INTERACTIONS BETWEEN THE BRASSICACEAE *Brassica napus* AND *Arabidopsis thaliana* AND THE PHYTOPATHOGENIC FUNGUS *Verticillium longisporum*

THE ROLE OF VOLATILE ORGANIC COMPOUNDS

DISSERTATION

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SUBMITTED BY

MARIA VLAIC
BORN IN BERLIN

GÖTTINGEN, FEB. 13. 2012

1ST EXAMINER: PROF. DR. STEFAN SCHÜTZ
2ND EXAMINER: PROF. DR. ANDREA POLLE

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FÜR MICHAEL

CONTENT

General Introduction

PLANT – FUNGUS INTERACTIONS

Interactions between plants and fungi can be mutualistic or parasitic. Mutualism means advantages for both partners, such as known from mycorrhiza and lichens. If the relations between the participants are unbalanced, a parasitic interaction is the case. Parasitic fungi may injure the plant so much, that it is losing fitness (SITTE et al. 2002). During evolution, plants evolved appropriate defense strategies (DODDS and RATHJEN 2010) towards those pathogens, such as mechanical barriers or toxins and inducible defense reactions (i.e. phytoalexins; SITTE et al. 2002). General plant response to pathogen attack is caused by elicitors, molecules developing during degradation of cell walls and membranes. This elicitors trigger further defense reactions (SITTE et al. 2002). But specialized fungi are often able to suppress or to avoid them (DODDS and RATHJEN 2010).

VOLATILE ORGANIC COMPOUNDS

Volatile organic compounds (VOCs) are "organic chemical compounds that have high enough vapour pressures under normal conditions to significantly vaporize and enter the atmosphere" (GROSSMANNOVA et al. 2007). They are released by all organism as well as by several natural and artificial sources.

The emission of VOCs by plants might be unavoidable because of their physicochemical properties (PEÑUELAS and LLUSIA 2004, HOLOPAINEN 2004, NIINEMETS et al. 2004). Plants form VOCs in the frame of their daily metabolism and evolutionary processes could be responsible for an ecological usage (PEÑUELAS and LLUSIA 2004). Their role as infochemicals, VOCs carrying information, between organisms is undisputed today. Many of those VOCs play a role in inter- and intraspezific communication, such as attraction of pollinators and parasitoids or defense against pathogens and herbivors (DICKE et al. 2003, HOLOPAINEN 2004, BRUCE and PICKETT 2007, GERSHENZON 2007). Best investigated is the VOC-mediated ***interaction between plants and insects*** (BRUXELLES and ROBERTS 2001, GATEHOUSE 2002, PICHERSKY and GERSHENZON 2002, BRUCE et al. 2005, VAN POECKE 2007, UNSICKER et al. 2009). ***Interactions between plants and microorganism*** are also known (MENDGEN et al. 2006, SPIVALLO et al. 2007, TOOME et al. 2010, WENKE 2010). Plant VOCs often have antimicrobial qualities and might play a role in defense

against different microorganisms (DROBNICA 1967a, 1967b, KIM et al. 1995, OLIVIER 1999, SMOLINSKA and HORBOWICZ 1999, AGGARWAL et al. 2002, UTAMA et al. 2002, NERI et al. 2006, GARCIA et al. 2008). Therefore VOCs become more and more the focus of agricultural attention as potential agents in biocontrol (FERNANDO et al. 2005, NERI et al. 2006, CAMPOS et al. 2010). The third kind of interaction is the ***communication between plants*** (BALDWIN 2002, 2006, HEIL 2008). Especially this part of communication indicates their signaling function (HEIL and BUENO 2007). ***Multitrophic interactions*** connect at least three different organisms on VOC level. The best-known example is indirect defense of plants against herbivorous insects. Plants damaged by herbivores, emit VOCs attracting parasitoids of its herbivores (i.e. HEIL 2008). But the interaction between plants, mycorrhizal fungi and bacteria is also mediated by VOCs (BONFANTE and ANCA 2009).

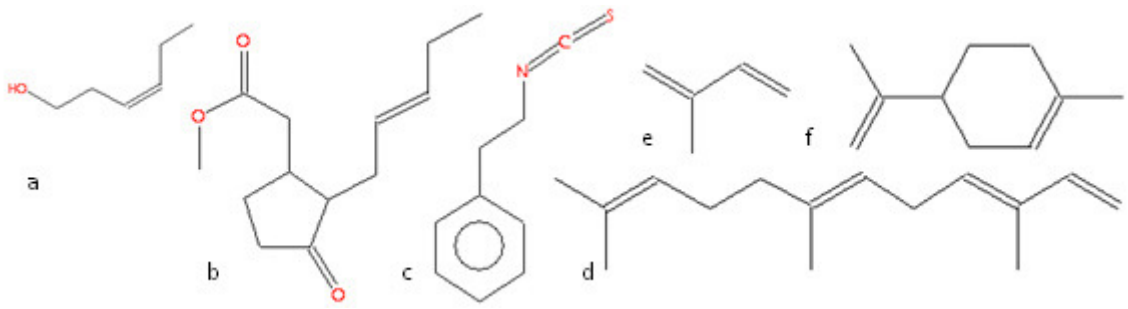

Fig. 0-1: examples for volatile organic compounds (VOC) emitted by plants; a) Z-3-hexen-1-ol, B) methyl jasmonat, C) phenylethyl isothiocyanat, D) farnesene (sesquiterpenoid), E) isoprene, F) limonene (monoterpenoid)

The emission of plant VOCs is influenced by biotic and abiotic factors, like herbivory, fungal pathogens, nutrient deficiency, ozone and many more (HANSEN and SEUFERT 2003, VALLAT et al. 2005, VUORINEN et al. 2004, TEUBER et al. 2008, GOUINGUENÉ and TURLINGS 2002, HOLOPAINEN and GERSHENZON 2010). Very common are ***green leave volatiles***, mainly composed of C6-alcohols (Fig.0-1a). They are emitted after tissue disruption, leading to the emission of *Z*-3-hexenol (apart from other compounds; CREELMANN and MULPURI 2002, MATSUI et al. 2006). Additionally, methyl jasmonate (Fig. 0-1b) develops, which is the methyl ester of one of the most important plant

hormones with an important role in defense (CREELMANN and MULPURI 2002, CHEHAB et al. 2005, HALITSCHKE and BALDWIN 2005, HEIL 2008). Brassicaceae plants (and few further plant families) are equipped with glucosinolates, which are cleaved by the enzyme myrosinase when plant tissue is damaged (SITTE et al. 2002). This leads to the emission of ***isothiocyanates*** (Fig. 0-1c), which are antibiotic (SMOLINSKA et al. 1997, SMOLINSKA and HORBOWICZ 1999). For ***terpenes*** (Fig.0-1d-f) two generating ways are known (DEWICK 2002, DUDAREVA et al. 2004, AHARONI et al. 2005, THOLL 2006): in the plastids the methylerythriol phosphate pathway is responsible for the development of isoprenes (C5), monoterpenes (C10) and bigger terpenes (C20). Sesquiterpenes (C15) and terpenes with 30 or 40 carbon atoms are synthesized in the cytosol (melvalonate pathway). Modifications of the base frame conduce towards ***terpenoids*****,** which are also quite common in the plant volatile spectrum (DUDAREVA et al. 2004). Many terpenoids have antibiotic properties (i.e. AGGARWAL et al. 2002, GARCIA et al. 2008). Some VOCs emerge after degradation of cell compounds. The cleavage of carotinoids for example leads to the release of terpenoids (AULDRIGE et al. 2006, SIMKIN et al. 2004).

STUDY ORGANISMS

PLANTS

Brassica napus L.

Brassicales; Brassicaceae

Brassica napus belongs to the family of Brassicaceae and is one of the most important crops in Germany. In 2010, 58.4 t oilseed rape were produced worldwide, 5.7% of that in Germany (LEF and LFL 2010). Many crops (and the most important model species in plant research, *Arabidopsis thaliana*) are members of that family. The scientific name gives suggestions to their special metabolites, the brassinosteroids, responsible for length growth and cell division, first discovered in Brassicaceae plants (SITTE et al. 2002, JÄGER et al. 2003). The usage of *Brassica napus* in the food industry and in research led to a huge number of different cultivars. In this work, the "Rapid Cycling Rape" (RCR) was used. Cultivated in the 1980s for research (WILLIAMS

and HILL 1986), it is characterized by a short developmental time (seven weeks from germination to seed production), which allows performing experiments to take as little time as possible (MUSGRAVE 2000). Variations due to the cultivation parameters are possible.

Brassica napus can be infested by several herbivores and microbial pathogens i.e. several fungal pathogens (such as *Sclerotinia sclerotiorum, Phoma lingam, Alternaria brassicae* and two *Verticillium*-species: *V.dahliae* and *V. longisporum*) (BÖRNER et al. 2009), which often lead to big losses in yield.

Arabidopsis thaliana(L.) Heynhold

Brassicales; Brassicaceae

Arabidopsis thaliana might be the best-known plant in research (SOMERVILLE and KOORNNEFE 2002). Because of its relative small and completely sequenced genome, the plant is often used to investigate biochemical or molecular biological issues. The genome can easily be manipulated and the plant has a short life cycle. Furthermore, *Arabidopsis thaliana* belongs to the family of Brassicaceae and is related to many important crop plants, such as *Brassica napus* (for review see SOMERVILLE and KOORNNEFE 2002). Many studies deal with the interaction between *Arabidopsis thaliana* and *Verticillium longisporum* (i.e. STEVENTON et al. 2001, VERONESE et al. 2003, TISCHNER et al. 2010). They proved that *A. thaliana* is susceptible for the *Verticillium* wilt caused by *Verticillium longisporum*. Infected plants show distinct symptoms up to 20 dpi, such as a significantly lower amount of chlorophyll in the leaves and a smaller leaf area (FLÖRL et al. 2010). At the time of 35 dpi, FLÖRL et al. (2010) observed a significantly smaller fresh weight of the rosettes of infested plants than of non-infested plants.

FUNGI

Verticillium longisporum (ex. *V. dahliae* var. *longisporum* Stark; comb. nov. Karapapa)

Ascomycota; Plectosphaerellaceae

Verticillium longisporum belongs to the ascomycota. As Eynck et al. (2007) noticed, it is still subject of discussion, whether *V. longisporum* is an own species (KARAPAPA et al. 1997, FAHLESON et al. 2004) or a variation of *V. dahlia* (STARK 1961, FAHLESON et al. 2003). *V. dahliae* has a broad host range and is responsible for a wilt disease on different plant species, while *V. longisporum* is specialized on *Brassica* species (KARAPAPA et al. 1997, ZEISE und TIEDEMANN 2002, JOHANSSON et al. 2006). Recent studies suggest the origin of *Verticillium longisporum* lies in hybridization of three different parental species, resulting in an increased virulence (INDERBITZIN et al. 2011).

Life cycle and morphology of *Verticillium longisporum*

Fig. 0-2: *Verticillium longisporum*, mycelium and microsclerotia on PDA

Verticillium longisporum is a fungal pathogen, infecting plants through the roots and initially colonizing the xylem. The *Verticillium* wilt is a typical disease of crop rotation (DAEBLER et al. 1988). There is no possibility of controlling the *Verticillium* wilt neither with fungicides nor with resistant cultivars or biological control (AMELUNG et al. 1996, ZEISE & STEINBACH 2004). The typical disease symptoms of a *Verticillium longisporum* infection are stunting and chlorosis of plants before ripening (EYNCK et al. 2007). A premature ripening can often be observed in infested plants (EYNCK et al. 2007). In laboratory experiments, the fungus develops a white

mycelium (Fig.Fig. 0-2) which changes to black after few days because of microsclerotia formation (personal observation). Microsclerotia are resting bodies, responsible for fungal survival over several years in the soil without a host plant (HEALE and KARAPAPA 1999). Root exudates of the host lead to the germination of the microsclerotia (MOL and VANRIESSEN 1995). As EYNCK et al. (2007) summarized, the development of *V. longisporum* infection is divided into three phases: (I) the dormant phase, (II) the parasitic and (III) the saprophytic phase (as illustrated in Fig. 0-3).

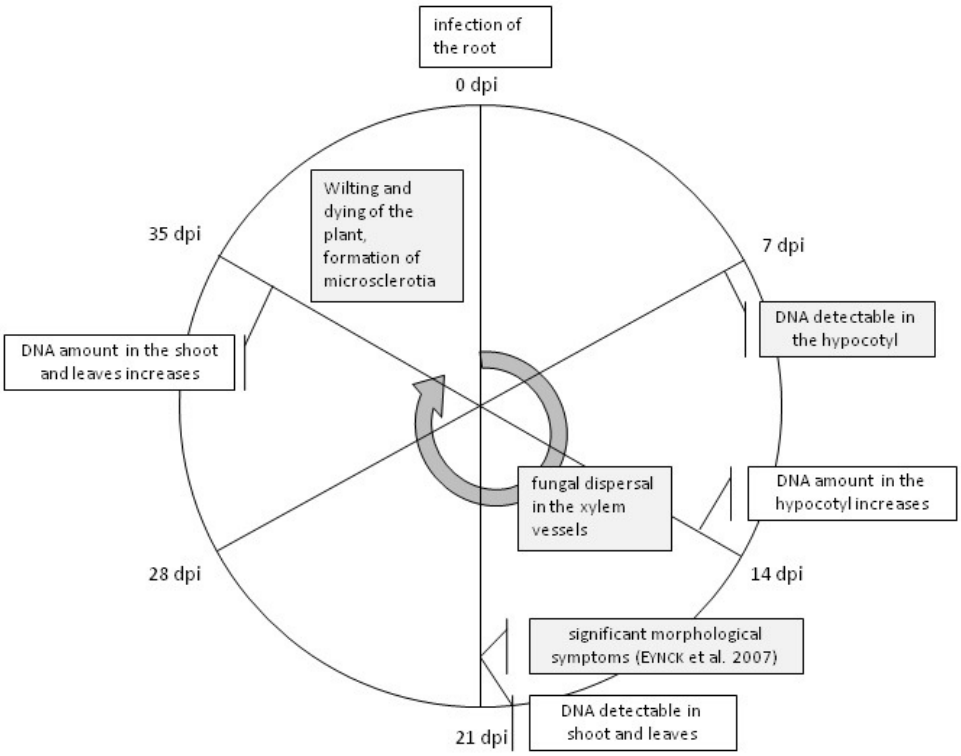

Fig. 0-3: infection development of *Brassica napus* with *Verticillium longisporum*, symptom formation and fungal dispersal in the plant. This figure orientates on observations of Eynck et al. (2007)

While the first phase implicates the storage of microsclerotia in the soil, the second phase starts with the penetration of epidermal root cells. Already 48 hours past inoculation (hpi), the hyphae of the young mycelium covers the roots like a net. The mycelium does not develop any special structures for penetration (at 60 hpi). The fungus colonizes the plant root inter- and intracellular until reaching the xylem vessels in the shoot at 21 days post inoculation (dpi). The formation of microsclerotia on dying plant tissue introduces the third, the saprophytic phase.

After the plant death, microsclerotia reach the soil and are able to infect again. The plant shows chlorosis and dark veins up to 14 dpi, but no typical wilting symptoms (EYNCK et al. 2007).

Gaeumannomyces graminis (Sacc.) Arx et Olivier *var. triciti* Walker

Ascomycota, Magnaporthaceae

Gaeumannomyces graminis var. *triciti,* the take-all fungus, is a specialist on wheat and other grain. The life cycle of *G. graminis* var. *triciti* is very similar to the life cycle of *Verticillium longisporum*. *G. graminis* var. *triciti* infects the host plants through the roots and disperses inside the plant via the vessels. The roots lose their function, which leads to interference of growth, followed by a disturbed uptake of water and nutrients (HOFFMANN and SCHMUTTERER 1999) *G. graminis* var. *triciti* causes the dark brown coloration of roots and leaf sheets, stunting of the shoot, bleaching and premature ripening (SMITH et al. 1988, BÖRNER et al. 2009). After harvest, resting mycelium reaches the soil. Further dispersal proceeds by ascospores. It is responsible for yield losses of wheat up to 70%. Changes in crop rotation and supporting antagonistic micro fauna in combination with Silthiofam® is suitable (SMITH et al. 1988, BÖRNER et al. 2009, Bayerischen Landesanstalt für Landwirtschaft).

Sclerotinia sclerotiorum (Lib.) de Bary

Ascomycota, Sclerotiniaceae

The white mold, *Sclerotinia sclerotiorum,* is a fungus with a huge host spectrum. Apart from *Brassica napus*, the fungus attacks several different crops belonging to over 64 different families including Solanaceae (i.e. tomato), Asteraceae (i.e. lettuce, chicory, sunflower), Brassicaceae (i.e. rape, cabbage), Apiaceae (i.e. carrot, celery) etc. (SMITH et al. 1988, BÖRNER et al. 2009). In contrast to the presented root borne fungi, *S. sclerotiorum* infects its host by penetrating plant surfaces on aboveground parts, above all the leaf axils (ADAMS and AYERS 1979, LUMSDEN 1979, BÖRNER et al. 2009). An infestation on oilseed rape causes chlorosis and premature ripening (BÖRNER et al. 2009). After harvest, sclerotia developed inside the plant reach the soil and can survive there for many years until infecting a new host (BÖRNER et al. 2009). The fungus is an important pathogen of agriculturally important crops as well as a model organism for scientific research (SMITH et al. 1988). In order to control the white mold, several appropriate methods are

known (changes in rotation and treatment with fungicides and calcium cyanamide) (HOFFMANN and SCHMUTTERER 1999, BÖRNER et al. 2009).

AIM OF THE WORK AND OBJECTIVES

The general focus of this study was to investigate whether an infection of *Brassica napus* with the root borne pathogen *Verticillium longisporum* effects the VOC emission of the plant. In order to observe the odor changes during the infection, a system for taking separate samples from the shoot and the root of a plant without damaging or stressing the plant was developed. Furthermore, possible ecological functions of different VOCs were investigated in the interaction with pathogenic organisms.

Hypotheses accompanying this study were:

I. The infection of *Brassica napus* and *Arabidopsis thaliana* with *Verticillium longisporum* affects the VOC emission of the plant shoot.

II. The infection of *Brassica napus* and *Arabidopsis thaliana* with *Verticillium longisporum* also affects the VOC emission of the plant root.

III. VOCs emitted by Brassicaceae have antifungal effects on generalists and fungi not specialized on Brassicaceae.

IV. VOCs emitted by Brassicaceae have limited antifungal effects on the specialized fungus *Verticillium longisporum*.

CHAPTER OUTLINE

In this work, the interaction between *Brassica napus* and *Verticillium longisporum* was observed with special interest in volatile organic compounds released by infested plants. This work is divided into two parts:

The first part: Changes in the VOC profile of *Brassica napus* and *Arabidopsis thaliana* during an infection with the root borne fungus *Verticillium longisporum* was investigated. Headspace of VOCs were sampled from shoot and root separately. Measurements were conducted on non-damaged and non-stressed plants (**chapters I, II and V)**.

The second part: Volatile organic compounds conspicuous during infection were tested on their antifungal activity. Therefore, a non-contact bioassay was improved and adapted on *Verticillium longisporum*. To clarify the ecological function of the applied volatile compounds in defense against pathogens, two further pathogenic fungi, *Gaeumannomyces graminis* var. *triciti* and *Sclerotinia sclerotiorum* were included in the experiments. The development of the bioassay is presented in **chapter III**. The experiments on all three fungi are shown in **chapter IV**.

