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ORIGINAL ARTICLE

Dactylonectria torresensis as the Main Component of the Black Root Rot Complex of Strawberries and Raspberries in Northern Germany

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Abstract In a long-term survey of black root rot of strawberries and raspberries in Northern Germany in 2007–2014, fungi with and without *Cylindrocarpon*-like anamorphs were isolated as potential pathogens. *Dactylonectria torresensis* was the most common species, being isolated from 18% of strawberry roots obtained from nursery plants and 37% of roots from production fields, as well as 21% and 29% (respectively) of raspberry roots. Less frequently isolated fungi with *Cylindrocarpon*-like anamorphs included *Ilyonectria crassa, Ilyonectria* sp. 2, *I. pseudodestructans, I. robusta, C. obtusisporium* and *Ilyonectria* sp. 1. Severe disease symptoms were reproduced by artificial inoculation of strawberries with *D. torresensis, I. crassa* and *Ilyonectria* sp. 2, milder symptoms with *C. obtusisporium*. A wide range of other root-pathogenic fungi such as *Fusarium oxysporum, Verticillium dahliae, Ceratobasidium fragariae, Gnomoniopsis fructicola, Hainesia lythri*, and species of *Cadophora, Leptodontidium, Pythium, Phytophthora, Plectosporella, Pestalotiopsis* and *Truncatella* were either isolated only sporadically or were not associated with black root rot symptoms, suggesting that they did not play any major role in this disease in Northern Germany. Visible disease symptoms and high frequencies of *D. torresensis* isolations in many batches of nursery plants indicated that these may comprise a major source of contamination of production fields. The previously unrecognised prominence of *D. torresensis* resolves a long-standing puzzle concerning the cause of the ongoing black root rot epidemic in Northern German strawberry and raspberry production.

Keywords Black root rot · *Cylindrocarpon* · *Dactylonectria torresensis* · Germany · *Ilyonectria* · Raspberry · Strawberry

Dactylonectria torresensis als wichtigste Komponente der Schwarzen Wurzelfäule an Erdbeeren und Himbeeren in Norddeutschland

Zusammenfassung In einer Langzeitstudie der Schwarzen Wurzelfäule an Erdbeeren und Himbeeren in Norddeutschland von 2007 bis 2014 wurden Pilze mit und ohne *Cylindrocarpon*-Sporenstadien als potentielle Krankheitserreger isoliert. Die häufigste Art, *Dactylonectria torresensis*, wurde aus 18 % der Wurzeln von Erdbeer-Jungpflanzen sowie aus 37 % der Wurzeln von Pflanzen aus Produktionsanlagen isoliert. An Himbeeren betrugen die entsprechenden Werte 21 % und 29 %. Weitere Pilze mit *Cylindrocarpon*-ähnlichen Konidienstadien waren *Ilyonectria crassa, Ilyonectria* sp. 2, *I. pseudodestructans, I. robusta, C. obtusisporium* und *Ilyonectria* sp. 1. Schwere Symptome der Schwarzen Wurzelfäule wurden durch künstliche Inokulation gesunder Erdbeerpflanzen mit *D. torresensis, I. crassa* und *Ilyonectria* sp. 2 reproduziert, mildere Symptome mit *C. obtusisporium*. Andere pathogene Pilze wie *Fusarium oxysporum, Verticillium dahliae, Ceratobasidium fragariae, Gnomoniopsis fructicola, Hainesia lythri* sowie Arten von *Cadophora, Leptodontidium, Pythium, Phytophthora, Plectosporella, Pestalotiopsis* und *Truncatella* wurden entweder nur sporadisch isoliert oder waren nicht mit den Krankheitssymptomen assoziiert, so dass kein ursächlicher Zusammenhang mit der Schwarzen Wurzelfäule in Norddeutsch-

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land angenommen werden kann. Sichtbare Krankheitssymptome sowie der häufige Nachweis von *D. torresensis* in Vermehrerware deuteten auf die Ausbreitung der Krankheit über diese Route hin. Die bislang nicht beschriebene Rolle von *D. torresensis* beantwortet lange bestehende Fragen zur Ursache der aktuellen Epidemie der Schwarzen Wurzelfäule in der norddeutschen Erdbeer- und Himbeerproduktion.

