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Characterisation and Identification of *Fusarium foetens*, Causative Agent of Wilting and Stem Rot of Begonia Elatior Hybrids (*Begonia* x *hiemalis*) by its Volatile Compounds

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Summary

Five morphologically different isolates of *Fusarium foetens* were cultivated on potato dextrose agar (PDA), and the fungal volatile compounds were detected and analysed by headspace sorptive extraction (HSSE) followed by gas chromatography / mass spectrometry (GC/MS). Ten sesquiterpenes and five cyclohexane derivatives were detected. Hierarchical cluster analysis enabled the differentiation between red coloured sesquiterpene producing strains and white or pink coloured cyclohexane derivative producing strains. This result has been confirmed by sensory characterisation carried out by sensory trained examiners. Sesquiterpenes 10, 11 and 18 were detected in all isolates of both groups, however, in the first group at a higher level with an average of 88 %-peak area. The patterns of a RAPD-PCR analysis did not show any differences between the isolates. However, RAPD-PCR enables a clear differentiation of *Fusarium foetens* from *Fusarium begoniae* and *Fusarium oxysporum* f. sp. *cyclaminis* isolates. The combination of both techniques, the HSSE and the RAPD-PCR, provides an improvement in identification of *F. foetens* and differentiation of its strains. Begonia elatior hybrids (*Begonia* x *hiemalis*), cultivar 'Barkos', were inoculated with a sesquiterpene producing strain of *Fusarium foetens* and incubated in a moist chamber. The volatile organic compounds were extracted and analysed by using the same methods as for the above-mentioned in vitro experiments. The three sesquiterpenes 10, 11 and 18, previously detected on agar plates, achieved an average of 63 %-peak area. Only traces of the main compounds were detected in extracts from whole infected plants. Therefore, practical application e.g. in the form of a so-called "e-nose" to help detect infected plants is currently not realistic, but holds promise for the future.

Key words. Headspace analysis – RAPD-PCR – sesquiterpenes – volatile organic compounds – SBSE – GC/MS – HSSE

Introduction

In 2001, a new severe tracheomycosis (*Fusarium foetens*) causing wilting, stem rot and total decay of begonia elatior hybrids (*Begonia* x *hiemalis*) had been observed in European greenhouses. On potato dextrose agar (PDA) or oatmeal agar (OA), a strong, pungent odour is released from *F. foetens* cultures (Schroers et al. 2004). Generally, odour from fungi colonies is often used in their identification procedure. However, mycologists are usually not well trained in the sensory characterisation of volatile compounds and, consequently, the description of specific odours is not always very precise. A more precise, objective and reproducible method for the detection and identification of volatile organic compounds is the in-vivo-Headspace-SBSE (stir bar sorptive extraction), also referred to as HSSE (headspace sorptive extraction). By this technique, volatile organic compounds accumulate in the headspace of a sample and are sorbed by polydimethylsiloxane (PDMS), which is applied onto a magnetic stir bar. After extraction, the compounds on the stir bar are desorbed by thermal desorption and analysed by gas chromatography / mass spectrometry (GC/MS).

Various studies have shown that emitted volatile compounds, especially sesquiterpenes, can be used for characterisation and taxonomic classification of microorganisms (Schnuerer et al. 1999; Demyttenaere et al. 2004; Momol et al. 2004). However, the microbial production of volatile compounds depends on various environmental factors, which include for example nutrition, temperature and light. In addition to taxonomic purposes, volatile compounds may also be useful in the development of techniques for monitoring diseases and pests in growing crops or stored plant products. Specific sensors, e.g. "electronic noses", may provide a perspective that enables the grower to detect infestations at an early stage and to react in time with adequate measures. By using biosensors, Schuetz et al. (1996) demonstrated the potential of such non-destructive techniques to detect plant diseases. Furthermore, in the quality control of stored food and feed, volatile compounds may be used as suitable indicators to predict spoilage (Schnuerer et al. 1999).

The main objective of this study was to evaluate the suitability of fungal volatile compounds for diagnosis and monitoring of *Fusarium foetens* in comparison to RAPD-PCR. Phenotypically different isolates of *Fusarium foetens* should be characterised by specific volatile compounds using the HSSE-technique. Additionally, it was to be clarified whether or not this pathogen produces the same characteristic compounds on agar plates, in excised infected plant tissue and also in infected intact whole plants.

