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Grapevine trunk disease in German viticulture: occurrence of lesser known fungi and first report of *Phaeoacremonium viticola* and *P. fraxinopennsylvanicum*

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Summary

Thirteen species of lesser known wood inhabiting fungi living on grapevine (*Vitis vinifera*) and/or in the vicinity of vineyards are presented with respect to systematics, life strategy and symptoms, host range, geographic distribution, transmission and occurrence on *Vitis*, identification, and diagnosis. Sampling has been performed during a three-year-survey covering nurseries, vineyards, and neighboring fruit trees in the viticultural area of southern Palatinate, Germany. The possible pathogenic significance and the relation to grapevine trunk diseases are discussed. The following species are reported for the first time in Germany: *Cadophora luteo-olivacea*, *C. fastigiata*, *Collophora paarla*, *Coniochaeta hoffmannii*, *Eucasphaeria capensis*, *Phomopsis cotoneastri*, and the Esca-related *Phaeoacremonium viticola* and *P. fraxinopennsylvannicum*. *Eucasphaeria capensis* and *Phomopsis cotoneastri* are reported for the first time from *Vitis* worldwide.

K e y w o r d s : fungal pathogens; geographic distribution; grapevine trunk diseases; molecular diagnosis; *Phaeoacremonium*.

Introduction

In nowadays viticulture, different species of *Vitis* are combined in grapevine. As a result of grafting processes, cultivars of *V. vinifera*, as the scion part, are combined with rootstock species such as *V. berlandieri*, *V. cinerea* or *V. rupestris*. The latter usually appear as hybrids. Both scions and rootstocks host numerous fungal organisms, showing a variety of different life strategies.

As with other perennial plants, also grapevine offers a vast range of ecological niches: fungal species may be isolated from leaves, berries, and – in increasing numbers with ongoing age – from the wood, both inside and on the surface. Classification of involved fungi encompasses a variety of taxonomic groups. Among these, anamorphic fungi and ascomycetes are most significant in relation to number of species and economic impact; well-known pathogens also are among the oomycetes and, in relation to the so-called grapevine trunk diseases (GTDs), the basidiomycetes. A unique role falls to the glomeromycetes, forming the essential VA-mycorrhiza with grapevine roots.

Anamorphic fungi, ascomycetes and basidiomycetes contribute to the complex of GTDs. The most important diseases in this context are Esca (including possible precursor diseases such as Petri Disease; for an overview see Mugnai *et al*. 1999, Surico *et al*. 2001, Bertsch *et al*. 2012), Eutypiosis, the "Bot cankers" (Lehoczky 1974, associated species worldwide are discussed in Úrbez-Torres 2011) and black foot disease (Nascimento *et al*. 2001, Fourie and Halleen 2004). Per definition, also Phomopsis cane and leaf spot (with *Phomopsis viticola* as the causing agent) as well as root rot of grapevine (species of *Armillaria*) are ranked within GTDs. For Europe, the economic significance of the latter two however is difficult to evaluate and related literature is rare (Aguín *et al*. 2006, Nascimento *et al*. 2007, Prodorutti *et al*. 2009); also, the exact taxonomic identity and pathogenic significance of the involved species partly remains unresolved or detailed knowledge is restricted to certain geographic areas (for an overview on *Phomopsis*, see Philipps 2000).

After a prolonged period of ongoing research worldwide, the exact composition of the fungal communities being involved in GTDs such as Esca, Petri disease or "Bot canker" is still not fully clear. In addition to the generally accepted taxa such as *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Botryosphaeria obtusa* (anamorph: *Diplodia seriata*), or species of *Fomitiporia*, just to name a few, a considerable number of new species have been detected and discussed in recent years. Among these are many species of *Phaeoacremonium* (Mostert *et al*. 2006, Gramaje *et al*. 2015), species of the teleomorphic genus of *Botryosphaeria* (Úrbez-Torres 2011), or members of the lesser known genera, *Cadophora* (Halleen *et al*. 2007, Gramaje *et al*. 2011) or *Neofusicoccum* (Amponsah *et al*. 2014).

To which degree these "new" species can be found in viticultural areas of Central Europe remains mostly unknown with the data at hand. Also, no statement is possible if these taxa would act as serious pathogens under Central European conditions and if, in some way, they contribute to the development of GTDs and related symptoms.

Fischer and Kassemeyer (2003) published an annotated list of esca related fungi in Germany, which was limited to a total of seven species, including the well known *Eutypa lata*, *Phomopsis viticola*, *Botryospahearia obtusa*, and *Cylindrocarpon destructans*. Subsequent research was limited, without taking much notice of other fungi. Fungal colonies not fitting into the realm of Esca-related

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fungi were mostly ignored and/or discarded. In 2013 we initiated a monitoring program on the possible existence of GTD-related fungi, including less known ones, in German nurseries as well as in vineyards and surrounding areas, with particular emphasis on fruit trees. By the end of 2015 we had collected and characterized numerous samples derived from different sources: i) nurseries, both indoor and outdoor; here, the wooden parts of the plant material and the different substrata related to the production of grafted vines such as water and callusing media were examined; ii) vineyards of different age and representing different cultivars; here, samples were derived from leaves, berries and wood; and, iii) air trapping was performed both in vineyards and nursery fields, and spore traps were also placed in selected stands of *Prunus* fruit trees, all of them in the vicinity of vineyards.

Fungi were identified by, i) culturing followed by microscopical and / or molecular based analysis of the derived mycelia; ii) molecular analysis without a pre-culturing step. The latter approach was used for some of the air samples in vineyards. The presented results are a selection only of our findings. With reference to the available literature and our own experience a total of 13 species is presented. For each species we provide information on life strategy and symptoms, host spectrum, geographic distribution, and diagnosis. General notes, with particular reference to the possible pathogenic impact, are added for each species. Cultural phenotypes are illustrated by photographs.

We have omitted well known species such as *Fomitiporia mediterranea* (*Fmed*), *Phaeomoniella chlamydospora* (Pch), *Phaeoacremonium viticola* (Pal), *Eutypa lata* (*Elata*) or *Phomopsis viticola* (*Diaporthe ampelina*). Plenty information on these taxa has been provided over the years and nothing new was added by our studies. It has to be mentioned though that in concordance with previous studies (Fischer and Kassemeyer 2003) *Fmed*, *Pch* and *Elata* appeared as the most common GTD related fungi, at least so in the vineyards.