Schlüsselwörter *Cylindrocarpon* · *Dactylonectria torresensis* · Deutschland · Erdbeere · Himbeere · *Ilyonectria* · Schwarze Wurzelfäule

Introduction

Strawberries (*Fragaria* × *ananassa*) are a major fruit crop in Germany. In the Northern German state of Lower Saxony, which has the largest production of all German federal states, strawberries are grown by some 200 farms with a combined acreage of approx. 4000 ha. Regional practice has it that plantlets are set before the end of May and cropped later during that year as well as during 1–2 subsequent years. Raspberry production comprises about 60 ha in Lower Saxony, plants being cropped annually for 10 years or even longer. These management periods exceed those in other regions such as Southern Germany or the Netherlands. Almost all strawberries and raspberries are currently grown in the open field rather than in glasshouses or tunnels. In recent years, an increase in biofuel production has compromised land availability, putting an increasing pressure on farmers towards replanting strawberries or raspberries on the same fields. At the same time soil fumigants, commonly used in the past prior to the replanting of a strawberry or raspberry crop, have been banned from use in Germany as from January 2007. These circumstances are suitable for promoting soil-borne diseases.

Root diseases due to fungi are a major constraint to strawberry production in Germany and worldwide. These include wilt caused by *Verticillium dahliae* (Zinkernagel 1970a; Harris and Yang 1996), red root rot caused by *Phytophthora fragariae* (Montgomerie 1967; Seemüller and Riedel 1980), rhizome rot caused by *Phytophthora cactorum* (Zinkernagel 1970b; Eikemo et al. 2004), and black root rot. A multitude of fungi has been associated with black rot, including *Ceratobasidium* (*Rhizoctonia*) *fragariae, Pythium* spp., *Fusarium* spp., *Cylindrocarpon* spp. and *Gnomoniopsis fructicola* (syn. *Gnomonia fragariae*) (Seemüller 1970; Zinkernagel 1970b; Yuen et al. 1991; Maas 1998). Free-living nematodes (*Pratylenchus* spp.) may predispose strawberries to infection by fungi causing black root rot as well as other root diseases (McKinley and Talboys 1979; LaMondia and Martin 1989; LaMondia 2003). In raspberries, red root rot and Verticillium wilt are the best-known diseases (Zeller 1936; Ellis et al. 1991; Wilcox et al. 1993). Verticillium wilt was prominent in Northern German strawberry and raspberry fields in the 1960s (Zinkernagel 1970a, b; Matthies and Lankes 1993) whereas red root rot became a problem in the 1980s following the accidental introduction of *P. fragariae* (Seemüller and Riedel 1980; Matthies and Lankes 1993). Throughout this time black root rot was present but of minor relevance in Germany. However, a dramatic increase in severity has been observed by regional strawberry and raspberry producers and their consultants since the mid-1990s. Initially assumed on the basis of earlier observations by Zinkernagel (1970a) to be caused by an interplay between *Verticillium* and nematodes, this explanation became increasingly untenable because of poor correlations between disease severity and either or both suspected causes (A-P Entrop, unpublished observations). We therefore initiated a long-term study in 2007 in which potential pathogens were isolated and identified from samples collected across Northern Germany. The results showed that fungi with *Cylindrocarpon*-like anamorphs were the main cause of black root rot, and that high levels of infection were already present in nursery material. The implications of these findings are discussed.

Material and Methods

Isolation and Maintenance of Fungi

All reagents were supplied by Carl Roth (Karlsruhe, Germany) unless indicated otherwise. Plants were collected from batches of nursery material prior to explanting, or uprooted from production fields, and stored at 2 °C for a maximum of 7 d prior to analysis. Following washing of the plants in running tap water and photographic documentation of symptoms, roots with signs of incipient rot were selected for further analysis. A 3–5 cm long segment from each of up to five main roots (strawberries) or lateral roots up to 5 mm diameter (raspberries) per plant was excised and subjected to surface sterilisation for successive 30 s intervals in dilute hypochlorite bleach (6% w/v NaOCl), ethanol (70% v/v) and hypochlorite bleach, followed by a 2 min rinse in sterile distilled water (Weber et al. 2004). From each root segment three cross-sections (2–3 mm long) were plated onto 1% malt extract agar (MEA) augmented with 200 mg penicilling G and streptomycin sulphate l^{-1}. For isolating *Pythium* and *Phytophthora* spp., a further three sections were placed on cornmeal agar (CMA; Neogen, Lansing, USA) augmented with 50 mg nystatin, 100 mg pentachloronitrobenzene (Sigma-Aldrich, St. Louis, USA) and 200 mg vancomycin l^{-1} (derived from Erwin and Ribeiro 1996). Strawberry rhizomes were also analysed. After scraping off the outermost layer of dark

epidermal tissue with a scalpel, rhizomes were washed in running tap water and surface-sterilised as described above. From each rhizome, three segments were placed on each of the two isolation media described above.