Materials and Methods

Sample preparation and extraction of volatile compounds

The study included five isolates of *F. foetens* and one isolate of each *F. oxysporum* f. sp. *cyclaminis*, *F. begoniae*, *F. culmorum*, *Phytophthora cryptogea* and *Botrytis cinerea* (Table 1). The sample preparation varied according to the three principal sources:
(1) in vitro growth of fungal colonies on agar plates (PDA),
(2) excised infected plant tissue in a moist chamber and
(3) whole infected plants.
For HSSE extraction, a 10 mm magnetic stir bar (Twister®, Gerstel, Mühlheim/Ruhr, Germany) on a sterile watchglass (d=20 mm) was placed onto the agar plate, into the moist chamber or under the glass cover close to the infected plant (Fig. 1). The stir bar was coated with a 0.5 µm layer of polydimethylsiloxane (PDMS), which is equivalent to 55 µl PDMS. After a defined extraction time, the stir bar was removed using magnetic forceps, rinsed with distilled water, then dried with lint-free tissue paper and placed in a glass thermal desorption tube (Gerstel) for further processing.

(1) Extraction of volatile compounds from in vitro cultures

A cylindrical 5 mm mycelium grown agar plug of each isolate was taken and inoculated onto PDA plates (9 cm diam.). After five days at 24 °C in the dark, a Twister® was placed onto a watchglass and put onto the agar surface. The plates were sealed with Parafilm® and incubated under the same conditions for another 48 h. A non-inoculated agar plate and an empty polystyrene Petri dish were used as controls. Colour, growth and various morphological characteristics of the isolates were recorded. The experiment was repeated twice.

(2) Extraction of volatile compounds from excised infected plant tissue

One week after potting, begonia elatior cultivar 'Barkos', were drench inoculated with 80 ml conidia suspension (ca. 10^6 cfu ml^{-1}) of a highly pathogenic *F. foetens* strain (isolate 63). After cultivation for 21 to 24 weeks under standard greenhouse conditions, lower stem parts up to the second internode of the plants showed typical symptoms but no obvious stem rot. The stem parts from one plant per treatment were surface sterilised, cut into pieces and placed together with a Twister® on a watchglass into a moist chamber (sterile 9 cm polystyrene Petri dish). After incubation for 4 days at 24 °C in the dark, stir bars were removed and processed as described above. A moist chamber with healthy plant material and one without plant material were used as controls.

(3) Extraction of volatile compounds from intact infected plants

For this experiment, the same cultivar and isolate were used as for the extraction from excised tissue in moist chambers. In addition to the drench inoculation (see above), root balls of the young plants were immersed in a conidia suspension (ca. 10^6 cfu ml^{-1}) for 30 minutes directly before potting. Typical symptoms (growth reduction, leaf necrosis and basal stem rot) developed within 4 weeks. Single plants with typical symptoms, together with a Twister® on a watchglass, were covered with a 250 ml sterile glass beaker (Fig. 1) and placed on a laminar flow bench under fluorescent light to ensure an odourless environment. The stir bars were removed after 4 days.

GC/MS analysis

Analysis of volatile compounds was performed according to the method of Bicci (2000) and Baltussen (1999),

Table 1. Differentiation of the fungal isolates by growth rate, colour and odour.

Fungal isolate	Colony diameter		Colour	Odour (sensory characterisation)
	5 dpi[1)]	7 dpi		
Botrytis cinerea	8.5 cm	8.5 cm	grey	mouldy, dusty, earthy, wet soil
Phytophthora cryptogea	3.5 cm	4.9 cm	white	sweetish, bacterial, caseous
Fusarium oxysporum f. sp. *cyclaminis*	5.2 cm	7.0 cm	white	bloomy, dusty
Fusarium culmorum	8.5 cm	8.5 cm	orange	odourless
Fusarium begoniae	4.3 cm	5.8 cm	white	odourless
Fusarium foetens 4	5.5 cm	8.5 cm	red	bloomy, mouldy, citric, dissolver-like
Fusarium foetens 10	5.8 cm	8.5 cm	white	mouldy, pungent, dissolver-like
Fusarium foetens 63	6.0 cm	8.5 cm	red	bloomy, mouldy, citric, dissolver-like
Fusarium foetens 140	5.8 cm	8.5 cm	white	mouldy, pungent, dissolver-like
Fusarium foetens 148	5.5 cm	8.5 cm	pink	fermenting, lightly pungent, mouldy, dissolver-like