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CHAPTER I

BRASSICA NAPUS AND THE ROOT BORNE PHYTOPATHOGENIC FUNGUS *VERTICILLIUM LONGISPORUM* – ALTERATIONS IN THE PATTERN OF SHOOT VOLATILES

ABSTRACT

Verticillium longisporum is one of the most important phytopathogenic fungi for *Brassica napus* in agriculture. Little is known about their interaction. A close-to-nature sampling method of shoot volatiles was designed in order to avoid changes in volatile pattern due to damaging or stressing the plant during measurements. The samples were analyzed by way of GC-MS. Infection specific symptoms and released volatiles have been monitored for four weeks. In the fourth week significant alterations in the volatile pattern were observed. Infected plants release significantly more ß-ionone, ß-cyclocitral and dimethyl disulfide than healthy plants.

INTRODUCTION

The interaction of phytopathogenic fungi and their hosts is not yet fully understood. One interesting system from the agricultural point of view is the "*Verticillium* wilt". This study focuses on the interactions between the pathogenic fungus *Verticillium longisporum* and the economically important crop plant *Brassica napus*. *V. longisporum* is specialized on *Brassica* (ZEISE and VON TIEDEMANN 2002). Infected *Brassica* plants show stunting, chlorosis and premature ripening. Thus, *Verticillium* is causing big yield losses every year (JOHANSSON et al. 2006 a, EYNCK et al. 2007). No effective *and* ecologically agreeable method is known to us which allows to prevent or control this disease (FRADIN and THOMMA 2006, FAN et al. 2008). The disease is conquering the plant via the root system and spreads through the vascular system into the shoot. Fungal DNA is detectable one week after infection in the hypocotyls, significant amounts of DNA in the leaves were shown at 28 dpi. (FLÖRL et al. 2008). When the plant dies, the fungus forms microsclerotia which serve for further dispersal (KARAPAPA et al. 1997, JOHANSSON et al. 2006 a, EYNCK et al. 2007). Infected *Brassica* plants show a higher level of salicylic acid (and its glycosides) at least at 14 days post inoculation (dpi) while jasmonic acid and abscisic acid are not affected (RATZINGER et al. 2009). There are also some indications for a participation of ethylene (JOHANSSON et al. 2006 b on *Arabidopsis thaliana*). Several proteins (such as ß-1,3 – glucanase and pathogenesis related proteins) are found in higher amounts when *Brassica napus* is infected. The net photosynthesis and transpiration of infected plants is not affected until 21 dpi (FLÖRL et al. 2008). In *Arabidopsis thaliana,* several genes involved in defense, cell wall degradation, proteolysis, defense, signaling and more are up-regulated already 50 minutes after roots contact fungus conidia (isolate 40; TISCHNER et al. 2010).

Plant surfaces release volatile organic compounds (VOCs) as a result of plant metabolism (PEÑUELAS and LLUSIA 2004) mediating diverse ecological functions (i.e. PICHERSKY and GERSHENZON 2002, HOLOPAINEN 2004, GERSHENZON and DUDAREVA 2007). Any change may indicate defense, senescence, herbivory, pathogenic activity, nutrient deficiency etc. (recently reviewed by HOLOPAINEN and GERSHENZON 2010), providing a tool to monitor and to understand the interactions between organism. A lot of compounds released by plants are known to be effective

infochemicals in the interaction with insects (i.e. BRUCE et al. 2005) and other plants (BALDWIN et al. 2006, GERSHENZON 2007). Moreover, communication with microorganisms is imaginable as MENDGEN et al. (2006) were able to show, using the example of the pathogenic rust fungus *Uromyces fabae* on *Vicia faba*. Antimicrobial properties of several volatiles such as terpenoids (AGGARWAL et al. 2002, MIMICA-DUKIE et al. 2002, GARCIA et al. 2008) and isothiocyanates (ANGUS et al. 1994, OLIVIER et al. 1999, RHAMANPOUR et al. 2009 and other) are documented.

In this study we focus at possible changes in volatile patterns released by *Brassica napus* following an infection by *Verticillium longisporum*.

MATERIAL AND METHODS

CHEMICALS

Applichem (Germany, Darmstadt): Sodium hypochlorite

Fluka (Germany, Steinheim): $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, FeO_4S

Merck AG (Germany, Darmstadt): $CaCl_2$, KNO_3, H_2BO_3, KH_2PO_4, EDTA, $(NH_4)_2SO_4$, K_2HPO_4

Carl Roth GmbH & Co. KG (Germany, Karlsruhe): Na_2MoO_4, Agar-Agar, Tween 20

Sigma-Aldrich Chemie GmbH (Germany, Steinheim): Czapek-Dox media, Potato-Dextrose broth

PLANT MATERIAL

Seeds of *Brassica napus* (RCR, AG von Tiedemann, University of Goettingen) were sterilized with Ethanol, Sodium hypochlorite and Tween-20 (Roth; Karlsruhe, Germany) (CLOUGH & BENT 1998) and germinated on 0,5% Agar (10mM KPP-buffer, 125µM Fe-EDTA, 2mM $MgSO_4$, 1mM $CaCl_2$, 2mM$(NH_4)_2SO_4$, 3mM KNO_3, 125µM H_2BO_3, 30µM $MnSO_4$, 2.5µM $ZnSO_4$, 2.5µM $CuSO_4$, 0.5µM Na_2MoO_4). One week old plants were infected with the root burning pathogen *Verticillium longisporum* (isolate 43) via root dipping for 30 min in a suspension of 10^6 spores/ml (according to KOIKE et al. 1994). Control plants were treated with sterile tap water. They grew up in climate chambers (16h light; 19°C±2) in single clay pots (Ø 8cm), filled with a sterilized mixture of soil

(Archut Früstorfer Erde; Germany Vogelsberg) and sand (Vitakraft; Germany, Bremen) (1:1) and watered with sterile tap water. When planting the seedlings in the pots, plants were placed in a clean and sterilized collar of PTFE (Polytetrafluorethylen; see Fig. AI-2). Clay pots and sand were cleaned before with ethanol (70%) and distilled water, heated at 120°C for 4 h and sterilized by autoclaving. The soil was also sterilized by autoclaving.

FUNGAL MATERIAL

Verticillium longisporum (isolate 43; Dept. of Crop Science, University of Goettingen) was grown in Czapek-Dox liquid media (Sigma Aldrich Chemie GmbH; Munich, Germany) and horizontal shook (app. 100 rpm) in an incubator (at room temperature and darkness). After harvesting, spores were diluted to 1 x 10^6 spores /ml.

SUCCESS OF INFECTION

The success of infection was evaluated by measuring shoot length and fresh weight of leaves and roots at the end of each experiment (28 dpi).

SAMPLING

Brassica napus plants were measured at 7, 14, 21 and 28 dpi - eight infected plants and eight control plants. During sampling, each plant was surrounded by a glass vessel. Headspace of *Brassica*-shoots was separated from the soil by a PTFE plate without damaging the plant (see Fig. AI-2). In this way it was possible to sample the volatiles only from the above ground headspace without producing artifacts by damaging or stressing the plant (VLAIC and SCHÜTZ 2009). Synthetic air, cleaned by a charcoal filter, was sucked by a pump through the glass vessel and onto the adsorbents (Tenax®- TA type; 175-180 mg; Gerstel; Mühlheim an der Ruhr, Germany; air flow: 270 ml/min, 2h sampling time). The whole experiment was repeated once.

ANALYSIS

Samples trapped on Tenax® TA were analysed by using gas chromatography (6890N) coupled with a 5973 mass spectrometer (both: Agilent Technologies; Santa Clara, USA). To insert the volatiles in the measuring system, a thermo-desorption system with cold injection system (TDS-

CIS, Gerstel; Mühlheim an der Ruhr; Germany) was used. Sampled volatiles emerge from the adsorbens by heating to 280°C and lead with helium (carrier gas) through a trap (-100°C) to the column (non-polar, HP – 5MS column; 30 m x 0.25 mm, i.d., 0.25 µm film thickness; Agilent Technologies; Santa Clara, USA). Beginning with 40 °C held for 3 min, the samples were heated 7.5°C/min up to 200°C (held for 5 min). The scanning mass range of the mass spectrometer was 11 – 300 amu.

Compounds were preliminary identified with Enhance Chemstation version D00.00.38 (Agilent Technology; Santa Clara, USA) and National Institute of Standards and Technology (NIST; Gaithersburg, USA) Mass Spectral Search Library. The identification was confirmed by matching of mass spectra and the linear retention index (VAN DEN DOOL AND KRATZ 1963) with those of commercially available authentic standards (for further information, see Tab. I-2). To quantify the compounds, the peak area of single ion chromatogram (SIC) from characteristic masses was used.

STATISTICS

Data was statistically analysed by using Statistica 7.0 (StatSoft; Tulsa, USA) by using the Mann-Whitney-U test, respective the HSD test. Differences were tested on significance with *P*-values < 0.05.

RESULTS

MORPHOLOGICAL DETAILS

Infected plants showed significantly reduced fresh weight of leaves and roots at 28 dpi (Fig. I-1). At 28 dpi shoots of infected plants were significant smaller compared to those of the control plants. Further measurements were only performed with plants, showing the described infection symptoms as “infected plants” and with plants not showing any of the symptoms as “healthy plants”, respectively.

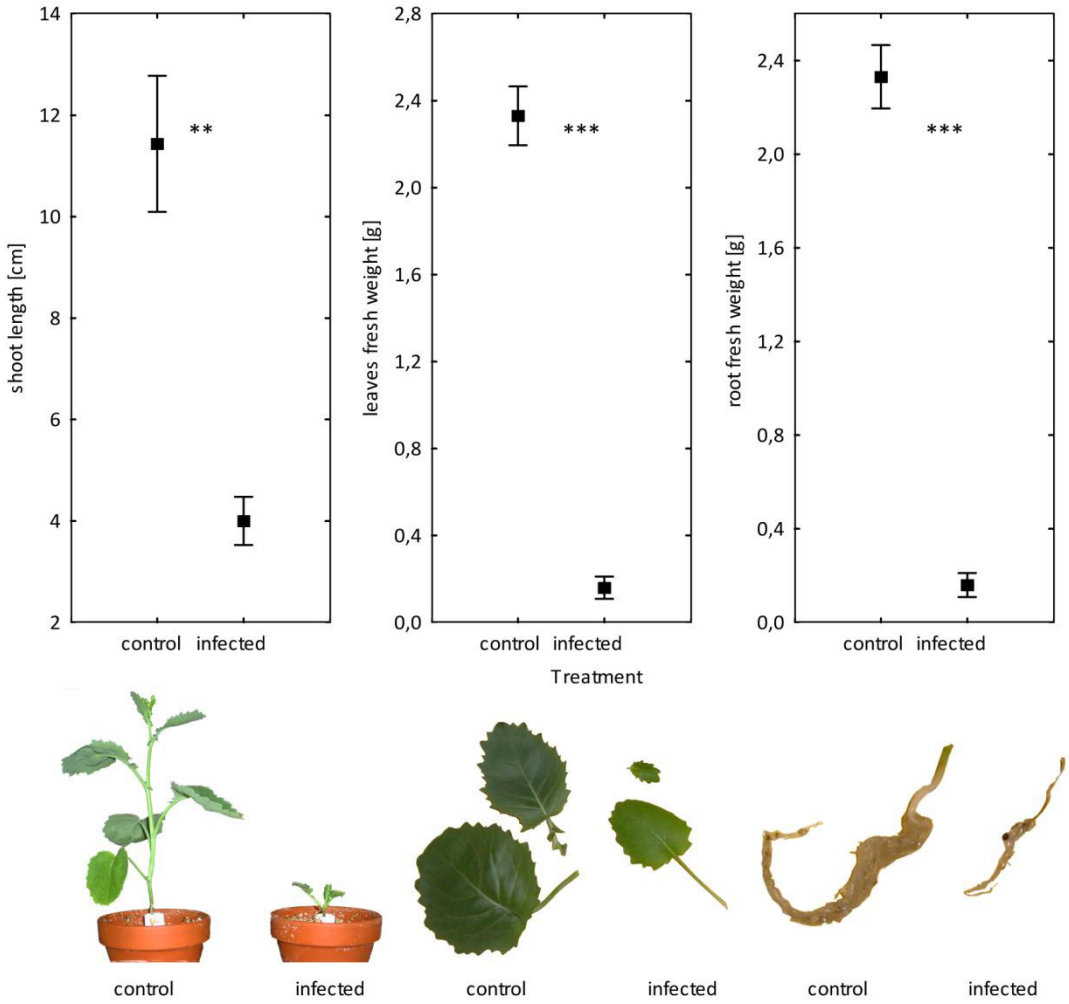

Fig. I-1: Differences in shoot length and fresh weight of leaves and roots of *Brassica napus* due to the infection with *Verticillium longisporum*, 28 dpi; mean ± SE; n= 16; * marks data significantly different to the control plants (HSD; $P < 0,05$); photos below diagram: comparison of control (left) and infected plants (rigth; at 28 dpi)

VOLATILE PATTERN

TIC (Total ion Chromatogram)

The headspace of the *Brassica napus* shoot contained more than 120 compounds – such as terpenoids, aromatic compounds, alicyclic compounds, alcohols, aldehydes, ketones, carboxylic acids, esters and alkanes. The emission amount changes during infection. Fig. I-2 contains the time course of the representative TIC of healthy, respective infected plants. While the emission of infected and non-infected plants showed a significant decrease of the total amount of VOCs in

the first phase (7 to 14 dpi), the significant increase was in the second phase (14 to 28 dpi) confined to the infected plants (for *P*-values please see Tab. I-1). The emission of the healthy plants still remained on a low level.

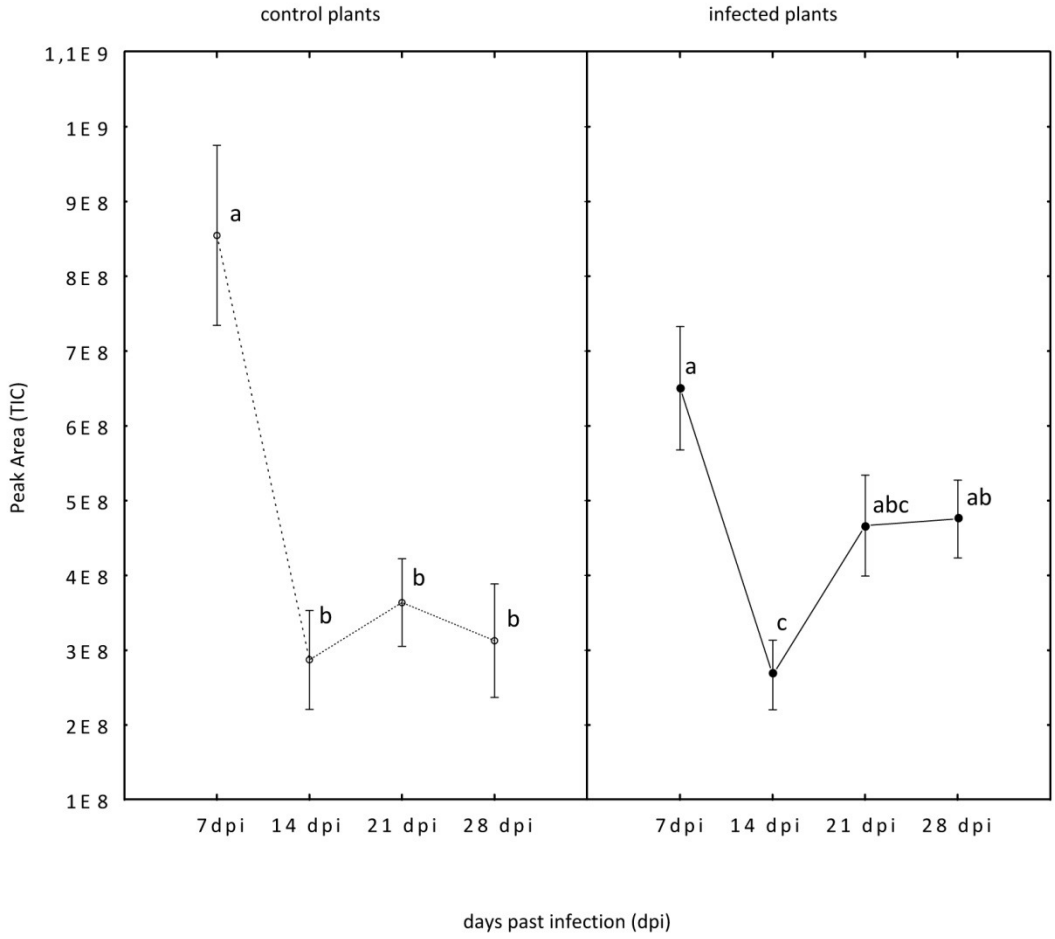

Fig. I-2: Comparison of the total abundance of comounds (total peak areaof TIC; 5-20min; manual integration) of *Brassica napus* plants during the experiment (7, 14, 21 and 28 dpi; mean ± SE), lines only serve for better visualisation, the letters mark significances; for statistical details see Tab. I-1

Tab. I-1: Results of Mann-Whitney-U Tests (*P*- and z- values in accordance to Fig. I-2) Comparison of control versus infected plants at a given dpi (dark grey boxes); Comparison of the dpi among control plants (white boxes); Comparison of the dpi among infected plants (light grey boxes). Significant differences are marked with *($P < 0,05$), tendencies with a · (near significant), (n=4 – 8 per treatment and sampling).

	7 dpi		**14 dpi**		**21 dpi**		**28 dpi**	
	***P*-value**	**z-value**	***P*-value**	**z-value**	***P*-value**	**z-value**	***P*-value**	**z-value**
7 dpi	0.201	1.28	0.034*	2.12	0.017*	2.28	0.021*	2.31
14 dpi	0.006*	2.74	0.881	0.15	0.414	-0.82	0.723	-0.35
21 dpi	0.125	1.53	0.072·	-1.80	0.211	1.25	0.734	0.34
28 dpi	0.201	1.23	0.027*	-2.20	0.877	-0.15	0.083·	1.73

altered volatiles

After analyzing the data in detail, VOCs significantly changing during infection were listed (see Tab. I-2). Infected *Brassica napus* plants emitted significantly more ß-Ionone, ß-cyclocitral and dimethyl disulfide than non-infected plants at 28 dpi (Fig. Fig. I-3). Dimethyl disulfide was detected in the highest amounts, while ß-cyclocitral was always detected in low amounts. Both compounds were detectable throughout the whole experiment, both in control and infected plants, whereas ß-ionone appeared in both cases (control and infected plants) only in the last phase (28 dpi).

Marker compounds for mechanical damage such as (Z)-3-hexen-1-ol, isothiocyanates and others were not detected, neither in control nor in infection treatments (see Fig. AI-1; Appendix I).

