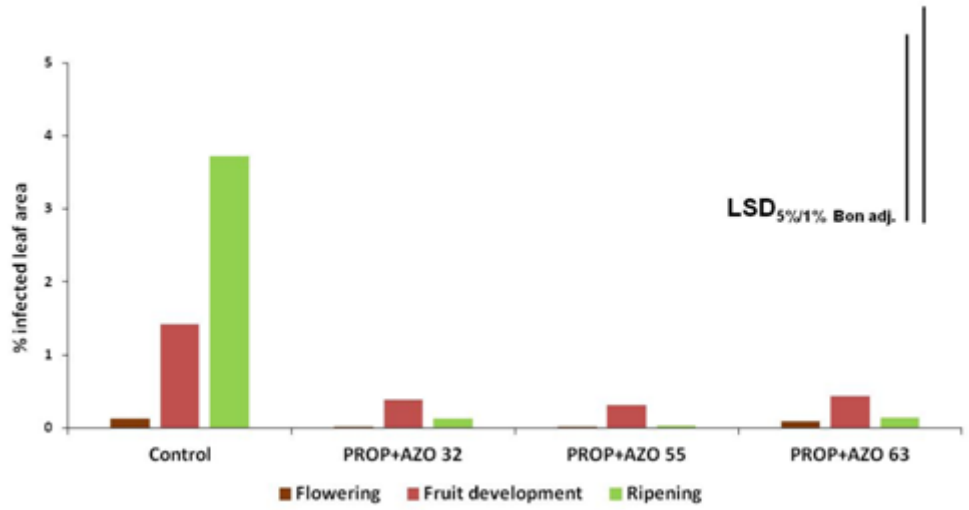

Fig. 67. Ostenfeld 2014. *K. zeae* infected leaf area on maize variety Kalvin at three growth stages – flowering, fruit development, ripening. Comparison of control and different fungicide applications at different times. 32 (BBCH)= applied at vegetative stage (early application); 55 (BBCH)= applied at tasseling (middle-late application); 63 (BBCH)= applied at flowering (late application). PROP= propiconazole; AZO= azoxystrobin; Least significance difference (LSD) according to Bonferroni test: LSD= 2.64 ($p<0.05$); LSD= 2.98 ($p<0.01$).

Although no significant differences were observed at the three assessed stages across the season, the disease was slightly more prevalent in the ear and upper leaves in the final disease assessment (Figure 68).

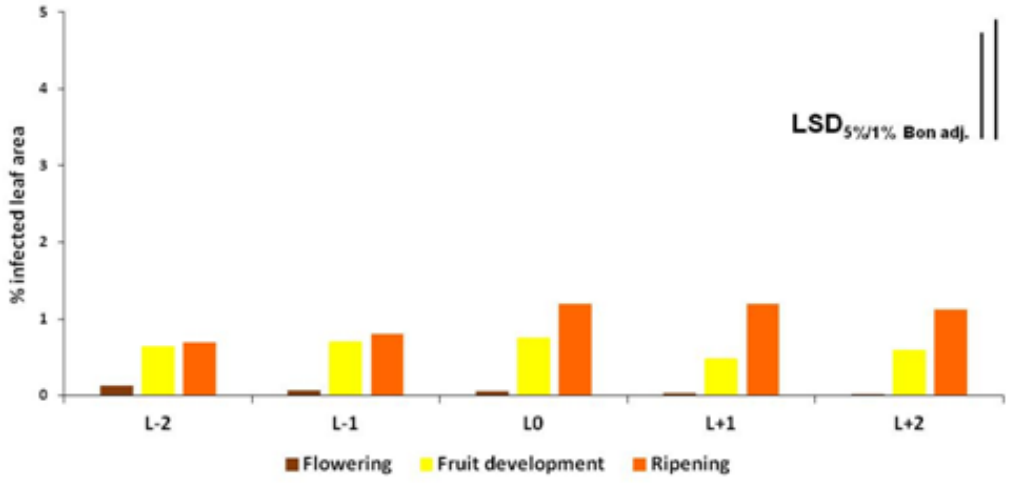

Fig. 68. Ostenfeld 2014. *K. zeae* infected leaf area on maize variety Kalvin according to the leaf position on the plant at three growth stages - flowering, fruit development, ripening. L0= main ear leaf; L-1 and L-2= two leaves below main ear leaf; L+1 and L+2= two leaves above main ear. Data obtained from control and different fungicide applications sprayed at different times. Least significance difference (LSD) according to Bonferroni test: LSD= 1.40 ($p<0.05$); LSD= 1.57 ($p<0.01$).

3.4.4. Effect of fungicide treatments on yield in Mittich 2013 and Inzing 2014

In 2013 in Mittich, although significant differences were observed in the infected leaf area among treated and non-treated plots, none of the fungicides provided significant benefits to the yield compared with the non-treated control (Figure 69). Although statistically non-significant, all treatments had a slightly positive effect on yield compared to the non-treated control, except for epoxiconazole + pyraclostrobin applied at BBCH 32, which had a lower yield than the control. High data variation was observed among repetitions within the same treatment.

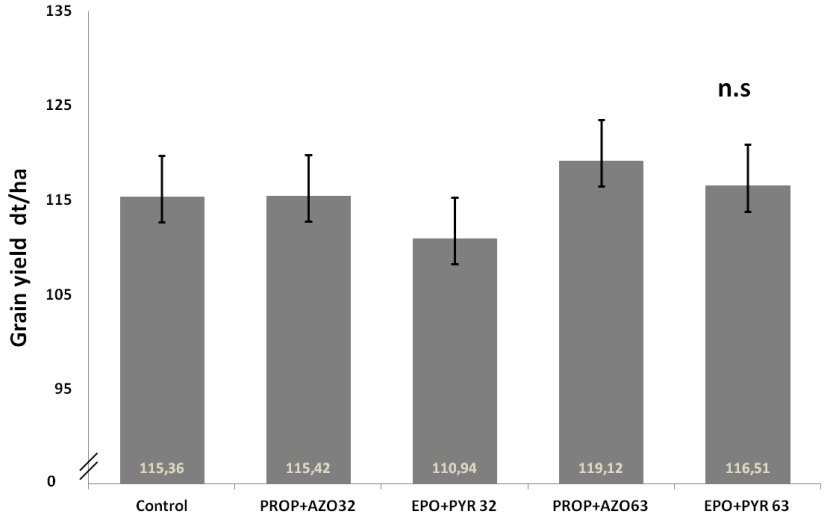

Fig. 69. Mittich 2013. Mean maize grain yield of variety Zidane. Comparison of control and different fungicide applications sprayed at different times to control Turcicum leaf blight. PROP = propiconazole; AZO= azoxystrobin; EPO= epoxiconazole; PYR= pyraclostrobin; 32 (BBCH)= applied at vegetative stage (early application). 63 (BBCH)= applied at flowering (late application). Error bars ($p<0.05$) represent the Bonferroni's standard error.

In 2014, no significant difference between the control with and without seed treatment was observed (Figure 70). Furthermore, no significant effects were observed between the two non-treated variants (control, control + seed treatment) and all fungicide applications. Nevertheless, the application of propiconazole + azoxystrobin at flowering (BBCH 65) obtained a significantly higher yield compared to the non-treated control without seed treatment, increasing the average grain yield by approx. 15 dt/ha.

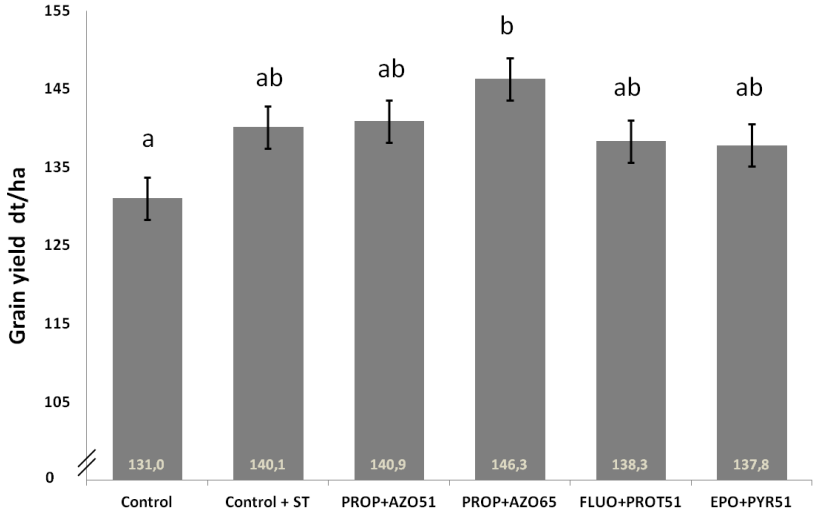

Fig. 70. Inzing 2014. Mean maize grain yield of variety NK Silotop. Comparison of control and different fungicide applications sprayed at different times to control Turcicum Leaf Blight. ST= applied seed treatment; PROP= propiconazole; AZO= azoxystrobin; FLUO= fluopyram; EPO= epoxiconazole; PYR= pyraclostrobin. 51 (BBCH) = applied at tasseling (middle-late application); 65 (BBCH)= applied at flowering (late application). Error bars ($p<0.05$) represent the Bonferroni's standard error.

3.4.5. Effect of fungicide treatments on yield in Ostenfeld 2013 and 2014

In 2013 in Ostenfeld, fungicide treatments applied at tasseling (BBCH 55) and flowering (BBCH 63) resulted in higher yields compared with the non-treated control (Figure 71). With respect to the control (192.8 dt/ha), propiconazole + azoxystrobin applied at flowering provided the highest yield (213 dt/ha), with an approximate increase of 20 dt/ha (10%). Conversely, the same mixture (propiconazole + azoxystrobin) applied at the vegetative stage (BBCH 55) yielded 12 dt/ha less (201 dt/ha). No increase in yield with respect to the control was observed when propiconazole + azoxystrobin was applied at the early vegetative stage BBCH 33. Fluopyram + protioconazole and carboxamin + flusilazole, both applied at BBCH 55, increased the yield by approx. 7 dt/ha (4%) and 14 dt/ha (7%), respectively. Significant effects among treatments and with respect to the control were not observed. High data variation was observed among repetitions within the same treatment.

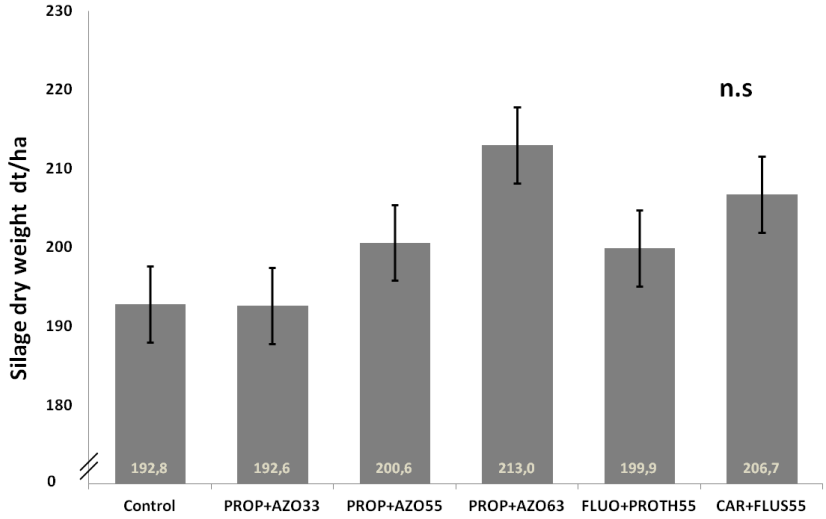

Fig. 71. Ostenfeld 2013. Mean maize grain yield of variety Ronaldinio. Comparison of control and different fungicide applications sprayed at different times to control Kabatiella eyespot. PROP= propiconazole; AZO= azoxystrobin; FLUO= fluopyram; PROT= prothioconazole CAR=carbendazim FLUS= flusilazole. 33 (BBCH)= applied at vegetative stage (early application); 55 (BBCH)= applied at tasseling (middle-late application); 63 (BBCH)= applied at flowering (late application). Error bars ($p<0.05$) represent the Bonferroni's standard error.

In 2014, biomass yield in the non-treated control was not significantly different from the fungicide treatments (Figure 72). An increase in the yield (3 dt/ha) was only obtained with the application of propiconazole + azoxystrobin applied at BBCH 55 (219 dt/ha).

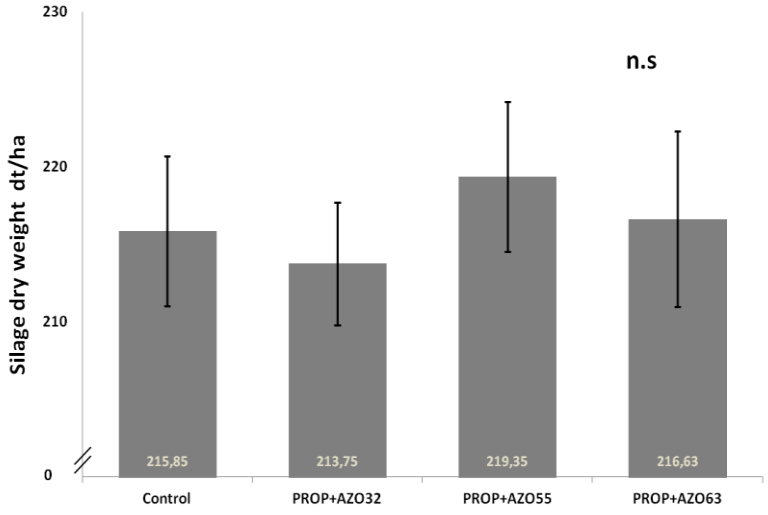

Fig. 72. Ostenfeld 2014. Mean maize grain yield of variety Kalvin. Comparison of control and different fungicide applications sprayed at different times to control to control Kabatiella eyespot. PROP= propiconazole; AZO= azoxystrobin. 32 (BBCH) = applied at vegetative stage (early application); 55 (BBCH)= applied at tasseling (middle-late application); 63 (BBCH)= applied at flowering (late application). Error bars ($p<0.05$) represent the Bonferroni's standard error.

3.4.6. Statistical correlation between yield and disease

Inzing 2014

The data for Inzing 2014 (Figure 73) showed a significant negative effect of the infected leaf area on yield ($p<0.05$). The negative regression reveals that for each percentage point increase in the infected leaf area, a yield loss of 1.59 dt/ha occurs, on average. The 95% CI indicates that the yield reduction could fall within the range of -0.02 to -3.15 dt/ha.

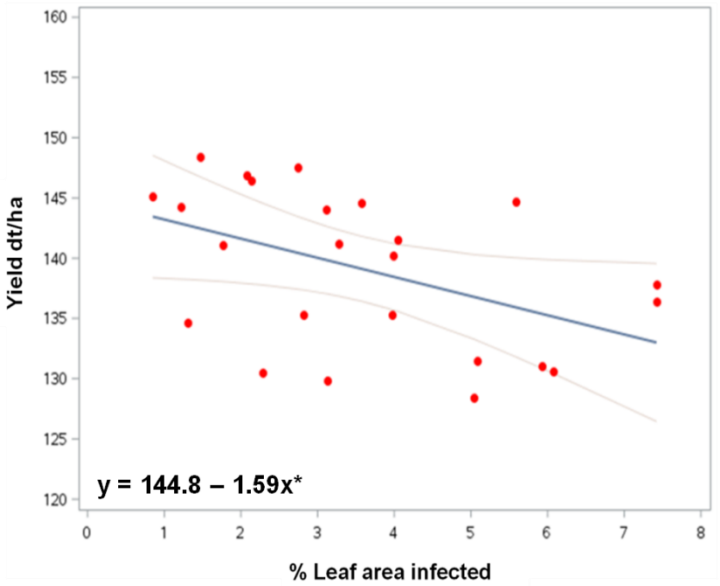

Fig. 73. Relationship between yield and infected leaf area with Turcicum leaf blight on maize variety NK Silotop in Inzing 2014. *Linear model significant ($p<0.05$). ▬ = regression (144.8 -1.59x). ▬ = 95% confidence limits. •= observations (n=24) from yield (y) and corresponding percentage of leaf area infected (x) per plot in the trial.

Mittich 2013

Although common rust was also present, only the effect of the Turcicum leaf blight was considered for the regression analysis for yield losses (Figure 74). The reasoning here is that the formation of Turcicum leaf blight lesions on the leaf was much greater and common rust was of secondary importance in terms of damage. Although a similar infected leaf area was recorded for both diseases in accordance with the different scales used to carry out the rating in the field, 6% of common rust is not comparable to 6% of Turcicum leaf blight due to the large difference in the size of the lesions. Nevertheless, a negative relationship between yield and infected leaf area could not be confirmed, possibly due to the high yield data variation. The regression coefficient of 0.2 implies the infected leaves actually had a positive effect on yield. This is counterintuitive. However, taking the lower 95% CI limit of the regression coefficient “– 1.95 (red dotted line)”, we might assume a negative influence.

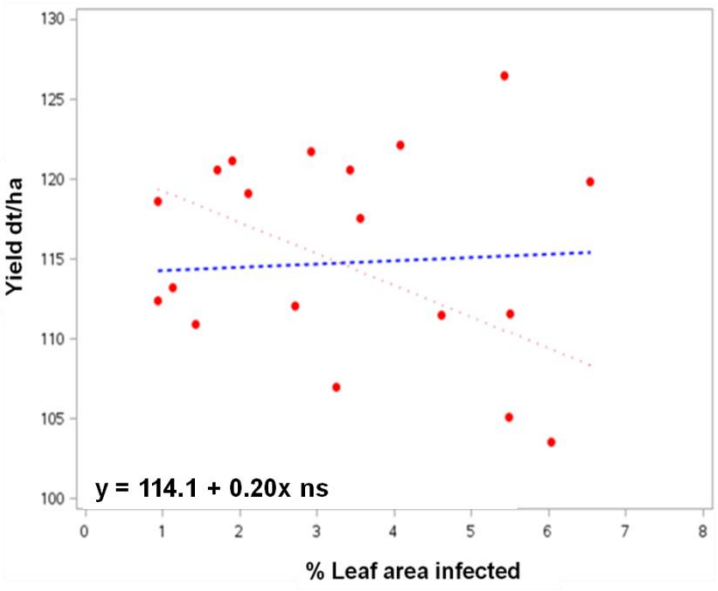

Fig. 74. Relationship between yield and infected leaf area with Turcicum leaf blight on maize variety Zidane in Mittich 2013. ns= linear model was not significant. •••= regression (114.12 + 0.20x). •••= lower limit of confidence interval (95%), which shows a negative influence •= computed observations (n=19) from yield (y) and corresponding percentage of leaf area infected (x) per plot in the trial.