Following incubation of the isolation plates for 14 d at room temperature, colonies were enumerated by morphology. Representative colonies were isolated by transfer of growing mycelial margins onto fresh MEA. Sporulating fungi were preserved as lyophilised conidial suspensions (Smith and Onions 1983), non-sporulating fungi on potato dextrose agar (PDA) slopes under light paraffin oil at 4 °C.

Identification of Fungi

Fungi were identified by microscopy, using an Axio Scope A1 fitted with differential interference contrast optics, and photographed using the digital camera ICc 3 (all from Carl Zeiss, Jena, Germany). For DNA sequence analysis, isolates with *Cylindrocarpon* anamorphs were further examined by amplifying and sequencing the histone H3 subunit, using primers CylH3F (5′-AGGTCCACTGGTGGCAAG) and CylH3R (5′-AGCTGGATGTCCTTGGACTG) according to Crous et al. (2004) and Cabral et al. (2012a). The PRC reaction mix (total vol. 50 µl) contained 0.5 µM of each primer, approx. 1 ng template DNA, and 25 µl of 2 × Dream Taq PCR Master Mix (Applied Biosystems, Foster City, USA). Cycling conditions comprised one period of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C, and one period of 10 min at 72 °C. For all other fungi, the internal transcribed spacer region (ITS1–5.8S–ITS2) of the ribosomal RNA gene cluster was amplified and sequenced using primers ITS4 (5′-TCCTCCGCTTATTGATATGC) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG), and cycling conditions of one period of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, and one period of 10 min at 72 °C (White et al. 1990; Weber and Zabel 2011). Following purification of PCR products with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), sequencing was conducted by Eurofins Genomics GmbH (Ebersberg, Germany). Sequence chromatograms were edited manually using Chromas 2.6.2 (Technelysium Ltd, Australia). All sequence searches were conducted in GenBank using the Blastn function.

Pathogenicity Tests

Aliquots of 200 ml perlite (Perligran G0/3, Knauf Perlite, Dortmund, Germany) soaked in 100 ml nutrient solution (1% malt extract and 0.1% yeast extract) were autoclaved, inoculated with selected fungal strains, and incubated at room temp. for 7 d. Strawberry plants visually free from symptoms were surface-sterilised by dipping the roots in 2% (w/v) NaOCl for 30 s, and washed in sterile distilled water for 2 min. Plants were then inoculated by placing approx. 25 ml colonised perlite medium in the centre of the root system directly underneath the rhizome, and planted into 1 l pots with autoclaved garden soil. Control plants were inoculated with sterile perlite medium. All strains and the control were set up as five replicate pots. Pots were placed in an unheated greenhouse (temperature range 18–25 °C). After 8 weeks, plants were removed from the pots, washed, photographed, and examined for root rot. From each of three plants, five root segments were subjected to surface sterilisation and isolation of fungi as described above. The identity of isolated fungi was confirmed by comparing their macroscopic and microscopic features with the original strains used for inoculation.

Results

Symptoms of Black Root Rot

Characteristic symptoms were observed on strawberry and raspberry plants throughout Northern Germany during all years of the survey (2007 to 2014). On strawberries, above-ground symptoms were visible as a somewhat retarded plant growth and smaller fruit size, followed by leaf reddening and chlorosis (Fig. 1a). In severe cases plants died within the same season in which the symptoms were first observed (Fig. 1b). There was an obvious effect of environmental stress in the sense that symptoms were most severe in areas of the field previously subjected to drought or waterlogging. By the time of appearance of the first above-ground symptoms, advanced black root rot had already developed (Fig. 1c). Black root rot attacked the feeder roots first (Fig. 1d). Later the entire root system became blackened, new lateral roots devoid of feeder roots being produced only from a small region at the apex of the rhizome (Fig. 1e). The rhizome itself remained free from symptoms even where black root rot was severe (Fig. 1f). A typical feature of this disease on lateral roots was the disintegrated cortex which could be pulled back between thumb and index finger to reveal the unstained, whitish root stele (Fig. 1g). Black root rot was also present in nursery plants delivered to fruit farmers (Fig. 1h).