Tab. I-2: VOCs of *Brassica napus*, significantly different between plants non-infected with *Verticillium longisporum* and their identification parameters (Retention Index, source of authentic standards and purity)

Substance	**CAS number**	**RI**	**Standard (purity, origin)**
Dimethyl Disulfide	624-92-0	746	98%, Merck AG; Germany, Darmstadt
ß-Cyclocitral	432-25-7	1231	90%, SAFC; Germany, München
ß-Ionone	79-77-6	1499	96%, ABCR; Germany, Karlsruhe

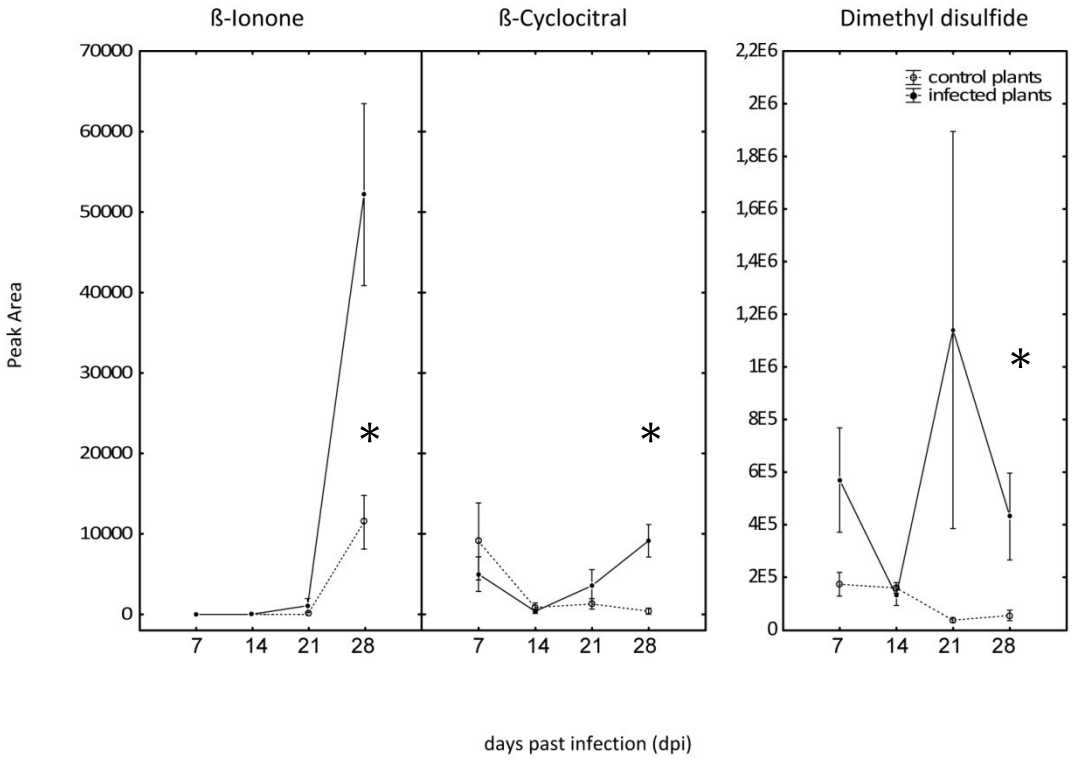

Fig. I-3: Time course (dpi) of abundance of ß-ionone, ß-cyclocitral and dimethyl disulfide, emitted by *Brassica napus* during the experiment; black line: control plants, interupted line: infected plants (mean ± SE), lines only serve for better visualisation; for statistical information see Tab. I-3.

Tab. I-3: Results of Mann-Whitney-U Tests (*P*- and z- values in accordance to Fig. I-3) contrasting abundance of ß-ionone, ß-cyclocytral and DMDS. Comparison of control versus infected plants at a given dpi (dark grey boxes); Comparison of the dpi among control plants (white boxes); Comparison of the dpi among infected plants (light grey boxes). Significant differences are marked with *($P < 0,05$), tendencies with a · (near significant), (n = 4 – 8 per treatment and sampling).

		7 dpi		14 dpi		21 dpi		28 dpi	
		***P*-value**	**z-value**	***P*-value**	**z-value**	***P*-value**	**z-value**	***P*-value**	**z-value**
ß-Ionone	**7 dpi**	1.00	0.00	1.000	0.00	1.000	0.00	0.014*	-2.45
	14 dpi	1.000	0.00	1.00	0.00	1.000	0.00	0.021*	-2.31
	21 dpi	0.480	-0.71	0.505	-0.67	0.441	-0.77	0.007**	-2.72
	28 dpi	0.011*	-2.56	0.014*	-2.45	0.005**	-2.78	0.021	-2.31
ß- Cyclocitral	**7 dpi**	0.597	0.53	0.826	1.74	0.062·	1.87	0.066·	1.84
	14 dpi	0.097	1.66	0.775	0.23	0.841	-0.20	0.715	-0.37
	21 dpi	0.526	0.64	0.113	-1.58	0.279	-1.08	0.533	0.62
	28 dpi	0.062·	-1.87	0.007**	-2.72	0.021*	-2.31	0.013	-2.48
DMDS	**7 dpi**	0.110	-1.599	0.897	0.13	0.029*	2.19	0.056·	1.91
	14 dpi	0.064·	1.85	0.475	-0.71	0.0009***	3.32	0.015*	2.43
	21 dpi	0.131	1.51	0.626	0.49	0.159	-1.41	0.651	0.45
	28 dpi	0.722	0.36	0.203	-1.27	0.248	-1.16	0.015	-2.43

DISCUSSION

The infection of *Brassica napus* with the soil-born pathogenic fungus *Verticillium longisporum* caused significant alterations in morphology and emission. Our observations concerning morphological disease symptoms confirm already published data (EYNCK et al. 2007, FLÖRL et al. 2008). Shoot length, shoot and root fresh weight of infected plants was significantly smaller than those of the control plants at 28 dpi. We investigated the alterations of the plant emission patterns during the infection. A significant increase of the total amount of VOCs, released by infected plants, was observed. While healthy plants stayed on a minimum level of VOC emission, dimethyl disulfide and the terpenoids ß-ionone and ß-cyclocitral were released by infected

plants at 28 dpi in significantly higher amounts. These observations might show plants reaction towards fungal pathogens. The terpenoids belong to the usual volatile emission of several plants in different compartments (KNUDSEN et al. 1993, MCEWAN et al. 1998, BALDWIN et al. 2000, ROHLOFF and BONES 2005, MORAGA et al. 2009, FERNANDES et al. 2009, TAVEIRA et al. 2009). Enzymatic cleavage of ß-carotene is most probably the source of ß-ionone and ß-cyclocitral (SIMKIN et al. 2004 a and 2004 b, AULDRIGE et al. 2006). The antimicrobial effect of several ß-ionone derivatives on *Fusarium solani*, *Botrytis cinerea* and *Verticillium dahliae* has already been proven in the 1980s (MIKHLIN et al. 1983). DENNIS and GUEST (1995) showed an inhibiting effect of ß-ionone on the infection of *Nicotiana tabacum* by *Phytophtera parasitica* var. *nicotianae*. Moreover, *Peronospora tabacina* (SCHILTZ 1974) and *Colletrotrichum musae* are inhibited by ß-ionone (UTAMA et al. 2002). Fungi are also able to cleave ß-carotene by enzymes (ZELENA et al. 2009) and release ß-ionone and ß-cyclocitral (CAMPOS ZIEGENBEIN and KÖNIG 2010). Recently it could be shown that ß-cyclocitral is released by the cyanobacterium *Mycrocyctis* NRC-1 (JÜTTNER et al. 2010) as a repellent to grazers like *Daphnia magna*. Additionally, they could prove that this substance almost do not occur in intact cells but only in disrupted ones. Dimethyl disulfide, a sulfur containing VOC, is known as a degradation product of enzymatic hydrolyzed proteins (GUO et al. 2010). ROUSEFF et al. (2008) detected its emission following artificial wounding of Guava leaves and ascribed it to defense potential against insects. Dimethyl disulfide is known to be highly neurotoxic on insects (DUGRAVOT et al. 2003). However, adapted organisms are able to detoxify or metabolize this sulfur compound (DUGRAVOT et al. 2004, FERNANDES et al. 2009). Microorganisms are also affected by dimethyl disulfide. This volatile compound inhibits the mycelium growth of *Paecilomyces lilacinus*, *Pochania chlamydospora*, *Fusarium culmorum*, *Rhizochtonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* (ZOU et al. 2007, KAI et al. 2009). Furthermore, *Chlonostachys rosea, Paecilomyces lilacinus*, *Pochania chlamydospora* are inhibited in their spore germination (CHUANKUN et al. 2004). The antibiotic effects of sulfur and related compounds (as isothiocyanates) suggest a role of dimethyl disulfide in plant defense.

The increasing total amount of VOCs (TIC) leads to the assumption, that VOCs are generally increasing under infection. But we did not detect the alteration or additional occurrence of any other known stress induced VOC. ß-Ionone, ß-cyclocitral and dimethyl disulfide seem to indicate decay or tissue disruption and probably play a role in defense against pathogens. Their enhanced emission of *Brassica napus* plants infected with *Verticillium longisporum* might show the degradation of cell compounds such as carotinoids and proteins in the last phase of infection (28 dpi). This suggestion has to be proven by appropriate methods. At least ß-cyclocitral and dimethyl disulfide seem to belong to the usual emission during plants life, because they can also be detected in the early infection phases and in controls.

CONCLUSION

First statistically significant alterations of volatile compound emission by infected rape plants were detected after 28 dpi. Thus, *Verticillium longisporum* seems to penetrate and colonize the root tissue of the plant without being recognized. Passing the hypocotyl and colonizing the leaves, the pathogen triggers the observed significant increase in volatile emission most likely related to the degradation of ß-carotene and proteins. We suggest that the emission of those VOCs is not induced by the infection with *Verticillium longisporum*. Nevertheless, ß-ionone, ß-cyclocitral and dimethyl disulfide are known to play a role in the interaction between organism. The release of these compounds may help the plant to avoid further infection by pathogenic microorganisms.

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APPENDIX

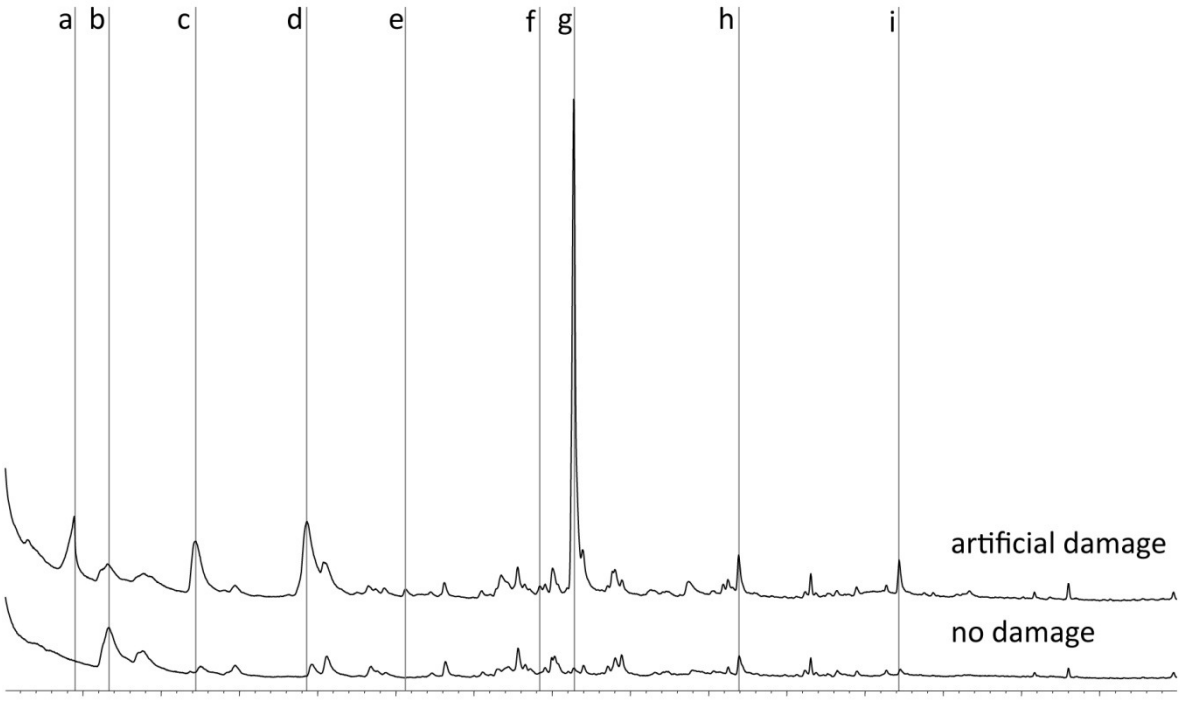

Fig. AI-1: Total Ion Chromatogram (TIC) of *Brassica napus*, non-infected. The figure compares plants artificial damaged by cutting the leaves and non-damaged plants; sampling directly after damaging; changing VOCs (a) acetic acid #, (b) dimethyl disulfide #, (c) 2-methyl-4-pentanal, (d) (*Z*)-3-hexen-1-ol #, (e) 2-penten-1-ol acetate, (f) 4-isothiocyanato-1-butene (g) (*Z*)-3-hexen-1-ol acetate #, (h) nonanal #, (i) decanal; compounds marked with a # are identified by artificial standards, non-marked compounds were tentatively identified by matching mass spectra and linear retention indices

Fig. AI-2: *Brassica napus* plants grown in a PTFE-collar (left) and inside a glass vessel during sampling (air gets lead into the vessel and sucked through a TDS-Tube (rigth))

CHAPTER II

DEVELOPMENT OF A VOLATILE SAMPLING SYSTEM FROM PLANT ROOTS

ABSTRACT

Plant roots and surrounding soil can be regarded as a specific compartment in soil with inter- and intraspecific interactions, biotic and abiotic influences – the rhizosphere. In order to analyze these compounds in the rhizosphere of *Brassica napus* plants, a sampling system for root volatiles was designed allowing damage free in-situ measurements under defined conditions. The system was established on *Brassica napus*. The results emerging from these in-situ samples were compared with results obtained by ex-situ methods. In order to evaluate whether the new method is suitable to monitor dynamic processes in the rhizosphere, plants were infected with the root borne pathogenic fungus *Verticillium longisporum* and tested at two different points of time after inoculation.

INTRODUCTION

Plants are able to interact with other organisms belowground and aboveground, like pollinators and herbivores (VAN POECKE 2007), often mediated by volatile organic compounds (VOC), released by the plant (BRUCE et al. 2005, DICKE et al. 2003, GERSHENZON 2007, PICHERSKY and GERSHENZON 2002). These infochemicals are often mirroring the metabolism of the plant, changes depend on biotic and abiotic factors as HOLOPAINEN and GERSHENZON (2010) recently reviewed. Plant roots interact in several ways with their environment: they release inorganic compounds like CO_2 (JOHNSON & GREGORY 2006), exudates (BERTIN et al. 2003) and VOCs (HITPOLD and TURLINGS 2008, WENKE et al. 2010). Root VOCs seem to be important for the orientation of soil inhabiting insects or other organisms. For instance, *Hylobius abietis* is affected in its oviposition by VOCs from conifer roots (NORDLANDER et al. 1986, LEATHER et al. 1999) and di- and trisulfides attract larvae of *Delia antique* (MATSUMOTO 1970).

Roots are surrounded by soil particles, water, air and (micro-) organisms, having an own flavor (LIEBMANN and EPSTEIN 1994, CHUANKUN et al. 2004), which could overlay plants odor. To avoid this, plants are usually removed from the substrate before measuring in order to describe this special root VOC spectrum. Several of the few publications, which are working with root VOCs, describe this method. The plants are removed from the pot and washed gently with water before headspace is sampled under airflow (i.e. LIN et al. 2007, RASMANN et al. 2011, WEISSTEINER & SCHÜTZ 2006). However, while removing the plant from the pot and preparing the roots, they will be damaged and taken from their familiar environment. In this way, plant tissue may still be alive, but the sampling process means stress, damages and most likely a change in humidity, temperature and other ecological factors. In some plant species an alternated root VOC spectrum was already shown after damaging. WEISSTEINER & SCHÜTZ (2006) documented the release of certain root VOCs after "massive root damaging" on *Daucus carota* and *Quercus* sp.. In contrast to these methods of gentle mechanical damage, hardly destructive methods are also used to describe roots bouquet. Distillation of dried pieces (ROHLOFF 2007, rhizome volatiles), methanol extraction (LIN et al. 2007, at 45°C), supercritical fluid extraction at non-natural temperatures

and pressure with solvents (TAPIA et al. 2007, roots in hexane) are some examples. Additionally, substrate influences root VOCs. This is not only because of the substrate own VOC spectrum, but because of the conditions it provides to the plant such as humidity, air, nutrition and the edaphon (GRIERSON and SCHIEFELBEIN 2002, WALTER et al. 2009, BONKOWSKI et al. 2009). LIN et al. (2007) designed an experiment in order to proove the effect of root preparation and growth conditions (substrate and watering) on the VOC spectrum. STEEGHS et al. (2004) used root cultures (*Arabidopsis* on PDA). To the best of our knowledge, no VOC measurements in hydroponically systems were performed yet. The results suggest the importance of a damage free and nature near measuring system in order to describe plant emission. This problem could be one reason that there is so little work on roots VOCs.

In this work, we present an approach to take volatile samples of plant roots minimizing damage and stress. Plants are grown in a substrate, which is poor on own volatiles, which serves important plant requirements regarding water, nutrients and air conditions and allows a volatile sampling inside the pot without removing the plant from its natural environment. Furthermore, this method allows realizing time series of single plants in order to observe their dynamic processes of VOC emission.

MATERIAL AND METHODS

CHEMICALS

Applichem (Germany, Darmstadt): Sodium hypochlorite

Fluka (Germany, Steinheim): $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, FeO_4S

Merck AG (Germany, Darmstadt): $CaCl_2$, KNO_3, H_2BO_3, KH_2PO_4, EDTA, $(NH_4)_2SO_4$, K_2HPO_4

Carl Roth GmbH & Co. KG (Germany, Karlsruhe): Na_2MoO_4, Agar-Agar, Tween 20

Sigma-Aldrich Chemie GmbH (Germany, Steinheim): Czapek-Dox media, Potato-Dextrose broth

PLANT MATERIAL

Seeds of *Brassica napus* (RCR) were sterilized with Ethanol, Sodium hypochlorite and Tween-20 (CLOUGH and BENT 1998) and germinated on 0.5% Agar (10mM KPP-buffer (K_2HPO_4 and KH_2PO_4), 125µM Fe-EDTA (FeO_4S and EDTA), 2mM $MgSO_4$, 1mM $CaCl_2$, 2mM$(NH_4)_2SO_4$, 3mM KNO_3, 125µM H_2BO_3, 30µM $MnSO_4$, 2.5µM $ZnSO_4$, 2.5µM $CuSO_4$, 0.5µM Na_2MoO_4).

PLANT CULTIVATION

The plants were grown in climate chambers (d/n: 16/8h; 19°C ± 2°C; 60% r.h.) in single pots (filled with sand-vermiculite mixture – 1:1) and watered with nutrition solution (same recipe as the previously described agar). After inoculation with the pathogen, each seedling was placed in a collar of PTFE (Polytetrafluorethylen) and planted in a clay pot. Sand (Vitakraft, Germany, Bremen), vermiculite (Deutsche Vermiculite Dämmstoff GmbH; Germany, Sprockhövel) and clay pots (Ø 8cm) were cleaned before with ethanol (70%) and distilled water, heated at 120°C for 4 h and sterilized by autoclaving. Plants were watered daily with sterilized nutrient solution.

FUNGAL MATERIAL

Verticillium longisporum (isolate 43; Dept. of Crop Science, University of Göttingen) was grown in Czapek-Dox liquid media and horizontal shook (app. 100 rpm) in an incubator (at room temperature and darkness). After harvesting, spores were diluted to 1 x 10^6 spores/ml.

INFECTION

One week old plants were infected with the root born pathogen *Verticillium longisporum* by root dipping (30 min in 1 x 10^6 spores /ml; according to KOIKE et al. 1994). Control plants were treated with sterile tap water.

SAMPLING OF ROOT VOLATILES

Undisturbed root method: A system consisting of a clay pot with a removable PTFE lid for separating roots from the shoot (see Fig. II-1) was developed.

The pot was filled by a sterilized and (with Aqua dest. and 70% ethanol) cleaned mixture of sand and vermiculite (1:1). This provides aeration, grip to the roots and constant humidity. Seedlings

were planted directly after infection, standing in a PTFE collar. During the sample taking, the plants were not removed from the pots and left untouched. The PTFE lid was placed on the collar to separate the air shoot headspace from the rhizosphere. The hole in the bottom of the clay pot was connected with a glass tube, filled with glass wool (siliconized; Merck AG; Germany, Darmstadt). The flow (270 ml/min; 1 hour) of charcoal cleaned air (filter: Messer; Germany, Griesheim) was lead through the glass tube inside the clay pot around the roots. This air was sucked through an outlet in the PTFE lid of the pot into the adsorbents (Tenax® - TA tubes; Gerstel; Mülheim an der Ruhr, Germany). After each measurement the PTFE lid was cleaned with Aqua dest. and 70% ethanol and dried at 60°C. Seven control plants and seven infected plants were measured at 14 days past infection (dpi) and 28 dpi. Between the time points, plants grew in the climate chambers.