Ostenfeld 2013

The tendency of a negative relation between yield and infected leaf area was found (Figure 75). The regression coefficient, with a p-value of 0.15, carries a certainty of 85%, but not 95%. At this lower level of significance, there might be a certain degree of yield loss occurring between the range of 1.18 and 2.8 dt/ha per percentage point of infected leaf area. The red dotted line indicates the upper 95 % CI limit of the regression coefficient.

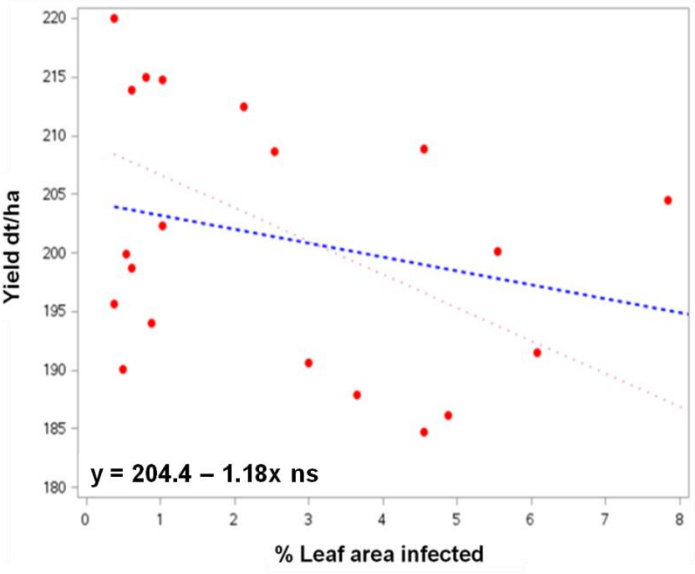

Fig. 75. Relationship between yield and infected leaf area with Kabatiella eyespot on maize variety Ronaldinio in Ostenfeld 2013. ns= linear model not significant. •••=regression (204.39 -1.18x) •••= upper limit of confidence interval (95%), which shows a negative influence •= computed observations (n=20) from yield (y) and corresponding percentage of leaf area infected (x) per plot in the trial.

Ostenfeld 2014

The number of data and their distribution do not permit a conclusion to be drawn about the relation between yield and infected leaf area. According to the linear regression, the disease did not have any influence on yield. The obtained certainty of p>0.2 for the regression coefficient and the corresponding CI limit states that there was no effect of diseased leaves on the yield.

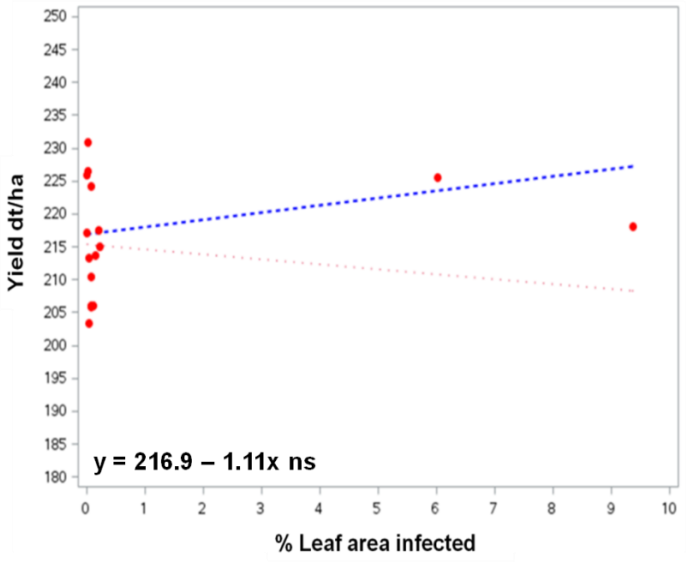

Fig. 76. Relationship between yield and infected leaf area with Kabatiella Eyespot on maize variety Ronaldinio in Ostenfeld 2013. ns= linear model not significant. •••=obtained regression (216.9 + 1.11x) •••= upper limit of confidence interval (95%), which shows a negative influence •= computed observations (n=15) from yield (y) and corresponding percentage of leaf area infected (x) in the trial.

3.4.7. Theoretical thresholds

As part of the control strategy with fungicides, economic damage thresholds are used to establish the lowest severity level of the disease (% infected leaf area) that will cause economic damage, i.e. the point at which the cost of fungicide application is equal to the expected economic loss caused by the respective leaf disease at a certain percentage of infected leaf area. Thus, if the economic losses associated with a certain percentage of infected leaf area are higher than the cost of fungicide application, an application would be economically justified. As shown above, a negative correlation between disease and yield only occurred in Inzing in 2014 for *E. turcicum* and in Ostenfeld in 2013 for *K. zeae*, of which only Inzing 2014 was statistically significant ($p>0.05$). Therefore, the economic threshold was only calculated for these two experiments. Data for the calculation are summarised in Table 37.

Tab. 37. Data for calculation of economic thresholds for fungicide application

Price of silage maize*	32 €/t
Price of grain maize*	160 €/t
Cost of fungicide application**	67.5 €/ha
Loss per 1% infected leaf area of *E. turcicum*	1.59-3.15 dt/ha (0.159-0.315 t/ha)
Loss per 1% infected leaf area of *K. zeae*	1.18-2.80 dt/ha (0.118-0.280 t/ha)

* Maize price in regions with high technological production (actual market rate April 2016)

** Fungicide application Quilt Excel (azoxystrobin + propiconazole)= **52 €/ha.**
Agricultural labour costs: **13.50 €/ha**. (Tractor, sprayer and driver remunerations)
Fuel: **2 €/ha** (Agricultural Chamber Nordrhein-Westfalen 2015).

In order to calculate the threshold, the average and maximum possible yield losses (dt/ha) obtained from the confidence intervals in the regression analysis for Inzing 2014 and Ostenfeld 2013 were taken. The average and maximum values allowed a calculation of two possible thresholds, where the maximum represents the upper limit of the confidence interval, i.e. the highest value within a range of possible outcomes. It should be noted that taking the minimum value (lower limit) for yield losses, i.e. the lowest value within a range of possible outcomes, would lead to yield losses which are equal or very close to zero. This would imply that no fungicide application is necessary and does not allow a threshold to be calculated.

These average and maximum values for yield losses were converted into t/ha and multiplied by the product price in order to estimate the monetary loss per 1% of infected leaf area. Note that the product price depends on the type of maize used in the respective locations, i.e.

grain maize in Inzing (*E. turcicum*) and silage maize in Ostenfeld (*K. zeae*). The costs of fungicide application (67.50 €/ha) were divided by the above value to determine the economic threshold (as percentage of infected leaf area). Table 38 presents the thresholds calculated for *E. turcicum* and *K. zeae* in Mittich 2014 and Ostenfeld 2013, respectively.

Tab. 38. Economic thresholds for fungicide application (percentage of leaf area infected) according to the potential yield losses (t/ha) (average and maximum value) obtained from calculated confidence intervals in the regressions in section 3.4.6.

	E. turcicum (Inzing 2014)	*K. zeae* (Ostenfeld 2013)
Average potential yield loss	0.159 t/ha	0.118 t/ha
Threshold	**2-3% (2.6%)**	**17%**
Maximum potential yield loss	0.315 t/ha	0.280 t/ha
Threshold	**1%**	**7%**

With regard to the average yield loss levels for *E. turcicum* in 2014, the economic threshold was 2-3%, while for the maximum possible yield loss the threshold is only 1%. Taking the average yield loss, this means that a fungicide application would be economically beneficial at an infected leaf area of >2.6% for *E. turcicum.* For the average yield loss level of *K. zeae,* the economic threshold was 17%, while for the maximum possible yield loss the threshold is only 7%. Note that the threshold would increase for *E. turcicum* if the significantly lower price for silage maize (32 €/t) compared to grain maize (160 €/t) was taken, and vice versa for *K. zeae.* However, due to the fact that the regressions were run in accordance with the type of maize used in the respective locations, calculating alternative thresholds would not be accurate.

4. Discussion

Despite the increase in the acreage of maize in Central Europe in recent years, there continues to be a lack of knowledge about associated fungal pathogens and their increasing presence in the region's maize fields. A monitoring of fungal maize leaf diseases in selected Central European regions was therefore deemed necessary to gain a greater understanding of the occurrence and severity of these diseases and the climatic conditions that determine their epidemiological pattern.

Based on the level of severity and the timing of the appearance of the disease, decisions can be made about the application of fungicides. As results in this work and related studies have confirmed, diseases such as Kabatiella eyespot and Turcicum leaf blight have the potential to cause yield losses. In this case, the application of fungicides can be an effective control method. The possible reasons for differences among variants of treated and untreated plots in the field trials carried out in this work are discussed here, as well as decisions regarding timing of application and the suitability of fungicide applications in relation to disease severity. The potential economic benefits of fungicide application and the calculation of thresholds are also evaluated. Of course, decisions regarding fungicide application should be considered within an integrated management of the disease, where decisive epidemiological factors such as inoculum pressure correlated with climatic conditions in the field need to be considered. This is discussed based on the results obtained via spore trapping in three locations. Potential latent periods are also looked at in greater depth in this context.

4.1. Occurrence of leaf pathogens on maize in Central Europe

4.1.1. Monitoring and prevalence of diseases in 2012 and 2013

Main diseases *E. turcicum* and *K. zeae*

The maize leaf spot pathogens *Exserohilum turcicum* and *Kabatiella zeae* dominated across the different monitored regions and are the causal agents of the Turcicum leaf blight and Kabatiella eyespot.

E. turcicum is described in the literature to be most damaging at temperatures varying between 18 and 27°C, with an optimum of 20-25°C (Berger 1970, White 2010). This correlates with the observations made during the monitoring in 2012 and 2013, where the main regions in which *E. turcicum* is widespread are situated in southern Germany (Bavaria, alpine foreland), northern Austria (alpine foreland) and southern France[8]. In these regions,

[8] Southern France was not visited but information was obtained via observations from Mainka (2012, pers. comm., September).

higher temperatures are recorded in summer compared with northern regions of Germany (Schleswig-Holstein, northern part of Lower Saxony), where plants infected with *E. turcicum* were scattered.

The other main pathogen, *K. zeae*, was found to be widespread in northern regions of Germany (Schleswig-Holstein, northern part of Lower Saxony) and the Netherlands due to it being favoured by lower temperatures (14-17°C) (Reifschneider & Arny 1980a). Thus, the temperature is the decisive factor which separates the regional occurrence of these two main pathogens. Both pathogens also need extended periods of high humidity for their development, and while it is of secondary importance in relation to the temperature, it remains essential.

The interaction between climatic conditions and *E. turcicum* and *K. zeae* will be analysed in greater depth in section 4.3.

***Phoma* spp.**

Several pathogenic and saprophytic *Phoma* species, or other pathogens related to this genus, can occur on maize. In this work, morphological analyses revealed different *Phoma* species. Further molecular analysis could not provide sufficient information to confirm the species of most of the obtained isolates. Furthermore, three representative isolates, 13.2B (considered saprophytic), 12.20 (pathogenic) and 12.13 (saprophytic) were sent to the CBS-KNAW Fungal Diversity Center in Utrecht for molecular analysis. Following these analyses and comparisons with internal sequence databases, the three isolates were confirmed as three putative new species within the genus *Peyronellaea* (syn. *Didymella*; anamorph related to the genus *Phoma*).

Prior to this, a first attempt to accurately identify these species turned out to be difficult due to the numerous micromorphological characters of *Phoma* species which had to be taken into account. A comparison of these morphological features with descriptions in the literature was not very helpful either. This is probably because *Phoma* species tend to vary greatly among different *in vitro* growth conditions (Aveskamp et al. 2010), making morphological comparisons extremely difficult for some species.

Pathogenic *Phoma spp.*

The lesions formed by the pathogenic isolates analysed in this work were very similar to those described for *Ph. zeae-maydis* in the literature (Arny & Nelson 1971; Punithalingam 1990; White 2010) and those produced by the isolate *Ph. zeae-maydis* (CBS 586.95) in the greenhouse after spray inoculations. Nevertheless, micromorphological observations revealed differences between *Ph. zeae-maydis* (CBS 586.95) and the pathogenic isolates obtained during the monitoring.

In *Phoma* spp., conidial shape and size are considered to be two of the most useful parameters for identification up to species level (Aveskamp et al. 2008). The size of the spores of *Ph. zeae-maydis* (CBS 586.95) is much larger when compared with other species. In fact, until reclassification by Aveskamp et al. (2010) and Chen et al. (2015), it was classified within *Phoma* section *Macrospora*. Species included in this section are characterised by the production of large conidia, both *in vivo* and *in vitro* (Boerema et al. 2004). According to the first description of *Ph. zeae-maydis* by Arny & Nelson (1971) (at the time as *Phyllosticta maydis*), while the spore size could vary considerably between 8-20 x 3-7.5 µm, most spores fell within the range of 12-15 x 4-6 µm. These measurements clearly represent larger conidia than those of the pathogenic strains isolated in this work (4.1-7.9 x 1.5-3.2 µm).

Variations in morphological characters can occur in *Phoma* spp., depending on the *in vitro* culture conditions (Boerema et al. 2004). Therefore, *in vivo* descriptions may resemble the initial morphology of fungi more accurately. Due to the large amount of *Phoma* isolates obtained during the monitoring and the extensive descriptions required for identification, an *in vivo* description was impractical. Thus, whether or not a variation in spore size could have occurred was not investigated further. Nevertheless, the *in vitro* descriptions for the pathogenic reference isolate *Ph. zeae-maydis* (CBS 586.95) coincided with those described in the literature *in vivo* by Arny & Nelson (1971) and by Punithalingam (1990). This is an indication that conidia of *Ph. zeae-maydis* also remain characteristically large under *in vitro* conditions.

Further *Phoma* species occurring on maize for which a smaller conidia size is reported are not extensively described in the literature. One of these species, *Phoma zeae (*described as *Phyllosticta zeae),* was described by Stout (1930) from samples collected in Illinois (USA). The size for conidia of *Ph. zeae* (4.5-7.5 x 2-3.5 µm) described by Stout (1930) matches those obtained from our isolates. On the contrary, the shape of the conidia differs. Although pathogenicity tests are not reported, the symptoms described by Stout (1930) are similar to those described for *Ph. zeae-maydis*. This species has also been reported in the Czech Republic (Saccas 1952, Cejp 1967 cited by Arny & Nelson 1971) and Romania (cited by Scheifele & Nelson 1971). McFeeley (1971) and Frezzi (1972) also considered *Ph. zeae* (at that time *Phyllosticta zeae*) as causal agent of the yellow leaf blight on maize in Ohio, USA and Argentina, respectively. However, Frezzi (1972) mentions that although descriptions of the lesions and other observations coincided with those described by Stout (1930) for *Ph. zeae*, the spores were larger in size (8 8 to 16.5 x 3,5 to 8.5 µm). Thus, this report still causes some confusion. It is therefore possible that the *Phoma* described by Frezzi (1972) was, in fact, the new pathogen *Ph. zeae-maydis* described by Arny and Nelson (1971) months earlier, which had a greater conidial size. A further mention about the presence of

Phyllosticta zeae G.L. Stout (later classified in the genus *Phoma*) was recently updated in a checklist of *Phyllosticta* species in China (Zhang et al. 2015). The descriptions and size of the conidia for the specimen studied (4 to 7 x 2 to 2.6 µm) were similar to our isolates.

A further *Phoma* species reported on maize by Stout (1930), which is also similar in shape and size to some of the isolated samples in this work, is *Ph. zeicola* (Ellis & Everh.). Unfortunately, only descriptions for the size and shape are given by the author (similar to those described for *Ph. zeae*). Further comparisons were therefore not possible. Confirming whether the pathogenic *Phoma* isolates correspond to the description of *Ph. zeae* or *Ph. zeicola* will be difficult to prove because specimens of these species are not easily accessible and DNA sequences are not available in the sequence databases.

In the monitoring, pseudothecia resembling those from *Mycosphaerella zeae-maydis.* (syn. *Didymella maydis*) described by Mukunya & Boothroyd (1973) were also found in one location. This teleomorph corresponds with the anamorph *Ph. zeae-maydis.* This fact would support the initial hypothesis that the pathogen corresponds to *Ph. zeae-maydis,* although molecular analysis contradicted this.

Other related teleomorphs for similar *Phoma* species on maize have been also reported in the literature such as *M. zeicola*, (Stout 1930), *M. maydis* (Pass.) Lobik, and *M. zeae* (Sacc.) (Mukunya & Boothroyd 1973).

Additional pathogenic *Phoma*-related species reported on maize such as the possible disease complex *Phaeosphaeria maydis* and *Ph. sorghina (syn. Epiccocum sorghinum)* were disregarded. These differ from the descriptions of symptoms and morphological characteristics given in this work and are mostly reported in tropical regions (Do Amaral 2004; Gonçalves et al. 2013).

Non-pathogenic *Phoma* species

In this work, *Phoma* species which were considered non-pathogenic on maize such as *Ph. pomorum (syn. Didymella pomorum)*, *Ph. subherbarum (syn. D. subherbarum)*, *Ph. glomerata* (*syn*. *D. glomerata*) and *Ph. tropica* (syn. *Allophoma tropica*) are also reported to be present on maize (Payak et al. 1987; De Gruyter et al. 1993; Demirci & Kordali 2000; Cervelatti et al. 2002; Sørensen et al. 2010). While the representative isolate 12.13 (this also implies isolates similar to 12.13) is very similar to *Ph. subherbarum*, this species is only reported in North and South America. Furthermore, the analyses by the CBS confirmed that isolate 12.13 does not correspond to either of these species or any of the others mentioned.

Isolates 12.36 and 12.37 show very similar in vitro characters to *Phoma pomorum* isolate CBS (838.84). This coincides with the molecular analysis carried out in this work, where both

isolates (12.36 and 12.37) showed 100% similarity with *Phoma pomorum*. *Phoma pomorum* was reported to be isolated from Danish maize by Sørensen et al. (2010).