On raspberries above-ground symptoms typically became visible between flowering and harvest and were prominent as a wilting of floricanes whilst young primocanes continued to grow (Fig. 2a, b). Infection foci showed a sharp boundary within rows, whereas adjacent rows often remained visually unaffected (Fig. 2a). In established fields, a season with rapid enlargement of infection foci was often followed by one or several years of apparent standstill. Farmers' attempts to uproot dead plants and replant the gaps inevitably resulted in the death of the young plants

Fig. 1 Symptoms of black root rot on strawberries. **a** Early above-ground symptoms; **b** death of affected plants; **c** plant with incipient above-ground symptoms and severe root decay; **d** incipient black rot on feeder and lateral roots; **e** typical appearance of advanced black root rot with new lateral root production confined to a small upper portion of the rhizome; **f** plant severely attacked by black root rot with rhizome remaining free from symptoms; **g** removal of the root cortex (*arrow*) to reveal the unstained stele; **h** nursery material attacked by black root rot

within a few weeks or at latest at the start of the next growing season (not shown). The appearance of black rot in the root system of raspberries was most prominent in the lowest regions where even the larger roots (5 mm diameter) showed a sloughing-off of the cortex (Fig. 2c). Any residual feeder roots were confined to the uppermost 1–3 cm of the soil. Batches of raspberry nursery plants were also commonly affected by black rot symptoms (Fig. 2d).

Isolation and Identification of Fungi

A wide diversity of species without (Table 1) and with (Table 2) *Cylindrocarpon*-like anamorphs was found in strawberry and raspberry roots and strawberry rhizomes. They were identified by microscopy and analysis of diagnostically relevant gene sequences.

The most frequent group of fungi lacking a *Cylindrocarpon* state comprised colonies of dark green mycelium sometimes producing inconspicuous hyaline conidial states

Fig. 2 Symptoms of black root rot on raspberries. **a** Progression of the disease as a sharply delimited focus of infection within a single raspberry row in the absence of symptoms in the neighbouring row; **b** typical above-ground appearance with floricane wilting and new primocane growth; **c** affected root system with the most severe symptoms in the lowest regions; **d** nursery material attacked by black root rot

resembling *Phialophora*. These were characterised by ITS sequence analysis as belonging to *Cadophora* and *Leptodontidium* spp. (Table 1). Among species with *Fusarium* anamorphs, isolates of *F. oxysporum* were noticeably more frequent than any other species that were identified, i. e. *F. culmorum* and *F. avenaceum* (identification by microscopy) as well as *F. acuminatum* and *F. solani* (identification by ITS sequence analysis). *Rhizoctonia*-like isolates belonged to *Ceratobasidium* anastomosis group I, as exemplified by ITS sequences of two typical colonies (Table 1). Isolates recognised microscopically as *V. dahliae* were confirmed as such by ITS sequence analysis. No attempt was made to characterise *Pythium* isolates morphologically, although they were shown to belong to *P. sylvaticum* and *P. irregulare* by ITS sequence analysis. Fast-growing colonies with *Acremonium*-like conidium formation were identified by ITS sequence analysis as *Plectosphaerella* spp. Further potential pathogens were isolated only sporadically, including *Phytophthora* spp., *Gnomoniopsis fructicola, Hainesia lythri, Leptosphaeria coniothyrium, Pestalotiopsis guepinii, Truncatella angustata, Botrytis* sp., *Colletotrichum* spp., *Diplodia* sp., *Phoma* spp. and *Phomopsis* spp. Other putatively saprotrophic fungi of sporadic occurrence included *Acremoniella atra, Chrysosporium pannorum, Geotrichum candidum, Pseudeurotium zonatum, Varicosporium elodeae* and *Verticillium tenerum* as well as species of *Absidia, Acremonium, Alternaria, Arthrinium, Chaetomium, Cladosporium, Coemansia, Doratomyces, Gilmaniella, Gliocladium, Mucor, Paecilomyces, Sphaeronaemella, Trichoderma* and *Zygorhynchus* (not shown).

Several species with *Cylindrocarpon*-like anamorphs were isolated. Based on their colony appearance and microscopic details of conidia and chlamydospores, these were grouped into five morphotypes which were confirmed as discrete species or species complexes by analysis of histone H3 sequences (Table 2). The most frequently isolated type I corresponded to *Dactylonectria torresensis* (24 sequences analysed); type II to *Ilyonectria crassa* (18 sequences),

Table 1 Species identification of various fungi associated with black root rot by comparison of ribosomal RNA-ITS sequences with published sequences and corresponding literature references