Material and Methods

Sampling and culturing: Samples from grapevine wood were collected from the southern Palatinate area of Germany from 2013 through 2015. Isolations were made by plating surface sterilised symptomatic grapevine material onto Potato Dextrose Agar (PDA) or malt extract (ME) medium (for details see Fischer and Kassemeyer 2003, Cloete *et al*. 2016). Samples from grafting tools in nurseries were taken by scraping residues from tools into sterile 50 mL tubes; samples from callusing media were collected in April/May 2014 during callusing stage from various callusing boxes. Three spatula tips (appr. 200 mg) each of grafting residues and callusing media were suspended in 500 µL sterilised tap water containing Tween®80 (Carl Roth), respectively. Dilution series (undiluted, 1:10 and 1:100) were prepared in sterile tap water and were plated on PDA containing Chloramphenicol at a concentration of 25 $\mu g \cdot mL^{-1}$. In addition, liquid samples from various dipping baths (water baths, baths containing fungicides) were taken prior to grafting. Dilution series (undiluted, 1:100, 1:1.000 and 1:10.000) were plated out as described above. Dishes were incubated at 25 °C under permanent dark conditions. Fungal growth was checked once a day; resulting colonies were transferred onto separate Petri dishes containing PDA. Fungal isolates are maintained in the culture collection of the Institute for Plant Protection in Viticulture at the Julius Kühn-Institut, Geilweilerhof, and are stored in tubes at +4 °C conditions. Growth studies of mycelia were conducted on Petri dishes (9.5 cm in diam.) containing PDA at approx. 23 °C under daylight conditions.

Spore trapping: Spore trapping was conducted from July through December in 2014 and from March through December in 2015. One vineyard of *Vitis vinifera* 'Phoenix' planted in 1996 and located at the Geilweilerhof area as well as five fruit trees belonging to different species of *Prunus* were chosen for monitoring. All trees showed some signs of external decline and were located next to vineyards. Sampling of the spore traps was based on the filter method described by Eskalen and Gubler (2001). Traps consisted of glass slides covered with Chesebrough Vaseline (Ponds Hamburg, Germany) affixed on tree branches and in the canopy of vines. Slides were exchanged on a weekly basis.

DNA extraction, PCR and sequencing: Whole cell DNA was isolated from cultured mycelium as described by Tillett and Neilan (2000). Prior to DNA extraction, cultures were grown on PDA at appr. 23 °C under daylight conditions. Quantity and quality of the DNA were examined using a Spectrophotometer (Nanodrop 2000c, Thermo Scientific, Waltham, USA). Isolated DNA was diluted to a final concentration of maximum 100 $\mu g \cdot mL^{-1}$ in distilled water. The polymerase chain reaction (PCR) was used to amplify a portion of the nuclear encoded ribosomal DNA unit defined by the primer combination prITS5 and prITS4 (for primer sequences, see White *et al.* 1990). The fragment spans the entire ITS1 region, the 5.8S rRNA gene, and the ITS2 region. The PCR reactions were set up in 20 µL volumes. Hot start PCR was applied throughout using a KAPAHiFi™ Hot Start Polymerase (PEQLAB Biotechnologie GmbH, Erlangen, Germany): twenty five cycles were performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems, Darmstadt, Germany) using the following parameters: 95 °C initial denaturation step (5 min), 98 °C denaturation step (20 sec), 53 °C annealing step (15 sec), 72 °C primer extension (20 sec). A final incubation step at 72 °C (1 min) was added after the final cycle. Five µL of each PCR reaction were electrophoresed on 1.5 % agarose gels. A 100 bp+ DNA ladder (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was used as standard. The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was suspended in 30 µL Tris-HCl buffer (10 mM, pH 8.5). PCR primers, *i.e.* prITS5 and prITS4, were used for sequencing. Cleaned products were sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA). Products were then analyzed on an ABI Prism 3130XL DNA sequencer

(Perkin-Elmer, Norwalk, CN, USA). Sequences were processed as described before (Cloete *et al.* 2016) and have been deposited at GenBank (www.ncbi.nlm.nih.gov/genbank/).

Results

Fungal descriptions (in alphabetical order):

Aureobasidium pullulans (De Bary) G. Arnaud ex Cif., Ribaldi & Corte

Classification: Ascomycota, Dothideomycetes, Dothideales, Aureobasidium.

Life strategy and symptoms: *Aureobasidium pullulans* is a ubiquitous fungus; it can be found on soil, in the air, in liquids such as water or on/in any plant part. Occurrence may be as epiphyte or endophyte. On plants, no disease symptoms are related to the species. On humans however, it has a certain clinical significance: among other things the phenomenon of hypersensitivity pneumonitis, due to mold contamination with a high frequency of *A. pullulans* conidia, has been reported in a few cases (for instance Temprano *et al*. 2007).

Host plants: On a wide range of plant species, both angiosperms and gymnosperms, both annual and perennial. The species has been reported from *Vitis* in Australia, South Africa, Greece and Spain (Gonzalez and Tello 2011).

Geographic distribution: Essentially in all regions worldwide, including both temperate and cool regions; however, exact classification of the respective specimens is partly uncertain (Zalar *et al*. 2008).

Transmission and occurrence on *Vitis*: One-celled conidia are readily formed by the fungus and are thought to act as the primary source of dissemination (for instance, see Barnett and Hunter 2006). In our studies, the fungus was revealed from different sources: i) in nurseries, it was isolated from wood of plant material and from waste water; ii) in vineyards, it was detected on the leaf surface, in wood next to the grafting union, and in xylem sap during springtime; iii) on spore traps located outside of vineyards. Air, water etc. therefore are considered as the main vectors for the fungus. All in all, *A. pullulans* was among the most frequently isolated fungi in our monitoring.