Due to the high diversity of *Phoma* species occurring on maize confirmed in this work, further phylogenetic and possibly *in vivo* morphological analyses are required. These are necessary in order to discover related species of the putative new species and clarify the *Phoma* complex (*Didymella* complex) of unidentified pathogenic and saprophytic species occurring in maize. This clarification could be helpful in understanding more about the phytosanitary state of maize in Central European regions, the importance of the *Phoma* spp., and comparisons with other maize-growing regions worldwide.

Bipolaris zeicola

The pathogen was found on a scattering of plants in northern and southern locations. Due to limited distribution of the pathogen across all locations, it was not possible to establish a primary region of occurrence. A race 3 isolate was found in central Czech Republic (characterised by a warm and dry climate in summer). Other isolates which produced similar spots to those described for race 2 were found in several locations with differing climatic conditions, e.g. southern and northern Germany and the Netherlands.

According to the literature, optimal temperatures favouring race 3 are generally cool, evidenced by several reports from regions with cool and mild temperatures in the summer (White 2010; Liu et al. 2015). In contrast, races 1 and 2 favour warm conditions (White 2010). This could not be confirmed, however, as race 2 was found in several locations with differing climatic conditions such as southern and northern Germany and the Netherlands.

Unlike other races or pathotypes of *B. zeicola*, the virulence and increased prevalence of race 3 has been observed in temperate regions (Xiao et al. 1992; Welz et al. 1993; Zitter 2012), as well as recently being considered an important limiting factor to corn production in hilly and mountainous areas of China (Liu et al. 2015). This race produces host-selective toxins called BZR-cotoxins I-IV, which facilitate the colonisation of the plant tissue and make it the determinant factor of virulence and host selectivity (Xiao et al. 1992). *B. zeicola* race 3 is also reported to be of pathogenic importance in rice plantations (Xiao et al. 1991), which is another example of the importance of its virulence.

The importance of race 3 in the literature coincides with the results of the pathogenicity test in the greenhouse, where the isolate described as race 3 showed the highest virulence of all isolates tested. It was able to cover large areas of the leaf tissue with long lesions, which led to an early ripening of the leaves that other isolates could not produce.

B. zeicola is considered to behave more prevalently as necrotroph than other *Bipolaris* spp., sporulating abundantly until maturing of the leaf tissue (White 2010). This could also explain the importance of the pathogen as a saprophyte but not as a main pathogen in the regions.

Two other *Bipolaris* spp. were found in leaves originating from the Czech Republic. These could not be classified as *B. zeicola* because their morphological characteristics differed *in vivo* and *in vitro*. Some authors (Hooker et al. 1973; Levic & Pencic 1980) have reported that the medium could have an effect on the conidial morphology of new pathotypes of *B. zeicola,* and atypical conidia of *B. zeicola* could be formed in agar. In this study, spores were similar when isolated from initial leaves and the *in vitro* medium. These *Bipolaris* spp. isolates were able to infect maize leaves and produce some tiny chlorotic spots which developed into lesions. In terms of virulence, however, these isolates were less virulent than the isolates confirmed as *B. zeicola*. Therefore, the behaviour of these *Bipolaris* spp. could be predominantly saprophytic and remain less virulent than *B. zeicola*. Further comparisons between these *Bipolaris* spp. and *B. zeicola* with different hybrid varieties and lines of maize under controlled climatic conditions are necessary to confirm this.

A confirmation of the species of these two isolates was not possible through morphological analysis. Besides *B. zeicola*, several *Bipolaris* spp. are reported to be pathogenic on maize. Parallel to *B. zeicola*, the most important is *B. maydis* (White 2010; Singh & Srivastava 2012). Based on the descriptions for *B. maydis* by White (2010), this possibility was disregarded due to the clearly distinguishable leaf symptoms of *Bipolaris maydis* (elongated or oval, pale lesions delimited along the veins) and its morphology (increased width and length of up to 160 µm) and more pronounced curvature compared to *B. zeicola*, which sometimes stretches out towards the bottom, resembling a long tail. The unidentified conidia are smaller and darker than *B. zeicola* and mostly not curved.

Other *Bipolaris* spp. which are less frequent, but also described in the literature as producers of typical spot blotch symptoms on maize leaves, are *B. spicifera* (Mendes et al. 1998; Li et al. 2016) and *B. sorokiniana* (Iftikhar et al. 2009). Although the spores of *B. spicifera* are smaller (15-32 x 9.9 -12.4) (Li et al. 2016) than those of *B. zeicola* (25-100 x 7-18 µm) (White 2010) and lighter in colour towards the terminal cells, resembling the unidentified isolates, other parameters such as three-septate, rounded ends and clustering of the conidia (resembling the form of a bottlebrush) in *B. specifera* (Navi et al. 1999) exclude this pathogen as a causal agent. *B. sorokiniana*, in contrast, exhibits more similar characteristics to both unidentified isolates (12.8 and 12.9), including curved or straight, conidia shape, black and bright to brown olivaceous in colour, with a characteristically spindle-shaped form, terminal and subhyaline cells (Warham et al. 1997).

Further morphological and pathological comparisons with confirmed *B. sorokiniana* isolates from the same regions, but possibly isolated from different crops, could provide more information. Nevertheless, the possibility of other *Bipolaris* species affecting maize leaves cannot be ruled out. Due to the high number of *Bipolaris* spp. described in the literature, molecular analysis could more accurately identify the species.

P. sorghi

During the monitoring, *P. sorghi* was observed in Holland, central Germany and Eastern Europe (Poland, Czech Republic), but the primary locations affected were situated in southern Bavaria. The affinity of the pathogen to moderate-high temperatures and high humidity, also necessary at night, could explain the higher prevalence in the southeast of Bavaria (Passau district). Summers are moderately warm (19°C avg.) and rainy, with frequent periods of dew formation at night and in the morning.

In the literature, a range of temperatures is described for the development of common rust, but sporulation of *P. sorghi* is higher at 20-25°C than at lower temperatures of around 15°C, and more common at a relative humidity of at least 98% (Hooker 1985). Night temperatures are also important for the development of the disease because low temperatures (approx. 8°C) prolong the latent period (Headrick & Pataky 1986).

C. graminicola

In the monitoring, *C. graminicola* was found in southern France, where average temperatures in the summer of 2012 (July, August, September) varied between 19 and 22°C (max. average of 24-28°C)[9], explaining the presence of the pathogen in accordance with the literature. Nevertheless, the pathogen was also found in northern areas of Lower Saxony (Nordholz) and Holland (Princepeel), where, on average, moderate temperatures of 14 to 18°C (max. average 19 to 24°C)[10] are assumed to retard the development of the disease. These unfavourable climatic conditions could explain the limited presence of the pathogen in the locations.

Although the fungal pathogen *C. graminicola* has been reported in various temperate, tropical and subtropical areas, it is particularly established and severe in warm, humid conditions (Wheeler et al. 1973; Gatch & Munkvold 2002; Jackson-Ziems et al. 2014; Da Costa et al. 2014; Zhang et al. 2014; Wise 2015). Furthermore, high temperatures (30°C) are considered optimal for lesion elongation and dew periods (Leonard & Thompson 1976; White 2010).

[9-10]Data obtained from climatic database of Syngenta GmbH.

After pathogenicity tests in the greenhouse in this work, the ability to infect both varieties of maize in only a few days (lesions observed after five to seven days), the high virulence, and the ability to produce stalk rot (also in the greenhouse) showed that *C. graminicola* could be one of the most devastating maize leaf pathogens if optimal conditions of high temperatures are present.

The pathogen is usually easy to recognise due to the acervuli produced on the leaves. These structures are reported to be produced on infected maize residues in the field (Bergstrom & Nicholson 1999). Nevertheless, other fungal organisms like *Menispora ciliata* Corda also colonise debris (CBS 2015), producing similar acervuli. This was observed in maize debris in the northern location of Ostenfeld. This could lead to a false identification of the pathogen in the debris.

***Fusarium* spp.**

Fusarium species are described as frequently occurring pathogens in maize, primarily causing stalk and ear rots, reducing yield and producing mycotoxins (Bottalico 1998; D'Mello et al. 1999; De Venter 2000; Munkvold et al. 2000, 2003; Desjardins 2006; White 2010; Eckard et al. 2011; Antonissen et al. 2014). However, infection on maize leaves producing symptomatic foliar spots or lesions is less common.

In this work, the isolates were obtained from leaf lesions produced by other pathogens or from *Fusarium* lesions produced on stalk and corn husks (tan spots with black border and pink to white sporulation in the centre, necrosis on the leaf sheath) or from maize residues from the previous season. Although the spraying of spore suspensions on the leaves did not produce any symptoms for any of the isolates tested in this work, some evidence that the pathogen can produce lesions on maize leaves is available in the literature.

Moya-Elizondo et al. (2013) reported *F. sporotrichoides* to cause circular, white to brown spots and necrotic tissue on the margins and tips of leaves in maize fields in Chile. In order to fulfil Koch's postulates, the author inoculated the leaves of healthy plants through spraying of spore suspensions and was able to confirm the capability of one *Fusarium* species to colonise the inner maize leaf tissue and produce lesions.

Further studies evaluating the role of *Fusarium* spp. on maize leaves were carried out by Nguyen (2014), who tested different leaf inoculation methods. The author showed that while coating fungal inoculum of several *Fusarium* species on the fourth leaf did not produce symptoms, dropping of inoculum into the whorl, combined with coating of the developed fourth leaf produced symptoms on the new emerging leaves (6^{th} and 7^{th}). The capacity of the pathogen to penetrate unfolded leaves of the whorl was also described by Schieber and Müller (1968) in plants infected with *F. moniliforme.* In their experiments, plants developed

water-soaked spots with brown border, inhibiting further opening of the unfolded leaves and a downward turning of the leaf tip very similar to those described by Nguyen (2014). This is possibly due to accumulated water in the whorl leaves of the plant, favouring the infection. Therefore, the inability to produce lesions on leaves could be more influenced by non-optimal conditions (e.g. low humidity) than the inability of the pathogen to infect different structures per se. Non-optimal conditions would restrict the colonisation of certain tissues, thus allowing the plant more time to respond (Oren et al. 2003).

Other fungi colonising maize leaves

Two of the most frequently found fungi in maize leaf microflora in all the sampled locations were species belonging to the *Alternaria* genus and *Epiccocum nigrum*. Both of these are also reported in the literature as belonging to the most frequent fungi isolated from maize leaves and kernels, along with *Cladosporium* and *Fusarium* species (Müller 1991; Fisher et al. 1992; Broggi et al. 2007; Remesova et al. 2007; Gonçalves et al. 2013).

In this work, one *E. nigrum* isolate and two *Alternaria* spp. isolates were tested through inoculation on maize leaves and no symptoms were produced. Thus, the fungi could not be considered capable of infecting healthy leaves.

While *E. nigrum* is considered to be an endophyte in several crops besides maize (Remesova et al 2007; Martini et al. 2009; Favaro et al. 2011), there is a lack of reliable evidence that it plays a parasitic role on maize leaves (Yoshizawa et al. 1994). The *Alternaria* species *A. alternata* and *A. tenuissima* have been reported as the most frequent on maize leaves (Müller 1991). *A. alternata* is considered a “weak pathogen” of maize, which is only capable of producing leaf blight after injury in optimal temperatures (20°C) in the absence of prolonged dry periods (>24 hours) and during senescence (Trainor & Martinson 1981). This is also likely to be the case for *E. nigrum,* which is mainly considered an endophyte and can also colonise the maize tissue under certain conditions of wounding.

Disease incidence in 2012 and 2013

In the monitoring results, it is notable that disease incidence in 2013 was higher compared to 2012. Looking at the weather data in more detail provides further insight into the reasons for this occurrence. Here, the weather data for the respective northern and southern German locations will be taken as representative as the vast majority of samples originated from here (especially in 2013). This allows for an in-depth comparison between the two years. Furthermore, the northern and southern German locations exhibited the highest pressure for the respective diseases that frequently occur in these regions (i.e. Turcicum leaf blight and common rust in the south, and Kabatiella eyespot in the north).

Looking first at the weather data for the Mittich/Inzing region (southern Germany), it immediately becomes apparent that a severe lack of precipitation in July 2013 appears to have inhibited the development of the diseases during the vegetative to reproductive stage of the plant. This value of 23.7 mm was considerably lower than in July 2012 (78.9 mm). Furthermore, while *E. turcicum* and *P. sorghi* theoretically flourish at higher temperatures (20-25°C), a 1.3°C temperature difference between July 2012 (19°C) and July 2013 (20.3°C) could also have played a role, albeit a less important one.

Regarding the northern region and taking the weather data for Ostenfeld (northern Germany) as a reference, the severe reduction in precipitation in July 2013 (55.4 mm) compared to July 2012 (145.5 mm) again appears to have inhibited the development of the pathogen *K. zeae*. Additionally, July 2013 was considerably warmer (18.1°C) than July 2012 (16.4°C). This, along with the low level of precipitation, is likely to have greatly inhibited the development of *K. zeae* as it is a pathogen which thrives in lower temperatures (14-17°C) (Reifschneider & Arny 1980).

4.1.2. Aspects of isolation and inoculum production

The maize leaves presented a rich fungal spectrum, which, in some cases, made the targeted isolation of certain pathogens difficult. *K. zeae,* for example, occasionally required several isolation techniques. Of these, the Waring Blendor technique is a reliable option when there is a small amount of lesions on the leaf. This was the case as the pathogen had not colonised the leaf tissue to a sufficient extent to provoke slimy masses of sporulation on the leaf surface. Also, due to the slow growth of *K. zeae* and the production of short conidiophores, saprophytes can easily grow in abundance over *K. zeae* colonies, rendering the isolation of the pathogen difficult. The Waring Blendor technique allows the pathogen to grow separately from other fungi in agar medium in the first days. By contrast, pathogens belonging to the *Helminthosporium* species such as *E. turcicum* and *Bipolaris* spp. have their conidia attached to long conidiophores. This separates the pathogen from saprophytes that could potentially inhibit sporulation and thus its isolation.

Another difficulty was that some fungi hardly sporulated *in vitro*. *Phoma* species and *C. graminicola* are able to produce pycnidia after various subcultures on solid medium. However, other organisms like *E. turcicum* lost their ability to sporulate after subcultures. This, in addition to the loss of pathogenicity during subsequent subcultures, has been reported for *E. turcicum* by several authors (Robert 1952; Hooker 1973; Chang & Fan 1986).

Some *K. zeae* isolates presented low sporulation on agar and also in the liquid medium for spore production described by Reifschneider (1979). To date, the modification and completion of this latter method by Algermissen (2014, pers. comm., 24 March) is the most

successful (lower quantities of required materials, economical, reliability) for the production of spores *in vitro*.

Another aspect to clarify with respect to the *in vitro* growth of *K. zeae* is that although the formation of sectors with both dark and light colours frequently occurs, subcultures of these sectors resulted in normal colonies, indicating the absence of mutations (Chinchilla 1985). The darkening of an initially light-coloured colony was also observed on the leaves after incubation in a humidity chamber. According to the descriptions of Dingley (1973), the pigmentation of the colony is also observed on leaf samples when it grows within epidermal cells.

4.1.3. Preservation techniques

The preservation of the isolated fungal species should guarantee not only their survival, but also maintain virulence and capacity to sporulate.

A simple method like autoclaved water and glycerol guarantees survival, virulence and capacity to sporulate for several years for *Phoma* spp. Nevertheless, for the rest of the fungi isolated and analysed (*K. zeae*, *Bipolaris* spp., *C. graminicola*, *Fusarium* spp.), this method did not ensure their survival. Consequently, an additional method based on preservation in silica gel granules with a skim milk solution (Perkins 1962; Vaillancourt Laboratory 1995 modified from Tuite & Lutrell 1969) was used for *K. zeae*, *C. graminicola* and *Bipolaris* spp. This method allows for the preservation of the fungal organisms within a solid medium, inhibiting the further development of the spores and therefore degradations or possible mutations produced in liquid medium. Nonetheless, some *K. zeae* and *Bipolaris* spp. could not be obtained from the silica gel granules after several months. This was probably due to the low survival rate of the species or, alternatively, because only a proportion of the granules were impregnated with the spore suspension. Thus, for the purpose of further selection and incubation on solid medium, the filter paper method described by Formento (2015) was more accurate when it came to identifying the part of the material in which the spores were situated. The continuous difficulties involved with storage reflect the specificity and difficulty of selecting the correct preservation method for any of the fungi. The simple method of fungal preservation in water is not recommended for any of these organisms. This contrasts with the scarce information for *K. zeae* in the literature, where preservation in water at 4°C should be a suitable method (Camochena 2009). A further step of testing the virulence before and after storage over several years should help establish the most adequate method for the preservation of each organism.

4.2. Development of *E. turcicum* and *K. zeae* across seasons (2011-2014)

The development of the associated disease is dependent on a combination of several climatic conditions, namely temperature, precipitation and humidity. None of these should be looked at in isolation, but rather in combination with the other factors to ascertain the reason for lower or higher prevalence of a disease.

K. zeae is favoured by mild (14-17°C) and humid weather (Narita & Hiratsuka 1959; Arny et al. 1970). Besides the temperature, long periods of high humidity favour the infection and sporulation (Narita & Hiratsuka 1959; Arny et al. 1970). If we compare this to the results in this work, several conclusions can be drawn. As observed during the monitoring in 2012 and 2013, *K. zeae* was also the most prevalent pathogen in the Ostenfeld fungicide trials in seasons 2013 and 2014. Nevertheless, the disease pressure was lower than in 2012 and especially 2011, where an epidemic outbreak occurred[11]. In line with the literature, it can be assumed that this noted outbreak in 2011 was caused by lower temperatures in July (16.4°C) and extremely high levels of precipitation in both July and August (355.5 mm in total) compared to 2013 (100.3 mm) and 2014 (145.7 mm). While the disease established itself in the field to a certain extent in 2012, where the average temperature for July was identical and precipitation was actually higher (145.5 mm as opposed to 110.7 mm in 2011), the lack of subsequent precipitation in August (60.2 mm) might not have favoured further development of the disease as did the high level of precipitation in 2011 (244.8 mm). Slightly higher temperatures in August 2012 (17.2°C) compared to 2011 (16.5°C) may also have played a role here. In 2015, the situation was rather similar; while in July a higher level of precipitation was recorded (186.6 mm) than in 2011 (110.7 mm), the level fell drastically in August (56.5 mm) compared to 2011 (244.8 mm). Furthermore, the average temperatures in July 2011 and July 2015 only varied slightly, while the average temperature in August 2015 (18.2°C) was higher than in August 2011 (16.5°C). Despite 2012 and 2015 following a similar pattern when compared to 2011, the warmer conditions in August 2015 (avg. temperature 18.2°C) inhibited the development of the disease to a much larger extent than they did in August 2012[12] (avg. temperature 17.2°C). This, coupled with the possibility of accumulation of inoculum in 2013 and 2014 being insufficient for the initial appearance (and subsequent establishment) of the disease in the field in the early season, led to the absence of *K.* zeae in 2015. In this context, the reverse applies to 2012, where the epidemic in 2011 seemingly provided high amounts of inoculum for 2012. In fact, it is possible that the presence of sufficient inoculum in 2012 (and the absence thereof in 2015) could have played a more important role than the aforementioned temperature difference in August.