[1)]days post inoculation

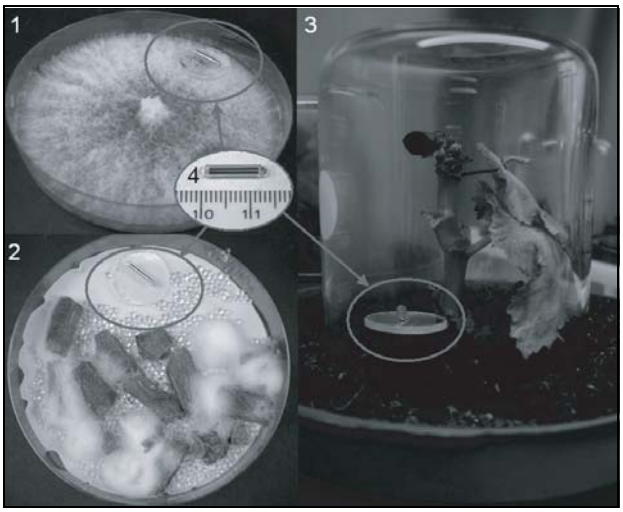


Fig. 1. Experimental setup of the headspace sorptive extraction (HSSE) of volatile compounds from agar plates (1), moist chambers (2) or whole plants (3) by using a Twister® (4).

which was slightly modified. The stir bars in thermal desorption tubes were introduced into a thermal desorption unit (TDS 2 and CAS 4, Gerstel), where the stir bar was thermally desorbed (temperature programme TDU: 30 °C; 720 °C min^{-1}; 250 °C (10 min) to release the extracted compounds into the cryogenically pre-cooled PTV (temperature programme of the CAS: −150 °C; 12.0 °C s^{-1}; 250 °C (10 min) for subsequent GC/MS analysis. GC/MS analysis was performed using a GC (GC 6890, Agilent Technologies, Little Falls, USA) equipped with a ZB-WAX column (30.0 m x 250 µm x 0.25 µm; Phenomenex, Aschaffenburg, Germany) and a mass selective detector (MSD 5973, Agilent Technologies). GC/MS conditions were as follows: temperature programme GC 130 °C; 4 °C min^{-1}; 250 °C (10 min); carrier gas helium with a constant flow of 1.3 ml min^{-1}; split-less time 1 min; transfer line temperature 250 °C; MS source temperature 230 °C; MS Quad temperature 150 °C; MSD total ion.

For data analysis, the program Enhanced Chem Station (G1701DA Version D 00.00.38) was used. The area of the important peaks was totalised and the sum was set to 100 %-peak area. Since there are no sesquiterpene standards available, a classification of the volatile compounds was done by mass spectra with the help of the NIST 98 library (US National Institute of Standards and Technology) and by determination of Kovàts retention indices.

Extraction of fungal DNA

Isolates of *F. foetens*, *F. oxysporum* f. sp. *cyclaminis*, *F. begoniae* and a re-isolate of *F. foetens* were cultivated in Czapek-Dox media and potato dextrose broth. DNA extraction was carried out with the E.Z.N.A Fungal DNA Mini Kit (peqlab Biotechnologie GmbH) and after a modified protocol of Hering (1997).

RAPD-PCR

For RAPD-PCR 11 ten-base oligonucleotide primers (Random-Primer-Kits A, B, C, ROTH GmbH + Co, Karlsruhe) with GC 60–80 % were used. PCR was performed in the PCR Systems 2400, 9600 and 9700 (Perkin Elmer Gene Amp) using the primers A01, A02, A03, A07, A10, A11, A13, A18, B01, C02 and C05. Denaturation started at 94 °C for 5 min in a first cycle, followed by further forty thirty second cycles. Primer annealing occurred at 40 °C and elongation at 72 °C both for 30 s. Finally an elongation at 72 °C for 7 min was performed. The reaction volume of 50 µl contained 1 µM l^{-1} primer, 200 µM l^{-1} of dNTPs, 5 µl "10x standard reaction buffer (15 mM l^{-1} $MgCl_2$), 1.5 U Taq polymerase and approximately 25 ng DNA. The amplicons and a molecular weight marker (1 Kb Plus DNA Ladder, Gibco BRL, Life Technologies) were separated by electrophoresis in a 1.4 % agarose gel.