Identification (Figure, a): The fungus is easy to cultivate and is fast-growing. Colonies on PDA are usually yeast-like; slightly pink when young, but turning dark with ongoing time; surface is more or less slimy due to production of conidia. We detected some variation in cultural appearance among our isolates, but this is in congruence

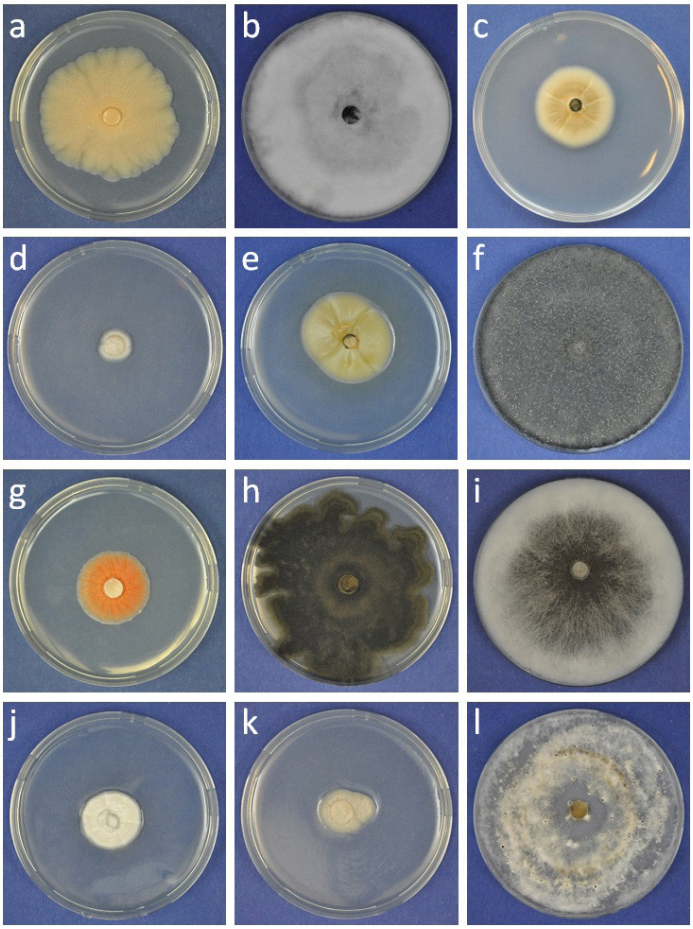

Figure: Fungi related to grapevine trunk disease in Germany. Cultures on PDA after 14 d of incubation at approx. 23 °C under daylight conditions. **a**: *Aureobasidium pullulans*; **b**: *Botryosphaeria dothidea*; **c**: *Cadophora luteo-olivacea*; **d**: *Collophora paarla*; **e**: *Coniochaeta hoffmannii*; **f**: *Diplodia seriata*; **g**: *Eucasphaeria capensis*; **h**: *Leucostoma persoonii*; **i**: *Neofusicoccum luteum*; **j**: *Phaeoacremonium fraxinopennsylvanicum*; **k**: *Phaeoacremonium viticola*; **l**: *Phomopsis cotoneastri*.

with former observations (summarized in Slepecky and Starmer 2009).

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX034050. No species-specific primers exist.

Notes: The species is significant under different aspects: i) it is of considerable importance in biotechnology, producing several enzymes and the edible polysaccharide polymer pullulan, and ii) several strains of the fungus are used as biocontrol agent on a variety of fruits or, in general, against postharvest pathogens such as *Botrytis cinerea* or *Penicillium expansum* (Ippolito *et al*. 2000). The species is more or less omnipresent in relation to grapevine and viticulture; its eventual impact on the plant is uncertain, but no real damage of the host is to be expected. For further evaluation of an antagonistic behavior tests on the possible efficacy against GTD related fungi would be essential.

Botryosphaeria dothidea (Moug. ex Fr.) Ces. & De Not.

Classification: Ascomycota, Dothideomycetes, Botryosphaeriales, Botryosphaeria.

Life strategy and symptoms: A number of disease symptoms have been related to *B. dothidea* in the past, among which dieback and canker on shoots and trunks are the ones most often cited (Larignon *et al*. 2001, under the designation "Black Dead Arm"; Phillips 2002, Úrbez-Torres *et al*. 2009). Besides, also berries might be affected (Millholland 1991). The species is saprobic, parasitic or endophytic (for an overview on related literature, see Farr and Rossman 2015), and the transfer between these different stages is still not fully clear. Rolshausen *et al*. (2010) have evaluated pruning wound susceptibility of grapevine using *B. dothidea* and other species.

Host plants: Both on angiosperms and gymnosperms, trees and shrubs; *Vitis* is just one of numerous hosts of *B. dothidea*. The species was redefined more recently (Slippers *et al*. 2004) and so the reported host range might be actually somewhat more limited. Phillips *et al*. (2013) should be consulted for actual data on the host range of *B. dothidea*.

Geographic distribution: Probably worldwide and cosmopolitan (Phillips *et al*. 2013).

Transmission and occurrence on *Vitis*: In general, infection by Botryosphaeriaceae is thought to occur *via* wounds (von Arx and Müller 1954, Smith *et al*. 1994; for an overview see Slippers and Wingfield 2007). Accordingly, affected wood takes its origin from wounds on branches or stems.

In our studies, the fungus was revealed from the following sources: i) in the nursery, where it was isolated from wood of plant material and waste water; ii) in the vineyard it was isolated from leaf surface and from wood of dead and living vines. In the wood of older vines the species was always co-occurring with other GTD-related fungi such as *Fmed*, *Elata* or *Pch*.

Identification (Figure, b): Colonies on PDA are variable in appearance; mostly whitish when young, then gradually turning dark grayish to olivaceous and finally blackish with age; reverse side always is darker. Colony surface is mat-like, aerial hyphae are abundant. Mycelium is fast-growing, covering a Petri dish (9.5 cm in diam.) within a week (see also Phillips 2002).

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037446. No species-specific primers exist.

Notes: Most prominent species of *Botryosphaeria* in Europe are *B. obtusa* (anamorph: *Diplodia seriata*; Larignon *et al*. 2001, Phillips *et al*. 2007, see below), *B. stevensii* (anamorph: *Diplodia mutila*; Lehozky 1974) or *B. parva* (anamorph: *Neofusicoccum* parvum; Phillips 2002). The pathogenic significance of the less common *B. dothidea* is still questionable. While the species was shown to have some impact in pathogenicity tests (Larignon *et al*. 2001, Úrbez-Torres *et al*. 2009, Rolshausen *et al*. 2010), it should be considered that such tests usually are based on mycelial plugs inoculated to artificial wounds. In the field, spores transported by air and / or water will act as the natural infection source however (for instance, see Kuntzmann *et al*. 2009).