[11] 2011 observations: Prof. Schlüter and Dr. Ute Kropf, personal communication, FH Kiel Ostenfeld (Lindenhof) trial location
[12] 2012: own observations made during monitoring (without rating data).

With regard to *E. turcicum,* the temperatures at which the pathogen is favoured can range from 15 to 30°C, with an optimum between 20 and 25°C (Casselman & Berger 1970; Malaguti & Subero 1971, Berger 1973; Levy & Cohen 1983; Díaz et al. 2012; De Rossi et al. 2015). As is the case for other pathogens like *K. zeae*, the interaction of the temperature with other climatic factors such as precipitation and humidity needs to be considered (Berger 1970; Levy & Cohen 1983). For example, in 2010, where an outbreak was recorded in the studied location of Mittich in southern Germany (Urban 2012), the average temperature in July (16.8°) was significantly lower than in subsequent years (19°C to 21°C from 2012 to 2015), as well as the long-term average (18.7°C). Initially, this may seem counterintuitive as *E. turcicum* is generally favoured by higher temperatures. However, precipitation in July 2010 was significantly higher (172 mm) than in all other years and the long-term average (112 mm). Rain and high humidity (>90%) is considered to play a decisive role in the development of epidemics of *E. turcicum,* whereas the extended range of temperatures (15 to 30°C) probably plays a secondary role if inoculum is present in the location (Berger 1973; Levy & Cohen 1983). In line with this, although the average temperature (19.2°C) and precipitation (105.3 mm) were optimal in July 2014, for example, further development of the disease was probably principally inhibited by infrequent rain, and secondly by comparatively low temperatures in August (16.7°C)

4.3. Epidemiological studies through spore trapping

Management of fungal maize leaf diseases is based on the use of resistant varieties, tillage practices, crop rotation and, in recent years, application of fungicides. The latter should be regarded as a last option when the other measures are not sufficient or cannot be included as a suitable method for management of the crop. According to Van der Plank (1963), the application of fungicides should be part of an integrated management, in which the epidemiology determines the adequate strategy.

The method of estimation for the latent periods for *E. turcicum* in Inzing and Göttingen is discussed in detail in this section. No latent periods could be determined for *K. zeae* in either location and will be discussed separately in section 4.3.2.

4.3.1. Estimation of the latent period of *E. turcicum*

Analysis of weather factors (humidity and temperature), increases in the infected leaf area, and spore release revealed a correlation among these variables for *E. turcicum* in Inzing (2014) and Göttingen (2015). In general, forecasting of weather conditions combined with spore trapping can be effective in predicting when the pathogen is present and when the

prerequisite conditions for an epidemic are met. In this way, a fungicide application can be made at the appropriate time (Manners 1993).

Among other factors, the appropriate time for application is correlated with the development of the pathogen based on its latent period. This latent period comprises the time from when the spore lands on the leaf surface to the manifestation of symptoms and sporulation (Rapilly 1991). This latent period should provide the time frame on which the application of fungicides can be based. This should not be confused with the incubation period, which comprises the period (days) from infection until the manifestation of the first symptoms.

Despite some differences in the development of Turcicum leaf blight and the origin of inoculum (natural vs. artificial), the latent periods estimated for *E. turcicum* turned out to be very similar for both locations.

Analysis of the latent period through spore trapping is based on the time of spore release (detachment of a spore or related propagule from the parent tissue), which can be correlated with weather conditions. Spore release is based on two mechanisms, passive and active. When the spore is attached to a conidiophore, the active mechanism is predominantly caused by extreme variations in relative humidity, which permits separation of the spore from its supporting tissue (in this case the conidiophore). This is produced by a gaseous phase in the lower part of the conidiophores, which first bends and then quickly straightens when the bubble reaches the top of the conidiophore (Rapilly 1991). Although this detachment mechanism is also considered for *E. turcicum* in the literature (Meredith 1963; Leach et al 1976), the mechanism of passive force based on wind, humidity and rain is much more relevant for the spore release and dispersal. These climatic conditions ensure the dissemination of inoculum across larger distances (Casselman & Berger 1970; Rapilly 1991). In this work, wind or rain were generally clear causes of spore release. In some cases, however, the decrease or increase in humidity was notable and this option was also considered.

The calculation of latent periods is not without its difficulties. Complications exist when lesions increase in size and the area of spore production expands (Van der Plank 1967). This was the case for *E. turcicum* in our trials. Nonetheless, while the expansion of the lesion provides large potential areas of inoculum, this inoculum is not responsible for the lesion itself; thus it cannot be directly considered for the calculation of the latent period. Therefore, according to the concept proposed by Van der Plank (1967):

> *A lesion must be considered as a whole which can be traced back to the spore from which it started; and one must measure the latent period of all parts of the lesion from a single zero time: the time the lesion started from the spore (1967; p. 84).*

The concept of latent periods provided by van der Plank (1967) was also applied to the results in this work - the first day of spore release in the infection period under consideration is probably closer to the day of first sporulation (i.e. the initial detection of spores) than the highest peak of spore release.

Favourable conditions for the development of *E. turcicum* were considered to be high relative humidity (henceforth RH) of 100%, frequent rain (Berger 1970; Casselman and Berger 1970; Leach et al. 1976) and temperatures in the range of 15°C-25°C (Casselman & Berger 1970; Malaguti & Subero 1971, Berger 1972; Levy & Cohen 1983; Diaz et al. 2012; De Rossi et al. 2015).

Inzing

In Inzing, although notable precipitation was registered in July, this was only concentrated on a few days. A favourable day for the development of Turcicum leaf blight in the field occurred on 22 July, when 17 hours of 100% RH occurred. On these days, while some spores were trapped, no lesions were observed in the trial. The existence of inoculum, which probably originated from other fields in the region, revealed the presence of the pathogen in the area. Nevertheless, due to the constantly low number of spores registered in the subsequent weeks, it is likely that weather conditions were not favourable enough to maintain the chain of secondary re-infections.

Several authors state that germination, penetration and infection could occur within five to 18 hours at a temperature range of 15-30°C. However, if the latent period is considered (infection and sporulation) the complete cycle will take longer. A minimum of 11-14 hours of dew is required for abundant sporulation, which is responsible for epidemic outbreaks (Berger 1973; Levy & Cohen 1983). Therefore, it is likely that several favourable days have to pass during summer in order for these requirements to be met.

At the beginning of August, the first scattered lesions with sporulation were observed in the field. These lesions appeared after a prolonged, relatively favourable period for infection at the end of July and beginning of August. This consisted of several days of high humidity and rainfall. Although this fulfilled the requirements for further development of the disease, further infection cycles are required to provoke higher infection rates and abundant spore release. This was reflected by the low disease severity observed in the location (<1%) and the low quantity of trapped conidia. Considering the days which elapsed between the favourable conditions for infection (starting from 30 July) and the first symptoms of the disease in the field with sporulation (approx. 7 August), eight days elapsed. This could represent the first latent period diagnosed in the season although the spore trap could not confirm an increase in sporulation during this period (see Figure 77, point A). Furthermore, on proceeding days, a

dry and warm period (max. 26-30°C) probably inhibited further possibilities for additional sporulation during the following weeks. This was correlated with the low number of trapped spores.

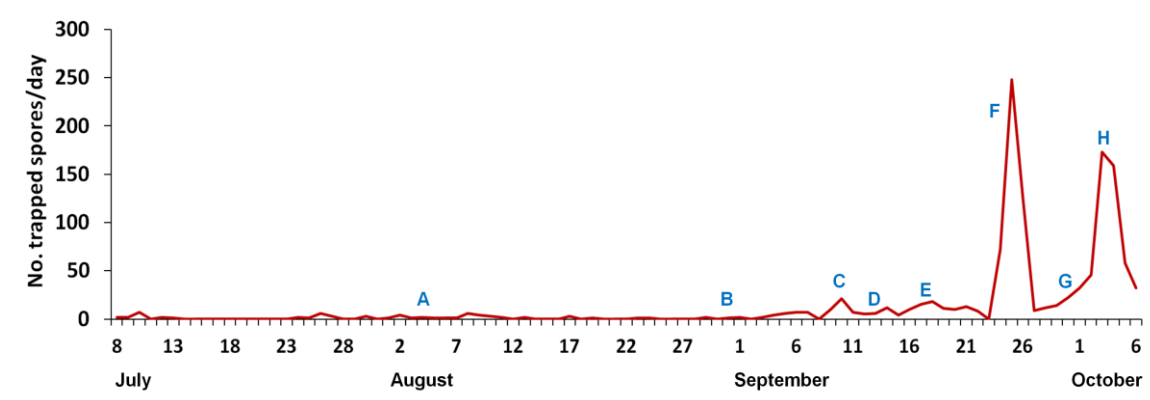

Fig. 77. Fluctuation of trapped conidia of *E. turcicum* during the sampling period in Inzing 2014. A-H: Periods of spore release discussed in this section correlated with latent periods.

With the onset of the late season, favourable conditions of high humidity and moderate temperatures were more regularly recorded and spore release was detected at a high incidence. Peaks of spore release occurred after rainy periods, combined with at least 29 hours of 100% RH. For example, after a notable period of favourable weather conditions from 28 August to 2 September, the number of trapped spores progressively increased during the following days. This culminated in a single, albeit small, peak on 10 September, probably favoured by heavy rainfall on the preceding day. From the day when the most favourable conditions for sporulation were recorded (30 August – Figure 77, point B) to the peak on 10 September (Figure 77, point C), 11 days elapsed. These 11 days could represent the latent period, with some slight variation possible with regard to the actual point of spore release. This could have occurred between the 9 and 10 September (10-11 days), or even slightly earlier. Considering that the release of spores into the atmosphere is dependent on favourable weather conditions, the recorded spore release can be separated from the point at which spore formation occurred by several days (Casselman & Berger 1970). At this time, the infected leaf area was still lower than 1%.

The spore release on the 9-10 September coincided directly with a period of high humidity from 11 to 14 September, where 87 hours of 100% RH greatly favoured germination and infection. While, in theory, this prolonged period of high humidity was almost certainly sufficient for high rates of infection and further sporulation, this process could have been inhibited by the coinciding low temperatures (avg. 12-16°C). This was reflected by the low number of trapped conidia (Figure 77, point D). Levy and Cohen (1980) maintain that at low temperatures, the length of the sporulation period could be prolonged for *E. turcicum* by

several days. Consequently, the increase in the amount of trapped conidia 14 days later (24-26 September) should be considered.

Nevertheless, another option would be to consider the small peak of spore release on 18 September (Figure 77, point E). Assuming that these spores originated from the first sporulation of the lesions, the period between 11 September (first day of favourable conditions for infection) and the day of slight spore release (18 September) could be considered as the latent period, i.e. approx. seven days. In this case, the peaks of abundant sporulation from 24 to 26 September (Figure 77, point F) would not be a result of a first sporulation but rather of massive sporulation in existing lesions, i.e. lesions which had already formed beforehand caused by prior spore release. The abundant sporulation was evidently favoured by the relatively warm (15-18°C) temperatures and high humidity on preceding days (19-23 September).

Thus, it is possible that the peaks of abundant spore release on 24 to 26 September represent the closest period to sporulation. In this case, 13 days elapsed from the first day of favourable conditions on 11 September to the first release of spores on 24 September. These 13 days (as opposed to seven) could instead be considered as the latent period. This would also be supported by the fact that favourable conditions for spore release occurred on the days before (19 to 23 September) and an increase in spore numbers was not registered until 24 September.

From the 24-26 September, masses of spores were again deposited on the leaves, and the cycle of infection was repeated once again. On the days following the peak to the 1-2 October, a progressive increase in the number of spores was observed (Figure 77, point G). In this case, an accurate analysis of the subsequent latent period becomes difficult because some older lesions could have sporulated again under favourable conditions, overlapping with spore release from new lesions. Based on the fact that new lesions were observed on the days immediately thereafter, the spores detected at the beginning of the new release period can be considered the first spores produced after a latent period of seven to eight days, which culminated in an abundant spore release on 3 and 4 October (Figure 77, point H). These last infection periods were reflected by an increase in the infected leaf area (3%).

Göttingen

In Göttingen, the Turcicum leaf blight epidemic followed a different pattern than in Inzing. This was due to the artificial conditions that provoked a much earlier epidemic than in Inzing under natural conditions.

Following artificial inoculation of the plants at the end of tasseling (BBCH 59-63), plots were covered with plastic, providing a saturated atmosphere (100% RH) to favour the first

establishment of the pathogen in the plant. After 24 hours, the first small chlorotic spots were visible on the plant, confirming the successful germination and penetration of the pathogen in the leaf tissue. By contrast, in Inzing in 2014, the establishment of the first natural inoculum of *E. turcicum* took several weeks due to the low quantities of inoculum and absence of rain and favourable RH (100%) over prolonged periods.

Once the pathogen had been established on the leaf, the plastic cover was removed (after two days) and the subsequent development of the fungus occurred under natural climatic conditions. Following the removal of the plastic cover, first conidia were trapped. However, these initial and inconsiderable quantities of conidia (1-4) were directly related to the sprayed inoculum. First lesions were observed after, on average, 12 days and were produced by the initially induced establishment of the pathogen. Thus, this time can be considered as the incubation period (time from germination to lesion formation) but not the latent period.

A dry and warm period occurred at the end of July and beginning of August. The existing lesions expanded markedly, covering large areas of the leaves (8% avg. infected leaf area). However, at this stage, sporulation in the lesions was still not observed. This mechanism of prominent expansion of lesions was described by Bergamin Filho & Amorim (1996), and is a variation of the classical concept for epidemics described by Van der Plank (1963). The theory put forward by Bergamin Filho & Amorim (1996) is based on differences in infection cycles between tropical and temperate pathosystems. Temperate pathosystems present a clockwise infection chain with so-called “sites”, i.e. potential areas of infection. These are defined as “healthy sites, latently infected sites (both with no symptoms), lesions (infectious sites), removed lesions (non-infectious sites)” (Van der Plank 1963, cited in Kranz 2003, p.63).

The tropical pathosystem defined by Bergamin Filho and Amorim (1996) provides an alternative method of infection, namely an anti-clockwise infection chain. This is based on a high rate of lesion expansion, which allows the pathogen to counteract the unfavourable conditions in tropical climates. Through this mechanism, the infected sites can generate new infectious sites without the need for production of new spores. This mechanism has been observed for *E. turcicum* by several other authors (Berger 1970; Vitti et al. 1995; Bergamin Filho & Amorim 1996; De Rossi 2015). According to Vitti et al. (1995), the increase in the lesions could reach a rate of >43 mm^2 day^{-1}. A prominent manifestation of this mechanism was also observed personally during visits to various maize growing regions of Argentina in 2015 in the scope of this work. Here, plants presented lesions which were expanded across the length the leaf. In this case, the establishment of the pathogen in the early season was probably favoured by high amounts of rainfall which occurred in 2015 (caused by the “El Niño” weather phenomenon), with interjecting periods of dry and warm weather.

In Göttingen, favourable conditions for the first stages of infection were artificially provoked. In Inzing, the high humidity and precipitation (as well as sufficient inoculum) required for the initial establishment of *E. turcicum* was absent, reflected by the scarcity of lesions. This counteracted the warm and dry weather which could have favoured the enlargement of lesions at the beginning of the season. Given the fact that high levels of humidity and dew are generally rare in the early season in the region of Inzing, it can be surmised that it is unlikely the mechanism described above plays an important role here.

After the dry and warm period at the end of July and beginning of August which promoted the enlargement of the lesions in Göttingen, favourable conditions increased during the middle of August. This was confirmed by the sporulation observed on the leaf lesions in the trial on 17-18 August. It is likely, however, that some lesions were already sporulating as the first peak of notable conidia release was detected on 13 August (see Figure 78, point A). Considering the concept of latent periods, which end upon the commencement of sporulation, in this case it took around 30 days (13 July to 13 August) due to the aforementioned dry and warm period. The subsequent amounts of conidia which could be counted daily from 16-22 August (Figure 78, point B) are probably attributable to the continuous, abundant sporulation on the large blighted leaf area. This was caused by high RH (90-100%) and a high amount of precipitation. The highest peak of the season (recorded on 23 August – Figure 78, point C) is probably attributable to the favourable temperature and RH which occurred during the previous days, leading to a massive sporulation of the blighted area.

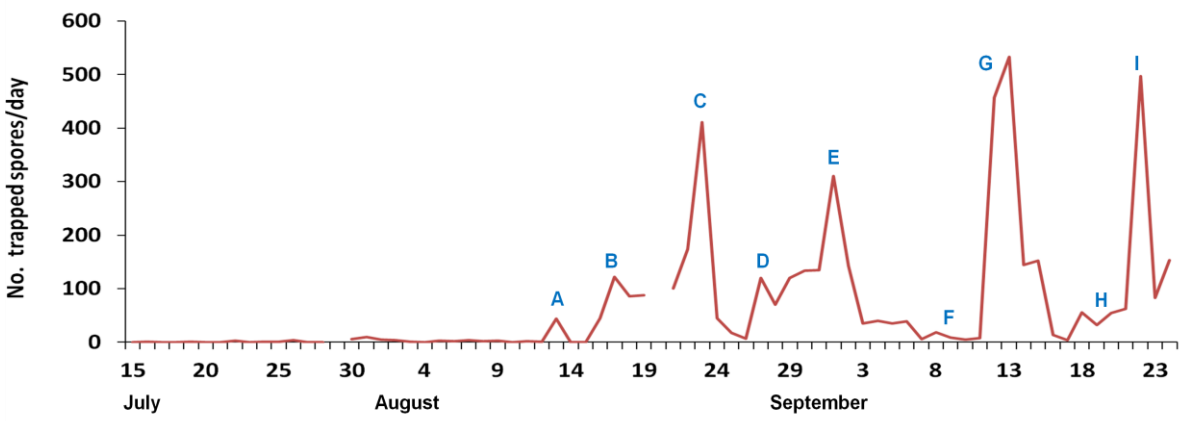

Fig. 78. Fluctuation of trapped conidia of *E. turcicum* during the sampling period in Göttingen 2015. A-I: Periods of spore release discussed in this section correlated with latent periods.