Washing root method: Roots were carefully removed from the pot and washed with water free of the substrate. The roots were enveloped with a PET enclosure (Melitta Haushaltsprodukte GmbH & Co. KG; Germany, Minden) – with the shoot outside (see Fig. II-1). The airflow (270 ml/min; 1 hour) was wetted and lead into the PET enclosure. VOCs were sampled with a Tenax® - TA tube, in the end of the PET enclosure. The PTFE collar was not removed before measurement. Four control plants and four infected plants were measured after the last measuring time point of the undisturbed method.

Reference samples: In the case of the undisturbed method, a clay pot filled with substrate, watered and standing next to the plants in the climate chambers was measured in the described way. For the root washing method, the air inside an empty PET enclosure was sampled.

MICROORGANISM IN THE SUBSTRATE AND THEIR VOLATILES

In order to identify VOCs having their origin not in the plant roots but in microorganism from the rhizosphere, water (approx. 20 ml) rinsing through the sand and plants roots was collected in sterile test tubes and 100 µl were plated on PDA filled Petri dishes. After incubation at room temperature and darkness the colonies were separated and transferred to a new one. The VOCs

of the different morphotypes were measured inside a PET enclosure with a clean air flow at 270 ml/min by using Tenax® - TA tubes for one hour. The samples were analyzed using GC-MS.

Reference samples: For control measurements, VOCs of the pure agar were sampled.

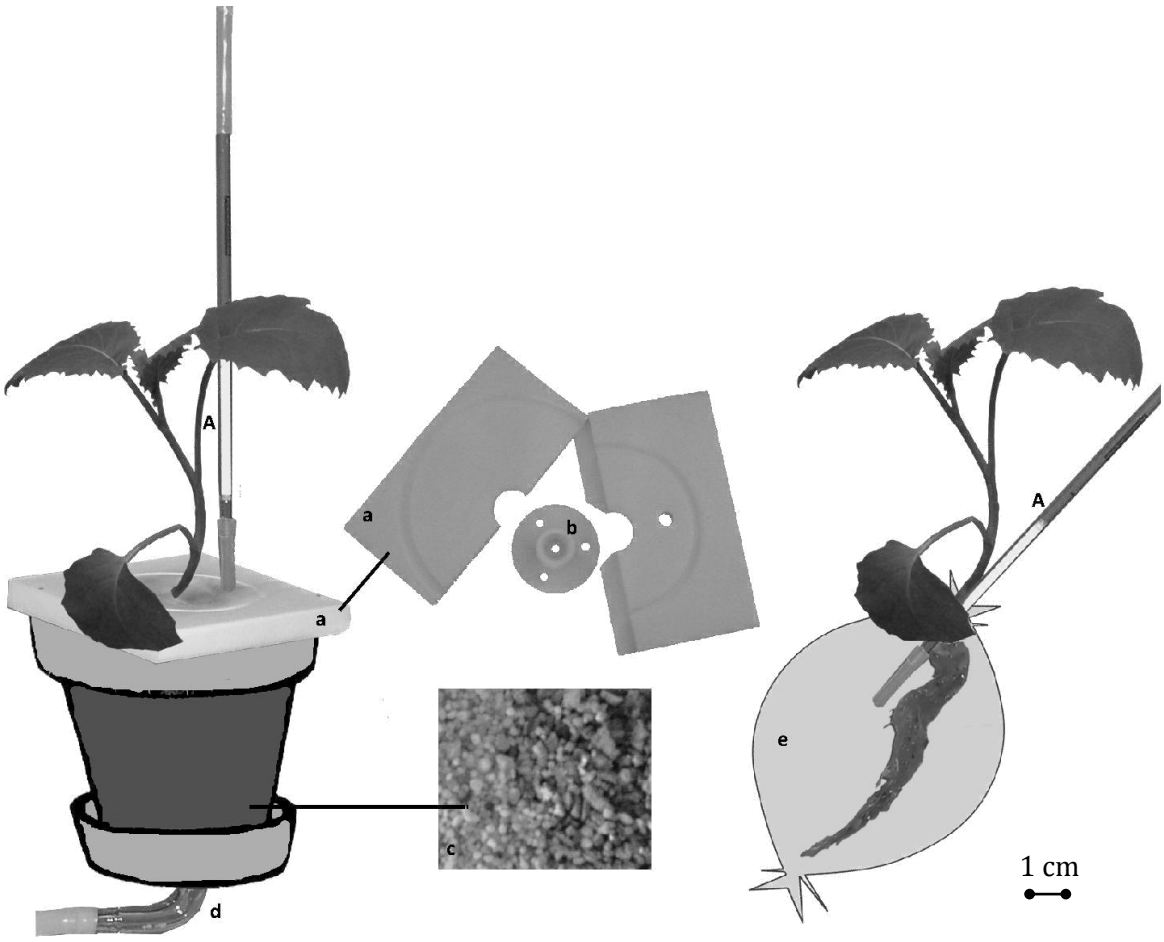

Fig. II-1: left: A plant grows up in a PTFE collar (b). Subtrate is a mixture of sand and vermiculite (c). During measurement air flows through a glass tube (d) through the bottom of the pot, passes the roots and the substrate nd gets sucked through a TDS-tube (A). A PTFE lid (a) separates the shoot's headspace from its rhizosphere. Right: A plant was removed from its substrate, gently washed and sampled in a PET enclosure (e). (Arrows illustrate airflow)

ANALYSIS OF VOLATILE COMPOUNDS

Samples trapped on Tenax® TA were measured using gas chromatography (6890N) coupled with a 5973 mass spectrometer (both Agilent Technologies; Santa Clara, USA). To insert the VOCs in the measuring system, a thermo-desorption system with cold injection system (TDS-CIS,

Gerstel; Mühlheim an der Ruhr; Germany) was used. Sampled volatile emerge from the adsorbens by heating to 280°C and lead with helium (carrier gas) through a trap (-100°C) to the column (non-polar, HP – 5MS column; 30 m x 0.25 mm, i.d., 0.25 µm film thickness; Agilent Technologies; Santa Clara, USA). Beginning with 40 °C held for 3 min, the samples were heated 7.5°C/min up to 200°C (held for 5 min). The scanning mass range of the mass spectrometer was 11 – 300 amu.

Tab. II-1: Identification parameters of detected VOCs

Substance	**CAS number**	**RI**	**Standard (purity, origin)**
Dimethyl Disulfide	624-92-0	748	98%, Merck AG; Germany, Darmstadt
Ethylbenzene	100-41-4	864	Mass spectra comparison
2,4-Dithiapentane	1618-26-4	895	Mass spectra comparison
Dimethyl Trisulfide	3658-80-8	976	98%, SVFC/Aldrich
3-Carene	13466-78-9	1009	100%, Roth
Limonene	138-86-3	1027	100%, Merck AG; Germany, Darmstadt
Eucalyptol	470-82-6	1032	100%, Merck AG; Germany, Darmstadt
Menthol	89-78-1	1180	Mass spectra comparison
Menthon	89-80-5	1171	Mass spectra comparison
Phenylethyl Isothiocyanate	2257-09-2	1465	99%, Sigma Aldrich; Germany, Steinheim

Compounds were identified with Enhance Chemstation version D00.00.38 (Agilent Technology; Santa Clara, USA) and National Institute of Standards and Technology (NIST; Gaithersburg, USA) Mass Spectral Search Library. The identification was confirmed by matching of mass spectra and the linear retention index (VAN DEN DOOL and KRATZ 1963) with those of commercially available authentic standards (see Tab. II-1) and from literature. To quantify the compounds, the peak area of single ion chromatogram (SIC) from characteristic masses was used.

STATISTICS

Data was statistically analyzed by using Statistica 7.0 (StatSoft; Tulsa, USA) with the Mann-Whitney-U Test (MWU). Differences were tested for significance with *P*-values < 0.05.

RESULTS

EMISSION OF *BRASSICA NAPUS* ROOTS (NON-INFECTED PLANTS)

The total amount of volatile compounds (TIC) measured from plants inside the clay pots was significantly higher than in the PET enclosure (see Fig. II-2). Following compounds were identified using standards: Dimethyl disulfide (RI 794), dimethyl trisulfide (RI 976), 3-carene (RI 1009), limonene (RI 1027) and eucalyptol (RI 1032). Ethyl benzene (RI 864), 2,4 – dithiapentane (RI 895), menthone (RI 1171) and menthol (RI 1180) were identified by matching the mass spectra with the NIST database and comparing the retention indices with those from literature. Dimethyl disulfide, ethyl benzene, 2,4 – dithiapentane and eucalyptol were found in both approaches. Dimethyl trisulfide, 3-carene, limonene, menthone and menthol were not detected in the root washing method. Further compounds found in the undisturbed method could not be identified or most likely did not have their origin in natural sources (such as Si-containing compounds and other). Limonene was only present in the undisturbed samples (see Fig. II-3). Eucalyptol was found in higher amounts when sampled with the undisturbed method (Fig. II-3). Phenylethyl isothiocyanates (RI 1465) was detected only in 1/7 of the undisturbed method samples, but in 50 % of the samples measured with the root washing method (because of the few samples, statistical analysis using the χ^2-Test was not possible). All other compounds did not differ significantly comparing both methods. Besides the described compounds, the VOC spectrum of plant roots measured with the undisturbed method contains several not identified benzenes.

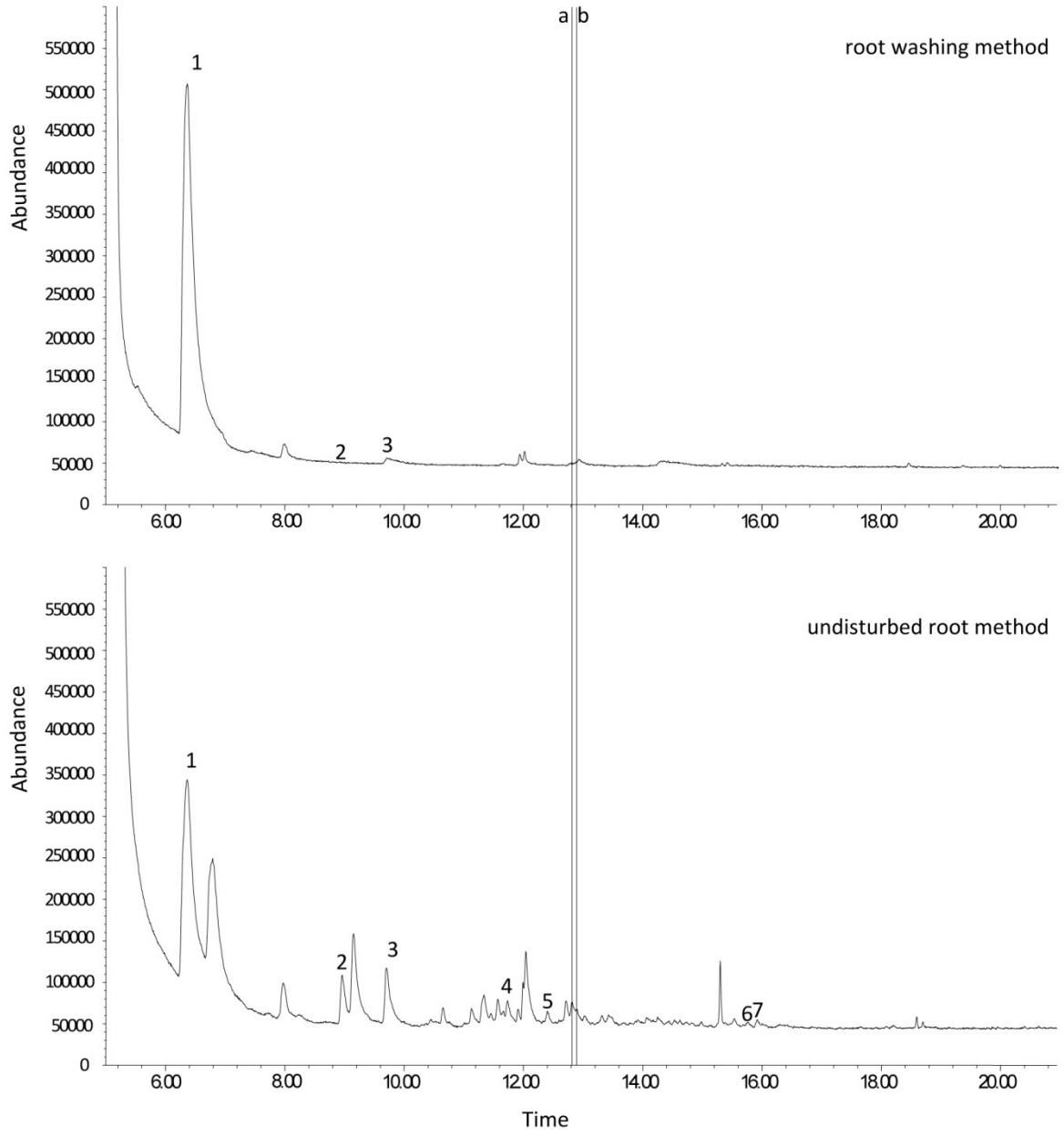

Fig. II-2: Total Ion Chromatogram (TIC; 5 – 20min) of root volatiles measured at 28 dpi by the root washing method (upper graphic) and the undisturbed method (lower graphic). Vertical lines mark limonene (a) and eucalyptol (b). Further volatiles: dimethyl disulfide (1) ethylbenzene (2), 2,4-dithiapentan (3), dimethyl trisulfide (4), 3-carene (5), menthone (6) and menthol (7).

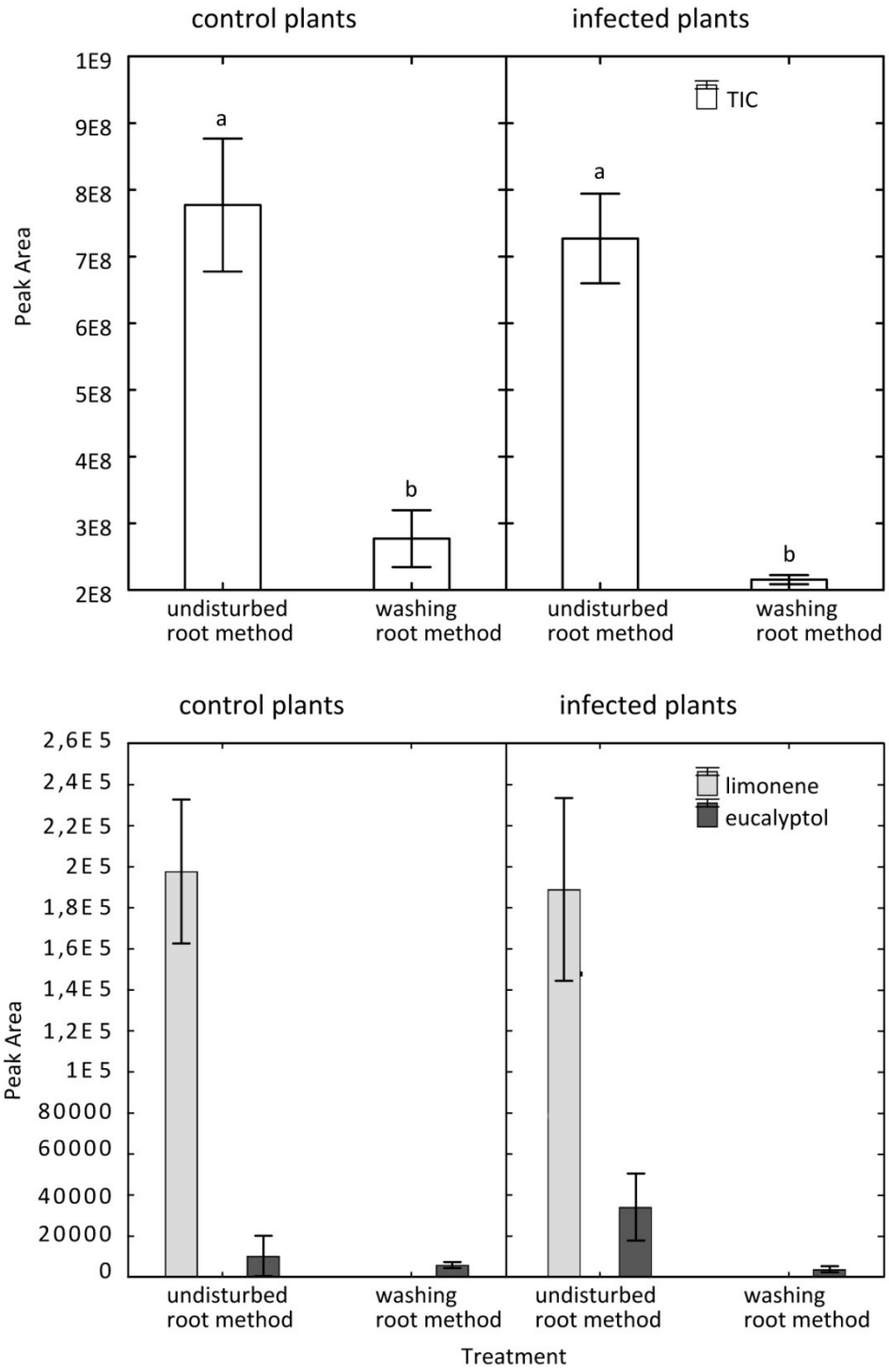

Fig. II-3: Comparison of the total abundance of compounds (upper diagram; total peak area of TIC; 5-20 min; manual integration) and of limonene and eucalyptol (lower diagram) released by *Brassica napus* roots (28 dpi). Each with two different sampling methods (undisturbed and root washing method) and two different treatments (healthy plants and plants infected with *Verticillium longisporum*); (mean ± SE, for statistical details please see Tab. II-2 and Tab. II-3)

Tab. II-2: Comparison of infected and non-infected plant root volatiles within one method; statistics were performed by using the Mann-Whitney-U Test; *marks compounds with significant difference ($P < 0,05$); n=22

Treatment	compound	Control plants		Infected plants			
		average	Standard error	average	Standard error	*P*	z
Undisturbed root method	TIC	777174551	104802422	727032802	70683629	0.655	0.45
	DMDS	10379056	3319010	9102041	2192315	0.848	0.19
	Limonene	197691	36913	188859	46780	0.949	0.06
	Eucalyptol	10319	10319	34295	17092	0.338	-0.96
	Phenylethyl-Isothiocyanate	1132299	843491	0	0	0.371	0.89
Washing root method	TIC	277159949	44922880	215514711	7105979	0.564	0.58
	DMDS	14267035	3795075	8034891	3156499	0.396	0.87
	Limonene	0	0	0	0	1.0	0.0
	Eucalyptol	5922	1534	4014	1548	0.386	0.87
	Phenylethyl-Isothiocyanate	4563609	4563609	1917470	1477066	0.773	-0.29

Tab. II-3: Comparison of the root volatiles sampled by two different methods within one treatment (infected and non-infected plants); statistics were performed by using the Mann-Whitney-U Test; *marks compounds with significant difference ($P < 0,05$), tendencies are marked with a •; n=22

Treatment	compound	Undisturbed root method		Washing root method			
		average	Standard error	average	Standard error	*P*	z
Control plants	TIC**	777174551	104802422	277159949	44922880	0.0082	2.65
	DMDS	10379056	3319010	14267035	3795075	0.3447	-0.94
	Limonene**	197691	36913	0	0	0.0082	2.65
	Eucalyptol•	10319	10319	5922	1534	0.059	-1.89
	Phenylethyl-Isothiocyanate	1132299	843491	4563609	4563609	0.925	-0.09
Infected plants	TIC**	727032802	70683629	215514711	7105979	0.0082	2.65
	DMDS	9102041	2192315	8034891	3156499	0.850	0.19
	Limonene**	188859	46780	0	0	0.0082	2.65
	Eucalyptol	34295	17092	4014	1548	1.0	0.0
	Phenylethyl-Isothiocyanate	0	0	1917470	1477066	0.186	-1.32

COMPARISON BETWEEN HEALTHY AND INFECTED PLANTS – IN BOTH APPROACHES

While the TIC at 14 and 28 dpi in the samples measured with the undisturbed method was almost the same in control and in infected plants, the PET enclosure approach at 28 dpi showed a slightly less emission of infected roots than healthy roots (see Fig. II-3; 14 dpi: data not shown). Neither limonene nor eucalyptol differed significantly under plant infection (Fig. II-3).