Endophytic appearance of Botryosphaeriacae has often been reported in the past (comprised in Slippers and Wingfield 2007), and no symptoms may appear unless the host plant is under stress.

Cadophora luteo-olivacea (J.F.H. Beyma) T.C. Harr. & McNew

Classification: Ascomycota, Leotiomycetes, Helotiales, Cadophora.

Life strategy and symptoms: The type material of *C. luteo-olivacea* has been isolated from waste water of a "Schleiferei" in Sweden, 1939. Species of *Cadophora* have been recovered from a large variety of environments, such as decaying wood (most interesting, Blanchette *et al*. 2010 report occurrence of *Cadophora* in Antarctica, where it was isolated from E. Shackleton's wooden hut erected in 1907), soil, or plants such as grapevine. In the latter, the species might be related to general decline (Halleen *et al*. 2005, 2007, Rooney-Latham *et al.* 2005) and also was isolated from young vines affected by Petri disease, from nursery material and related environmental samples (Gramaje *et al*. 2011). *Cadophora* spp. are also found in healthy vines.

Host plants: Uncertain with the scattered data at hand, but possibly occurring on many deciduous plant genera.

Geographic distribution: Probably worldwide and cosmopolitan. Our finding of the species is the first documented one in Germany.

Transmission and occurrence on *Vitis*: The species readily produces conidia that are dispersed by wind or water and may infect any wounds in the wood (Gramaje *et al*. 2011). *Cadophora luteo-olivacea* in our studies was frequently found in nurseries, where it was isolated from grafting tools, wood of plant material (rootstocks, both grafted and non-grafted), waste water as well as from callusing media, *i.e.* sawdust and peat. In the vineyard, on one occasion it was isolated from xylem sap; otherwise, it was mostly derived from the wood next to the grafting union both in dead and living vines.

Identification (Figure, c): Mycelium is slowly growing on PDA, reaching maximum 1.5 cm of diam. per week. Colony surface in the centre is slightly cottony, olivaceous-greenish; at the margin it is usually whitish-grayish, smooth and shiny; reverse side is darker.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037447. Species-specific forward primers have been published by Spadaro *et al.* (2011).

Notes: Pathogenicity trials have been performed with *C. luteo-olivacea* and *C. melinii* by Gramaje *et al*. (2011). The former species produced lesions in selected rootstocks and consequently was considered a potential pathogen (also see Halleen *et al*. 2007, Travadon *et al*. 2015). Trials were based on mycelial plugs though and in this way do not fully represent natural conditions where conidia will act as source of infection. Based on conidial suspensions, postharvest pathogenicity was documented on kiwifruit, apple and pear (Spadaro *et al*. 2011). In our studies C. *luteo-olivacea* was the species most commonly isolated from wood showing signs of brown wood streaking (bws) and gummosis. While it usually appeared "single-handed" in affected areas of plant material, it was co-existing with other wood fungi such as *Pch* or *Pal* in older vines in the field. Further attention will be necessary to evaluate the pathogenic significance of *C. luteo-olivacea* on grapevine.

Another member of *Cadophora* new for Germany is *C. fastigiata* Lagerf. & Melin which was recovered on one opportunity from wood of rootstock material. To our knowledge (compare with Blanchette *et al*. 2010, Gramaje *et al*. 2011, Travadon *et al*. 2015) this is the first report of this species on *Vitis* as a host plant worldwide. GenBank accession is: KX037448.

Collophora paarla Damm & Crous

Classification: Ascomycota, Leotiomycetes, uncertain order, Collophora.

Life strategy and symptoms: Little is known about the life strategy of the recently described *C. paarla* (Damm *et al*. 2010). The type material has been collected from dark brown wood necrosis in the wood of *Prunus persica* (Peach) or *P. salicina* (Japanese plum) in the Western Cape region of South Africa and no further findings of the species have been documented so far. Related species have been derived exclusively from the same host genus, usually next to pruning wounds but also from discolorations inside tree branches, and often co-existing with other fungi such as *Alternaria* or *Phaeoacremonium*.

Host plants: Both *Prunus persica* (in the formal description) and *P. salicina* (Table) are mentioned in the original paper by Damm *et al*. (2010).

Geographic distribution: Original description from South Africa; with our finding now also demonstrated for Germany. Distribution probably larger, but records are most likely incomplete because of poor knowledge and/or misidentification.

Transmission and occurrence on *Vitis*: Conidia are formed in masses by the fungus and by wind or water may be transported to infection sites such as injured wood. Occurrence on grapevine is uncertain with the data at hand.

Identification (Figure, d): Mycelium is slowly growing on PDA, under 25 °C conditions reaching appr. 1 cm in diam. per week. Colony surface is without aerial mycelium; with irregular wrinkles in the centre; white to gray in the beginning, and slowly turning pale reddish with ongoing time. Margin is irregular, concolorous with centre.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037449. No species-specific primers are known.

Notes: In pathogenicity trials performed on a variety of *Prunus* cane sections, *C. paarla* caused lesions significantly longer than the negative control but significantly shorter than those caused by the pathogen control, *Eutypa*

Table

Grapevine Trunk Diseases in Germany: selected fungal species in alphabetical order and their occurrence in different environments

Species	Isolated from			
	nursery	vineyard	[3] outside vineyard	environment
Aureobasidium pullulans	+	+	+	wood, xylem sap, air sample
Botryosphaeria dothidea	+			wood
[1] *Cadophora luteo-olivacea*	+	+		wood, tool debris, sawdust, peat
[1] *Cadophora fastigiata*	+			wood
[1] *Collophora paarla*			+	air sample
[1] *Coniochaeta hoffmannii*		+		wood
Diplodia seriata		+		wood
[1, 2] *Eucasphaeria capensis*	+		+	wood, water, air sample
Leucostoma persoonii			+	air sample
Neofusicoccum luteum	+			wood
[1] *Phaeoacremonium fraxinopennsylvanicum*		+		wood
[1] *Phaeoacremonium viticola*		+		wood
[1, 2] *Phomopsis cotoneastri*	+			wood

[1] First reports for Germany; [2] First reports for *Vitis* worldwide; [3] from spore traps attached to trees of *Prunus* spp.

lata (Damm *et al*. 2010). Existence of the species on grapevine is uncertain; however, isolates might be simply overgrown on Petri dishes or they might be discarded due to their non-familiar appearance not resembling more widespread pathogens. In our studies, the species was revealed twice, August and September 2014, from spore traps attached to trees of *Prunus persica* respective *P. dulcis*, located next to vineyards.