Sensory characterisation

A cylindrical 5 mm mycelium grown agar plug of each isolate was taken and inoculated onto potato dextrose agar plates (9 cm diam.). After one week incubation at 24 °C in the dark, four sensory-trained examiners performed sensory analysis of odour. Classification follows the standard terminology of wine tasting (Fliedner and Wilhelmi 1989).

Statistics

Statistical analysis of 19 volatile compounds produced by the fungal isolates on agar plates was performed by using the statistical software SPSS (SPSS for Windows v.12.01). For grouping the various isolates according to their volatile compounds, a hierarchical cluster analysis was applied by using squared Euclidean distances.

Results

Morphological and sensory characterisation of the fungal isolates

The colonies of the various *F. foetens* isolates did not differ in their growth rate, however, they could clearly be distinguished by colour, which was red (isolates 4 and 63), white (isolates 10 and 140) or pink (isolate 148). Table 1 describes the groups of coloured colonies. *F. oxysporum* f. sp. *cyclaminis* showed a similar growth rate as the *F. foetens* isolates, whereas *F. culmorum* could be distinguished by its faster and *F. begoniae* by its slower mycelium growth.

All *F. foetens* isolates were characterised by a mouldy and dissolver-like odour. However, the red coloured isolates 4 and 63 produced additionally a bloomy and citric odour, the white coloured isolates 10 and 140 a pungent and the pink coloured isolate 148 a fermenting odour. Similar to the *F. foetens* isolates 4 and 63, *F. oxysporum* f. sp. *cyclaminis* released a bloomy odour but no mouldy or dissolver-like odours. *F. begoniae* and *F. culmorum* showed no remarkable odour. The odour of *Botrytis cinerea* was characterised as mouldy, dusty, earthy and wet soil like and that of *Phytophthora cryptogea* as sweetish, bacterial and caseous.

Volatile compounds

The most relevant volatile organic compounds of the various isolates detected by GC/MS analysis are presented in

Table 2. Relevant volatile compounds from various sources detected by GC/MS-analysis (n=2)

Volatile compounds		RI	%-peak area (extraction from agar plates)						Detection (+/-) in extracts from various sources		
			Fusarium foetens isolates					Foc	AP	MC	PL
			4	63	10	140	148				
cyclohexane derivative 1		1105	–	–	9.2	7.0	0.4	–	+	–	–
cyclohexane derivative 2		1110	–	–	25.6	19.2	–	–	+	–	–
cyclohexane derivative 3		1143	–	–	4.0	3.0	–	–	+	–	–
cyclohexane derivative 4	2-norbonene-5-ethylidene ?	1148	–	–	11.1	8.4	–	–	+	–	–
cyclohexane derivative 5		1216	–	–	19.4	22.0	–	–	+	–	–
sesquiterpene 1	δ-elemene ?	1431	1.8	3.6	1.9	2.6	0.2	2.6	+	+	–
sesquiterpene 2		1449	–	–	–	–	–	–	–	+	–
sesquiterpene 3		1523	–	–	–	–	2.6	–	+	–	–
sesquiterpene 4		1539	–	–	–	–	–	–	–	+	–
sesquiterpene 5	α-copaene ?	1552	1.4	0.7	–	–	0.5	–	+	–	–
sesquiterpene 6	β-caryophyllene ?	1563	–	0.7	–	–	20.7	–	+	+	–
sesquiterpene 7		1571	–	–	–	–	–	–	–	+	–
sesquiterpene 8		1578	–	–	–	–	–	–	–	+	–
sesquiterpene 9	δ-cadinene ?	1626	–	–	–	–	–	–	–	+	–
sesquiterpene 10	α-gurjunene ?	1629	65.0	75.7	24.4	32.2	8.2	96.1	+	+	+
sesquiterpene 11	acoradiene ?	1637	8.4	5.0	1.0	1.2	5.4	–	+	+	+
sesquiterpene 12	β-cubebene ?	1648	2.6	0.8	–	–	22.6	–	+	–	–
sesquiterpene 13		1655	–	–	–	–	–	–	–	+	–
sesquiterpene 14		1673	–	–	–	–	–	–	–	+	–
sesquiterpene 15		1722	–	–	–	–	–	–	–	+	–
sesquiterpene 16		1798	–	–	–	–	–	–	–	+	–
sesquiterpene 17		2054	–	0.1	–	–	10.5	–	+	–	–
sesquiterpene 18		2103	12.8	9.0	3.4	4.1	13.9	–	+	+	+
sesquiterpene 19		2146	4.6	1.9		0.4	6.3	–	+	–	–
sesquiterpene 20		2253	–	–	–	–	–	–	–	+	+