MICROORGANISMS IN RINSING WATER AND THEIR VOLATILES

Microorganisms isolated from rinsing water were characterized as morphotypes. Their distribution did not correspond with the treatments. Volatiles released by microorganisms isolated from rinsing water are listed in Tab. II-4. Only five of the VOCs were also detected in plant root samples. Acetic acid (RI 725) α-pinene (RI 936), 2-ethyl-1-Hexanal (RI 1027) and nonanal (RI 1113) were often found in the undisturbed method samples with a homogenous distribution between the treatments. Furthermore, benzaldehyde (RI 965) could be detected in the root washing method samples. *Verticillium longisporum* has never been cultivated from the rinsing water.

Tab.II-4: detected VOCs, emitted by the cultures isolated from the rinsing water

Chemical group	Alcohols	Acids	Other VOCs
Detected volatiles	2,3-dimethyl-1-Butanol	2-methyl-ethyl-ester Butanoic acid	2,4-dimethyl-Heptane
	1-Octen-3-ol	2-methyl Propanoic acid	2ethyl-1-hexanal
	1-Pentanol	2-methyl-3methyl-butyl-ester Butanoic acid	3-Hydroxy-2-Butanon
	2,3-Butanediol	3-methyl Butanoic acid,	3methyl-1-Butanol-acetate
	2-ethyl-1-Hexanol	Acetic acid	Benzaldehyde
	2-methyl-1-Butanol		Ethylbenzene
	2-methyl-Propanol		Nonanal
	2-Phenylethyl alcohol		α-Pinene
	3-methyl-1- Butanol		
	3-methyl-2-Butanol		
	4-Penten-1-ol		

DISCUSSION

In this work the "root washing method" of root volatile sampling (removing the roots carefully from the substrate and washed with water) was compared with the alternatively developed method ("undisturbed root method") of leaving the plant inside the growth substrate during measurement. In the first case, plants may be stressed and mechanically damaged by removing the soil from the roots. Moreover, the roots are surrounded by air during sampling. This is an artificial situation prone to cause artifacts. The re-use of plants, which had been removed from soil, is problematic. Because of that, measurements via the washing root method could be performed only at 28 dpi. In the second case, the VOC samples were taken from the roots, left in the usual environment, minimizing stress and mechanical damage and allowing time series experiments (14 and 28 dpi measurements).

COMPARISON OF TWO DISTINCT METHODS FOR ROOT SAMPLING (CONTROL PLANTS)

The detected amount of VOCs (TIC) measured by using the undisturbed root method was significantly higher than measured with the washing root method. In nature (and under laboratory conditions) they are apart from the substrate as well as from other organisms, like yeasts, bacteria and fungi, which also release VOCs (i.e. GRIMME and ZIDACK 2007, CHIRON and MICHELOT 2005, COMBET et al. 2006, KAI et al. 2007, KAI et al. 2009, SPLIVALLO et al. 2007 and CAMPOS ZIEGENBEIN and KÖNIG 2010). CHIRON and MICHELOT (2005) listed several odor compounds of fungi, such as limonene and α- and β-pinene, but also different alcohols (i.e. 3-octanol), various substituted benzenes, phenols, ketones and aldehydes. Some of them, we found were released by the microorganisms, isolated from the rinsing water and also in the samples measured with the undisturbed root method (such as limonene and the not identified benzene compounds). Although dimethyl disulfide and dimethyl trisulfide are well known to be released by microorganisms (i.e. KAI et al. 2009), we could not sample them from the rinsing water colonies. This might be because of the media. Depending on the media, microorganism VOC patterns vary (FIDDAMAN and ROSSALL 1994, KAI et al. 2009). On the other hand, surely not all microorganism species grown in the sand of the *Brassica* plants are growing on PDA. Dimethyl

disulfide was detected in the root washing method and in the undisturbed samples. So most likely it was released by the roots.

Using the undisturbed root method, the VOCs of the whole rhizosphere including its microorganisms will be measured. Some of the detected VOCs seem to have this source, such as limonene, dimethyl trisulfide and probably further not identified compounds. But the VOCs of microorganism, detected in our experiment, did not disturb our measurements. After visual control there was no distinct distribution of the microorganisms' morphotypes corresponding to the treatment. So no mistakable VOC distribution due to the microorganisms was detected.

The most important aim for this work was a damage free measurement. Damaged Brassicaceae plants are releasing isothiocyanates. They occur after tissue disruption as a product of myrosinase hydrolysis of glycosinolates, which are common in Brassicaceae species (GREENHALGH and MITCHEL 1976, KIRKEGAARD and SARWAR 1998, VAN POECKE 2007). One of the most common isothiocyanates in *Brassica* roots is phenylethyl isothiocyanate (ANGUS et al. 1994). Damaging causes the emission of phenylethyl isothiocyanates in the roots of *Brassica napus* (ANGUS et al. 1994; own data not shown). In both approaches (undisturbed root method and washed root method) damaging due to measurement could not be completely avoided, like the detection of this compound showed – but distinctly reduced.

Further identified volatile compounds are the monoterpenes limonene and eucalyptol. Both of them are common plant volatiles, found in several plant species and plant tissues, analyzed by different methods. ROHLOFF and BONES (2005), STEEGHS et al. (2004) and VAN POECKE et al. (2001) found them released by *Arabidopsis thaliana*. LIN et al. (2007, headspace sampling of washed roots) detected limonene released by *Pinus* roots. The amount of this compound was decreasing when plant suffered drought stress. RASMANN et al. (2011, headspace sampling of washed roots) also sampled limonene from undamaged and damaged roots of *Asclepias syriaca*, significantly increasing, when roots were damaged by an herbivorous insect. Although limonene is a well known plant VOC, as previously shown, we found this compound only in those samples,

measured undisturbedly. Plant roots, measured in the PET enclosure did not emit that compound in detectable amounts.

Eucalyptol was just slightly less detected in the PET enclosure samples. Compared with the VOCs released by the microorganisms isolated from the rinsing water, it has no microorganism origin, like the literature investigations of CHIRON and MICHELOT 2005 and KAI et al. (2009) confirm. Additionally CHEN et al. (2004) provided genetic evidence for the eucalyptol synthesis of *Arabidopsis* roots, as they characterized its synthase.

In our experiments, VOCs of additional microorganisms, which are unavoidable in a near nature approach, did not influence the results, but they enhanced the amount of detected VOCs, which are probably not plant originated.

COMPARISON OF ROOT VOLATILES OF HEALTHY AND INFECTED PLANTS

Both methods (undisturbed root method at both points of time or washing root method) did not provide significant different volatile emissions between healthy plants and those infected with the pathogenic fungus *Verticillium longisporum*. This pathogen conquers the plant through the roots and moves inside the vessels into the plant shoots. At the end of the experiment, plants showed significant stunting and reduced leave and root fresh weight. At 7 dpi fungal DNA was already detectable in significant amounts in the hypocotyl. 14 dpi the fungus inhabits still mainly the hypocotyl. During sampling the root VOCs at 28 dpi the fungus already inhabits shoot and leafs of infected plants (time course after EYNCK et al. 2007). Indeed, interactions within one plant between below- and aboveground parts are common (BEZEMER and VAN DAM 2005) and expected. But with the used methods effects of pathogen infection on the emission of root volatiles at 14 respective 28 dpi could not proven. During first days after infection, the fungus interacts with plants surface, 7 dpi it already conquered the hypocotyl (EYNCK et al. 2007). Until that time, a change in the volatile emission can be expected. But the methods have to evolve to be more sensitive, because the root mass of the seedlings is quite small at that time.

CONCLUSION

A new method to measure root VOCs in its natural environment without damaging or stressing the plant is much needed. To study the metabolism products is a great chance and an important field in ecological research to investigate interactions between and within organisms (BEZEMER and VAN DAM 2005). Destructive methods, like distillation, solvent extraction and other methods previously presented, may lead to an increased amount of detectable volatiles, but are not mirroring the natural circumstances. The pooling of individual plants to one sample may help providing more volatile compounds from small plants, but it entails the risk of artifacts. The (even slight) damaging of roots leads to a distinct VOC emission. The presented method we worked on is a possibility to describe the natural odor of roots in a surrounding near nature in time series, but is yet not sensitive enough. Root damaging could be reduced but not excluded completely. However, the almost damage free measurement of root emission inside the substrate and the possibility to monitor emissions of a single plant for a longer time, might be a good base for further developments.

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CHAPTER III

FUNGI-VOLATILE BIOASSAYS AND THE INFLUENCE OF THE USED SOLVENT

ABSTRACT

The presented work will investigate one of the factors leading to different results and unforeseen effects in fungus-volatile bioassays – the used solvent. We tested three different solvents (Tween 20, paraffin oil, hexane) in order to show their influence on growth and development of the phytopathogenic fungus *Verticillium longisporum* (Ascomycota) under laboratory conditions. While paraffin oil and hexane were significantly supporting fungus growth within experimental observation time, Tween 20 showed the smallest influence. Microsclerotia formation was not affected significantly by the tested solvents. Furthermore, the naturally occurring plant volatile compound ß-ionone, diluted in the previously named solvents in two different concentrations, was tested on its inhibitory effects. The best durability and most constant quantity within the experimental observation time was provided by paraffin oil, while Tween 20 and hexane showed an enhanced amount of ß-ionone in the headspace during the first days.

INTRODUCTION

Naturally occurring VOCs often have antimicrobial activities. One example are the odor compounds released by bacteria (FERNANDO et al. 2005, KAI et al. 2007). Antifungal effects of isothiocyanates are also well known (DROBNICA 1967a, 1967b, OLIVIER 1999). Additionally, monoterpenes have antimicrobial activities (AGGARWAL et al. 2002, GARCIA et al. 2008). Science and agriculture know this effect for a long time and may be able to use VOCs as biofumigation agent in the future (ANGUS et al. 1994, Neri et al. 2006). However, the scientific approach to providing evidence to this fact is very divers. In 2009, KAI et al. reviewed the literature concerning the biofumigation potential of bacterial VOCs – and pointed out contradictions between the publications, resulting from differing set-ups, used concentrations or other experimental conditions. While some scientists use fresh (RHAMANPOUR et al. 2009, KOITABASHI et al. 2007) , dried (SMOLINSKA and HORBOWICZ 1999), powdered (FAN et al. 2008) or crushed (OLIVIER et al. 1999) plant material or in the case of microorganism a second culture (i.e. KAI et al. 2007) as repelling agent, others use the synthetic compounds, to which the effect is ascribed. Those compounds are applied pure (FERNANDO et al. 2005), in ethanol (GARCIA et al. 2008), methanol (SARWAR and KIRKEGAARD 1998), pentane (KAI et al. 2009), Tween 20 (KIM et al. 1995) or Tween 80 (SCHILTZ 1974), Ch_2Cl_2 (SPLIVALLO et al. 2007) or 1.4-Dioxan (ANGUS et al. 1994) or together with Aqua dest. (CHUANKUN et al. 2004). The offered applied concentrations range from 0.1 to 0.000001g/g and lead, depending on the solvent and the employed amount of the agent, to different final concentrations in the headspace surrounding the considered organism. Even the used units in publications about the topic are not always similar, so a comparison is often not possible.

With the knowledge about toxicity of different organic solvents (see STRATTON & SMITH 1988) towards organisms which could produce artifacts or reduce the dissolution of the results, and the experiences from the previously presented studies, a bioassay was developed which allows to investigate the antifungal activities of VOCs from plant origin to the soil borne pathogenic fungus *Verticillium longisporum*. The focus of this study was to choose the solvent in order to

have the best combination of VOCs durability and concentration inside the system without solvent artifacts and experimental results with best dissolution. Paraffin oil, hexane and Tween 20 (0.05% in Aqua dest.) were tested as solvents towards possible influences on growth and development of *Verticillium longisporum*. Because of the property of paraffin oil to release the dissolved volatile compounds over a long time in similar amounts, it is often used in the olfactory research (VISSER 1979, WEIßBECKER et al. 2004, JOHNE et al. 2006, THAKEOW et al. 2008). In addition, hexane is known from electroantennographic studies (ALTUZAR et al. 2007) and frequently used because of its fast evaporation. Therefore, it is possible to present the pure volatile compound in exactly prepared concentrations. Tween 20 is a detergent, mostly used in microbiology and biochemical research (i.e. RHEM and LETZEL 2010). In the food industry, Tween 20 is used for stabilization and a common food additive (E 432). There are indications that some microorganism can use that substance as a carbon source (WOERTZ et al. 2004). Afterwards the naturally occurring volatile ß-ionone, dissolved in Tween 20, paraffin oil and hexane, was tested on its supposed antifungal properties in the investigated bioassay. Based on the discussed results, suggestions are made to improve similar experiments.

MATERIAL AND METHODS

FUNGAL MATERIAL

Verticillium longisporum was grown in Czapek-Dox liquid media (Sigma Aldrich Chemie GmbH, Germany) and shook gently (app. 100U/min) in an incubator (at room temperature and darkness). After harvesting, spores were washed with sterile tap water and diluted to $1x10^6$ spores/ml.

BIOASSAY I – THE INFLUENCE OF SOLVENTS

200µl diluted spore suspension of *Verticillium longisporum* were distributed homogeneously on PDA (20 ml Potato Dextrose Broth; Sigma-Aldrich Chemie GmbH; Steinheim, Germany) and 1% Agar agar (Carl Roth GmbH & Co. KG; Karlsruhe, Germany). Afterwards, petri dishes were sealed

with Parafilm and incubated at room temperature and darkness for three days until mycelium was grown.

A mycelium disc (0.7 cm diameter) was placed in the middle of a PDA-Plate. A filter paper (approx. 1.5 x 1.5 cm; Mackery-Nagel; Düren, Germany) with 50 µl of the experimental dilution was placed in petri dishes top (Aqua dest., paraffin oil (Uvasol®, Merck, Darmstadt, Germany), hexane (Merck, Darmstadt, Germany), Tween 20 (0.05%; Polyoxyethylene sorbitan monolaurate; Carl Roth GmbH & Co. KG; Karlsruhe, Germany) or only filter paper). As a control, we used the pure colony without any treatment. All in all, 52 samples were used (Control, filter paper, water and paraffin oil: each 10 samples, Tween 20: 8 samples and hexane: 4 samples). After treatment, petri dishes were sealed with Parafilm and stored at room temperature and darkness, lying upside down (Fig. III-1).

Six days after starting the experiment, petri dishes were controlled by measuring the colony diameter. The colony diameter was measured by cross lines on the petri dishes' bottom in which center the mycelium disc was placed. In order to find out if the formation of microsclerotia had taken place, the color of the mycelium was examined two weeks after treatment. A sample was positive, that means microsclerotia were formed, when a black ring around the mycelium disc could be clearly seen.

BIOASSAY II – ß-IONONE DILUTED IN DIFFERENT SOLVENTS

In a second experiment, the ß-Ionone was diluted in paraffin oil (P4 means ß-ionone 0.0001 g/g in paraffin oil, respectively P6 means ß-ionone 0.000001 g/g in paraffin oil), Tween 20 (0.05%; Tw4 means ß-ionone 0.0001 g/g in Tween 20, respectively TW6 means ß-ionone 0.000001 g/g in Tween 20) and hexane (He4 means ß-ionone 0.0001 g/g in hexane, respectively He6 means ß-ionone 0.000001 g/g in hexane). After treatment, petri dishes were sealed with Parafilm and stored at room temperature and darkness, lying upside down (Fig. III-1a).

Six days after the treatment, growth was measured by using the same system already described. In order to find out if the formation of microsclerotia had taken place, the color of the mycelium

was examined two weeks after treatment. A sample was positive, that means microsclerotia were formed, when a black ring around the mycelium disc could be clearly seen.

A commercially available standard of ß-ionone was used (96% pureness; ABCR; Karlsruhe, Germany).

VOLATILE SAMPLING

The stability of ß-ionone dissolved in paraffin oil, Tween 20 and hexane (0.0001 g/g) inside empty petri dishes (without PDA and fungus) during one experiment was investigated using the SPME®-techniques (solid phase microextraction). Carboxen™/Polydimethyl-siloxane (Car/ PDMS) StableFlex™ fibre (85 µm; Supelco, USA) was used in all experiments. Directly before sampling, they were prepared by inserting them into a 250°C injection port for 15 min according to the manual.

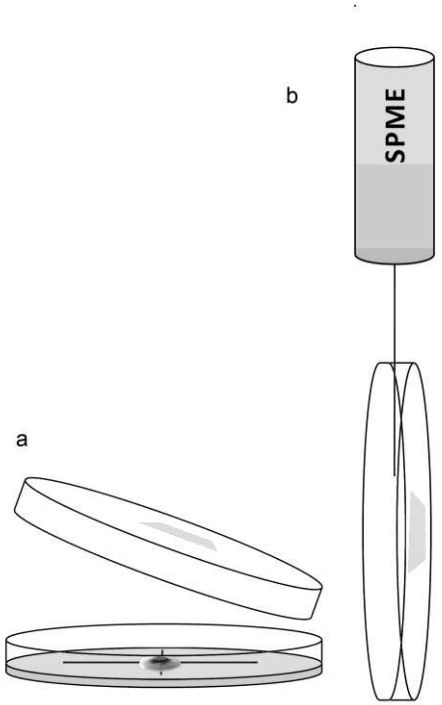

Fig. III-1: Setup of the presented experiments: a) bioassay - a mycelium disc is placed in the center of a petri dish filled with PDA (20 ml). The bottom is marked with cross lines for growth measuring. In the lid a filter paper with the experimental dilution is placed. b) VOC-stability - a SPME®-fiber is exposed to the headspace inside the petri dish.

Samples were taken as follows: At four points of time (0d, 3d, 5d, 14d after treatment), the headspace inside the petri dish was measured. Therefore, a hole (0.7 mm) was drilled into the

side of the petri dishes. Immediately afterwards, a SPME-fiber was gently placed inside the petri dish without contact to the petri dish or the filter paper (Fig. III-1b). The fiber was exposed to the sample for 20h to adsorb the VOCs and analyzed directly afterwards. Each experiment was repeated once.

ANALYSIS

SPME® samples were analyzed using gas chromatography (Agilent Technologies, Santa Clara, USA; model 6890N) coupled with a 5973 mass spectrometer (Agilent Technologies, Santa Clara, USA; model 5973N). To separate the volatile compounds a HP – 5MS polar column (INNOWAX; 30 m x 0.25 mm, i.d., 0.25 µm film thickness; Agilent Technologies, Santa Clara, USA) was used. As carrier gas, helium was used (1ml/min). For desorption of the adsorbens, the needle was inserted into the GC injection port and exposed. The analysis started at 40°C (1.5 min). Afterwards, temperature increased to 200°C (6°C/min) and was held for 5 minutes. The mass spectrometer had a mass range of 15 – 300 amu, a source temperature of 230°C and an EI mode at 70 eV.