Coniochaeta hoffmannii (J.F.H. Beyma) Khan, Gené & Guarro

Classification: Ascomycota, Sordariomycetes, Sordiales, Coniochaeta.

Life strategy and symptoms: Species of *Coniochaeta* and their anamorphs, *Lecythophora*, are ubiquitous and have been reported from substrata such as animal dung, wood-pulp, bark or wood of trees, water, soil etc. (see compilation given in Damm *et al*. 2010). Casieri *et al.* (2009) detected *C. hoffmannii* (as *Lecytophora hoffmannii*) on pruning wounds of young *Vitis vinifera* 'Humagne' in Switzerland. This finding was the first report of the species on grapevine; no particular symptoms were related to the fungus.

Host plants: Unknown, but probably occurring on numerous host plants both coniferous and deciduous (for instance, see Sieber *et al*. 1999).

Geographic distribution: Unknown, but probably widespread. Existing data are most likely incomplete because of poor knowledge and/or misidentification. In our study reported for the first time in Germany.

Transmission and occurrence on Vitis: By wind, water etc. The species has been revealed from pruning wounds of young vine (Casieri *et al*. 2009) and, on one occasion, from the grafting union of older vines in our study.

Identification (Figure, e): Colonies are moderately-growing on PDA, with colony diam. of appr. 3.0-4.0 cm in two weeks; appearance is flat and smooth at the margin, more wooly and with distinct aerial hyphae at the centre; margin is grayish to yellowish; centre is orange to dark gray, with slimy heads producing huge amounts of broadly ellipsoidal to cylindrical conidia; colony colors are less pronounced on reverse side.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX158197. No species-specific primers are known.

Notes: Under certain conditions, *C. hoffmannii* might act as a soft rot fungus. Different types of hyphae have been described for the species (Hale and Eaton 1985), with the so-called T-branch hyphae being able to penetrate the wood cell wall. Cellulase is one of the enzymes usually segregated by soft rot fungi. No external symptoms were observed in young vines infested by *C. hoffmannii* in the study of Casieri *et al*. (2009). In contrast, the sampled vine in our study showed weak Esca symptoms on the leaves; age of the vine was over 15 years, however, and it contained both *Pch* and *Fmed*. After all, the role of *C. hoffmannii* as a potential pathogen is not clear at the moment.

Diplodia seriata De Not.

Classification: Ascomycota, Dothideomycetes, Botryosphaeriales, Diplodia.

Life strategy and symptoms: *Diplodia seriata* (with the teleomorphic state, *Botryosphaeria obtusa*; Shoemaker 1964) is potentially related to several diseases such as black rot of fruits, Frogeye leaf spot on leaves, and canker of wood on numerous plant species. In grapevine, Black Dead Arm (BDA) was first described in 1974 in Hungary (Lehoczky 1974) and originally was associated with one particular species only, namely the close relative, *Diplodia mutila* (*Bot. stevensii*). Up to now, however, more than 20 Botryosphaeriaceae species have been related to grapevine trunk diseases (for instance, van Niekerk *et al*. 2006), and therefore the name "Botryosphaeria dieback" has been proposed recently (Úrbez-Torres *et al*. 2012). Essentially it is an open question if species of *Botryosphaeria* should be considered true pathogens, endophytes or, most likely, as opportunistic organisms switching back and forth between these two strategies (Phillips 2002).

Host plants: Apparently on numerous plant species, both coniferous and deciduous (Phillips *et al*. 2013).

Geographic distribution: Apparently worldwide (Phillips *et al*. 2013). The species is probably widespread in Germany, but has been usually overlooked or ignored until more recently. In contrast, it has been commonly isolated in France some 15 years ago already (Larignon *et al*. 2001).

Transmission and occurrence on *Vitis*: Pycnidia have been shown to develop on infected wood or shoots (Phillips, 2002, Bertsch *et al*. 2013). Airborne conidia are formed by pycnidia and are spread with or without rainfall (Kuntzmann *et al*. 2009; van Niekerk *et al*. 2010). Invasion of host plants most likely is via pruning wounds and/or wounds caused by other mechanical damage.

Identification (Figure, f): For detailed culture characteristics see Phillips (2002). On PDA, colonies are fast growing under 25 °C conditions, covering a Petri dish (9.5 cm) within one week; they are grayish brown to nearly black, with a dense aerial mycelium. Reverse side is almost getting black with ongoing time. No conidial structures are formed on PDA.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037450. For multi-species primer pairs see Ridgway *et al*. (2011).

Notes: In our studies, *Diplodia seriata* was only detected in older vines in the vineyard; no isolates were obtained from nursery material or from grafting tools. In a pathogenicity study based on mycelial plugs, *D. seriata* was among the less pathogenic species, causing lesions between appr. 18 and 32 mm long on lignified canes of the cultivars Redglobe and Cabernet Sauvignon after four weeks of incubation (Úrbez-Torres e*t al*. 2009). With the data at hand it is still difficult to evaluate the exact pathogenic potential of *D. seriata* in Germany; in our findings the species always was in co-existence with other GTD

related fungi such as *Pch* or *Fmed* and therefore should be rather considered as an opportunist.

Eucasphaeria capensis Crous

Classification: Ascomycota, Sordariomycetes, Hypocreales, Eucasphaeria.

Life strategy and symptoms: No particular symptoms are known to be related to *E. capensis*, possibly due to the sparse information available on this fungus. All isolates mentioned in the original description had been derived from leaves or leaf litter of *Eucalyptus* trees and their relative importance remains largely unknown (Crous *et al*. 2007). Very recently (Azeem *et al*. 2015) the species was isolated from *Hylobius abietis* ("Pine weevil") feces and frass in Sweden. Volatile organic compounds are thought to be produced by the fungal community associated with the feces, some of which are known to influence the orientation of pine weevils.