RI = Kovàts Index, linear retention index based on a series of n-hydrocarbons
Foc = *Fusarium oxysporum* f. sp. *cyclaminis*
AP = agar plates, MC = moist chamber, PL = plant
+ = detected, – = not detected

Table 2. Because of missing standards for classification, the compounds were numbered serially according to their Kovàts retention indices. Some of the volatile compounds could be named with the help of the NIST library but without screening by references.

Altogether 20 important compounds could be detected, including five cyclohexane derivatives and 15 sesquiterpenes. Thereof 15 compounds were emitted by the fungal isolates on agar plates. The %-peak area of these compounds was used as basis for a hierarchical cluster analysis. *F. begoniae* and *F. culmorum* were neglected in the calculations, because they did not release any volatile compounds. Fig. 2 shows a main cluster summarising all isolates of *Fusarium* and distinguishing it from *Botrytis cinerea* and *Phytophthora cryptogea*. Furthermore, the isolates of *Fusarium* are separated in two sub-clusters. The first sub-cluster includes the red coloured isolates 4 and 63 of *F. foetens* and the isolate of *F. oxysporum* f. sp. *cyclaminis*. Nearly all volatiles of this group were identified as sesquiterpenes. Hence, these isolates will be referred to as sesquiterpene producing strains in the following. The white coloured *F. foetens* isolates 10 and 140 and the pink coloured isolate 148, belonging to the second cluster, additionally released various cyclohexane derivatives. These isolates will be referred to as cyclohexane derivative producing strains in the following. The *F. foetens* isolates 10 and 140 are very similar in their pattern of volatile compounds while the isolate 148 is different.

Table 2 shows that all isolates of *F. foetens* emitted sesquiterpenes 10, 11 and 18 in different amounts. The sesquiterpene producing strains 4 and 63 emitted 65.0–75.7 %-peak area of sesquiterpene 10 and *F. oxysporum* f. sp. *cyclaminis* even 96.1 %. Moreover, 5.0–8.4 %-peak area of sesquiterpene 11 and 9.0–12.8 %-peak area of sesquiterpene 18 were detected for the sesquiterpene producing strains. In conclusion it can be pointed out that sesquiterpenes 10, 11 and 18 totalised could be detected with an average of 88 %-peak area for the sesquiterpene producing strains on agar plates. The cyclohexane deriv-