The chromatograms were preliminarily interpreted with Enhance Chemstation version D00.00.38 (Agilent Technology) and National Institute of Standards and Technology (NIST, Gaithersburg, USA) Mass Spectral Search Library (comparison of mass spectra).

STATISTICS

The mycelium growth was analyzed using the Mann-Whitney-U Test (MWU). To show changes in the formation of microsclerotia, the χ^2 Test was used. Differences are significant with *P*-values < 0.05.

RESULTS

THE INFLUENCE OF SOLVENT TO GROWTH AND THE FORMATION OF MICROSCLEROTIA

Possible influences of the solvents were observed over a period of 15 days. At four points of time, the colony diameter was measured and statistically analyzed. At these points of time, all solvents led to an increased colony diameter compared to the control colonies although none of the data points differed significantly from the control (presented by the horizontal line at 100%, Fig. III-2). However, the increase during the whole experiment was significantly higher in colonies treated with paraffin oil, hexane or only filter paper than the control colonies (see Tab. III-1).

Tab. III-1: Growth within 11 days of observation [cm] and microsclerotia formation (MS) of *Verticillium longisporum* after 15 days exposition to different solvents; n= 52; * marks data significantly different to the control (mean ± SE; growth: Mann-Whitney-U Test, microsclerotia formation: χ^2-Test; $P < 0.05$)

Treatment	growth [cm]	*P*- value	z- value	MS [%]	*P*- value	χ^2
Control	1.38 ± 0.061	-		80	-	-
Filter paper	1.78 ± 0.097	**0.005	-2.82	40	**0.002	10
Water	1.61 ± 0.104	0.241	-1.17	60	0.114	2.5
Paraffin oil	1.68 ± 0.070	*0.010	-2.57	70	0.429	0.63
Hexane	1.69 ± 0.125	*0.048	-1.98	75	0.803	0.06
Tween 20	1.56 ± 0.085	0.143	-1.47	75	0.724	0.13

Those colonies which were treated with water or Tween 20, did not show a significant increase of the colony diameter over the time. In addition, the solvents did not influence the formation of microsclerotia (Tab. III-1). Only the treatment with filter paper leds to a significant inhibition of the formation of microsclerotia.

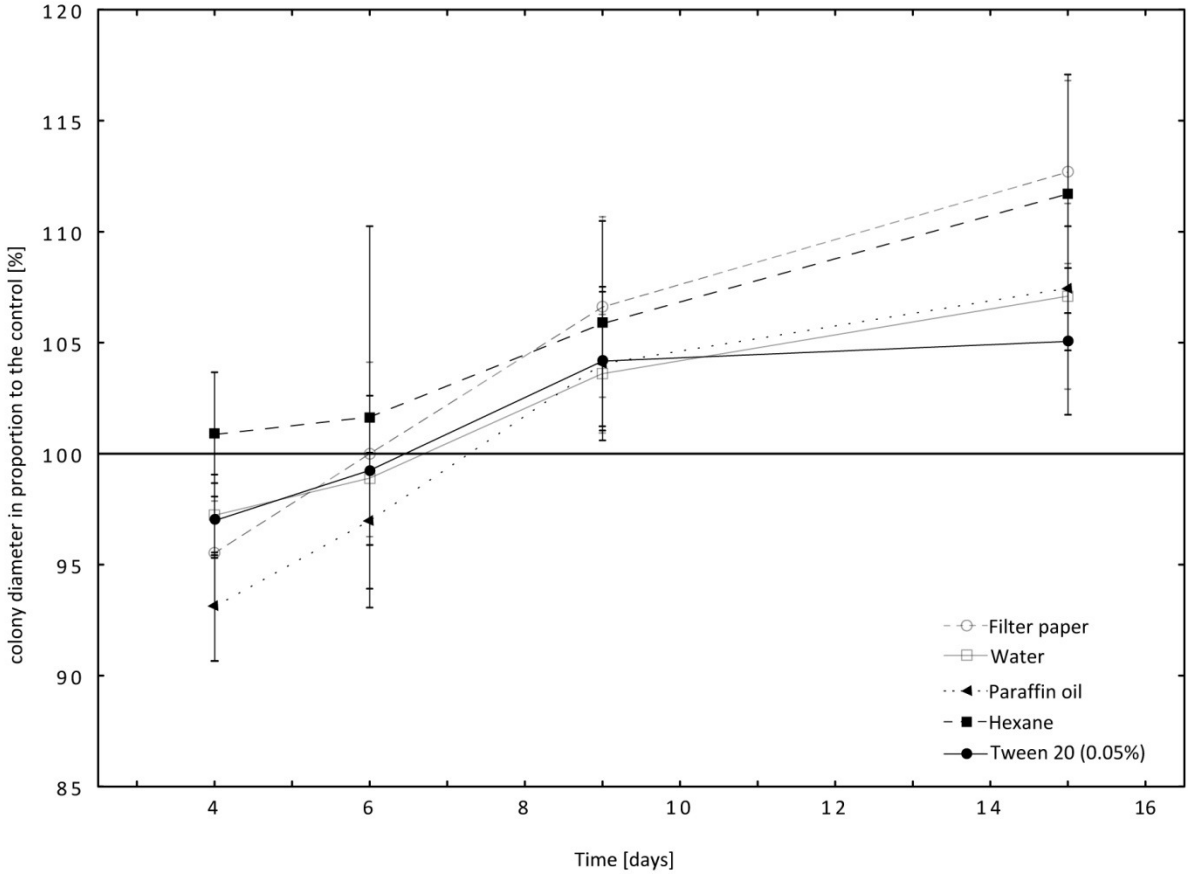

Fig. III-2: Mycelium growth of *Verticillium longisporum* colonies after exposing to different solvents at 4, 6, 9 and 15 days after starting the experiment. Presented data describes the colony diameter in proportion [%] to the non-treated control (black line at 100%), (mean ± SE), lines only serve for better visualisation; n=52

STABILITY AND DURABILITY OF ß-IONONE INSIDE A PETRI DISH

Paraffin oil released ß-ionone most homogenously in the observed period of time. After two weeks, the amount of ß-ionone in the headspace inside the petri dishes was almost the same. In the case of Tween 20 and hexane, during the first days we measured a two to four times bigger amount of ß-ionone, which approximately reached the paraffin oil sample's amount of ß-ionone from the third day after treatment (see Fig. III-3).

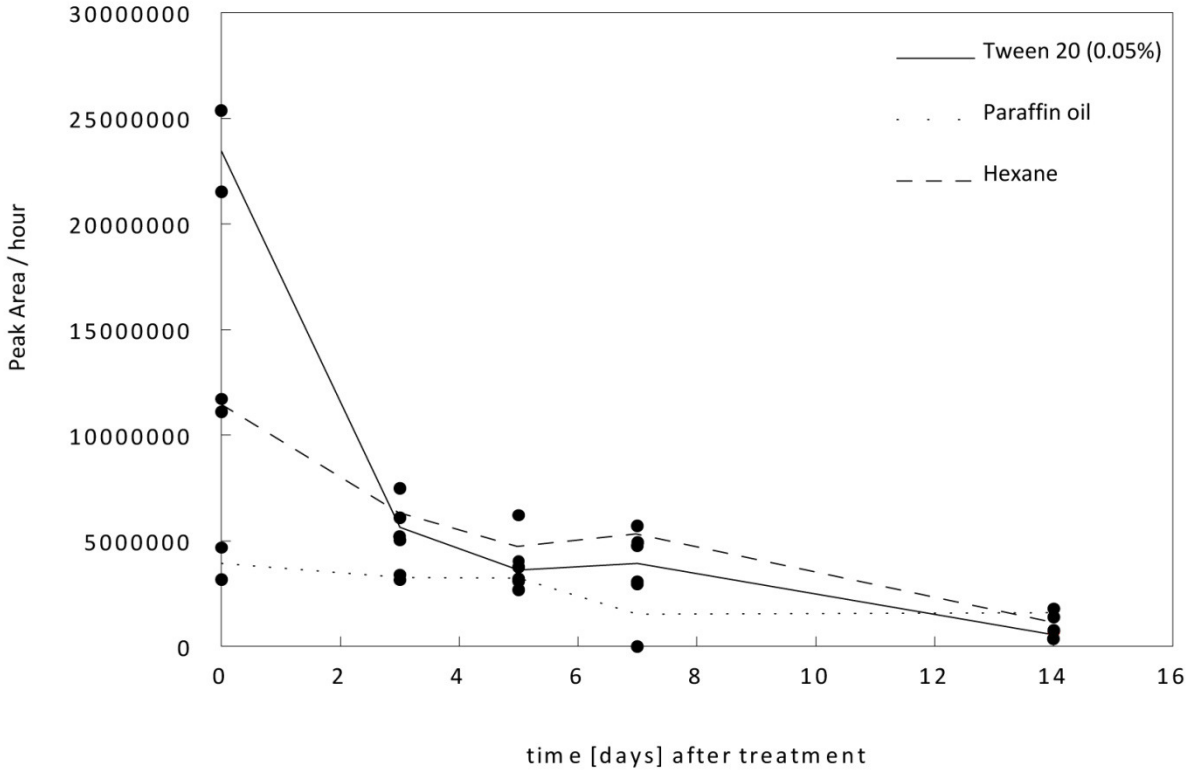

Fig. III-3: Stability of ß-ionone (0.0001 g/g) dissolved in Tween 20 (0.05%; black line), Paraffin oil (pointed line) and Hexane (interrupted line) in petri dishes over 14 days (samples taken via SPME ® technique). Data point mark the peak area per hour (m/z = 177), lines only serve for better visualisation (n=2).

Certain volatile compounds could be found additionally in very low amounts. Those were detected directly after starting the experiment. It was not the intention to identify those substances exactly, but the mass spectra indicated their origin in the degradation of ß-ionone. In Tab. III-2 those VOCs are listed with the name of the most similar mass spectra. Apart from ß-ionone and its supposed degradation products, we detected samples of many low molecular VOCs in paraffin oil which are most likely not related to the applied ß-ionone (Fig. III-4).

Hexane and Tween 20 showed solvent-related contaminations in the high molecular VOC range. It was not the intention to identify those substances exactly, but the mass spectra indicate predominantly alkanes and aldehydes of different substitution, furthermore naphthalenes, pyrazins, acid ethylester and many more.

Tab. III-2: Volatile Organic Compounds, detected in the headspace of petri dishes, containing ß-ionone dissolved in Tween 20 (0.05%), paraffin oil or hexane, which are suggested to be degradation products of the applied substance ß-ionone (compounds only detected in samples with paraffin oil are marked with a #; each treatment n=2).

substances	CAS	RI
3-methyl-3-cyclohexen-1-ol #	53783-91-8	955
4,7,7-Trimethylbicyclo[4.1.0] heptan-3-on #	4176-04-9	1047
4-methyl-2,3- hexadien-1-ol	k.A.	1331
1,3- Dimethylcyclohexane	2207-03-6	1381
Alpha - Ionone	127-41-3	1413

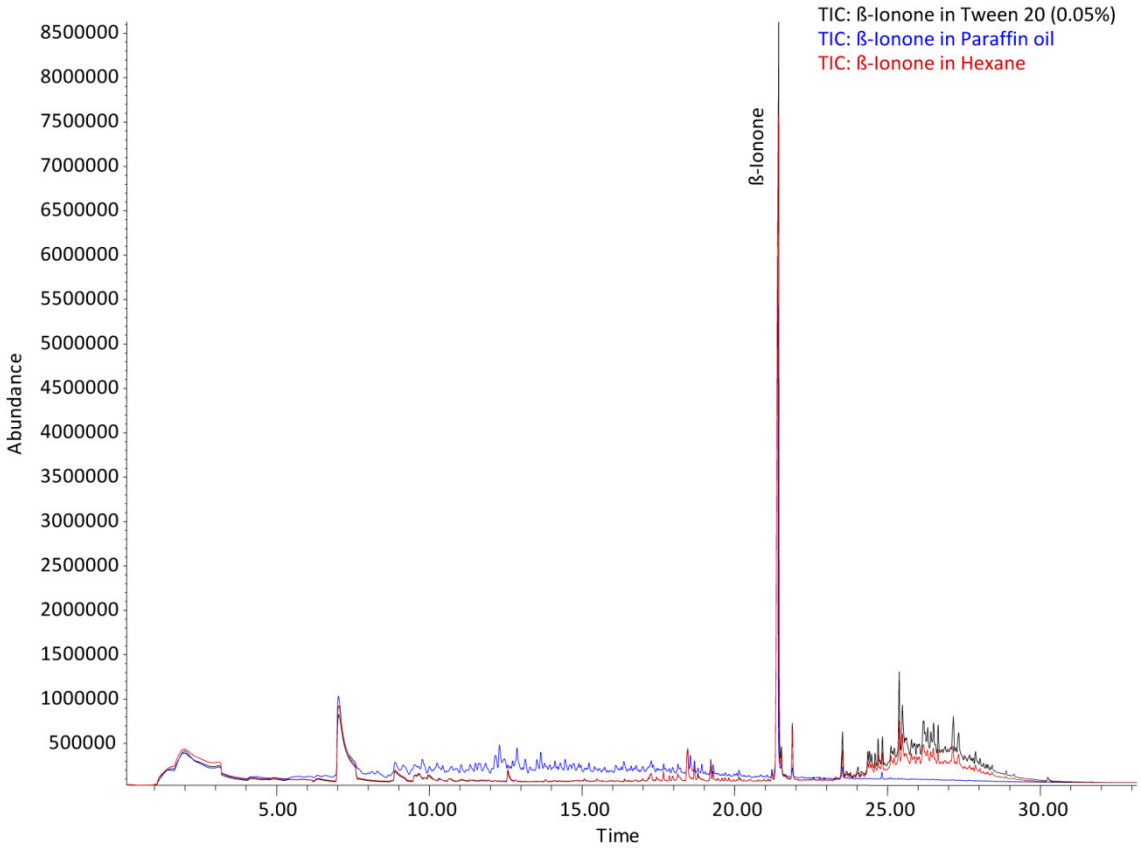

Fig. III-4: Comparison of the Total Ion Current of petri dishes headspace containing ß-Ionone dissolved in Tween 20 (0.05%; black line), paraffin oil (blue line) or hexane (red line) after 3 days of exposition.

EFFECTS ON VERTICILLIUM LONGISPORUM AFTER EXPOSITION TO ß-IONONE DILUTED IN DIFFERENT SOLVENTS

After analyzing the effects of different solvents on growth and development of *Verticillium longisporum*, ß-ionone was diluted in Tween 20, paraffin oil and hexane. Based on the durability experiment, the diameter was measured six days after treatment, because at this time approx. the same VOC amount in all three solvent samples could be expected.

Tab. III-3: Mycelium growth after 6 days of exposition to ß-Ionone and microsclerotia formation (MS) of *Verticillium longisporum* after 12 days of exposition, diluted in Tween 20, paraffin oil or hexane. Data describes the colony diameter in [%] according to the solvent control and the amount of colonies formed microsclerotia (MS) [%]; n= 82; * marks data significantly different to the solvent control (mean ± SE; growth: Mann-Whitney-U Test, microsclerotia formation: χ^2-Test; $P < 0.05$)

Treatment		growth [%]	*P*- value	z- value	MS [%]	*P*- value	χ^2
Tween 20	TW				60		
	TW6	107.79 ± 3.760	0.143	-1.47	70	**0.009	6.8
	TW4	105.63 ±3.649	0.345	-0.95	80	**0.002	9.3
Paraffin oil	P				50		
	P6	105.63 ± 3.338	0.307	-1.02	33	*0.010	6.7
	P4	110.82 ± 2.424	*0.017	-2.38	33	*0.010	6.7
Hexane	He				83		
	He6	107.61 ± 2.242	0.083	-1.73	83	1.000	0
	He4	104.35 ± 1.633	0.168	-1.38	67	0.273	1.2

While ß-ionone diluted in Tween 20 and hexane had no significant inhibiting effect on mycelium growth, the same compound diluted in paraffin oil showed a significant growth support at a concentration of 0.0001 g/g (Tab. III-3). ß-Ionone dissolved in Tween 20 significantly supported the formation of microsclerotia. When the compound is dissolved in paraffin oil, the formation of microsclerotia was inhibited significantly. ß-Ionone dissolved in hexane did not lead to significant alterations (Tab. III-3).

DISCUSSION AND CONCLUSION

The presented study shows the influence of the solvent on the development of the fungus *Verticillium longisporum* in a non-contact bioassay to investigate antimicrobial activities of VOCs. Paraffin oil, hexane and Tween 20 (0.05%) were applied to *Verticillium longisporum* colonies in petri dishes sealed with Parafilm. Experiments showed that **Tween 20** has the smallest influence on fungus growth and no influence on the formation of microsclerotia. However, directly after applying ß-ionone diluted in Tween 20, this solvent released amounts of that compound more than four times bigger than paraffin oil does. Stimulatory effects of Tween 20, observed by WOERTZ and KINNEY (2004, *Exophiala lecanii-corni*) and MIEHLE & LUKEZIC (1972, *Colletortrichum trifolii*) could not be confirmed by our experiments. Hexane did not provide an alternative to Tween 20 as a solvent. Because of its toxicity and its "inferior solvent capabilities" STRATTON & SMITH (1988) did not recommend **hexane** as suitable solvent in bioassays, either. **Paraffin oil** showed indeed the best releasing properties for ß-ionone over a long time, but also leads to significant enhancing of fungal growth. Additional VOC analysis of the headspace inside a petri dish showed, that paraffin oil also released much more pollution compounds in the low molecular range than Tween 20 and hexane.

In conclusion, Tween 20 (0.05%) is the most suitable solvent for bioassays with the fungus *Verticillium longisporum*. According to the observations during experiments, we suggest three days after applying ß-ionone as the best point of growth measurements. At that time, the effect of the solvent is negligible and the amount of ß-ionone dissolved in Tween 20 (0.05%) is comparable to that dissolved in paraffin oil. Early measuring also helps to decrease the possibility of artifacts caused by degradation products of the experimental VOCs.

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CHAPTER IV

VOLATILE ORGANIC COMPOUNDS OF *BRASSICA NAPUS*, EMITTED AFTER INFECTION OF *VERTICILLIUM LONGISPORUM*, ARE INHIBITING GROWTH OF OTHER FUNGAL PATHOGENS.

ABSTRACT

In the last years, interactions between organisms via volatile organic compounds (VOC) have been investigated more and more. With the results of this work, we show the possibility to use VOCs, emitted by *Brassica napus*, for defense against fungal pathogens. Three plant pathogenic fungi of different degrees of specialization were exposed to a series of different dilutions of two VOCs of plant origin. In order to investigate fungus susceptibility towards the antimicrobial activity of dimethyl disulfide and the terpenoid ß-ionone, we tested three fungal pathogens in a bioassay. *Verticillium longisporum* is a specialist to *Brassica*, *Gaeumannomyces graminis* var. *triciti* is a specialist on grain and *Sclerotinia sclerotiorum* is a generalist, also infecting Brassicaceae.

Both VOCs show antifungal activities – detected as reduced mycelium growth within the first three days. Dimethyl disulfide inhibits the growth of the generalist and the grain specialist already at lower concentrations than it does for the *Brassica* specialist. ß-Ionone needs to be applied in high concentrations to influence growth, but is the only tested compound influencing the formation of microsclerotia of the *Brassica* specialist.

INTRODUCTION

Volatile organic compounds (VOCs) as potential agents in biocontrol become more and more the focus of agricultural attention (FERNANDO et al. 2005, NERI et al. 2006, CAMPOS et al. 2010). Antimicrobial effects of several VOC species have already been demonstrated, such as monoterpenes (AGGARWAL et al. 2002, GARCIA et al. 2008) and isothiocyanates (DROBNICA 1967a, 1967b, OLIVIER 1999).