Host plants: Essentially unknown; in the literature described from living leaves or leaf litter of *Eucalyptus* sp. (Crous *et al*. 2007), from feces and frass of the beetle *Hylobius abietis* (in this way possibly indicating the fungus´ existence on conifers), and from leaves of the Asteraceae genus *Espeletia* (Miles *et al*. 2012).

Geographic distribution: Possibly worldwide; so far known from South Africa (Crous *et al*. 2007), Columbia, Canada and the Far East. One single European finding has been published recently, from Sweden (Azeem *et al*. 2015).

Transmission and occurrence on *Vitis*: In the original description (Crous *et al*. 2007) subepidermal ascomata and conidiomata had been observed on infested leaves of *Eucalyptus* and both ascospores and conidia will act as spreading agents in the field. In our studies we demonstrated *E. capensis* from a spore trap attached to a tree of *Prunus avium* next to a vineyard, from waste water and rootstock wood derived from a single nursery, and in one case from the stemhead of a 20 year old trunk of living vine.

Identification (Figure, g): On PDA, colonies reach appr. 2 cm of diam. in one week. Appearance is slightly variable in subcultures. Usually, cultures are with sparse or without aerial mycelium; centre is ochraceous to reddish, margin is thin, cream; reverse side is more or less identical; older colonies are becoming increasingly dark reddish.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037451. No species-specific primers are known.

Notes: Our isolate from wood of rootstock was isolated from a zone of dark discoloration typical for Esca related pathogens; the possible pathogenic significance of *E. capensis* however remained unknown in our studies. Despite its distinct colors in culture conditions the fungus has been completely overlooked in the past, but apparently is more widespread both in vineyards and in nurseries. To our knowledge our finding is the first in Central Europe and the first worldwide from *V. vinifera*.

Leucostoma persoonii (Nitschke) Höhn.

Classification: Ascomycota, Sordariomycetes, Diaporthales, Leucostoma.

Life strategy and symptoms: The species causes the so-called Leucostoma canker on a variety of stone fruit trees, which is considered as one of the most serious stone fruit diseases worldwide (Ogawa 1991). Different wooden parts of the trees might be affected, such as twigs, branches or trunks. Symptoms include discoloration, necrosis, and, with ongoing time, gummosis, *i.e.* amber gum ooze is apparent on infected sites.

Host plants: Most prominent on *Prunus* spp. and *Malus* spp.; besides occurring on numerous deciduous trees.

Geographic distribution: Probably worldwide. Very few reports exist for Germany, all of them in the eastern part.

Transmission and occurrence on *Vitis*: The fungus infects through injured, dying or dead tissues of the tree (Tekauz and Patrick 1974). Conidia will be released from pycnidia especially during cooler and moist weather periods and will be spread by wind or water (Bertrand and English 1976). After infection, the resulting mycelium is able to actively invading healthy tissue. The role of ascospores in the disease cycle is unknown. With our data, the possible occurrence on *Vitis* remains uncertain.

Identification (Figure, h): On PDA, colonies are fast growing under 25 °C conditions, covering a Petri dish (9.5 cm) within one week. They are grayish, with a greenish touch especially on the reverse side; centre always is slightly darker. Aerial mycelium is well developed, dense and flat. No conidial structures are formed on PDA after 14 d.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037452. No species-specific primers are known.

Notes: Overall life strategy as well as pattern of host invasion of *L. persoonii* are strikingly similar to those observed in GTD related fungi (Bertrand and English 1976). Production of the amber-colored gum corresponds with the phenomenon of gummosis which is so common in vines affected by GTD. Leucostoma canker is very difficult to control, with no biological and chemical management approach known to be truly effective (Ogawa 1991, Biggs and Grove 2005). With pruning, it is recommended to be performed during warmer and dry periods of weather, in this way adjusted to the pathogen's biology. Our sole isolate of *L. persoonii* has been derived from a spore trap attached to a peach tree situated within vineyards.

Neofusicoccum luteum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips

Classification: Ascomycota, Dothideomycetes, Botrysphaeriales, Neofusicoccum.

Life strategy and symptoms: While the species (as *Botryosphaeria lutea*) was repeatedly reported as pathogen in grapevine, both incidence and related symp-

toms seem to differ between countries (van Niekerk *et al.* 2006, Úrbez-Torres 2011). Amponsah *et al*. (2011) report *N. luteum* to colonize both green shoots and trunks of grapevine; apparently the host´s physiological state affects the expression of symptoms on the plant. *Neofusicoccum luteum* has also been reported from olives (*Olea europea*) in Australia, causing fruit rot and leaf necrosis (Sergeeva *et al*. 2009). The species may also occur as endophyte or saprobe, mainly on woody hosts.

Host plants: On numerous deciduous and coniferous plants (Phillips *et al*. 2013). Among cultivated plants, *Actinidia*, *Malus*, *Olea*, *Pyrus* and *Vitis* are the ones most cited.

Geographic distribution: Probably worldwide (Phillips *et al*. 2013).

Transmission and occurrence on *Vitis*: Conidial concentration was found to be a crucial factor in developing of symptoms on artificially infected green shoots (Amponsah *et al*. 2014). Any wounds, including pruning wounds, are susceptible to infection by air or waterborne conidia, produced by more or less globose pycnidia. In the field, pycnidia together with the ascomata are frequently formed on the same stromata (Phillips *et al*. 2013). Susceptibility of pruning wounds against species of *Botryosphaeria* was found to vary depending on the age of the pruning wound and the time of pruning (Serra *et al*. 2008, Úrbez-Torres and Gubler 2011) and in general might be related to the geographic region, rainfall etc. In our studies, *N. luteum* was a comparatively rare species; it was isolated from different parts (trunk, grafting union) of vines older than 10 years.

Identification (Figure, i): See Phillips (2002) for a detailed description. Our colonies on PDA medium were fast growing, covering a 9.5 cm Petri dish within 4-6 d; colonies produce a slightly yellowish pigment visible in the agar next to the margin. Mycelium is grayish when young, gradually darkening to dark grey or nearly black with age; surface is becoming increasingly wooly. No pycnidia are formed on PDA.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037453. No species-specific primers are known.