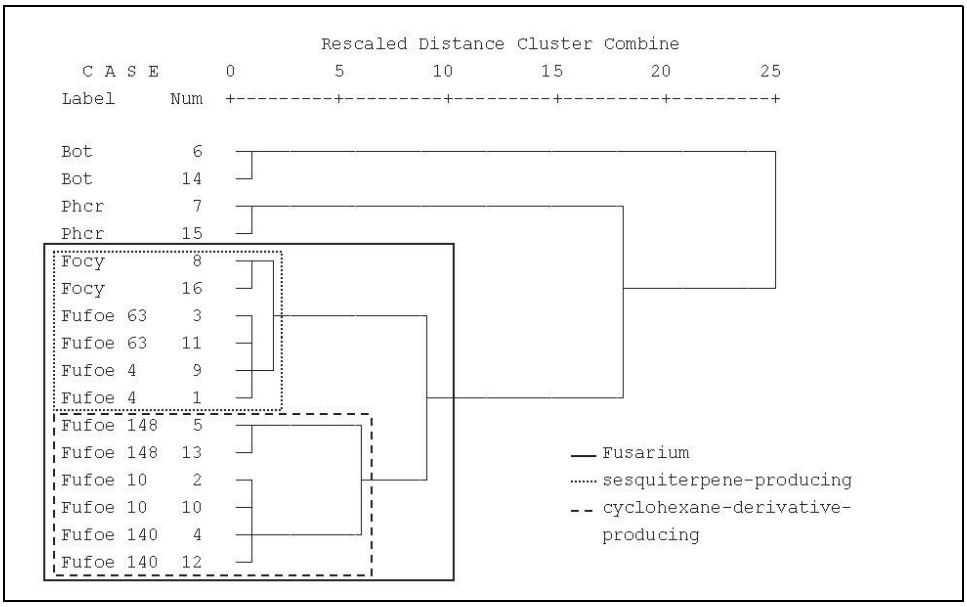


Fig. 2. Dendrogram (average linkage between groups) of the hierarchical cluster analysis based on %-peak areas of 19 volatile compounds from mycelium grown agar plates. Bot = *Botrytis cinerea*, Phcr = *Phytophthora cryptogea*, Focy = *Fusarium oxysporum* f. sp. *cyclaminis*, Fufoe = *Fusarium foetens*.

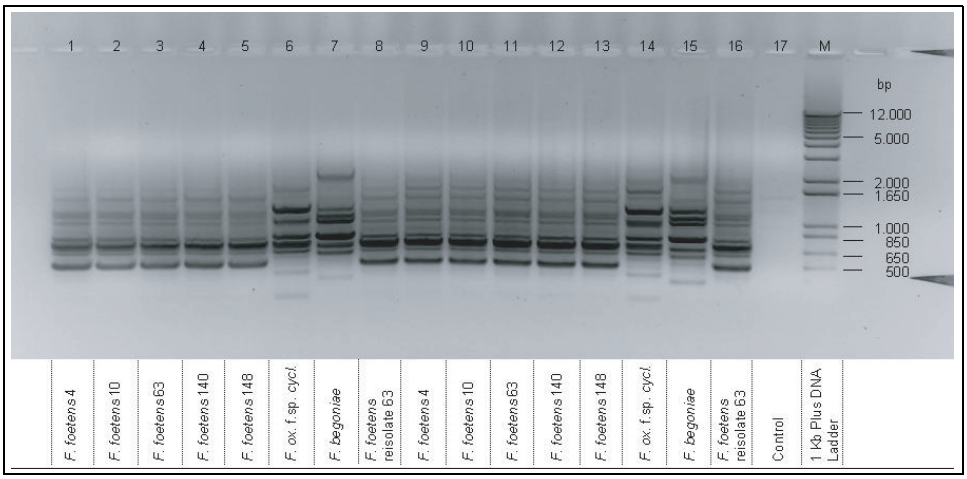


Fig. 3. RAPD-PCR patterns of *Fusarium begoniae*, *Fusarium oxysporum* f. sp. *cyclaminis* und various strains of *Fusarium foetens*. DNA isolation for lane 1 to 8 by E.Z.N.A fungal DNA MiniKit and for lane 9 to 16 by a modified protocol acc. HERING (1997).

ative producing *F. foetens* strains 10, 140 and 148 emitted sesquiterpene 10 to 8.2–32.2 %-peak area, sesquiterpene 11 to 1.0–5.4 %-peak area and sesquiterpene 18 to 3.4–13.9 %-peak area. Finally, for the cyclohexane derivative producing strains an average of 31 %-peak area was detected.

Extraction and analysis of volatile compounds from excised infected plant material in moist chambers revealed 15 different sesquiterpenes. Five of these sesquiterpenes (1, 6, 10, 11, 18) were detected on agar plates as well. Sesquiterpenes 10, 11 and 18 were the main compounds averaging to 63 %-peak area. Detection of volatile compounds was related to the superficially developed mycelium.