Towards the end of August and at the beginning of September (27 August- 2 September), the daily conidial release corresponded with the fluctuations in the relative humidity, although only one day reached the minimum level of 90% RH (daily average) required for sporulation. Nonetheless, it is possible that dew periods in the subsequent days of at least 5-9 hours (at

night) were sufficient for sporulation in this case. As Levy and Pataky (1992) argue, if the hours of 100% RH are not sufficient for the development of the conidia, the subsequent morning dew permits the completion of the formation of these conidia.

The peaks produced in the middle of August (Figure 78, points A & B) could be considered to be the first outbreak of the epidemic which led to the subsequent cycle of infection commencing on 27 August (Figure 78, point D). The peak on 27 August is the first of an ascending curve and could be considered a direct product of the latent period, which probably commenced with the first sporulation between 13-16 August, i.e. 11-14 days. The culmination of the curve in a peak of abundant sporulation (1 September – Figure 78, point E) can be considered the result of substantial secondary sporulation on expanded lesions after a period in which RH increased.

From this period to the next notable spore release, an ascending curve similar to that of the last two periods of spore release was not observed. This was probably attributable to unfavourable weather conditions of frequently low humidity, coinciding with a plateau in conidial release (Figure 78, point F). The fourth peak of abundant conidial release on 12-13 September (Figure 78, point G) coincided with a five-day period of favourable conditions. In this case, different possibilities exist for this massive sporulation. The peak could be the product of new lesions formed, for example, as a result of the spore release on 1 September (11-day latent period). In contrast, the peak could also be a product of massive sporulation on the older lesions, produced by an overlapping of these preceding infection cycles.

In the fifth period of spore release, a similar pattern to that of the second and third periods was observed, i.e. an ascending curve of spore release. Initially low spore release from 18-21 September (Figure 78, point H) was followed by a sharp peak of abundant spore release on 22 September (Figure 78, point I). This is in line with the hypothesis outlined above: the initial release of spores is the product of first sporulation after lesion formation, while the large peak represents secondary spores produced in abundance on the previously formed lesions. In this case, six to 10 days elapsed from the last period of abundant release (12 September) to the next (18-22 September).

Comparison of spore release pattern from Inzing and Göttingen

The latent periods ranged from seven to 13 days for Inzing and six to 14 days for Göttingen. This is more or less in line with the literature, which states a latent period for *E. turcicum* of eight to 14 days that can vary depending on the maize cultivars or climatic conditions (Malaguti & Subero 1971, De Rossi et al. 2015, Diaz et al. 2012). It is possible that the six or seven-day latent periods estimated in some cases are too short and the actual

time frame was closer to the maximum range calculated, e.g. 10 days (12-22 September) as opposed to 6 days (12-18 September) in Göttingen.

In both locations, the initial sporulation and subsequent spore release occurred after a long period of unfavourable weather conditions. The development of the disease in Inzing was especially retarded, with the first noticeable spore release not registered until September. In Göttingen, on the other hand, the first noticeable spore release occurred in the middle of August and a pronounced second mechanism of lesion expansion compensated for the unfavourable conditions. Furthermore, in Göttingen the artificial inoculation was carried out at a time (mid-July) when natural inoculum is usually not present at such a high level. This resulted in an infected leaf area of 46% by the end of the season, as opposed to only 2.8% in Inzing under natural conditions.

As discussed in the introduction to this section, latent periods can vary by several days depending on the estimation method used. Complications also exist when lesions increase in size and the zone of spore production expands (Van der Plank 1967), which was especially pronounced in Göttingen.

A number of other factors can also have an impact on the latent period. Carson (2005) found that varieties resistant to Turcicum leaf blight tend to be effective in prolonging latent periods. The opposite also applies: susceptible varieties advance the development of the disease, providing more inoculum and shortening the latent periods (Rapilly 1991). This argument can be tentatively applied to Göttingen as the variety used was susceptible and in the last infection cycle, the final latent period (six to 10 days) was shorter than in the first infection cycles (11-14 days). In this context, the length of the latent period is linked to partial resistance (Carson 2005). Furthermore, the latent period can also depend on variations in the resistance of the plant between the early and late stages (Kranz 2003).

Climatic conditions can also have a large impact on the latent period. This is one of the most plausible explanations for the shorter latent periods at the end of the season in both Inzing and Göttingen, where more favourable weather conditions such as 100% RH and dew periods for the pathogen occurred. Related to this, the duration of the incubation and latent periods of necrotrophic fungal pathogens can also be affected by the concentration of inoculum present (Rapilly 1991). The author presents the case of *Dreschlera teres* on barley, which is also considered within the "*Helminthosporium* complex", where higher concentrations of inoculum are correlated with shorter latent periods, and vice versa. This is in line with our results in Göttingen and Inzing, where higher concentrations of inoculum in the late season coincided with shorter latent periods.

With regards to the pathogen itself, the parasitic fitness could also play a role in the higher concentration of the spores. In a study by Levy (1991), isolates from different regions varied

in infection efficiency, sporulation and lesion size. While the development of Turcicum leaf blight in Inzing was limited to the natural inoculum in the region, in Göttingen different isolates (of different races) from several European regions (southern and western France, Hungary and Germany)[13] were inoculated. These pathogens had diverse levels of aggressiveness and the purpose of their combined use as inoculum was an attempt to achieve compatible interaction between the plant and the pathogen, as well as the adaptation of the pathogen to various conditions based on their origin. This might be another explanation for the larger amount of spores registered by the spore trap compared with Inzing under natural inoculum.

Overall, it is difficult to attribute the length of the latent period for *E. turcicum* to one single factor. Factors such as variety susceptibility, inoculum concentration and favourable climatic conditions all have an influence in this regard. The method of evaluation can also have an impact, and further studies could be carried out by focusing on newly formed lesions instead of the evaluation of the whole blighted area.

Based on the data obtained, spore trapping helped to understand the development of the disease across the season. As part of an integrated pest management, in combination with weather forecasting, spore trapping can be very useful in evaluating inoculum pressure during the season. This could help to decide whether a fungicide application to control Turcicum leaf blight is required and even the timing of the application.

4.3.2. Interpretation of *K. zeae* results

In contrast to *E. turcicum,* spore trapping did not reveal fluctuations within a season in Ostenfeld in 2013 and 2014 (natural inoculum) or in Göttingen in 2015 (artificial inoculation). Conidia of *K. zeae* are hyaline, normally non-septate and relatively small in size (average 3 x 27 µm). These characteristics hindered the recognition of spores among the mass of other material and spores found on the spore trap samples. This was even the case on the artificial spore trap tapes as the spores blended in with the vaseline on the tape.

In this case, DNA detection techniques become crucial. The protocol to extract DNA from the sampling tape followed in this work is based on the method described by Kaczmarek et al. (2009). The author also encountered difficulties with *L. maculans* and *L. biglobosa* as it was impossible to distinguish between trapped ascospores of the two species by microscopy.

[13] These isolates were obtained from a monitoring of *E. turcicum* races carried out in 2012 as part of the doctoral project of Hendrik Hanekamp ("Population structure of the pathogen *Exserohilum turcicum* and spatial efficacy of race specific resistances") at the Division of Plant Pathology and Plant Protection, Georg-August-University, Göttingen.

Nevertheless, the analysis through qPCR could not reveal seasonal fluctuations of *K. zeae* in any of the locations analysed. This failure in the detection of *K. zeae* through spore trapping and analysis of DNA from the samples via qPCR could be a result of an insufficiently optimised qPCR method. For possible future development of the qPCR method, further analyses are required in order to improve detection by testing possible inhibitions in the presence of non-target DNA and investigating the irregularity of the the melting curve of amplification. Furthermore, non-specific amplifications should also be considered. In this work, non-specific amplifications of the genetically related fungi *K. caulivora* DNA were obtained with the selected primers for *K. zeae*. Nevertheless, this may not affect the specificity of the test to a large degree because the pathogen is mostly described as infecting red clover (this is assuming that its presence is relatively low in maize fields). Nevertheless, other genetically related fungi to *K. zeae*, such as *Aerobasidium pullulans*, which is a ubiquitous fungus, should be tested.

On the other hand, the low levels of the disease observed in Ostenfeld during the 2013 and 2014 seasons could reflect a lack of inoculum in the field. The subsequently low number of *K. zeae* spores was probably insufficient for detection, both via microscopy and qPCR. Similar levels of disease to Ostenfeld in 2013 were registered in Göttingen at the end of August. This equally explains the absence of peaks in spore release in Göttingen. However, with the onset of the late season, a decrease in temperature favoured the development of the disease, with an infected leaf area of 20% being reached in Göttingen. This sharp increase in the infected leaf area was probable caused by two days of rain on 13 and 14 September, providing sufficient inoculum to be detected by the spore trap. In fact, this was the only point at which a positive amplification via qPCR assay was registered at this location.

Nevertheless, the failure of the spore trap to detect inoculum could explain a decisive factor related to the development of epidemics of *K. zeae*, i.e. the primary transport mechanism is based on rain and not wind. While some authors (Arny et al. 1970; Smiljakovic & Pencic 1971) refer to the possible distribution and dispersal of the pathogen via air currents, this theory has never been tested scientifically. This probably reflects the difficulty of analysing the infection cycle of *K. zeae* focusing solely on wind dispersal. Rapilly et al. (1975) studied the dispersion of *K. zeae* spores via splashing produced by rain and sprinkler irrigation. This was achieved by using a device designed for the collection of water and estimating the time at which the pathogen sporulated based on the number of spores collected per ml of water. To our knowledge, this is the only report that analysed the sporulation and dispersal of *K. zeae* with quantifiable data.

Rapilly et al. (1975) concluded that *K. zeae* can sporulate in high quantities on a water film on the leaf. However, the sporulation of *K. zeae* appeared to be inhibited by the sensibility of

the spores to conditions of high UV radiation and desiccation. The impact of a droplet of water onto the colony of a fungus ensures the dispersion of numerous pathogens, in particular those in which the conidia are produced on short conidiophores or are integrated in a mucilaginous matrix (Rapilly 1991). This is explained by the spreading of the water soluble substances of the mucilaginous matrix with the film of water which is formed. However, successive impacts of droplets of water lead to an exhaustion of the fructification. While the conidia attached to long conidiophores are exhausted faster (reflected during the first impacts), as is the case with species of the *Helminthosporium* complex (e.g. *E. turcicum*) Rapilly et al. (1975) found that this exhaustion process through rain is almost impossible in the case of *K. zeae* colonies. This is due to the abundant sporulative capacity of *K. zeae* and the short period of time necessary for the formation of new spores (5-6 hours). Observations made in the field also lend support to the theory that *K. zeae* largely depends on droplets of water for its dispersion. In the field, the disease is usually unevenly distributed, probably because the droplets of water are distributed from one plant to another when there is contact between their respective leaves. This uneven distribution pattern could be observed in both Ostenfeld and Göttingen, especially when the first infections occurred.

An optimised qPCR assay might demonstrate the importance of wind in *K. zeae* epidemics, rejecting or accepting its role in the dispersal of the pathogen.

4.3.3. Further considerations for qPCR and PCR assays

The possibility of a molecular biological detection of *E. turcicum* propagules was investigated in order to accelerate the assessment. Molecular methods based on spore detection have been described in the literature for maize leaf pathogens such as *P. polysora* and *P. sorghi* (Crouch & Szabo 2006), as well as for several fungal pathogens of other crops (Kaczmarek et al. 2009, Rogers et al. 2009; Vogelzang 2011, Meitz-Hopkins et al. 2014).

Performance conditions, limit of detection, specificity and reference microscopy were evaluated. The qPCR method permitted the detection and quantification of *E. turcicum* DNA and the data from the qPCR assay were positively correlated with the spore counts for seasonal fluctuation of conidial release by visual microscopy. The qPCR assay offered the detection of *E. turcicum* DNA at quantities of picograms. Nevertheless, lower concentrations were not always amplified. This was the case for several samples from Göttingen.

Some samples in which no spores were counted through microscopy were positive via qPCR, and vice versa. Nevertheless, most of these samples had low numbers of conidia ($0<x\leq10$). Possible explanations for this discrepancy could be a visual limitation in recognising the presence of a spore due to it being degraded, in the early formation stages

(where the septa are not yet formed), or hidden under other biological material on the tape. False amplification of non-target pathogens or material could be also discussed. In this work, non-specific *Alternaria* spp. amplifications were obtained. Nevertheless, extremely high amounts of DNA (100 pg μl^{-1}) need to be present to obtain positive amplifications. Such high amounts of DNA are improbable, however. On the other hand, although a specific test with 13 other fungi was carried out, the possibility that other fungi or biological material were amplified cannot be ruled out.

Regarding some samples in which spores were counted through microscopy but not detected through qPCR, possible inhibitions through other material not tested in this work could have limited further detections, such as high amounts of non-target DNA. These negative results mostly occurred for samples with a low number of spores. One exception was a sample with 152 spores counted through microscopy resulting in no amplification via qPCR. This was probably due to errors during the extraction method: as spore trap tapes were measured daily and one spore trap was situated in each location, only one sample per day was available for further analysis. Daily repetitions did not exist in this case.

According to the correlation between DNA yield and number of spores obtained from samples from Inzing and the artificial spore trap tapes, it was estimated that each spore contains about 1.15 - 1.24 pg DNA, whereas the same correlation resulted in 4.6 pg per spore for samples from Göttingen. This discrepancy could be explained by the presence of other inoculum (e.g. conidiophores) which was not counted by visual microscopy. This is most likely due to the close proximity of the spore trap to the plants. This was especially noticeable in Göttingen, where large amounts of similar conidiophores to those described for *E. turcicum* were observed on the tapes. This could have lead to a higher DNA yield (conidia and conidiophores) than that calculated for one spore through qPCR.

The most important purpose of the qPCR test, i.e. to differentiate between seasonal fluctuations of spore release, was achieved. Further optimisation of the method could improve its reliability. Therefore, the adapted protocol from Kaczmarek et al. (2009) could support integrated pest management programs by saving time and effort in the monitoring of the *E.turcicum* inoculum pressure. This would be more efficient for future modelling and forecasting of Turcicum leaf blight.

4.4. Application of fungicides

Management of fungal maize leaf diseases is based on the use of resistant varieties, tillage practices, crop rotation and, in recent years, application of fungicides. The latter should be regarded as a feasible option when the other measures are not sufficient or cannot be included as a suitable method for management of the crop.

To date, a lack of knowledge and information about the dynamic of the disease correlated with the use of fungicides on maize leads to confusion among organisations and farmers with regard to their correct use in Germany. Two foliar fungicides composed of mixes of QoI and DMI fungicide compounds were permitted in 2014. Nevertheless, their application in maize fields remains uncommon due to several factors limiting the necessity for their use: I) in some areas, the disease pressure is too low to benefit from an application, II) it is not defined at which level of disease severity the fungicide should be applied (thresholds are not defined), III) lack of knowledge about the dynamic of the diseases (latency period and further development mechanisms), which is important for the timing of application, and IV) the efficacy against different target diseases has not been sufficiently tested.

The fact that severe epidemics occur only sporadically, e.g. 2010 for Turcicum leaf blight and 2011 for Kabatiella eyespot (Urban 2012), makes it difficult to gain sufficient knowledge about the dynamic of the diseases in the field, and, in turn, the optimal management of these diseases through fungicides.

4.4.1. Fungicide effects on infected leaf area

In Mittich in 2013, foliar fungicides significantly (statistically) decreased the infected leaf area caused by Turcicum leaf blight and common rust compared with the untreated control. This reduction ranged between 3% and 4.7% for Turcicum leaf blight and 2.9% and 4.6% for common rust, demonstrating that the different applications were similarly effective against both pathogens.

The literature indicates that when a fungicide is applied to a plant infected with common rust, the pustules are eradicated and new infectious areas can no longer be produced (Berger et al. 1997). In contrast, Turcicum leaf blight is characterised by a fifteen-fold faster lesion expansion rate than common rust (Bergamin & Amorim 1996). Thus, combating the disease through fungicides may be much more difficult, or in some cases ineffective, once it is established in the field (Diaz et al. 2012; Couretot et al. 2013).

In the trials in Mittich, however, Turcicum leaf blight had yet to develop to a significant level in the field (less than 1%) when the fungicide was applied. This is likely to be the reason that a similar reduction in infected leaf area could be achieved for both Turcicum leaf blight and common rust.

In contrast to 2013, in 2014 only Turcicum leaf blight was assessed, while pressure of common rust was low. Here, a less consistent protection effect between treated and non-treated plots was observed, probably due to the low level of Turcicum leaf blight in the field. Only one treatment significantly reduced the infected leaf area (1.2% reduction) compared with the untreated plots.

For Kabatiella eyespot, statistically significant differences between treated and untreated plots were observed in Ostenfeld in 2013 and 2014 in terms of infected leaf area. This is in line with trials carried out in Denmark, which have found fungicides to be very effective against Kabatiella eyespot, especially at higher levels of disease severity. Treatment is not necessary every year, however (Jørgensen 2012).

4.4.2. Disease development

Protection of leaves that contribute most to the corn cob filling needs to be taken into account when applying foliar fungicides to maize. Pataky (1992) reported that leaves L-1, L0 and L+1 are an important production source for assimilates, which explain 30% of the corn cob filling. The author also noted that when severity in the upper 75% of the canopy was less than 8%, yield was not affected. This could explain the lack of a positive yield response in Inzing in 2014, where the highest levels of infected leaf area with Turcicum leaf blight were registered on the upper leaves of the plant, probably due to the late appearance of the disease. This can also be applied to Ostenfeld, where in both 2013 and 2014, Kabatiella eyespot was registered at higher levels on the upper leaves. Nevertheless, this hypothesis does not explain the results from Mittich in 2013, where the most affected leaves were situated in the middle of the plant (L-1; L0; L+1).