Notes: Currently 22 species are recognized for *Neofusicoccum*, based both on morphological and molecular characters (Phillips *et al*. 2013). The anamorphic *Neofusicoccum luteum* is linked with the well known teleomorph, *Botryosphaeria lutea*, the morphological and cultural characters of which have been discussed in detail by Phillips (2002). The pathogenic significance of *Botryosphaeria* species has been a matter of intensive discussion over the years; in general, related symptoms in the vineyard are only slowly developing, with the severity increasing with age of the vines (Larignon and Dubos 2001).

Phaeoacremonium fraxinopennsylvanicum (T.E. Hinds) D. Gramaje, L. Mostert & Crous

Classification: Ascomycota, Sordariomycetes, Diaporthales, Phaeoacremonium.

Life strategy and symptoms: See Mostert *et al*. (2006) and Gramaje *et al*. (2015) for detailed information. *Phaeoacremonium fraxinopennsylvanicum* (as *P. mortoniae* Crous & W. Gams) was found to be related to the typical Esca symptoms of grapevine in California; besides, on ash trees (*Fraxinus latifolia*) surrounding the vineyards the species was detected on declining parts of the host (Eskalen *et al*. 2005b). It remains an open question if the species should be considered a serious pathogen on non-*Vitis* hosts. Symptoms in the wood of grapevine comprise bws and/or gummosis.

Host plants: *Actinidia*, *Fraxinus*, *Malus*, *Prunus*, *Pyrus*, *Quercus*, *Vitis*.

Geographic distribution: Europe (Croatia, Germany, Hungary, Italy, Spain, Sweden), Iran, South Africa, Canada, USA; probably worldwide.

Transmission and occurrence on *Vitis*: As stated by Mostert *et al*. (2006), aerial infection will come from the production of anamorphic and teleomorphic structures developed on the surface of grapevine trunks, cordons etc., with the relation between conidia and ascospores fully unknown however. As an indication of a heterothallic life cycle, perithecia of *P. fraxinopennsylvanicum*, *P. aleophilum* (teleomorhic association with *Togninia minima* first demonstrated by Mostert *et al*. 2003) and *P. viticola* were observed on grapevines in the field (Rooney-Latham *et al*. 2005, Eskalen *et al*. 2005 a, b). Anamorphic structures can be isolated out of infected parts of the wood. In our studies, *P. fraxinopennsylvanicum* was isolated once, from a trunk of an approx. 20 years old *V. vinifera* 'Phoenix'.

Identification (Figure, j): Colonies of *P. fraxinopennsylvanicum* are characterized by a slightly yellowish color developed after several days on PDA medium; in the centre they are slightly felty; outer parts are flat, with entire margin. Reverse side is grayish yellow. Growth is slow under 25 °C conditions, with app. 2.5 cm in two weeks.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037455. No species-specific primers are known.

Notes: This is the first report of *P. fraxinopennsylvanicum* in Central Europe. To our surprise, we have found a total of three species of *Phaeoacremonium*, *i.e. P. aleophilum*, *P. viticola* and *P. fraxinopennsylvanicum*, in one single vineyard appr. 20 years old; rate of Esca related symptoms on the leaves was > 5 % in the year of isolation (2014). Still, with the background of sampling hundreds of vines in different parts of Germany throughout the years we consider *P. fraxinopennsylvanicum* as certainly rare in German vineyards. In a pathogenicity study based on nine *Phaeoacremonium* spp., *P. fraxinopennsylvanicum* was one of two causing a significant root weight reduction after five months of incubation (Aroca and Raposo 2009). Phylogenetically the species seems next related to a southern-hemisphere species, *P. novae-zealandiae* (Mostert *et al*. 2006).

Phaeoacremonium viticola J. Dupont

Classification: Ascomycota, Sordariomycetes, Diaporthales, Phaeoacremonium.

Life strategy and symptoms: For a comprehensive overview see Mostert *et al*. (2006) and Gra-

GRAMAJE *et al*. (2015). In general, species of *Phaeoacremonium* are commonly found in woody plants showing brown wood streaking (due to plugging and discoloration of vessels) and/or gummosis. In young grapevine, *P. viticola* in low frequency together with the more common *P. aleophilum* and *Phaeomoniella chlamydospora* is associated with Petri Disease causing stunted growth and dieback (DUPONT *et al*. 2000).

Host plants: *Actinidia*, *Prunus*, *Pyrus*, *Sorbus*, *Vitis vinifera.*

Geographic distribution: Europe (France, Germany, Italy, Spain), Iran, South Africa, USA; probably worldwide. For Germany, the only report is from a tree of *Sorbus intermedia* (coll. K. Weise, Feb. 1995); unfortunately, no further information about this finding is available. A mycelial culture has been deposited at the CBS culture collection (www.cbs.knaw.nl) though and was included in MOSTERT *et al*. (2006), under the designation CBS 428.95.

Transmission and occurrence on *Vitis*: *Phaeoacremonium viticola* is a heterothallic species, and in fertile crossings perithecia were formed after 12 weeks under laboratory conditions (MOSTERT *et al*. 2006). Mycelia isolated out of infected wood will produce abundant conidia after few days of incubation on artificial medium. The main source of infection in the field is essentially unknown, and it may be both anamorphic and teleomorphic structures. The respective conidia and ascospores will be transported by air, water etc., and may be obtained from spore traps attached to the vines in the field. As with related species of *Phaeoacremonium*, injured parts of the wood, mostly pruning wounds, will act as main entrance of the fungus. In our studies, *P. viticola* was revealed once, from the stemhead of an old vine showing typical Esca symptoms.

Identification (Figure, k): Several species of *Phaeoacremonium*, i.e. *P. viticola*, *P. angustius*, *P. rubrigenum* or *P. alvesii* show a reddish discoloration on PDA; the former species, however, is distinguished by a maximum growth temperature of 35 °C, while it is lower or, mostly, higher in the other species (MOSTERT *et al*. 2006). On PDA, the colony of our specimen was mostly flat, with a wooly centre turning reddish after prolonged incubation. Reverse side was turning reddish brown after 8 d, becoming slightly violet over time. A considerable cultural variation among different isolates has been noted by MOSTERT *et al.* (2006).

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX094557. Species-specific primers for the partial β-tubulin gene have been developed by AROCA and RAPOSO (2007).