Species (isolate)	Sequences[a]	GenBank sequences[b]	Reference
Cadophora luteo-olivacea (Cyl087)	1	AB725391 (577/577)	Nagano et al. (2016)
C. luteo-olivacea (Cyl123)	2	HM116748 (657/657)	Johnston et al. (2010)
Leptodontidium sp. (Cyl076)	2	KC007251 (602/602)	(Unpublished)
Fusarium oxysporum (Cyl009)	1	GU566301 (569/569)	López et al. (2009)
F. oxysporum (Cyl014)	1	GU445374 (558/558)	Glynou et al. (2016)
F. oxysporum (OVB11-017)	1	KT268852 (568/568)	Glynou et al. (2016)
F. acuminatum (Cyl118)	1	KT269065 (574/574)	Glynou et al. (2016)
F. solani (Cyl017)	1	KP265360 (567/567)	Stefańczyk et al. (2016)
Ceratobasidium AG-I (Cyl068)	1	AB196650 (560/560)	Hyakumachi et al. (2005)
Ceratobasidium AG-I (Cyl074)	1	AB196650 (559/561)	Hyakumachi et al. (2005)
Verticillium dahliae (Cyl091)	2	AF104926 (565/565)	Pramateftaki et al. (2000)
Pythium sylvaticum (Cyl079)	2	DQ528741 (970/970)	Klemsdal et al. (2008)
P. sylvaticum (OVB11-032)	2	AB108008 (907/907)	Matsumoto et al. (2000)
P. irregulare (OVB11-034)	1	KU208390 (935/936)	Rojas et al. (2017)
Plectosphaerella plurivora (OVB11-030)	1	HQ238969 (516/516)	Carlucci et al. (2012)
Gnomoniopsis fructicola (OVB12-042)	1	GU320816 (497/497)	Walker et al. (2010)
Varicosporium elodeae (Cyl094)	1	KU516615 (537/537)	Jankowiak et al. (2016b)

[a]Number of isolates with identical sequences from the present study
[b]The GenBank accession number of the best match as well as the number of matching nucleotides/number of total nucleotides in the alignment are given

Table 2 Species identification of *Dactylonectria* (*D.*) or *Ilyonectria* (*I.*) spp. or their *Cylindrocarpon* (*C.*) anamorphs by comparison of histone H3 sequences with published sequences and corresponding literature references

Microscopy	Species (isolate)	Sequences[a]	GenBank sequences[b]	Reference
Type I	*D. torresensis* (Cyl020)	18	JF735657 (500/500)	Cabral et al. (2012b)
Type I	*D. torresensis* (Cyl002)	1	JF735663 (500/500)	Cabral et al. (2012b)
Type I	*D. torresensis* (Cyl038)	1	JF735658 (500/500)	Cabral et al. (2012b)
Type I	*D. torresensis* (Cyl003)	4	JF735658 (499/500)	Cabral et al. (2012b)
Type II	*I. crassa* (Cyl060)	6	JF735534 (521/521)	Cabral et al. (2012a)
Type II	*I. crassa* (Cyl065)	4	JF735534 (520/521)	Cabral et al. (2012a)
Type II	*I. crassa* (Cyl028)	3	JF735534 (520/521)	Cabral et al. (2012a)
Type II	*I. crassa* (Cyl021)	5	JF735536 (521/521)	Cabral et al. (2012a)
Type II	*Ilyonectria* sp. 2 (Cyl012)	11	KM248606 (458/458)	Jankowiak et al. (2016a)
Type II	*I. pseudodestructans* (Cyl097)	1	JF35562 (473/473)	Cabral et al. (2012b)
Type III	*I. robusta* (Cyl026)	10	JQ860004 (459/459)	Erper et al. (2013)
Type IV	*C. obtusisporium* (Cyl024)	1	JF735609 (494/494)	Cabral et al. (2012b)
Type IV	*C. obtusisporium* (Cyl015)	1	JF735608 (494/494)	Cabral et al. (2012b)
Type V	*Ilyonectria* sp. 1 (Cyl095)	8	JF735610 (501/501)	Cabral et al. (2012b)
Unassigned	*I. europaea* (Cyl034)	1	JF735566 (520/520)	Cabral et al. (2012a)
Unassigned	*I. europaea* (Cyl069)	1	JF735565 (520/520)	Cabral et al. (2012a)
Unassigned	*I. europaea* (Cyl066)	1	JF735566 (520/521)	Cabral et al. (2012a)
Unassigned	*I. liriodendri* (Cyl061)	1	JF735508 (510/511)	Cabral et al. (2012b)
Unassigned	*I. liriodendri* (Cyl052)	1	JQ859988 (477/477)	Erper et al. (2013)

[a]Number of isolates with identical sequences from the present study
[b]The GenBank accession number of the best match as well as the number of matching nucleotides/number of total nucleotides in the alignment are given