The main sesquiterpenes 10, 11 and 18 were also detected in the extracts from 16 infected plants but only in low concentrations. Sesquiterpene 18 could be detected on 15 plants, sesquiterpene 11 on four and sesquiterpene 10 on one plant. Additionally, sesquiterpene 20 was extracted from four plants. At the time of extraction (5–6 weeks after inoculation), the collapsing plants showed basal rot covered with mycelium.

RAPD-PCR patterns

Using the 11 randomly selected RAPD-primers, no polymorphisms in PCR patterns could be detected amongst the *F. foetens* isolates. The grouping of the various *F. foetens* isolates by their colour, odour or volatile compounds could not be confirmed by their PCR patterns. However, it was possible to distinguish isolates of *F. foetens* from its sister taxon *F. oxysporum* f. sp. *cyclaminis*. *F. begoniae,* pathogenic to begonia elatior hybrids, could be clearly distinguished, too (Fig. 3).

Discussion

Headspace sorptive extraction (HSSE) of volatile compounds on agar plates has shown to be a suitable technique for the characterisation of *Fusarium foetens*. The hierarchical cluster analysis of the volatile compounds showed a division into red coloured sesquiterpene producing strains and in white and pink coloured strains producing cyclohexane derivatives. This classification has

been confirmed by sensory characterisation. SCHROERS et al. (2004), who first described *F. foetens*, also mentioned differences between the isolates with regard to colour but not to odour. They characterised the odour of *F. foetens* as strong, unpleasant and pungent. Usually, colony odour is not recognised as a highly valuable tool for discrimination. Characterisation by colony odour is often not very specific and mostly based on non-standardised terms. With the help of sensory trained examiners following the standard terminology of wine tasting, a more specific olfactory description of the colony odour has been possible demonstrating the additional value for characterisation of fungal isolates.

RAPD-PCR clearly differentiated *F. foetens* from other *Fusarium* spp., however, not between morphologically and olfactory different isolates of *F. foetens*. However, the randomly selected primers did not cover the complete genetic background. Perhaps applying other primers may result in genetically based groupings according to their phenotypic differences. Colony colour, odour and the production of volatile compounds may vary depending on growing media, growing conditions and degeneration (HERING 1997). However, the strains used in this study, regularly inoculated from nutrient poor onto nutrient rich agar, were very constant in colour, odour and emitted volatile compounds. Therefore, in addition to the molecular biological techniques, the detection of emitted volatile compounds and the advanced olfactory colony characterisation may prove to be useful tools for further discrimination of various strains or isolates of *F. foetens*.

By using the HSSE–GC/MS analysis, three characteristic volatile compounds (sesquiterpenes 10, 11 and 18) of *F. foetens* could be detected in extracts from agar plates, infected excised plant material and infected plants. Nevertheless it is undecided whether these main volatile compounds are responsible for the characteristic odour of *F. foetens* due to the fact that the odour strength of a substance does not primarily depend on the amount but on the odour threshold (OHLOFF 1990). To substantiate the three sesquiterpenes responsible for the pungent odour, these compounds must be first synthesised and then evaluated by GC regarding inherent odour and odour threshold at a sniffing-port.

The adapted methods for the extraction of volatile compounds showed to be most suitable for the characterisation of *F. foetens*. A possible use in practice is discussed in the following.

The extraction of volatile compounds emitted by infected plant material in a moist chamber or by *F. foetens* re-isolated on agar plates seems to be quite conceivable for practical use in diagnostic procedures. It allows a rapid identification of the pathogen by enrichment of the volatile compounds in the headspace and a fast detection by HSSE or electronic nose.

To use this method as an early detection system of diseased plants instead of isolating the pathogen or incubating infected plant material in a moist chamber is an exciting prospect, however, it is currently still too immature. The three main indicator volatile compounds of *F. foetens* in extracts from whole plants were detected in small amounts only. Moreover, at the time of extraction the plants showed already severe symptoms and superficially grown mycelium. However, based on the results presented in this study more sensitive detection systems based on HSSE or a specific electronic nose might be developed to allow monitoring of whole crops or packaging units of plants. Furthermore, the detection of volatile compounds from *Botrytis cinerea* and *Phytophthora cryptogea* may also encourage further research with the aim to develop non-invasive disease monitoring systems.

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