Common rust was also present in Mittich but the higher levels of infected leaf area (6%) were registered in the upper canopy. Therefore, due to the lower damage to the leaves and its location in the upper canopy, common rust was of secondary importance in terms of having a negative effect on yield in 2013 compared to Turcicum leaf blight. As explained in section 3.4.1, while common rust had a similar infected leaf area, the differences between the rating scales and the size of the lesions mean that the same percentage of common rust is not identical to Turcicum leaf blight. Adding the percentage of infected leaf area of both diseases together is a highly questionable method due to this discrepancy. In this case, a so-called “green rating” (a rating exposing the leaf area that is still not infected) may have been the most reliable option. Green ratings are commonly used when various diseases are present in the field as it is difficult to differentiate between the damage produced by each respective disease. However, green ratings fail to recognise the importance of the different foliar diseases on maize and the efficacy of fungicides against each specific disease. Furthermore, if the capacity of all foliar diseases to infect the leaf area were to be treated the same, the potential yield losses caused by each disease would be indeterminable, thus making it impossible to establish specific thresholds for each disease. This complicates the issue of finding a sustainable strategy for managing foliar diseases. Nevertheless, green ratings are

very reliable when other external factors, e.g. a “greening effect”, have to be evaluated. This will be explained in more depth below.

4.4.3. Timing of application

The efficacy of a fungicide depends to a large extent on the timing of application (Munkvold 1997; Ward et al. 1997). If severe defoliation is produced by the disease before or during the grain-filling period (two-three weeks after pollination, BBCH 71-87), the reduced photosynthetic area will translate into a reduction in the supply and assimilation of nutrients to the cell. This leads to a subsequent reduction in the grain yield (Tuleen & Frederiksen 1977; Agrios 2004). Therefore, fungicide application should provide a control effect during this critical period. Therefore, the most important stage in this context could be considered from tasseling to flowering (BBCH 65). Early applications reduce an initial early appearance of the pathogen, avoiding infections in the early stages where the plant tends to be more susceptible. An early application may also inhibit further infections which could continue to develop until the aforementioned critical stages.

Among treatments analysed in this work, no significant differences were observed between different timings of application for Turcicum leaf blight in 2013 and 2014. By contrast, significant disease control of common rust was observed when propiconazole + azoxystrobin was applied at flowering (BBCH 63). Regarding Kabatiella eyespot, a significantly superior level of control was only achieved in 2013 when treatments were applied at mid-late stages, i.e. from tasseling to flowering (BBCH 55 to 63). This result was achieved despite a relatively low severity of the disease in the untreated control (5.9%). Significant differences among timings of application could not be observed in 2014, however, probably due to the lower level of the disease (3.7%) compared to 2013. Urban (2012) investigated the effect of fungicides with early and late applications against Turcicum leaf blight and Kabatiella eyespot during the 2010 and 2011 seasons in the same regions as those studied in this work. For Turcicum leaf blight, significant differences between early and late applications could only be confirmed in 2010, when the disease severity was high (44%, non-treated control). The disease severity in 2011 (7%) reported by Urban (2012) was similar to that of 2013 (8%) and was not sufficient for differences to be registered among application timings. Similarly, Urban (2012) could only confirm significant differences among application timings when a high level of Kabatiella eyespot was recorded (2011, 19.8%).

It is important to note that the residual period for fungicides (i.e. the amount of time it provides control) is approximately 14-21 days. Most of the fungicides used in these trials contained two active ingredients, one of which was a triazole and the other a strobilurine.

While strobilurines offer protective control against sporulation, germination and penetration, propiconazoles offer curative protection.

Taking into account the latent periods for *E. turcicum* estimated in this work for 2013 (see section 4.3.1) and those provided in the literature (De Rossi et al. 2015), the first infection by the pathogen could have commenced 10-14 days prior to the first observation of the symptoms in the field (31 July). This date falls within the time range in which the fungicide applied at an early stage was still effective (application 3 July, growth stage: stem elongantion of the plant, BBCH 32). This would explain the efficacy reflected by the final results and implies that the early application was successful in providing effective protection against the initial cycles of infection. Nevertheless, this can be difficult to achieve as there is a progressive loss of efficacy of the fungicide, i.e. it is less effective 20-21 days after application compared to the days immediately following the application. Considering a window of efficacy of approx. 21 days, an early application on 3 July would imply a loss of efficacy around 24 July. Thus, while this was sufficient to cover the initial infection caused by primary inoculum, secondary infections (re-infection) which occurred in Mittich after this date would no longer have been controlled. Thus, while the late application would not have controlled against the initial infection cycle, this was probably compensated for by controlling these secondary infections. Unfortunately, this could not be confirmed in 2013 as there was no significant difference between early and late applications, likely owing to the low severity of the disease.

In general, however, secondary infections are considered more damaging in these regions as the diseases are favoured by more optimal conditions in the late season (higher humidity, moderate temperatures). Therefore, a late application should be more effective in seasons where the level of disease severity is sufficiently high. As stated above, the results for 2013 and 2014 in Mittich/Inzing and Ostenfeld were not convincing in this regard. However, Urban (2012) found that late applications (BBCH 51/55) were responsible for a significant reduction in the infected leaf area of Turcicum leaf blight in 2010 and Kabatiella eyespot in 2011, years in which the respective disease pressure was high.

4.4.4. Disease yield loss relationships

Although the infected leaf area was significantly reduced through a fungicide application in 2013 and 2014 for Kabatiella eyespot, as well as in 2013 for Turcicum leaf blight and common rust, this did not convert into a significant benefit in yield (in terms of biomass or grain production). Wise and Mueller (2011) maintain that the efficacy of a fungicide is dependent on the severity of the disease that is present in the field, with a low disease severity resulting in inconsistent yield responses. Nevertheless, this unpredictable correlation

has also been reported in some cases for high disease pressure. Bradley and Ames (2010), for example, found significant differences between treated and non-treated plots in terms of disease severity but without significant differences in yield for grey leaf spot (*Cercospora zeae-maydis*). The authors concluded that this could have been due to the high disease severity which was also present in the treated plots due to a late fungicide application.

In the trials conducted in Mittich and Ostenfeld, the lack of a positive yield response to a fungicide application may instead be related to the late occurrence of the disease. A noticeable increase in the infected leaf area only occurred when the plant was in the ripening stage and grain and biomass yield had almost formed (BBCH 85). This infers that although a statistically significant reduction in the infected leaf area was recorded at the end of September, this is not necessarily a driving factor or representative for yield losses. Despite a significant impact on the infected leaf area, Jørgensen et al. (2015) also reported no significant yield increases from fungicide treatments due to the minor severity and late arrival of the diseases in maize fields in Denmark. Thus, at late stages of plant development, the outbreak of the disease has to be severe enough to have a significant impact on yield. This is also supported by Urban (2012), who found that the outbreak of Turcicum leaf blight which occurred in the late season (BBCH 85-89) in 2010 led to significant losses in grain yield. For Kabatiella eyespot, the level of infected leaf area recorded at the end of September (≤6%) in our trials in 2013 and 2014 were recorded by Urban (2012) one month earlier in 2011, with a noticeable increase in the infected leaf area in September resulting in significant yield losses.

Another explanation for the results of our trials could be closely related to the resistance of the respective hybrid used. Hybrids with moderate resistance against Turcicum leaf blight, as was the case for the variety NK Silotop in 2014, can counteract the impact of the disease to a greater extent. Although higher levels of disease severity were registered in the untreated control compared to the treated plots, yield was not significantly affected. This explanation is less plausible for Inzing in 2013 as the sown variety, Zidane, is classified as susceptible. In this case, however, the low levels of disease are unlikely to have been sufficient to have a significant negative effect on yield, even with a susceptible hybrid. This reasoning also applies to the tested varieties in Ostenfeld in 2013 and 2014, Ronaldinio and Kalvin, which are considered moderately susceptible to Kabatiella eyespot (levels of resistance in commercial hybrids are not published). Significant yield losses were not observed here either. Despite the inconsistency of these results, likely owing to the aforementioned low disease severity, the importance of hybrids and variation in their susceptibility to leaf diseases should not be underestimated. Several authors report significant differences in performance among hybrids with respect to resistance levels for Turcicum leaf blight (Guiomar 2011; Khot et al. 2006). Furthermore, information about the susceptibility of the regional varieties sown is frequently provided by the regional body responsible for plant

protection and private companies (Hiltbrunner et al. 2015). For Kabatiella eyespot, significant differences in disease severity have also been reported among different maize hybrids (Prończuk 2004; Formento et al. 2014).

An additional reason for the inconsistencies between infected leaf area and yield response in our trials is related to the high variation in the data among repetitions of the same treatment. Although repetitions were analysed separately in the linear regression, the high variation in the data did not allow a significant negative relationship between yield and infected leaf area to be established in three of the four field trials. The trial in which a significant negative correlation was obtained (Inzing 2014) presented relatively harmonious results for repetitions of the same treatment, with lower standard deviations than the other trials in general. Thus, despite the fact that disease pressure was lower in 2014, a negative correlation between yield and infected leaf area could be determined due to the consistency of the data. At the same time, the high data variation could be due to external factors. In a field trial, these can include the soil, water supply and fertilisation, factors which can never be completely uniform (Schuster and Geidel 1978). In addition, for the trials in Ostenfeld, the distribution of Kabatiella eyespot in the field was inconsistent. This was especially evident in 2014, where certain areas of the trial were more infected than others. This was caused by external factors unrelated to the effect of the fungicide.

For example, higher levels of humidity may have been concentrated in certain zones of the trial due to its north-south orientation. Due to the height of the maize plant, prolonged radiation (from higher exposure to sunlight) in the first blocks of repetitions (southern end of the trial) would have caused a lower level of humidity. As a result, the plants dried out at a quicker rate than in other areas of the trial. In these blocks, the severity of the disease was noticeably lower. Thus, the orientation of field trials could be an important factor.

Finally, a potential, so-called “greening effect” could also have had an influence on the correlation. In addition to the control of pathogens through inactivation, fungicides from the strobilurine and triazole groups have secondary effects, stimulating physiological activity in the plants to which they are applied. The “greening effect” refers to an increase in photosynthetic activity through the higher production of chlorophyll, retarding the senescence of the plant and producing a higher yield (Gerhard & Habermeyer 1998; Bryson et al. 2000; Venancio et al. 2003; Häuser-Hahn et al. 2004). While an inhibition of the ethylene biosynthesis is reported for triazoles, which leads to a delayed senescence (Siefer & Grossmann 1996), strobilurins improve nitrogen metabolism, maintaining the green leaf area for longer (Häuser-Hahn et al. 2004).

A potential example of the above phenomenon is the trial in Ostenfeld in 2013. Here, two treatments applied at the vegetative stage (BBCH 55), namely fluopyram + prothioconazole

and carbendazim + fluxilazole, provided a better control against Kabatiella eyespot than propiconazole + azoxystrobin applied at flowering (BBCH 63) in terms of infected leaf area, yet propiconazole + azoxystrobin provided a yield increase which was 6 dt/ha higher, on average. A delay in the ripening could be observed in Inzing in 2014, where plots sprayed with propiconazole + azoxystrobin applied at flowering (BBCH 65) were notably greener than the other plots (Figure 79). Nonetheless, the infected leaf area recorded for all plots (non-treated and treated) was similar. This result could not be methodically confirmed because quantitative data on the green leaf area (green leaf area rating) were not registered.

Fig. 79. Mittich 2013. Example of greening effect observed between non-treated control and treated with propiconazole + azoxystrobin applied at flowering (BBCH 63).

4.4.5. Economic thresholds

There is a lack of consensus in the literature with regard to economic thresholds and their reliability in the decision-making process of applying fungicides to control maize leaf diseases. To date there are no clear thresholds established for individual diseases (Wise and Mueller 2011), making it difficult to quantitatively determine a level of disease severity at which fungicide application is profitable (Munkvold et al. 2001). For example, while Paul et al. (2011) established a difference between severity levels above and below 5% in relation to economic benefits of application, the authors themselves maintained that the results were unclear in terms of establishing fungicide thresholds. Studies such as these, however, may be useful for further research on calculating thresholds. In Europe, despite some studies on the relationship between yield and fungicide application, no thresholds have been developed

due to the variation in the severity of diseases like Turcicum leaf blight and Kabatiella eyespot across seasons, as well as the lack of research on fungicide application in general (Jørgensen 2012). Some research on thresholds has been done in other countries such as Argentina, establishing disease levels of 1.5% for common rust (*P. sorghi)* for a late application (Carmona et al. 2011), while an application is recommended for Turcicum leaf blight at a level of 1-5% (De Rossi 2015). In this case, the low thresholds are due to the optimal climatic conditions (e.g. high humidity) which exist in certain regions of Argentina for the development of foliar diseases, as well as favourable agricultural measures (e.g. non-tillage, late sowing) (Diaz et al. 2012, Couretot et al. 2013).

Smith (2015) maintains that while a threshold can be a useful tool in order to understand the disease levels which could be considered severe, the decision whether or not apply a fungicide is dependent on a number of other factors. These include an analysis of the symptoms in the field, susceptibility of the variety to the disease, prevailing weather conditions and history of the disease in the specific location. For Turcicum leaf blight, analysis of the initial symptoms is most important in the lower leaves as the disease can be passed on to the ear leaves, which have the largest impact on yield. For Kabatiella eyespot, on the other hand, Smith (2015) argues that management of the disease should involve the use of resistant varieties and tillage (debris management). Significant yield losses are only likely if the disease reaches levels above 50% and fungicide application may not be cost effective if no other diseases are present. Robertson et al. (2007) state that fungicide applications were rare until 2007 due to a lack of profitability, finding that the use of resistant hybrids was an effective control method for Grey leaf spot and common rust.

The overwhelming consensus is that while fungicide applications on maize are effective in increasing yields, this does not mean that there will always be a clear economic benefit. For example, while there was a positive impact on yield for approximately 80% of 472 fungicide treatments analysed in the US from 2008-2010, only 48% of these treatments resulted in a positive economic benefit (Wise and Mueller 2011). Robertson et al. (2007) report similar results, with a positive impact on yield for 77% of the studied locations, yet only a positive economic benefit in 27% of the locations. As stated in section 3.4.7 regarding the calculation of the threshold, the decision to apply a fungicide is dependent on whether the improvement in yield and associated monetary benefit is sufficient to offset the costs of the fungicide product and its application (Liu et al. 2015). The price of maize also has to be taken into account here. As the maize price decreases, the threshold increases, and vice versa. For example, taking the average yield loss of 0.118 t/ha for *K. zeae* from our calculations in section 3.4.7, the threshold at a silage maize price of €32/t was 17%, while it would rise to 19% at a maize price of €30/t. On the other hand, an increase in the maize price to €36/t would reduce the threshold to 15%. This is intuitive - if the price the farmer is receiving for his

maize is higher, he can justify the expense of a fungicide application at lower levels of disease severity. In fact, fungicide use on hybrid maize has risen in the US in recent years due to, among other factors, an increase in the market price of maize (Wise and Mueller 2011). As an example, while Liu et al. (2015) found that fungicide applications provided an overall economic benefit, this result was sensitive to the maize price used. The price (grain maize) used for their calculations was an average price over a number of years (2009-2012) and was higher ($5.24 / bushel)[14] than recent US market prices (approx. $4 / bushel)[15]. At this recent market price, the application of one of the fungicides used in their study would no longer have been economically beneficial.

This lack of a clear economic benefit is especially noticeable at lower severity levels of the disease. For an analysis of fungicide trials in 14 US states from 2002-2009, Paul et al. (2011) concluded that there was a higher probability of a fungicide application having a positive economic effect when disease severity was greater than 5% (the diseases present were grey leaf spot, Turcicum leaf blight and common rust). Based on their results, a fungicide application when disease severity was low (<5%) could not be recommended as the probability of a fungicide application returning an economic benefit was less than 50%. In addition, application at higher disease levels was economically beneficial if the maize price was relatively high, application costs were low, and it could be determined with some certainty that yields would be low without treatment. Furthermore, studies conducted in 2009 and 2010 found that only 3% of fungicide treatments resulted in a significant yield gain when the disease severity of Turcicum leaf blight was, on average, less than 1% and no other disease was present (Wise and Mueller 2011).

The above is pertinent for the fungicide trials carried out in 2013 and 2014 in Ostenfeld and Mittich/Inzing. As the disease pressure was low in both locations, it was only possible to obtain a negative correlation between disease and yield in Inzing 2014 (*E. turcicum)* and Ostenfeld (*K. zeae)*, of which only Inzing 2014 was statistically significant. Consequently, it is debatable whether these were ideal years to draw conclusions about the suitability of a fungicide application from an economic perspective, as well as the accuracy of the associated thresholds calculated.

It is also argued that the decision to apply fungicides is made with factors other than combating fungal diseases in mind, e.g. the increase in photosynthetic activity through production of higher chlorophyll content, the so-called “greening effect”. This is known to delay the senescence of the plant and produce higher yields (Venancio et al. 2003; Häuser-

[14] This corresponds to a price of $206.29 per ton (price per bushel x 39.36825 = metric ton price; CME Group 2014).
[15] This corresponds to a price of $157.47 per ton (CME Group 2014). Price used for calculations in this work (grain maize) was €160.

Hahn et al. 2004). While it has been established that this can increase yields, it does not necessarily provide an economic benefit. The delayed senescence can lead to a later harvest date, which is problematic when harvesting large fields and can also become an issue if bad weather in the late season has an adverse effect on the harvest. Furthermore, the delayed senescence may require the harvesting of maize with high moisture content, which can have a negative economic impact due to drying costs (Wise and Mueller 2011). The benefit of the “greening effect” will depend on whether the associated monetary gain from the increase in yield is sufficient to cover the cost of fungicide application.

To conclude, while there is a relatively clear positive relationship between yield and fungicide applications, the economic benefit of an application is significantly less obvious, especially at low disease levels. The use of economic thresholds to justify the application of fungicides needs to be considered with caution, with a lack of specific thresholds for individual diseases to date (Wise and Mueller 2011). Furthermore, overemphasis on economic thresholds may understate the importance of factors such as variety susceptibility, cultural practices (e.g. tillage) and climatic conditions. In the European context, while there is some research on the relationship between fungicide application and yield losses, there is a distinct lack of information on the economic decision-making process and thresholds related to the application of fungicides. Future research is needed here in order to gain a greater understanding of these relationships.