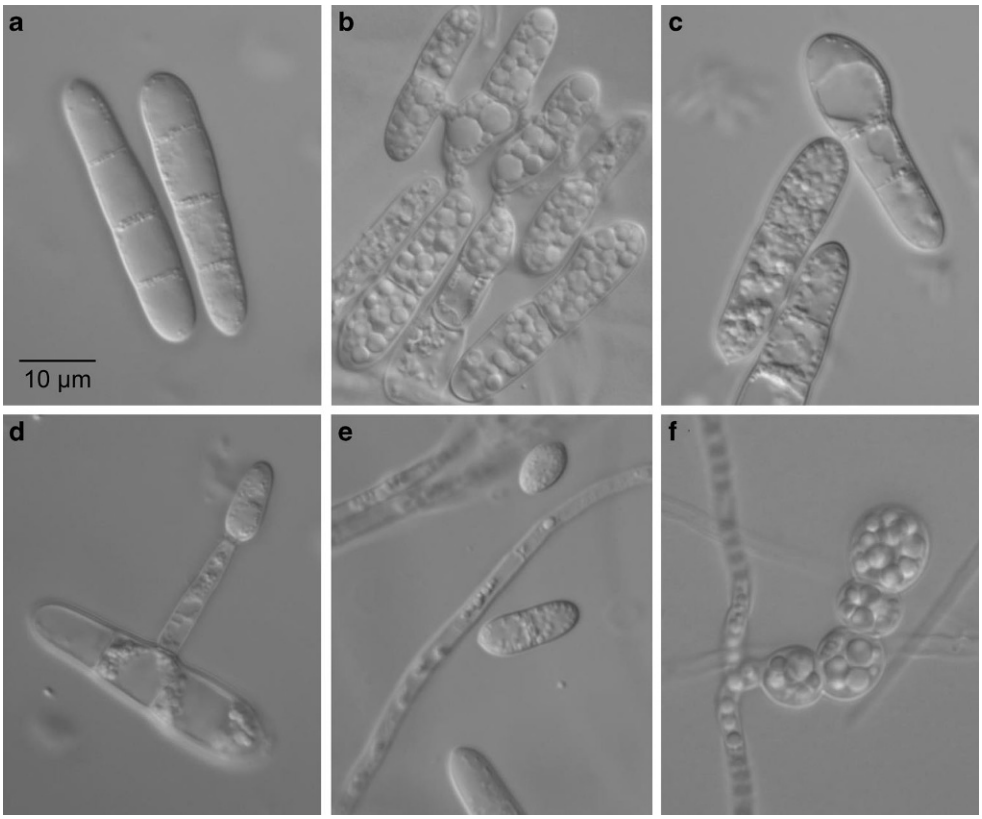

Fig. 3 Microscopy of *Dactylonectria torresensis*. **a** Three-septate macroconidia; **b** profuse anastomosis between aged macroconidia; **c** swelling of the terminal segment of an ageing macroconidium to produce a chlamydospore-like structure; **d** repetitious germination by formation of a new conidium; **e** microconidia; **f** hyaline chlamydospore-like structures embedded in the agar. All images to same scale

Ilyonectria sp. 2 (10 sequences) and *I. pseudodestructans* (one sequence) which could not be clearly separated from one another on the basis of routine microscopy; type III to *I. robusta* (10 sequences); type IV to *Cylindrocarpon obtusisporium* (2 sequences); and type V to an as yet unnamed *Ilyonectria* sp. 1 (10 sequences). On the basis of microscopic features, therefore, it was possible to assign colonies from different batches of roots to any of these five species or species groups. Isolates that fell outside were rare, belonging to *I. europaea* and *I. liriodendri* (Table 2) or in three cases to species not currently identifiable by reference to any published sequence (not shown).

Description of the Most Common Fungi with *Cylindrocarpon* Anamorphs

On 1% MEA, the most frequent morphotype I (*D. torresensis*) was most readily recognised by its one-septate (23.4–34.0 × 5.7–8.3 µm) to three-septate (31.0–40.2 × 6.5–8.4 µm) macroconidia which were elongate, widening slightly and asymmetrically towards the apex with one side minutely curved and the other almost straight (Fig. 3a). A second diagnostic feature was that older macroconidia displayed a prominent tendency to produce anastomosis tubes with one another (Fig. 3b). A third feature was an unusual ageing process in which after 2–3 weeks on MEA the apical conidial cell enlarged in a chlamydospore-like fashion (Fig. 3c), sometimes accompanied by its separation from the other conidial cells. Macroconidia were infrequently observed to undergo microcyclic germination by emitting a phialide which went on to produce a new conidium (Fig. 3d). Egg-shaped aseptate (7.5–11.9 × 4.2–5.4 µm) or ellipsoid one-septate (13.9–20.5 × 5.2–7.0 µm) microconidia were occasionally produced (Fig. 3e). Chlamydospores were rare; where present, they were submerged in the agar, hyaline, 8.3–13.1 µm diameter, filled with large lipid bodies, and typically produced in short chains (Fig. 3f).