Notes: After twenty years of intensive study fourty-six *Phaeoacremonium* species are presently recognized (CROUS *et al*. 1996, GRAMAJE *et al*. 2015). The very low number of *Phaeoacremonium* species so far detected in Germany might be due to incomplete sampling and/or false identification among con-generic taxa; however, similar results, even though based on young vines only, have been obtained for Switzerland (CASIERI *et al*. 2009). Even the most common species, *i.e. P. aleophilum*, is quite rarely found in infected wood of older vines, with a percentage of < 5 % among a sample of approx. 500 trunks including different cultivars, age and localities (FISCHER, unpubl. results). The pathogenic significance of *P. viticola* might be comparable to *P. aleophilum*.

Phomopsis cotoneastri Punith.

Classification: Ascomycota, Sordariomycetes, Diaporthales, Phomopsis.

Life strategy and symptoms: Only recently, the species has been acknowledged as a true pathogen, causing trunk cankers and death of young apple trees (ABREO *et al*. 2012). There is some evidence, though based on unidentified *Phomopsis* spp. that it might also be involved in cankers on peach, plum and Asian pear (UDDIN *et al*. 1998). As with other species of *Phomopsis*, the species also might occur as an endophyte in green shoots.

Host plants: Reported from several deciduous plants, such as *Cotoneaster*, *Malus* or *Sorbus*.

Geographic distribution: Documented for Europe and South America, probably widespread. Our finding represents the first report for Germany.

Transmission and occurrence on Vitis: As a genus specific character *P. cotoneastri* forms two types of conidia both in the field and under laboratory conditions (ABREO *et al*. 2012). The so-called α-conidia are thought to be responsible for infection processes, and are formed under sufficiently moist conditions in pycnidia developed on the bark of shoots. Infections occur under cooler and more humid conditions, with the conidia spread by wind or water. In our studies, *P. cotoneastri* was revealed from the wood of rootstock material in the nursery, where it was associated with the typical GTD related symptoms, *i.e.* gummosis and bws.

Identification (Figure, l): See ABREO *et al.* (2012) for a detailed description of mycelial cultures. Apparently two mycelial morphotypes exist within the species, with our isolate belonging to the so-called type II. On PDA, this type is characterized by a white to gray mycelial mat; colony diam. was appr. 3 cm after 14 d. Scattered pycnidia are formed after several weeks, producing both α- and β-conidia.

ITS sequence based on primer pair ITS5-ITS4: GenBank accession KX037454. No species-specific primers are known.

Notes: A pathogenicity assessment of strains of *P. cotoneastri* has been performed by ABREO *et al*. (2012). In this test, lesions of different length were produced on twigs of apple trees, in this way confirming the pathogenic potential of the species. Our finding represents the first report of the species in Germany, and the first report on grapevine worldwide. Probably the species is far more widespread, but has been misidentified and/or confused with the much better known *P. viticola*.

Discussion

Selection of species: A total of over 50 fungal species has been identified in a three-year-survey based on nursery material, vineyards and neighboring fruit trees in the southern Palatinate area of Germany. In the present

study a selection of 13 species is covered, all of them in some relation to wood diseases and not commonly reported in the literature. No extra information is provided on the well known *Eutypa lata*, *Phomopsis viticola*, *Phaeomoniella chlamydospora*, or *Fomitiporia mediterranea*, all of which frequently isolated during our survey.

Numerous "first reports": After consulting the specific references and data banks (Fungal databases at nt.ars-grin.gov, www.mycobank.org, etc.) we realize that eight of the above species are reported for the first time in Germany. With respect to host range, *Eucasphaeria capensis* and *Phomopsis cotoneastri* on a worldwide basis have not been reported from *Vitis* before. The following issues may contribute to these remarkable findings: i) our studies have been performed over a prolonged period of three years, including different locations, seasons and environmental conditions; ii) samples have been taken from widely diverse substrata, including air samples from spore traps (Table); iii) isolates of our fungi in fact have been obtained in former studies elsewhere, but were not carefully checked or even discarded. With this background we are quite convinced that additional taxa will be uncovered in future studies.

Species of pathogenic significance: With reference to the available literature we consider the following taxa as possibly relevant pathogens of grapevine: *Botryosphaeria dothidea*, *Cadophora luteo-olivacea*, *Diplodia seriata*, *Neofusicoccum luteum, Phaeoacremonium viticola* and *P. fraxinopennsylvannicum*. Among these, the commonly isolated and obviously widespread *C. luteo-olivacea* deserves particular interest. The species occurred both in the nursery (isolated from wood of plant material, tools, callusing media; Table) and in the vineyard, and consistently was related with the symptoms typical for GTDs. As for *P. viticola* and *P. fraxinopennsylvanicum* further testing both in different vineyards and localities will be necessary to evaluate their exact pathogenic impact on German viticulture.

Beyond doubt *Leucostoma persoonii* may act as an important pathogen on trees of *Prunus* (cherry, peach, plum, etc.), but to which degree - if at all - it "jumps" to other host plants such as *Vitis* remains an open question. At any rate, close spatial vicinity in the field might favor such transfers between host plants, as has been demonstrated for the Esca-related basidiomycete, *Fomitiporia mediterranea*, in different parts of Europe (Fischer 2002, di Marco *et al*. 2004).

Airborne inoculum: We tried to assess the occurrence and diversity of airborne inocula, *i.e.* spores or conidia, by taking air samples based on spore traps placed both in the vineyards and its vicinity. Two species, *Collophora paarla* and *Leucostoma persoonii*, were solely demonstrated by cultures derived from spore traps attached to *Prunus* trees situated next to vineyards (Table). Other species demonstrated by air sampling include the common *Aureobasidion pullulans*, the rare *Eucasphaeria capensis*, and all the significant Esca pathogens, *i.e. Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea* (the latter not specifically treated in the present manuscript). This clearly demonstrates the use of spore traps with respect to a more accurate evaluation of potential inoculum, both in space and over time (among others, see Larignon and Dubos 2000, van Niekerk *et al*. 2011).

Conclusion

Based on a limited geographic area we have been able to come up with a considerable number of "first reports" with respect to geographic region and host plant. We consider the success of the present study to be related to the particular sampling approach which was used over a prolonged period of time, covering a variety of agricultural background as well as different climate conditions throughout the year. Besides, a broad spectrum of measures has been used in order to collect and identify fungal organisms. As a future prospect, further studies are under way, for example with emphasis on species of *Cadophora* and their putative significance for German viticulture.

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