This work investigates the VOC-based interactions in the pathosystem *Brassica napus* and *Verticillium longisporum*. *Brassica napus* is one of the most important crop plants in the world. *Verticillium* species cause big losses in yield each year (KOIKE et al. 1994, STEVENTON et al. 2002, ZHOU et al. 2006). They infect the plant as a seedling by entering root tissue and spreading through the vessels into the upper parts of the plant. EYNCK et al. (2007) showed that *V. longisporum* is entering the plant via the epidermal cells of the plant root. Having passed the hypocotyl, a later stage of infection, the fungus also conquers leaf tissue outside the vessels and forms microsclerotia on plant surfaces (EYNCK et al. 2007). Typical disease symptoms are stunting of the shoot, yellowing of the leaves and black veins. They are clearly shown up to 21 days post inoculation (dpi). At this time of infection, the fungus is already spreading into shoot vessels (EYNCK et al. 2007). *Verticillium longisporum* is specialized on *Brassica* (ZEISE and VON TIEDEMANN 2002, JOHANSSON et al. 2006). Indeed, fungi often have been in the focus of Brassicaceae VOC research, but especially the antifungal activity of isothiocyanates, a typical VOC of Brassicaceae plants, has been in the center of research (i.e. DROBNICA 1967, ANGUS et al. 1994, SARWAR et al. 1998, SMOLINSKA and HORBOWICZ 1999, FAN et al. 2008, RHAMANPOUR et al. 2009). Only little is known about the role of other VOCs, emitted by *Brassica napus*. After an infection with *Verticillium longisporum*, *Brassica napus* plants release significantly more ß-ionone and dimethyl disulfide in shoots headspace than non-infected plants (see CHAPTER I).

ß-Ionone is a degradation product of carotinoids (SIMKIN et al. 2004, AULDRIDGE et al. 2006). There are few indications for its antimicrobial activity. DENNIS and GUEST (1995) showed an inhibiting effect of ß-ionone on the infection of *Nicotiana tabacum* with the Tobacco Necrosis

Virus. *Peronospora tabacina* is inhibited by the fumigation with ß-ionone (SCHILTZ 1974) and UTAMA et al. (2002) investigated germistatic effects of ß-ionone towards *Colletrotrichum musae*. In contrast, dimethyl disulfide is a neurotoxic molecule to non-specialized insects, affecting the mitochondrial chain reaction and the ATP dependent potassium channels (DUGRAVOT et al. 2003). Specialization of insects can lead to adaption (DUGRAVOT et al. 2004, FERNANDES et al. 2009). *Psidium guajava* emits dimethyl disulfide after wounding. It is assumed to play a role in defense (ROUSEFF et al. 2008).

Our hypotheses are I. antifungal activities of dimethyl disulfide and ß-ionone on *Gaeumannomyces graminis var. triciti*, a specialist on grain, and *Sclerotinia sclerotiorum*, a generalist, also infecting Brassicaceae plants. II. The specialized fungus *Verticillium longisporum* is less susceptible towards these antifungal *Brassica*-VOCs. The possible role in the plant-pathogen interaction of ß-ionone and dimethyl disulfide will be discussed.

MATERIAL AND METHODS

FUNGAL MATERIAL

Three fungi species were used for the experiment. *Verticillium longisporum*, a root borne pathogen, is specialized on *Brassica*. In comparison, we chose *Gaeumannomyces graminis* var. *triciti*, which is also a root borne pathogen, specialized on grain. As a generalist fungus we chose *Sclerotinia sclerotiorum*.

Verticillium longisporum (isolate 43) was grown in Czapek-Dox liquid media (Sigma Aldrich Chemie GmbH; Munich, Germany), shook horizontally (approx. 100 rpm) in an incubator (at room temperature and darkness). After harvesting, spores were diluted to $1x10^6$ spores/ml. 200 µl of the freshly prepared spore suspension was plated onto PDA (20 ml; 1%). Afterwards petri dishes were sealed with Parafilm and incubated at room temperature and darkness for three days until mycelium was grown.

Gaeumannomyces graminis var. *triciti* (Isolate TS 2133) and *Sclerotinia sclerotiorum* (Isolate Ss 1.5) were provided as mycelium cultures on Potato Dextrose Agar (PDA; 1%; Sigma Aldrich Chemie GmbH; Steinheim, Germany) in petri dishes by the Department of Crop Science, Plant Pathology and Plant Protection Unit, University of Goettingen.

BIOASSAY

One mycelium disc (0.7 cm diameter) was placed in the middle of a PDA-Plate (20 ml). A filter paper (app. 1.5 x 1.5 cm; Mackery-Nagel; Düren, Germany) with 50 µl of the experimental solution was put in the lid of the petri dish (Fig. IV-1). Two compounds of the *Brassica napus* volatile spectrum were selected. Dimethyl disulfide (98% purity, Merck; Darmstadt, Germany) and ß-ionone (96% purity, ABCR; Karlsruhe, Germany) were dissolved in Tween 20 (0.05%; Roth; Karlsruhe, Germany) in the following three dilutions: 0.001 g/g; 0.0001 g/g; 0.000001 g/g. As a control we used the pure solvent. Additionally, we made a double control in which we only used the fungus without any treatment. Each treatment was done with 15 to 20 replicates. Petri dishes were sealed with Parafilm and stored at 25°C and darkness. To prevent contact of filter paper with mycelium during the experiment, petri dishes were placed upside down. The fungi growth was measured three days after the treatment with chemicals. Because of the faster growth of *Sclerotinia sclerotiorum*, we measured this species at the second day after starting the experiment.

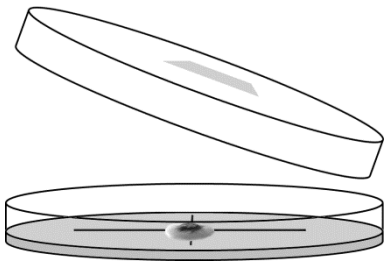


Fig. IV-1: Bioassay: A filter paper with the diluted VOC was placed in the lid. On the bottom under the agar disc a cross line was marked to measure fungus growth. Subsequently, the petri dish was turned upside down.

Using cross lines on the petri dish bottom, the colony diameter was measured. In its center, the mycelium disc was placed (Fig. IV-1). Because of the very different growth rates of the three different fungi (*Verticillium longisporum*: 0.19 cm/day; *Gaeumannomyces graminis* var. *triciti*:

0.6 cm/day und *Sclerotinia sclerotiorum*: 0.9 cm/day; average of untreated colonies) a comparison of absolute colony diameter did not seem to be meaningful. So all presented data was normalized to their solvent control (only Tween 20 (0.05%), without VOC treatment). Five days after starting the experiment, the formation of (micro-) sclerotia was inspected. Microsclerotia were visible in *Verticillium longisporum* (positive sample), when a black ring around the mycelium disc was clearly developed. Furthermore, *Gaeumannomyces graminis* var. *triciti* and *Sclerotinia sclerotiorum* were observed for one week towards the formation of (micro-) sclerotia.

STATISTICS

The statistical analyses were conducted using Statistica 7.0 (StatSoft; Tulsa, USA). The mycelium growth was analyzed using the Mann-Whitney-U Test (MWU). To show changes in the formation of microsclerotia, the χ^2 Test was used. Differences are significant with *P*-values < 0.05.

RESULTS

Two VOCs of *Brassica napus* were tested on their antifungal effect on three fungal pathogens. The used solvent did not influence colony growth or the formation of (micro-) sclerotia compared to the non-treated colonies (see CHAPTER III).

ROOT BORNE FUNGI –THE SPECIALISTS

Both of the root borne fungi (*Verticillium longisporum* and *Gaeumannomyces graminis* var. *triciti*) showed significant reactions towards the fumigation with dimethyl disulfide and ß-ionone.

Tab. IV-1: Colony diameter (% to the average of the Tween-control - 100%) three days after treating *Verticillium longisporum* and *Gaeumannomyces graminis* var. *triciti* respectively two days after exposition of *Sclerotinia sclerotiorum* with dimethyl disulfide (D6 – D3) or ß-ionone (J6 – J3) in different dilutions; mean ± SE; n= 160; * marks data significantly different to the solvent control, · marks tendencies (Mann-Whitney-U Test; $P < 0.05$)

Treatment		growth [%]	*P*- value	z- value
Verticillium longisporum	J6	98.07 ± 4.765	0.514	-0.653
	J4	94.33 ± 3.139	0.254	-1.140
	J3**	85.33 ± 2.549	0.002	-3.070
	D6	109.21 ± 5.68	0.337	0.960
	D4*	90.53 ± 2.750	0.049	-1.967
	D3	92.82 ± 2.680	0.108	-1.609
Gaeumannomyces graminis var. triciti	J6	97.39 ± 1.936	0.399	-0.843
	J4·	94.58 ± 1.553	0.068	-1.826
	J3**	89.86 ± 1.345	0.005	-2.838
	D6*	91.61 ± 2.191	0.028	-2.192
	D4**	90.36 ± 1.877	0.007	-2.701
	D3	95.11 ± 2.441	0.216	-1.236
Sclerotinia sclerotiorum	J6	94.62 ± 7.982	0.326	0.982
	J4	92.82 ± 8.291	0.589	0.540
	J3	90.33 ± 10.006	0.736	-0.337
	D6	98.28 ± 3.933	0.150	1.440
	D4*	93.83 ± 3.729	0.033	2.127
	D3·	96.15 ± 4.212	0.073	1.792

ß-Ionone was inhibiting mycelium growth in a dose-dependent manner. Lower concentrations (J6) resulted at the most in a slight growth inhibition, while higher concentrations (J3) led to significantly reduced mycelium growth (Tab. IV-1). For dimethyl disulfide, we observed a biphasic dose dependency. While low concentrations (D6 and D4) showed a reduced increase of

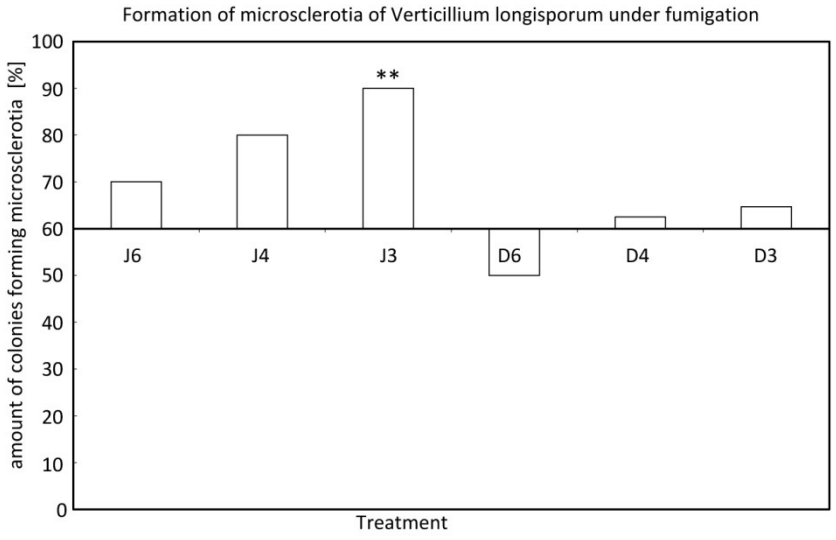
the colony diameters growth, high concentrations (D3) did not affect colony diameter in comparison to the control. The most important difference we detected between those two fungi by exposing them to dimethyl disulfide was the influence of low concentrations (D6) to the growth of *Gaeumannomyces*. This was significantly reduced, while *Verticillium longisporum* did not show any alterations in mycelium growth at that concentration.

AIR BORN FUNGUS – THE GENERALIST FUNGUS

The growth of *Sclerotinia sclerotiorum* treated with ß-ionone was not affected significantly during experimental time (Tab. IV-1). However, dimethyl disulfide was supporting growth at D4 compared to the solvent control.

FORMATION OF (MICRO-) SCLEROTIA

The formation of sclerotia in *Gaeumannomyces graminis* var. *triciti* is not known. During the week of exposing the fungus to the compounds, no formation was observed. *Sclerotinia sclerotiorum* formed black sclerotia on the petri dishes border, but we observed no influence of the fumigation. *Verticillium longisporum* formed black microsclerotia. The exposure to ß-ionone in higher concentrations (J3) significantly supported their formation (Fig. IV-2).



	P-value	χ^2 value
J6	0.361	0.83
J4	·0.067	3.33
J3	**0.006	7.5
D6	0.361	0.83
D4	0.838	0.04
D3	0.692	0.15

Fig. IV-2: Formation of microsclerotia under fumigation with ß-ionone and DMDS five days after starting the experiment; n=160; the horizontal axis shows the solvent control value; * marks data significantly different to the solvent control, · marks tendencies (χ^2; $p < 0.05$)

DISCUSSION

In the presented work, dimethyl disulfide showed growth inhibition effects on both root borne fungi, with different susceptibility. The fumigation with dimethyl disulfide supported the growth of *Sclerotinia sclerotiorum*. ß-Ionone showed at most slight inhibiting growth effects on the fungi, whereas this compound supported the formation of microsclerotia in *Verticillium longisporum*.

Although antimicrobial activity of ß-ionone was already shown (DENNIS and GUEST 1995, SCHILTZ 1974 and UTAMA et al. 2002), our results do not support these observations. As KAI et al. (2009) already determined, results in research may be contradictory regarding the same compound, which might be due to different experimental designs or other experimental conditions. However, our observations confirm indications to antimicrobial activities of dimethyl disulfide. This compound reduces growth of microorganism (KAI et al. 2009). Sulfur is an important element in defense against fungal pathogens of Brassicaceae and other plants (COOPER et al. 2004, NOVO et al. 2007). Deficiency leads to increased pathogen susceptibility (DUBIUS et al. 2005). The antimicrobial activity of sulfur volatile compounds (i.e. FAN et al. 2008), naturally occurring in Brassicaceae (KIRKEGAARD and SARWAR 1998, GREENHALGH and MITCHEL 1976, ROHLOFF and BONES 2005), is well known, such as isothiocyanates (DROBNICA et al. 1967 a & b, ANGUS et al. 1994, SMOLINSKA et al. 1997 SMOLINSKA and HORBOWICZ 1999, OLIVIER et al. 1999, RHAMANPOUR et al. 2009). While the *Brassica* specialist *Verticillium longisporum* is able to tolerate or even to detoxicate dimethyl disulfide in low concentrations, the grain specialist *Gaeumannomyces* is not. Our data confirms the hypothesis that specialized fungi on *Brassica* are less susceptible towards these VOCs than non-specialized.

In the analysis, the fumigation with dimethyl disulfide in different dilutions leads to a biphasic graph for all three fungi. CONOLLY and LUTZ (2004) suggest that different overlying effects are responsible for these phenomena, like some receptors, differing in i.e. the ligands threshold. We suggest that detoxification might be triggered up to a certain toxic concentration in the surrounding area. To survive, the fungus (specialized on *Brassica*) has to develop a tolerance towards lower, naturally occurring concentrations. This confirms our observations of growth

inhibition of *Verticillium longisporum* only at medium concentrations (D4) while the grain-specialist *Gaeumannomyces* already showed inhibitory effects at lower concentrations (D6 and D4). At D3, the highest used concentration, no significant growth reduction was observed, neither in *V. longisporum* nor in *G. graminis* var. *triciti*. Following the suggestions of CONOLLY and LUTZ (2004), a general detoxification mechanism started in both fungi at that concentration.
The generalist *Sclerotinia sclerotiorum* is also infecting Brassicaceae plants. RHAMANPOUR et al. (2009) showed the adaption of this species towards *Brassica*-VOCs and especially isothiocyanates within two to four days. After a short growth inhibition time, the fungus is said to adapt to the VOC. Our results however, only include measurements on the second day. The previous time of inhibition may therefore have been missed.

The appearance of (micro-) sclerotia was not detected in colonies of *Gaeumannomyces graminis* var. *triciti*. While *Sclerotinia* showed no differences, *Verticillium* developed significantly more microsclerotia when treated with ß-ionone (J3) than the control. ß-Ionone is a degradation product of carotinoids. It is developed by oxidative cleavage of ß-carotene (SIMKIN et al. 2004, AULDRIGE et al. 2006). Plants release this compound in later stages of infection in significantly higher amounts. At approx. 35 dpi, the tissue will be invaded (Eynck et al. 2007). Therefore, the release of ß-ionone might be a signal for the fungus, indicating the state of disease. We suggest that the compound ß-ionone triggers further development of microsclerotia only in specialized fungi. This suggestion is confirmed by the observations of UTAMA et al. (2002). In their experiments, ß-ionone showed inhibition effects only on decay microorganisms. HEIL and BUENO (2007) also investigated a role of VOCs in indirect defense by signaling within an individual plant.

Infected plants released ß-ionone and dimethyl disulfide in significantly bigger amounts than non-infected plants at 28 dpi (see CHAPTER I). At that time, *Verticillium longisporum* already spread into the shoot. DNA is detectable in the hypocotyl up to 7 dpi and in the leaves up to 21 dpi (EYNCK et al. 2007). To develop antifungal activities against a plant pathogen, which is already inside the plant, VOCs have to disperse inside in a water milieu. VOCs can be stored in

significant amounts in the lipid or water phase inside the plant (LORETO et al. 1998, NIINEMETS and REICHSTEIN 2002, NIINEMETS et al. 2004). The solubility in water is very low for both substances and amounts at 20°C to 0.11 g/l for ß-ionone and 2.5 g/l for dimethyl disulfide (corresponding to dilutions of approx. 0.001 resp. 0.0001 g/g (VOC/water)). We suggest that higher concentrations in plant xylem sap are not possible, but smaller concentrations might be possible in the natural system. So following the hypothesis of defense against the present pathogen *Verticillium longisporum*, a VOC-based control of the *Brassica* specialist *Verticillium longisporum* is unlikely. But if pathogens, not specialized on *Brassica*, reach the vessels, a growth inhibition is possible, as *Gaeumannomyces graminis* var. *triciti* shows. A distribution of the odor molecules in the air is easier to realize: the vapor pressure of ß-ionone amounts to 0.0013 hPa, of dimethyl disulfide to 28 hPa (both at 20°C). Both compounds did not significantly inhibit the growth of *Sclerotinia sclerotiorum*, other air borne fungi might be affected by those VOCs. Experiments with air borne fungi specialized on distinct plant families have to be done.

CONCLUSION

For the first time, this work showed antifungal activities of dimethyl disulfide and ß-ionone towards *Verticillium longisporum*, *Gaeumannomyces graminis* var. *triciti* and *Sclerotinia sclerotiorum*, three fungi of different degrees of specialization. Fungi, specialized on *Brassica*, are less susceptible towards dimethyl disulfide than others. Observations of an adaption to *Brassica* emissions of the fungal generalist *S. sclerotiorum* could be confirmed. Furthermore, our data indicates that ß-ionone triggers the formation of microsclerotia of *V. longisporum*.

To point out the specific role of these VOCs in the interaction between *Brassica napus* and *V. longisporum*, further research has to be done. Their defense or signaling function has to be investigated.

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CHAPTER V

ANALYSIS OF VOLATILE PATTERN IN A MODEL SYSTEM FOR PLANT-INSECT-FUNGUS INTERACTIONS

MARIA VLAIC, STEFAN SCHÜTZ

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ABSTRACT

Arabidopsis thaliana (L.) Heynh. (Brassicaceae; Capparales) is a model plant and a member of one of the most important families in agriculture - the Brassicaceae. Modern plant protection has to consider ecological interactions like tritrophic interactions of plants, fungi, and herbivores. These interactions might be mediated by volatile organic compounds. Here, we analysed volatile organic compounds released by single *Arabidopsis thaliana* plants as affected by *Verticillium longisporum* (Moniliaceae; Hyphomycetales) infection using Gas chromatography-Mass spectrometry. *Verticillium longisporum* is a soil born root pathogenic fungus causing stunting and premature senescence in Brassicaceae. Volatile organic compounds released from shoot and roots were examined separately. The releasing rate of terpenoids like limonene and geranylacetone was changed aboveground and belowground by fungal infection and can be perceived by insects attacking the plant.