4.4.6. Recommendations for application of fungicides on maize

Based on the points discussed above and the results in this study and related literature, a number of recommendations with regard to effective fungicide application can be made for the analysed regions (southern and northern Germany):

1) The use of resistant varieties and phytosanitary measures (tillage practices, crop rotation) are effective methods to control the development of Turcicum leaf blight and Kabatiella eyespot. If these measures are not sufficient or cannot be incorporated into the management of maize, the use of fungicides becomes a feasible option.

2) If a resistant variety is being used, it is recommended to wait until early flowering to ascertain whether the disease has developed to a sufficient level to apply a fungicide. Hence, end of July is a key date in which to make an application decision (middle-late application).

3) In most cases, an early application can be omitted as a viable option in southern and northern Germany. However, an application at flowering is more complicated due to the increased height of the plants.

4) An increase in infected leaf area in the late season (September) only produces significant yield losses in severe cases. Currently, the likelihood of this occurring in Germany is relatively low.

5) Compared to Turcicum leaf blight, the infected leaf area of Kabatiella eyespot will need to be considerably higher to cause yield losses as the lesions are noticeably smaller and cause less damage to the leaf.

6) The potential yield benefits of a fungicide application do not necessarily convert into economic profit. This will depend on additional costs associated with application compared to the monetary gain from yield increase (dependent on the maize price).

7) It is difficult to establish economic thresholds for individual maize leaf diseases due to a number of factors which vary on a case-by-case basis such as hybrid susceptibility, level of inoculum and climatic conditions. This illustrates the importance of a regional warning service such as those in several regions in Germany. The use of spore traps could be useful here to determine whether a fungicide application is necessary in the region and, if so, the optimal timing of application based on spore dispersal/inoculum pressure.

Summary

In the last decade, there has been a progressive increase in the acreage of maize in Central Europe due to its high yield and diverse uses such as food, feed and bio-energy. This has led to more intense maize cultivation in many regions with narrowed crop rotations, coinciding with an increased presence of certain maize leaf diseases. The knowledge about the occurrence and significance of fungal pathogens and their epidemic development in maize fields in Central Europe is however still limited. In order to gain a broader overview of the phytosanitary state of maize crops in Central Europe, a qualitative monitoring of potentially leaf infecting pathogens in maize was carried out in selected fields in Germany, the Netherlands, Czech Republic, Austria, France and Poland in 2012 and 2013. Fungal isolates obtained from collected leaf samples were analysed morphologically. For a number of isolates, pathogenicity tests were conducted in the greenhouse by inoculating healthy plants with spore suspensions prepared from single-spore cultures in order to fulfil Koch's postulates. The studies revealed that seven isolates of *Kabatiella zeae* (Kabatiella eyespot), five isolates of *Bipolaris zeicola* (northern corn leaf spot), two unidentified isolates of *Bipolaris* spp., two isolates of *Colletotrichum graminicola* (anthracnose) and six isolates of *Phoma* spp. (Phoma leaf spot) fulfilled Koch´s postulates. The resulting disease symptoms were described in detail. For three representative *Phoma* isolates (one pathogenic, two saprophytic) which could not be clearly identified through morphological analysis, a taxonomical assignment was carried out by the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands). Following this analysis and comparisons with internal sequence databases, the three isolates were confirmed as putative new species within the genus *Peyronellaea.* Turcicum leaf blight and Kabatiella eyespot were confirmed as the most important diseases in the monitored regions. In 2012 and 2013, a higher pressure of Turcicum leaf blight was observed in southern regions of Germany (Bavaria, alpine foreland) and northern Austria (alpine foreland). *K. zeae* was particularly widespread in fields of the Netherlands and northern Germany (northern and western Lower Saxony and Schleswig-Holstein). The importance of common rust was also confirmed in southern Germany.

The application of fungicides is considered an effective control method against the most common leaf diseases Turcicum leaf blight (causal agent *E. turcicum*), common rust (causal agent *P. sorghi*), and Kabatiella eyespot (*K. zeae*). Thus, a second part of the project involved an analysis of the impact of various fungicide compounds: propiconazole + axoxystrobin (Quilt Excel®), epoxiconazole + pyraclostrobin (Opera®), fluopyram + prothioconazole (Propulse®) and carbendazim + flusilazole (Harvesan®). These were tested under conditions of natural infection in two regions in Germany where the diseases are prevalent, namely Mittich (2013) and Inzing (2014) in southern Germany and Ostenfeld (2013 and 2014) in Northern Germany. For Turcicum leaf blight in (Mittich and Inzing),

statistically significant differences in disease severity between treated and untreated plots were only registered in 2013. A statistically significant reduction of the infected leaf area was also observed for common rust. In 2014, probably due to the low level of disease pressure in the field, only one treatment (epoxiconazole + pyraclostrobin), applied at the early stage, significantly reduced infected leaf area compared with the untreated control. Among treatments, timing of application did not produce significant differences in infected leaf area in both years. For Kabatiella eyespot in Ostenfeld, significant differences among all variants of treated fungicide plots and untreated plots were registered in 2013 and 2014. Although significant differences among timing of application was not registered in both years, a tendency for optimal application at middle to late stages of maize plants was observed in 2013, i.e. tasseling to flowering (BBCH 51-BBCH 65). Statistically significant differences in infected leaf area between treated and untreated plots did not translate into significant positive yield responses. This was probably due to the low level of diseases in both years and the high variation in data among repetitions.

In addition, linear regressions were run to determine the relationship between infected leaf area and yield losses. A significant negative correlation between infected leaf area and yield was only observed for Turcicum leaf blight in Inzing in 2014. Based on the regression equations, economic thresholds were calculated to evaluate the minimum percentage of infected leaf area which is necessary for a fungicide application to have a monetary benefit. These results were discussed with regard to published thresholds and should be interpreted with caution due to the difficulty of obtaining accurate thresholds for individual diseases. This is especially pertinent at low levels of disease severity, which prevailed in the trials.

Spore dispersion patterns were analysed using a Burkard spore trap in Inzing in 2014 (*E. turcicum*, natural infection), Ostenfeld in 2013 and 2014 (*K. zeae*, natural infection) and Göttingen in 2015 (*E*. *turcicum* und *K. zeae*, artificial inoculation). For *E. turcicum*, the daily amount of spores was examined via microscopy and real-time qPCR. The number of spores counted on tapes through microscopy was, on average, positively correlated with the DNA yields obtained from tapes and analysed with qPCR. Hence, qPCR is a reliable alternative to microscopic assessment for the evaluation of inoculum pressure and dispersal. On the contrary, the detection of *K. zeae* presented difficulties via microscopy and qPCR, hampering further analysis.

For *E. turcicum*, the data obtained from the spore trap correlated with disease development in the field and favourable weather conditions. Moderate temperatures and high humidity favoured sporulation in Inzing, while in Göttingen high temperatures and dry weather promoted the expansion of lesions on the leaves. Based on the results of spore trapping in Inzing and Göttingen and the estimation of the latent period, the epidemic of *E. turcicum* was analysed in detail. Despite differences in the development of Turcicum leaf

blight and the origin of the inoculum (natural vs. artificial), the latent periods estimated for *E. turcicum* turned out to be very similar in both locations (6/7-14 days) and similar to those calculated in the literature (8-14 days). Furthermore, the latent period was shorter at the end of the season, possibly due to the increased concentration of inoculum as the season progressed.

Overall, the spore trap helped in gaining a more in-depth understanding of the development of Turcicum leaf blight throughout the season. Thus, spore trapping can be very useful in evaluating inoculum pressure during the season as part of an integrated pest management. This could help to decide whether a fungicide application to control the disease is required and, if so, the optimal timing of application.

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Appendix

Tab. A1. Phytosanitary measures in field trials of 2013 and 2014.

Field trial/season	Mittich (2013)	Inzing (2014)	Ostenfeld (2013)	Ostenfeld (2014)
Seed treatment				
Insecticides Fungicides	Mesurol FLO 500 FS Aatiram 65 FS	Mesurol FLO 500 FS Aatiram 65 FS + Maxim Quattro[1]	Mesurol FLO 500 FS TMTD	
Herbicides	Laudis® OD 2 L/ha 17.05.2013	Terano® WG 0,7 kg/ha 25 g/kg Metosulam 600g/kg Flufenacet 25.04.2014	Calaris® SC 0,75 l/ha 70g/l Mesotrione 330g/l Terbuthylazin BBCH 11-12; 17.05.2013	Calaris® SC 0,5 l/ha 70g/l Mesotrione 330g/l Terbuthylazin 16.05.2014
	Gardo Gold® SC 3 L/ha 312,5g/l Metolachlor 187g/l Terbuthylazin 17.05.2013	Gardo Gold® SC 2 L/ha 312,5g/l Metolachlor 187g/l Terbuthylazin 25.04.2014	Dual-S-Gold® EC 1 L/ha 915g/l S-metolachlor BBCH 11-12; 17.05.2013	Dual-S-Gold® EC 1 L/ha 915g/l S-metolachlor 16.05.2014
			Trend® 90 SL (adjuvant) 0,3 L/ha BBCH 11-12; 17.05.2013	Milagro Forte® OD 0,2 L/ha 60g/l Nicosulfuron 16.05.2014
			Peak® WG 16g/ha 750g/kg Prosulfuron BBCH 11-12 17.05.2013	Peak® WG 16g/ha 750g/kg Prosulfuron BBCH 15-16 10.06.2014

[1]Maxim Quattro: Thiabendazole + metalaxyl-M, fluodioxonil and azoxystrobin. Seed- and soil-borne diseases protection (providing good effect against *Fusarium* species)

Tab. A2. Sowing and management in field trials of 2013 and 2014.

Fertilisers	**Mittich (2013)**	**Inzing (2014)**	**Ostenfeld (2013)**		**Ostenfeld (2014)**
Fertilisers I	Liquid manure 9 m^3/ha (45 kg N/ha) 25.04.2013	Liquid manure 25 $m^{3/}$ha (45 kg N/ha) 20.03.2014 Alzon 46 (64 kg N/ha) 141 kg/ha 2.04.2014	NP 20+20+0+4 22.04.2013	Others: Excello Basis 2.65% Cu; 3% Zn 0.18% Fe; 0.16% Mn 0.04% B; 53% CaCO3 27% MgCO3 1.20 dt/ha 22.04.2016	NP 20+20+0+4 1.50 dt/ha 16.05.2014
Fertilisers II	NP Underground 1.) 30 kg/ha 27.04.2013/ 2.) 108 kg/ha 15.05.2013	NPMg 40+10+10 187 kg/ha 21.04.2014	NPK 15+9+20+2+4 23.04.2013	Others: Kieserit granulate 25% MgO; 20% S 1.60 dt/ha 23.04.2016	NPK 17+6+18+2 +6 7.65 dt/ha 19.05.2014

Other Non-pathogenic Phoma Isolates

Isolate 13.2d

Growth rates of the colony between 53 and 59 mm in size (fast growing) after seven days, with a regular border. Production of aerial mycelium absent or scarce with flat/effuse and scattered, white to salmon areas. Pycnidia are produced in abundance and homogeneously distributed on the plate, situated on/ in the medium, either solitary or coalescing with other pycnidia. Pycnidia are cream to dark brown, pyriform and flask-shaped, with one to two conspicuous papillate ostioles and 75-200 x 100-400 µm in diameter. They are glabrous, in some cases developing hyphal outgrowths, with pseudoparenchymatous cell wall structure. Conidial matrix rosy-white-yellow and abundant. Conidia are extruded in very typical cirri. Conidia are ellipsoidal, single-celled, hyaline, typically biguttulate with small, polar guttules. Conidia size: 3-4.2 x 1.3-2 µm (average 3.56 x 1.55 µm).

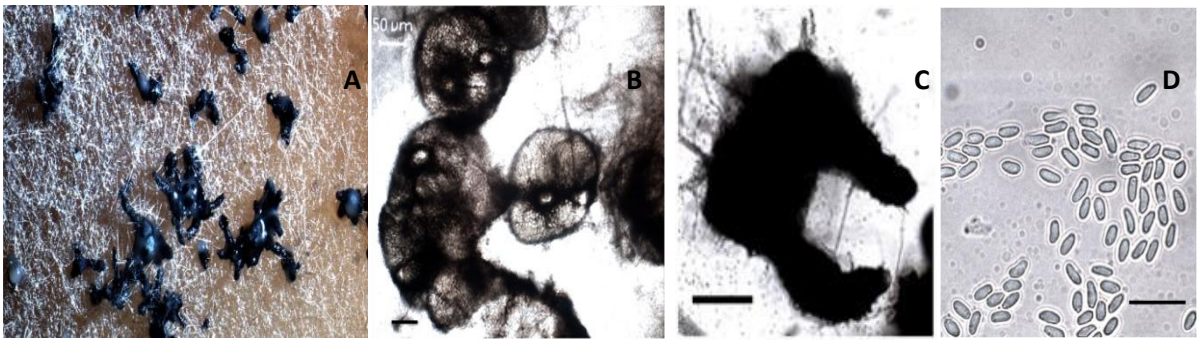

Fig. A1. Isolate 13.2a. A. Pycnidia on the agar medium surface. B. Globose to subglobose pycnidia. C. Irregular pycnidia. D. Conidia. Scale bars B-C=100 µm; C=10 µm.

Isolate 12.27

Growth rates of the colony between 49 and 52 mm after seven days with a regular border regular border. Aerial mycelium, sparse, in some cases absent, white, effuse and scattered in some areas. Mycelium are immersed and brown. Pycnidia are produced in abundance and evenly distributed on the plate, both on/in the medium. Pycnidia are brown to black, globose to subglobose, glabrous or with hyphal outgrowths, with pseudoparenchymatous cell wall structure. Size of the pycnidia range between 50-125 x 75-150 µm and are either solitary or confluent with other pycnidia. Pycnidia with a single ostiole, non or slightly papillate. Conidial matrix white, containing conidia in abundance. Conidia are ellipsoidal, single-celled, hyaline, non- or biguttulate with polar and medium-sized guttules. Size of the conidia: 3.4-4.7 x 1.3-1.9 µm (average 3.8 x 1.6 µm) with a few large conidia.

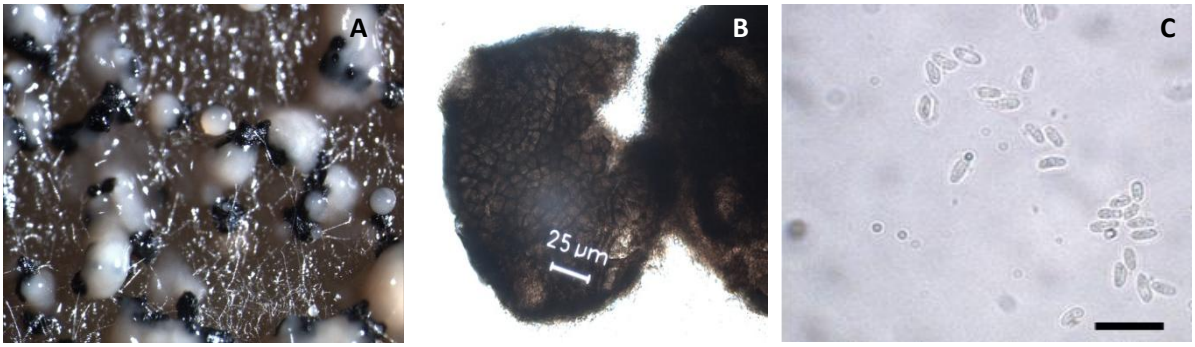

Fig. A2. Isolate 12.27. A. Pycnidia on the agar medium surface. B. Pycnidia. C. Conidia. Scale bars C=25 µm; D=10 µm.

Isolate 12.28

Growth rates between 45 and 47 mm after seven days with a regular border. Aerial mycelium sparse, white or salmon, flat to effuse. Immersed mycelium salmon. Pycnidia are produced in abundance, evenly distributed on the plate and disposed on both the agar surface and in the agar. Pycnidia are brown to black, globose to subglobose, glabrous, with pseudoparenchymatous cell wall structure. Size of the pycnidia within the range of 75-150 x

75-150 µm either solitary or confluent with other pycnidia. Presence of mycropycnidia. Pycnidia present a single, slightly papillate ostiole. In some cases, 'necks' were developed. Conidial matrix whitish to yellow containing conidia in abundance. Conidia are ellipsoidal, single-celled, hyaline, non- mono- or biguttulate, polar and small-medium size guttules. Size of the conidia: 3.1-4.6 x 1.5-2.4 µm (average: 3.9 x 1.7 µm). Chlamydospores present.

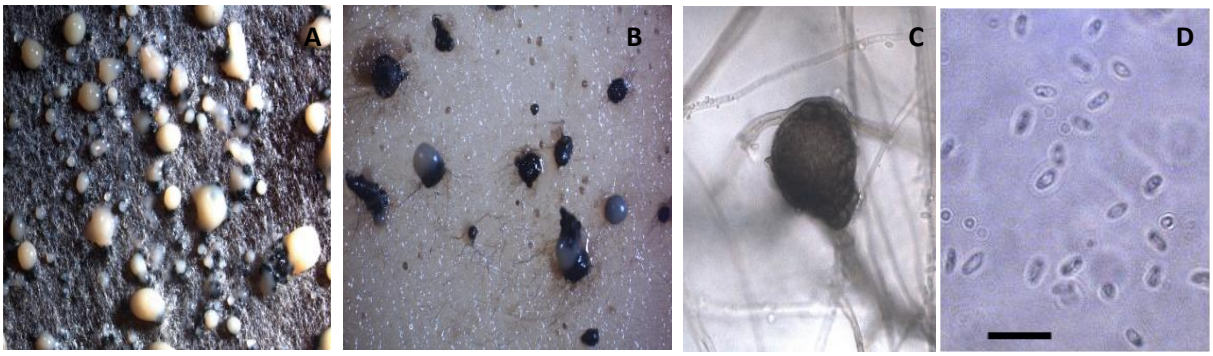

Fig. A3. Isolate 12.28. A. Pycnidia and conidial matrix on the agar medium surface. B. Presence of mycropycnidia structures within pycnidia. C. Mycropycnidium. D. Conidia. Scale bar=10 µm.