Isolates belonging to morphotype II were initially identified as '*Cylindrocarpon*' *destructans* on the basis of microscopic features. Following histone H3 sequence analysis, the two major species within morphotype II were re-examined by microscopy, revealing subtle differences. *Ilyonectria crassa* was characterised by abundant one-celled egg-shaped microconidia (3.9–8.0 × 3.4–4.5 µm) which were produced in slimy drops on solitary or loosely clustered phialides (Fig. 4a, b). These spores were also produced within the agar layer. Macroconidia were mostly aseptate (10.7–14.2 × 3.4–4.5 µm) or one-septate (19.3–31.2 × 4.9–5.8 µm), slightly curved, and densely filled with small lipid droplets (Fig. 4b). Thick-walled golden-brown chlamydospores (9.7–13.0 µm diameter) were produced in short chains especially from hyphae embedded in the agar medium (Fig. 4c). *Ilyonectria* sp. 2 closely resem-

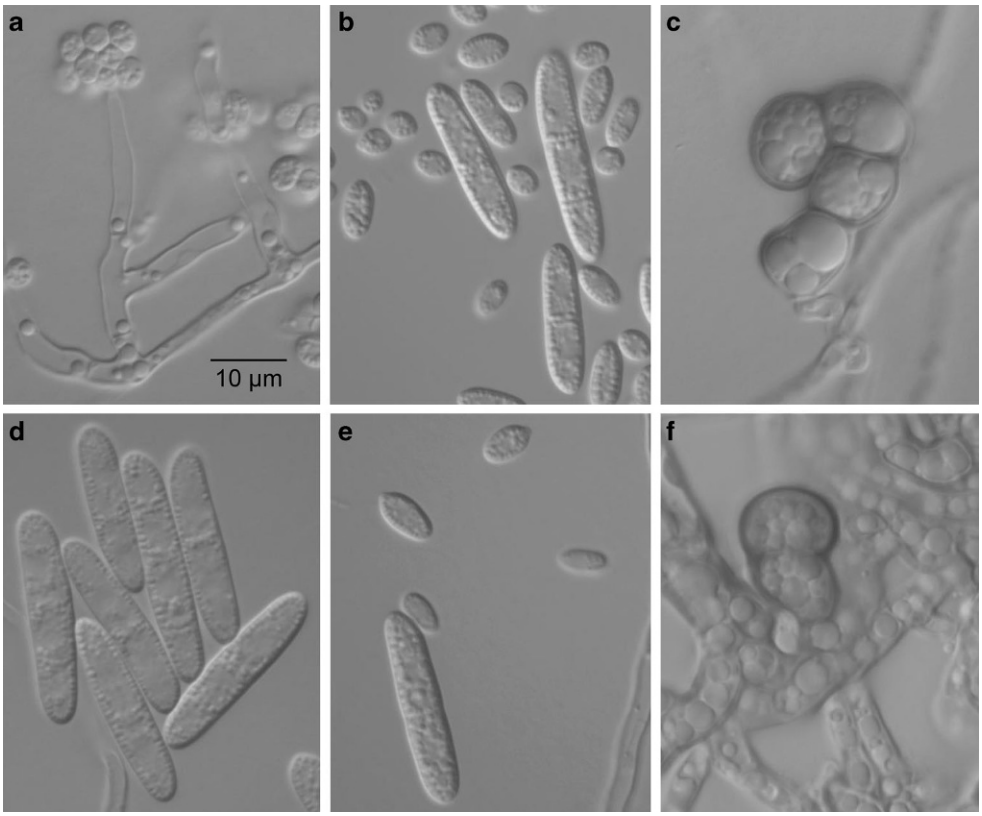

Fig. 4 Microscopy of morphotype II. **a–c** *Ilyonectria crassa*. **a** Microconidium formation on phialides; **b** macroconidia and microconidia; **c** melanised chlamydospores in a short chain. **d–f** *Ilyonectria* sp. 2. **d** Macroconidia; **e** microconidia in comparison to a macroconidium; **f** chlamydospores embedded in a tuft of thick-walled, partially melanised hyphae. All images to same scale

Fig. 5 Reproduction of typical black rot symptoms eight weeks after artificial infection of potted strawberry plants with a strain of *I. torresensis* (Cyl020, centre) or *Ilyonectria* sp. 2 (Cyl012, *right*) in comparison to an uninoculated control plant (*left*)

bled *I. crassa* in its macroconidia (Fig. 4d) which were of similar shape and mostly one-septate (18.0–26.9 × 4.7–6.0 µm) but with a higher proportion of two- and three-septate spores (25.0–31.2 × 5.1–6.0 µm). Microconidia were sparse. Where present, they were aseptate and short-cylindrical (5.3–9.1 × 3.2–4.5 µm) with a blunt abscission scar (Fig. 4e). Chlamydospores were of similar size and shape as in *I. crassa*. A distinguishing feature was their frequent association with aggregates of melanised hyphae embedded in the agar (Fig. 4f).