Keywords: *Arabidopsis thaliana*, *Verticillium longisporum*, tritrophic interactions, VOC

INTRODUCTION

Plant volatiles are not "rubbish" of plant biochemistry but metabolic products or by-products of ongoing processes contributing to growth, proliferation and defence (PEÑUELAS & LLUSIÀ 2004; NIINEMETS et al. 2004). Thus, they are a mirror of the biochemical processes in the plant. Emission of those infochemicals is often correlated to abiotic factors like light, temperature and nutrition (GOUINGUENE et al., 2002), and biotic factors like microbial infection and insect attack. Changes in the metabolism due to an infection, for instance, can cause changes in the volatile profile and results in interaction with the environment. Their functional role in intra- and interspecific communication between plants and non-plant organisms was examined (i.e. by PICHERSKY & GERSHENZON 2002; VAN POECKE et al. 2001) and discussed (by PEÑUELAS & LLUSIÀ, 2004). These modifications in volatile pattern of plants can be used by insects to assess the suitability of a host plant for feeding or oviposition. Moreover, in tritrophic interactions volatiles are used by the plant to attract antagonists of herbivores feeding on the plant. VAN POECKE and others already examined model systems to study several aspects of those connections (i.e. VAN POECKE et al 2001).

Due to the role of *A. thaliana* as a model plant the volatile profile was screened (see ROHLOFF & BONES 2005). Tritrophic interaction of this plant with a herbivore and a parasitoid were examined by VAN POECKE et al. (2001, 2003). Therefore, another kind of tritrophic interaction in the *Arabidopsis thaliana* system waits for examination: the plant-fungus-herbivore examination. We chose *V. longisporum* as the fungus because it causes economically important damages on oil seed rape (JOHANSSON et al. 2006) and because it links belowground attack of the fungus to above and belowground responses of insects mediated by volatiles released by the different compartments of the plants.

MATERIAL AND METHODS

PLANT MATERIAL

Seeds of *Arabidopsis thaliana* (Col) were sterilized (according to CLOUGH & BENT 1998) and germinated on 0.5% Agar (for receipt see nutrient solution). Plants were grown in growth cabinets (d/n: 8/16; 21 °C/16 °C; 60% r.h.) in sand/vermiculite mixture in clay pots. They were watered daily with nutrient solution (10mM KPP-buffer, 125µM Fe-EDTA, 2mM $MgSO_4$, 1mM $CaCl_2$, 2mM $(NH_4)_2SO_4$, 3mM KNO_3, 125µM H_2BO_3, 30µM $MnSO_4$, 2.5µM $ZnSO_4$, 2.5µM $CuSO_4$, 0.s5µM Na_2MoO_4). Plants were inoculated in the age of 4 weeks by watering with 10 ml spore suspension (10^6 spores/ml; according to DEBODE et al. 2005).

FUNGUS MATERIAL

Verticillium longisporum (43) was grown in PD-Broth at 23 °C on a rotary shaker (app. 100 U/min). After 14 days spores were harvested by filtering through glass wool and diluting with sterile tap water.

SAMPLING OF VOLATILES

Plant volatiles were sampled 14 days post infection, 5 replicates each treatment, by using Tenax®-TA adsorbent (175-180 mg; Gerstel, Germany) and charcoal cleaned synthetic air in an open system. Gas volume was replaced 3 times before collecting the volatiles for 2 (shoot) or 1 (root) hours at a flow rate of 1l/min. During sampling, the plants were not removed from the pots and left untouched. So they were not damaged or stressed, but measured under near-nature conditions. Shoot and root were measured separately.

ANALYSIS

For analyzing the samples a gas chromatograph (G890N GC) coupled with a mass spectrometer (5973 MS; both Agilent Technologies) was used. Adsorbed volatiles were disorbed via thermo-desorption (cold injection system; Gerstel, Germany) and separated on a HP-5MS (non-polar column; Agilent Technologies). For interpretation Enhance Chemstation version D00.0038 (Agilent Technologies) and National Institute of Standards and Technology (NIST, Gaithersburg,

USA) Mass Spectral Search Library was used. Identity of compounds was confirmed by coelution with authentic standards. Results were evaluated on a statistical basis (Post Hoc). Additionally principle component analysis (Pirouette, Gerstel, Germany) was used.

RESULTS

GENERAL OBSERVATIONS

Plants infected with the pathogen showed symptoms of early senescence, like yellowing of leaves, and smaller leaves. They did not show any symptoms of stress or damaging concerning the sampling. Moreover, we could confirm a reliable separation of root and shoot during sampling.

VOLATILE ANALYSIS

As summarized in Tab. V-1, alterations were found mainly for aldehydes and terpenoids. Isothiocyanates and "green-leaf" odour (i.e. 3-Hexen-1-ol) were not detected – neither in the shoot nor in the root.

Tab. V-1: Changes in volatile release rates of *Arabidopsis thaliana* in case of infection by *Verticillium longisporum*; 14 days post infection (* means: significant: $P<0.05$, ANOVA)

	Chemical classes	**Alterations in case of infection**
Shoot	Isothiocyanates	No compounds found
	"green-leaf" odour	No compounds found
	Terpenes	Decrease: Geranylacetone, Limonene*
	Aldehydes	Increase: Octanal, Nonanal
Root	Isothiocyanates	No compounds found
	"green-leaf" odour	No compounds found
	Terpenes	Increase: Limonene*
	Aldehydes	Increase: Octanal, Nonanal

DISCUSSION

Volatiles are products of plant metabolism and allow us to point out alterations in physiology of plants. Terpenes and oxylipins play an important role in plant-insect interactions being infochemicals by attracting pollinators and parasitoids or repelling herbivores (PICHERSKY & GERSHENZON 2002; VAN POECKE et al. 2001, 2003). In 2007 VAN POECKE reviewed the role of terpenoids in *Arabidopsis* insect interactions. Terpenes are reduced emitted in the shoot of infected *Arabidopsis* plants.

However, a reduction in releasing rate of limonene of the shoot is complemented by an increase of release rates from the roots. Limonene has antibiotic properties (AGGARWAL et al. 2001) and might be regarded as a defense response to *V. longisporum* attack. It is not clear yet, whether limonene is translocated from shoot into the roots or if it is de novo synthesized, induced in the roots.

Aldehydes are also emitted in higher amounts when the plant is infected. Those compounds are typically degradation products of membrane proteins (i.e. MATSUI et al. 2006). So they indicate damaging, stress or senescence of cells. The absence of isothiocyanates and "green leaf"-odor compounds, like 3-Hexen-1-ol, showed mechanically damage free sampling. This leads to the assumption that the fungus causes damage to plant membranes followed by a degradation of fatty acids without inducing the oxylipin pathway by wounding plants cells. This would end up with the production of jasmonic acid (and 3-Hexen-1-ol as by-product; CREELMAN & MULPURI 2002). But no 3-Hexen-1-ol emission was found.

Thus, the damage free sampling system proved to yield consistent VOC pattern from phyllosphere and rhizosphere under near nature conditions. This provides the opportunity to study multitrophic interactions on the model organism *Arabidopsis thaliana* with the benefits of a laboratory set-up reducing the artifacts known to occur in conventional laboratory set-ups. Working with this system, we could show that *Arabidopsis thaliana* is responding to fungal infection with symptoms of drought stress and early senescence. Gross terpenoid emissions are

reduced and degradation products of membrane lipids are increased. Rough handling artifacts can be excluded because of the absence of isothiocyanates and “green-leaf odour” compounds in the volatile samples.

Both the terpenoids and the aldehydes affected by *Verticillium longisporum* infection are perceived by root and shoot feeding herbivores as well as by parasitoids (i.e. BRUCE et al. 2005). Therefore, impact of *Verticillium* infection on host finding and host choice behaviour of these insects is likely but remains to be proven.

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SUMMARY

SUMMARY

Brassica napus is one of the most important crops in agriculture. Pathogens cause big losses in yield each year. In the frame of this work, the interactions between the crop *Brassica napus*, resp. the model plant *Arabidopsis thaliana* and the phytopathogenic fungus *Verticillium longisporum* were investigated. *V. longisporum* is specialized on *Brassica* and causes the "*Verticillium* – wilt". It penetrates the roots of the plant und spreads through the vessels inside the shoot. Infection symptoms are stunting of the shoot, chlorosis and premature ripening.

Brassica napus and *Arabidopsis thaliana* were infected with *Verticillium longisporum* and changes of their VOC emissions (Volatile Organic Compounds) of shoot and root were analyzed. We ensured damage free and stress reduced measurements. Therefore, plants were not removed from the clay pots during measurements of root VOCs, but sampled inside the substrate.

While the VOC pattern of the roots from *B. napus* did not change, the shoots emitted significantly more dimethyl disulfide, ß-ionone and ß-cyclocitral. These compounds were irrelevant in the infection of *A. thaliana*. Afterwards, we proved the suggestion that VOCs, changing under infection, might serve as defense molecules. Therefore, a non-contact bioassay was developed. Two further pathogenic fungi (*Gaeumannomyces graminis* var. *triciti* and *Sclerotinia sclerotiorum*) with different specialization were included. We fumigated them with dimethyl disulfide and ß-ionone in different dilutions. Both compounds showed a limited fungicidal effect, measured by the mycelium growth, against the *Brassica* specialist *V. longisporum*. *G. graminis* var. *triciti* was inhibited at lower concentrations. *S. sclerotiorum* was not or even positively influenced by those VOCs. The formation of microsclerotia of *V. longisporum* was supported by ß-ionone.

ZUSAMMENFASSUNG

Brassica napus ist eine der wichtigsten Pflanzen in der Landwirtschaft. Pathogene verursachen jährlich große Ertragsverluste. Im Rahmen dieser Arbeit wurden die Interaktionen zwischen der Nutzpflanze *Brassica napus* bzw. der Modellpflanze *Arabidopsis thaliana* und dem phytopathogenen Pilz *Verticillium longisporum* untersucht. *Verticillium longisporum* ist ein auf *Brassica* spezialisierter Pilz, der die sogenannte *Verticillium*-Welke verursacht. Er dringt in die Wurzeln der Pflanze ein und steigt durch die Leitgefäße in den Spross auf. Die Pflanze zeigt in Folge der Infektion vor allem einen stark verkürzten Spross, Chlorosen und eine Notreife.

Für die Experimente wurde *Brassica napus* und *Arabidopsis thaliana* mit *Verticillium longisporum* infiziert. Die Emissionen von Volatilen (volatile organic compounds (VOC)) der Pflanzen wurden sowohl ober- als auch unterirdisch bezüglich sich ändernder VOCs analysiert. Bei der Probennahme wurde darauf geachtet, die Pflanzen nicht zu verletzen und durch Messungen bedingten Stress zu minimieren. Auch während der Messungen der Wurzelemissionen wurden die Pflanzen nicht aus ihrem Substrat entfernt, sondern innerhalb dessen gemessen.

Während sich das Duftspektrum der Wurzeln von *Brassica napus* nicht änderte, emittierten die Sprosse infizierter *Brassica* Pflanzen signifikant mehr Dimethyldisulfid, ß-Ionon und ß-Cyclocitral. Bei der Infektion von *Arabidopsis thaliana* mit *Verticillium longisporum* hingegen spielten diese VOCs keine Rolle. Im zweiten Teil der Arbeit wurde die Vermutung überprüft, dass sich im Infektionsverlauf ändernde VOCs als Abwehrmoleküle dienen könnten. Hierfür wurde ein Bioassay entwickelt und zwei weitere pathogene Pilze, *Gaeumannomyces graminis* var. *triciti* und *Sclerotinia sclerotiorum*, unterschiedlicher Spezialisierung einbezogen. Wir setzten sie Dimethyldisulfid und ß-Ionon in unterschiedlichen Konzentrationen aus. Sowohl Dimethyldisulfid als auch ß-Ionon zeigten nur eine geringe fungizide Wirkung, gemessen am Myzelwachstum, gegen den *Brassica*-Spezialisten *Verticillium longisporum*. *Gaeumannomyces graminis* var. *triciti* wurde bereits bei geringeren Konzentrationen gehemmt. *Sclerotinia sclerotiorum* reagierte nicht oder sogar positiv auf die VOCs. Die (Mikro-) Sklerotienbildung

wurde nur bei *Verticillium longisporum* beeinflusst. ß-Ionon unterstützte diese Entwicklung signifikant.

SYNTHESIS

The presented work addresses the pathosystem *Brassica napus* and *Verticillium longisporum*. With gas chromatography and mass spectrometry, alterations in the emission pattern of the plant were investigated. During the infection, no VOC changes were detected in the root emissions. However, two terpenoids (ß-ionone and ß-cyclocitral) and one sulfur containing compound (dimethyl disulfide) were released in significantly higher amounts from the shoot of infected plants. In order to investigate their possible ecological role in the interaction between *Brassica napus* and *Verticillium longisporum*, a bioassay was developed. ß-Ionone and dimethyl disulfide, already reported to have antimicrobial activities, were tested using a non-contact bioassay for their antifungal activities against *Verticillium longisporum*, a fungus specialized on *Brassica*. Furthermore, *Sclerotinia sclerotiorum*, a generalist with a wide host spectrum, and *Gaeumannomyces graminis* var. *triciti*, a specialist on grain, were tested. *Verticillium longisporum* was found to be less susceptible to dimethyl disulfide than *Gaeumannomyces graminis* var. *triciti*. That compound supported the growth of *Sclerotinia sclerotiorum*, which showed adaption towards dimethyl disulfide. ß-Ionone did not affect the growth of that fungus, but had an impact on both specialists in high concentrations. Additionally, the terpenoid supported the formation of microsclerotia of *Verticillium longisporum*.

CHANGES OF THE VOC EMISSION IN *BRASSICA NAPUS* UNDER INFECTION

The presented work gives some indications of the role of VOCs in the interaction with pathogenic fungi. One characteristic of the pathosystem "*Brassica napus – Verticillium longisporum*" is that the fungus seems to invade the plant without being recognized. Significant infection symptoms, such as stunting and chlorosis, get visible only four weeks after inoculation, but no typical wilt symptoms can be observed (EYNCK et al. 2007, confirmed by own observations). Additionally, significant changes in the VOC pattern were detectable quite late. Starting three weeks after inoculation, the total emissions of infected plants increased. However, we did not detect stress induced VOCs in higher amounts, but instead the degradation products ß-ionone, ß-cyclocitral and dimethyl disulfide. They also occured in lower amounts in non-infected plants. So most likely the emission of those compounds was not induced by the infection

of *Verticillium longisporum.* We suppose that the disease leads to early degradation of cell compounds, such as carotinoids and proteins, followed by an enhanced emission of ß-ionone, ß-cyclocitral and dimethyl disulfide. This observation supports the suggestion to declare the *Verticillium* wilt (without wilt symptoms) as premature ripening (Eynck 2007). No alterations were found in the VOC emission of the roots four weeks after inoculation.

PLANT VOC IN PATHOGEN DEFENSE

VOCs are developed in the daily metabolism and their release might be unavoidable, but evolution assigned them special tasks (PEÑUELAS and LLUSIA 2004, HOLOPAINEN 2004, NIINEMETS et al. 2004). As HOLOPAINEN (2004) determined, plant VOCs could reduce or even prohibit further dispersal of a pathogen inside the plant. Among others, NIINEMETS et al. (2004) reported on storage possibilities for VOCs inside the plant, which also supports this assumption. We tested two of those degradation products towards their possibilities to inhibit the growth of *Verticillium longisporum* and two other fungi. Both of the tested compounds are hardly soluble in water, therefore we assume that only low concentrations (0.0001 g/g and lower) can diffuse within the plant. These concentrations did not affect the *Brassica* specialist, but inhibited fungi with distinct host specialization, indicating a partial role in general defense strategies of the plant against non-compatible pathogens. Ddimethyl disulfide and ß-ionone might accompany defense strategies but most likely they did not play the leading role, because their antifungal activity was limited towards the tested organisms. Furthermore, our observation of the support of microsclerotia formation under ß-ionone treatment might indicate a signaling role of this compound in the interaction between *Brassica napus* and *Verticillium longisporum*.

To confirm our assumptions and hypotheses, further research has to be done. Attention must be focused on the methodology which can influence results and conclusions significantly. Above all, applied concentrations should be adapted to mimic naturally occurring ones, to the best of our ability.

EMISSIONS OF *BRASSICA NAPUS* COMPARED TO *ARABIDOPSIS THALIANA*

In this study we sampled VOCs from two Brassicaceae plants. In contrast to *Brassica napus*, *Arabidopsis thaliana* emits only few VOCs, when undamaged (VAN POECKE 2007). Only 32 compounds were identified in the headspace of *Arabidopsis* plants (ROHLOFF and BONES 2005), compared to 120 compounds, which we found in the emissions of *Brassica napus*. In order to enhance the emission, many scientists damage or pool *Arabidopsis* plants and induce non-natural emissions by doing this.

In our damage-free shoot measurements of single *Arabidopsis thaliana* plants, we did not detect ß-ionone, ß-cyclocitral and dimethyl disulfide. ROHLOFF and BONES (2005) as well as VAN POECKE (2001) characterized those compounds as autolysis products of *Arabidopsis thaliana*, which occur when plant tissue is damaged artificially or by caterpillar feeding. The absence of those VOCs means only that the emitted amount might be too small to detect. In our samples, as well as in those of ROHLOFF and BONES (2005) and VAN POECKE (2001) another terpenoid was found. Geranylacetone has the same origin than ß-ionone and ß-cyclocitral (SIMKIN et al. 2004). Both, infected and healthy *Arabidopsis* plants emitted geranylacetone. The slightly smaller amount (not significant) in *Verticillium*-infected plants might be due to their smaller leaf area caused by the infection. ROHLOFF and BONES (2005) associate geranylacetone with the "wound-induced" VOCs ß-ionone and ß-cyclocitral, which supports the assumption that terpenoids deriving from carotinoid degradation are not induced by the infection with *Verticillium longisporum* but rather a signal of premature ripening. Observations by FLÖRL (2007, 2010) support the theory of carotinoid degradation. At 25 dpi, infected *Arabidopsis* plants showed a significantly lower amount of carotinoids, apart from characteristic infection symptoms (significantly smaller leaf area, lowered chlorophyll content, yellow veins and stunted petiols).

ADVANTAGES OF DAMAGE FREE SAMPLING METHOD

In this work we emphasized damage and stress free measurement and avoided destructive sampling methods. Therefore, the shoots of intact plants were separated from the soil by a PTFE lid and measured inside a glass vessel. The roots were not removed from the soil but VOCs were sampled inside the substrate. Our results showed that we succeeded in damage and stress free sampling of shoot VOCs. No damage or stress induced compounds were detected in the shoot. Measuring the root by using the non-disturbed method could reduce damaging but not avoid it completely. Furthermore, all VOCs from the rhizosphere were sampled. Nevertheless, this method might provide a good basis for further research.

CONCLUSION

The infection of *Brassica napus* and *Arabidopsis thaliana* leads to the degradation of carotinoids, the products of which are released by the plant as VOCs. We assume that the carotinoid cleavage is not induced by the infection exclusively. Instead, we suggest that it is a sign of premature ripening. Nevertheless, as the presented studies showed, the released VOCs might play a role in defense, at least against non-compatible pathogenic fungi.

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