Isolate 12.31

Growth rates between 49 and 52 mm after seven days with a regular border. Aerial mycelium, sparse, white, flat to effuse. Immersed mycelium salmon. Pycnidia are produced in a noticeably large quantity compared with other similar isolates. Pycnidia are homogenously distributed on the plate and located on both the surface of the medium and in the medium. Pycnidia are black, with globose, subglobose and irregular shape, glabrous, with pseudoparenchymatous cell wall structure. Size of the pycnidia falls within the range of 75-200 x 75-200 µm, either solitary or confluent with other pycnidia. Pycnidia present one or two slightly papillate ostioles. 'Necks' also frequently developed. Conidial matrix whitish to yellow, containing conidia in abundance. Conidia are ellipsoidal, single-celled, hyaline, non-mono- or biguttulate with polar and medium-sized guttules. Size of the conidia: 3.1-6.3 x 1.3-2 µm (average 3.90 x 1.73 µm). Large conidia were also observed.

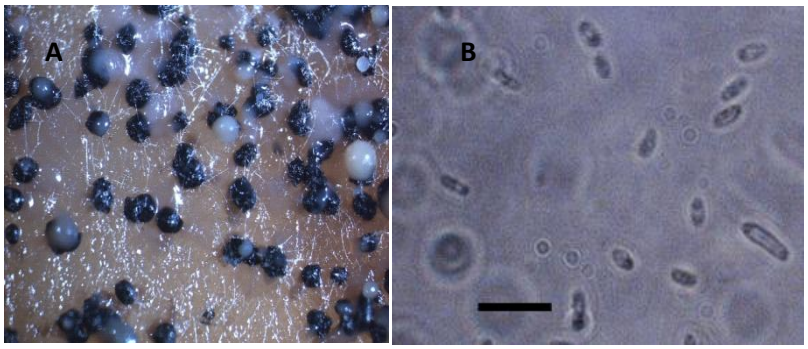

Fig. A4. Isolate 12.31. A. Pycnidia on the agar medium surface. B. Conidia.Scale bar=10 µm.

Isolate 13.37

According to the obtained data from the DNA sequencing: *Phoma pomorum* (syn. *Didymella pomorum*). Growth rates between 45 and 51 mm after seven days with a regular border. Moderate to abundant production of green/olivaceous buff, dark cyan floccose to wooly aerial mycelium with floccose white tufts. Under UV light, the culture develops a felty black centre and more frequently compacted white, grey, green/olivaceous aerial mycelium. Pycnidia are produced in abundance both on and in medium. Pycnidia are brown to black, globose to subglobose, solitary and confluent, glabrous with pseudoparenchymatous wall. Single and multiple ostioles could be developed from a single pycnidia. The pycnidia present a hyaline-pinkish matrix with abundant production of conidia. Conidia are ellipsoidal single-celled, hyaline, with two to four polar, small-sized guttules. Size of the conidia: 3.8-6 x 1.9-3.1 µm (average 5 x 2.5 µm). Identified as *Ph. pomorum* according to morphological characterisation.

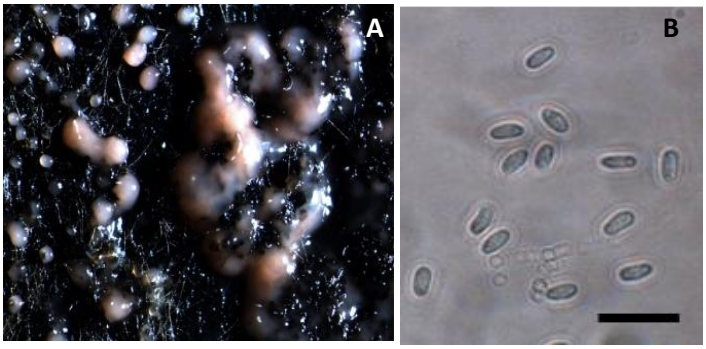

Fig. A5. Isolate 13.37. A. Pycnidia on the agar medium surface. B. Conidia. Scale bar =10 µm.

Isolate 13.47

Growth rates between 65 and 70 mm after seven days with regular border. Aerial mycelium sparse, and in some cases absent, white effuse and scattered in some areas. Immersed mycelium brown. Pycnidia are produced in abundance, evenly distributed on the plate and disposed both on and in the medium. Pycnidia are solitary or confluent, brown to black, globose to subglobose and glabrous. The cell wall structure could not be clearly defined. Size of the pycnidia falls within the range of 75-200 x 100-200 µm. Pycnidia present 1 to 3, slightly to conspicuously papillate ostioles with possible development of 'necks'. Conidial matrix hyaline to pinkish, containing conidia in abundance. Conidia are ellipsoidal, single-celled, hyaline, either without or with one to two guttules polar and small in size. Size of the conidia: 3.4- 4.6 x 1.4-2.3 µm (average 3.9 x 1.8 µm).

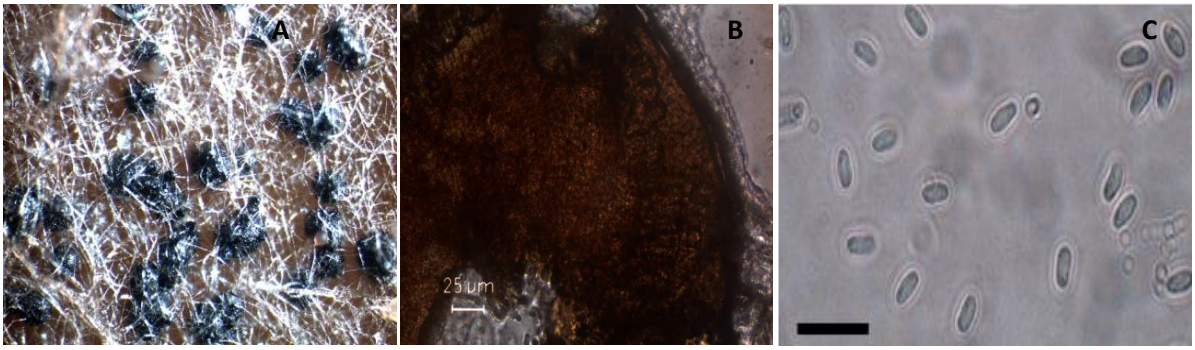

Fig. A6. Isolate 13.47. A. Pycnidia on agar medium surface. B. Pycnidia. C. Conidia. Scale bar= 10 µm.

Isolate 13.48

Growth rates between 60 and 73 mm after seven days with a regular border. Aerial mycelium sparse, and in some cases absent, white effuse and scattered in some areas. Immersed mycelium, brown to red/vinaceous. Pycnidia are produced in abundance, evenly distributed on the plate and disposed on and in the medium. Pycnidia are solitary, confluent or in chains, brown to black, globose to subglobose, glabrous and with pseudoparenchymatous cell wall structure. The size of the pycnidia range between 100-200 x 125-250 µm. Pycnidia present one to three, slightly to conspicuously papillate ostioles. Conidial matrix hyaline to pinkish, containing conidia in abundance. Conidia are ellipsoidal, single-celled, hyaline, either without guttules or one to two polar small guttules. Size of the conidia: 2.7- 4.1 x 1.5- 2.2 µm (average 3.5 x 1.7 µm). Large spores were also present.

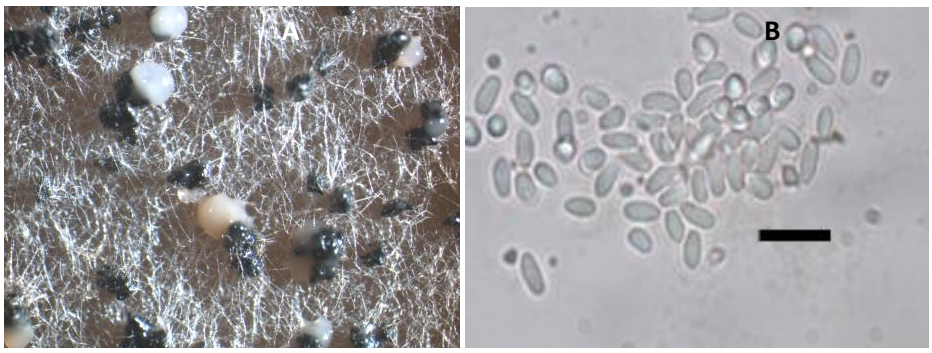

Fig. A7. Isolate 13.48. A. Pycnidia on the agar medium surface. B. Conidia. Scale bar= 10 µm.

Isolate 12.10

Growth rates between 39 and 43 mm after seven days, regular, presenting a scarce production of grey to brown flat/effuse, felty immersed dark mycelium, as well as black pycnidia which are produced in abundance. Both give the impression of dark discolouration of the agar medium. Pycnidia are globose to subglobose, glabrous, in some cases developing hyphal outgrowths, with pseudoparenchymatous cell wall structure. Size of the pycnidia ranges from 50-125 x 45-150 µm, either solitary or confluent with other pycnidia. In

every pycnidium, a single, slightly papillate ostiole was observed. Pycnidia present a white matrix with moderate production of conidia. Conidia are ellipsoidal to irregular, single-celled, hyaline, presenting one to five polar, small-sized guttules. Size of the conidia: 2.8-4.6 x 1.3-2 µm. Large spores were also observed.

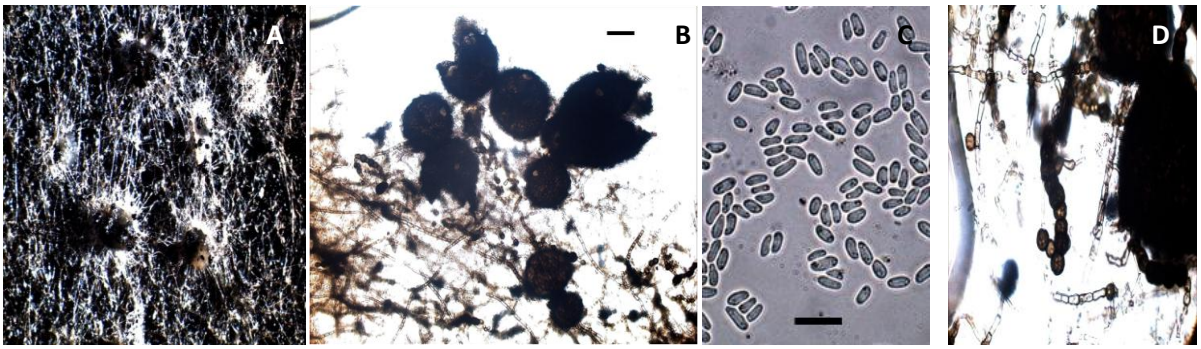

Fig. A8. Isolate 12.10. A. Pycnidia on the agar medium surface. B. Pycnidia. C. Conidia. D. Chlamydospores (63x). Scale bars B=50 µm; C=10 µm.

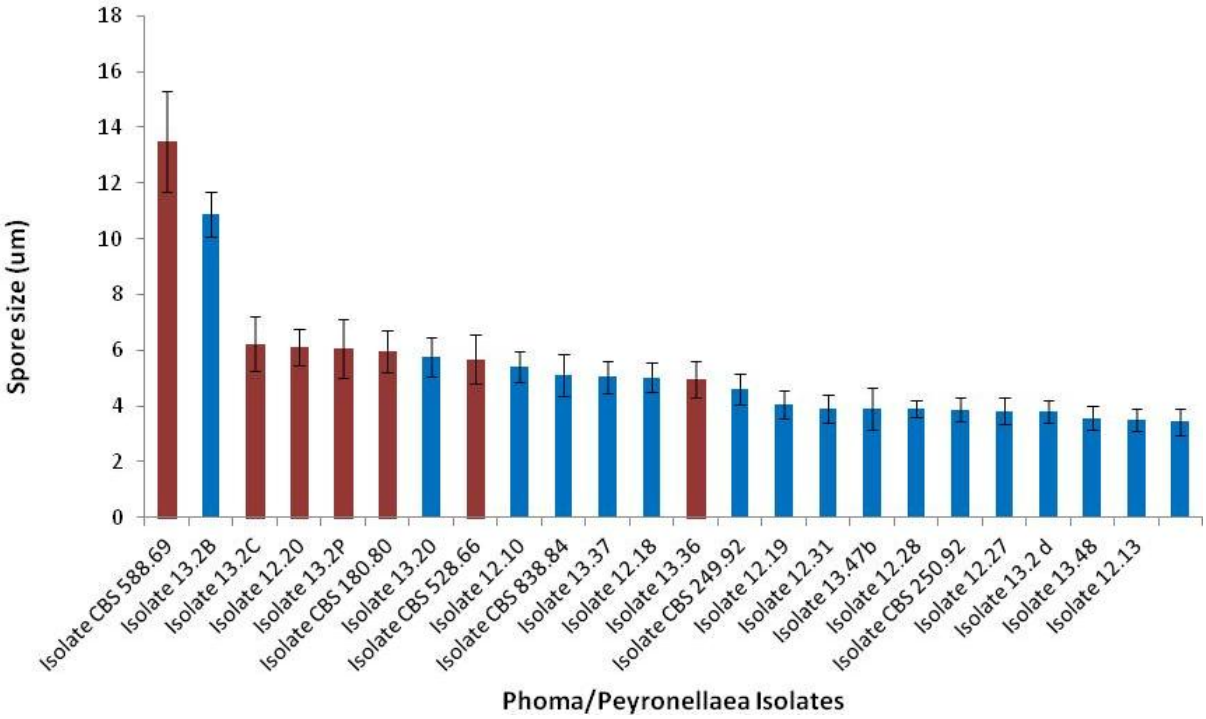

Fig. A9. Length of several *Phoma and Didymella* isolates investigated in this work. Red columns represent pathogenic isolates after pathogenicity tests. Blue columns represent isolates considered non-pathogenic after pathogenicity tests.

Tab. A3.: results obtained from NCBI database using BLAST analysis based on ITS 4 and ITS 5 sequences.

Isolate	Some of the resulted CBS species from BLAST	Score	Query covery	E. number	% similarity
12.18	*Didymella heteroderae* CBS 109.92	835	87%	0.0	98%
	Phoma pomorum CBS 838.84	835	87%	0.0	98%
	Didymella maydis CBS 588.69	824	87%	0.0	97%
12.20	*Didymella heteroderae* CBS 109.92	880	86%	0.0	99%
	Didymella nigricans CBS 444.81	876	88%	0.0	99%
	Didymella subherbarum CBS 305.79	876	88%	0.0	99%
	Didymella maydis CBS 588.69	857	88%	0.0	99%
13.2C	*Didymella pinodella* CBS 110.32	905	98%	0.0	99%
	Didymella heteroderae CBS 109.92	878	94%	0.0	99%
	Didymella nigricans CBS 444.81	874	94%	0.0	99%
	Didymella subherbarum CBS 305.79	874	94%	0.0	99%
	Phoma aliena CBS 379.93	872	94%	0.0	99%
	Didymella maydis	857	94%	0.0	99%
13.2P	*Didymella heteroderae* CBS 109.92	880	86%	0.0	99%
	Didymella nigricans CBS 444.81	876	86%	0.0	99%
	Didymella subherbarum CBS 305.79	876	86%	0.0	99%
	Didymella maydis	857	86%	0.0	99%
13.2B	*Didymella pinodella* CBS 110.32	963	99%	0.0	99%
	Phoma macrostoma var. incolorata CBS 300.36	937	95%	0.0	99%
	Didymella subherbarum CBS 305.79	893	89%	0.0	99%
	Phoma pomorum CBS 838.84	891	89%	0.0	99%
	Didymella dimorpha	885	89%	0.0	99%
	Didymella americana	885	89%	0.0	99%
12.13	*Didymella protuberans* CBS 381.96	891	88%	0.0	99%
	Didymella dimorpha CBS 346.82	891	88%	0.0	99%
	Didymella pinodella CBS 318.90	880	88%	0.0	99%
	Didymella subherbarum CBS 250.92	880	88%	0.0	99%
12.19	*Didymella pinodella* CBS 110.32	1000	98%	0.0	99%
	Didymella protuberans CBS 381.96	891	87%	0.0	99%
	Didymella dimorpha CBS 346.82	891	87%	0.0	99%
	Didimella subherbarum CBS 305.79	885	87%	0.0	99%
	Didymella maydis	874	87%	0.0	99%
12.36	***Phoma pomorum* CBS 838.84**	896	88%	0.0	**100%**
	Didymella pinodella CBS 110.32	977	100%	0.0	99%
12.37	***Phoma pomorum* CBS 838.84**	896	88%	0.0	**100%**
	Didymella pinodella CBS 110.32	976	88%	0.0	99%

Phoma sequences forward + reverse primers

Isolate 13.2B

AAAATCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAAGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACC
CATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCG
TCTGAAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCC
TGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGCGTAGACTCGCCTCAAAAC
AATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATT
TTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATTTAA

Isolate 13.2C only forward

ATGTCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTCTGTTTCCTCGGCGGGTCC
GCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCGTCTGAAAAAAATTAATAGTTACAACTTTCAACAACGG
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CTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGT
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ATCTCGCGCTTTGCATTCAGAACGACGACGTCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGC
TGAACTTAAGCATATCAAATG

Isolate 13.2P

TTGGAAAGTAAAAAATCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGC
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TGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGTGTAGAC
TCGCCTCAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCATTCAGAACGACGACGTC
CAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAA

Isolate 12.13

AAAAGCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTACC
CATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTTCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAGCG
TCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCT
GTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGCGTAGACTCGCCTCAAAAC
AATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCAGAACGACGACGTCCAAAAGTACATT
TTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATACTAA

Isolate 12.18

AAAAAACGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTAC
CCATGTCTTTTGAGTACCTTCTGTTTCCTCGGCGGGTCCGCCCGCCGATTGGACAATTTAAACCATTTGCAGTTGCAATCAG
CGTCTGAAAAAAATTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA
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Isolate 12.19

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Isolate 12.20

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Isolate 12.36

TAAAAATCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTTA
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CGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA
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CAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACAT
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Isolate 12.37

TAAAAAATCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCTGCTATCTCTT
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GCGTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
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ATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATACAAG

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DECLARATIONS

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.
Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen, 26.05.2016.

.....

Lucia Ramos Romero

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorised aid.

Göttingen, 26.05.2016.

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Lucia Ramos Romero

CURRICULUM VITAE (EDUCATION)

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