Pathogenicity Tests

Severe and typical symptoms of black root rot were induced by strains Cyl003 and Cyl020 (*D. torresensis*), Cyl021 (*I. crassa*) and Cyl012 (*Ilyonectria* sp. 2). Strain Cyl024 (*C. obtusisporium*) caused weaker symptoms than the other species whereas the uninoculated control plants remained free from symptoms (Fig. 5). Strains with colony and conidial morphologies identical to the respective original inoculum were isolated from the roots of all inoculated plants examined (Table 3).

Quantification of Fungi in Roots

During the eight years of our survey, a total of 3938 roots of strawberry plants collected from 101 production fields and 49 batches of nursery plants, as well as 1675 raspberry roots collected from 38 fields and 38 batches of nursery plants, were analysed for fungi associated with black root rot symptoms. The results, presented as percentage of total roots and total number of fields from which given species were isolated, are summarised in Figs. 6 and 7 for strawberries and raspberries, respectively.

Table 3 Fulfilment of Koch's postulates for isolates of *Dactylonectria torresensis* (*D. torr.*), *I. crassa* (*I. cra.*), *Ilyonectria* sp. 2 (*Ilyo.* 2) and *Cylindrocarpon obtusisporium* (*C. obt.*)

Treatment	Root rot (8 weeks)	Re-isolation from 15 roots			
		D. torr	*I. cra*	*Ilyo.* 2	*C. obt*
Control	Absent	0	0	0	0
Cyl003 (*D. torr.*)	Strong	15	0	0	0
Cyl020 (*D. torr.*)	Strong	14	0	0	0
Cyl021 (*I. cra.*)	Strong	0	13	0	0
Cyl012 (*Ilyo.* 2)	Strong	0	0	15	0
Cyl024 (*C. obt.*)	Moderate	1	0	0	14

On strawberries from nurseries as well as production fields, *D. torresensis* and dark green colonies representing species of *Cadophora* and *Leptodontidium* were by far the two most abundant fungal groups (Fig. 6). In nine batches of plants, altogether 193 healthy-looking and 193 diseased roots were processed separately in order to relate the frequencies of these two groups of fungi to the incidence of black root rot. *Dactylonectria torresensis* and sterile green colonies were isolated from 37.8% and 31.6% (respectively) of healthy-looking roots, and from 74.1% and 5.7% (respectively) of diseased roots, confirming several additional observations (RWS Weber, unpublished) that *D. torresensis* was strongly associated with black root rot whereas dark green colonies were not. *Fusarium* spp., fungi belonging to *Truncatella* and *Pestalotiopsis, Ceratobasidium* AG-I, *Pythium* spp. and *Hainesia lythri* were found in 10% or more of the nursery batches or production fields, but overall in 5% or less of all roots examined, indicating a widespread but rather sporadic occurrence. The diversity and abundance of fungi associated with black root rot on raspberries (Fig. 7) generally matched the data for strawberries.

Rhizomes from 205 strawberry plants collected from production fields were also analysed. The most frequent fungi, expressed as percent of rhizomes examined, were dark green colonies (14.6%), *Verticillium dahliae* (10.7%), *Phytophthora* spp. (10.4%), *D. torresensis* (9.8%), *Ceratobasidium* AG-I (5.4%), *F. oxysporum* (4.9%), other *Fusarium* spp. (4.4%), and species belonging to *Truncatella* and *Pestalotiopsis* (4.4%). Thus, the share of black root rot pathogens was generally lower than in the roots, corroborating our observations of a healthy appearance of rhizomes even in plants strongly affected by black root rot symptoms.

Discussion

The symptoms of black root rot in our long-term study of strawberries and raspberries in Northern Germany exactly matched those described in the older German literature (Seemüller 1970; Lutz and Lauber 1981) and elsewhere (Wing et al. 1994; Botha 2002). A correlation of this disease with stress factors such as replanting of strawberries or waterlogging of soil has also been noted before (Wing et al. 1995). Although we isolated a multitude of fungi, all available evidence points to species with *Cylindrocarpon*-like anamorphs – notably *D. torresensis* – as the principal cause of the disease. Firstly, these fungi but no other group were consistently isolated from every batch of plants analysed. Secondly, Koch's postulates were fulfilled for *D. torresensis* as well as for other species, viz. *I. crassa, Ilyonectria* sp. 2 and *Cylindrocarpon obtusisporium*; all these were able to cause typical root rot symptoms in inoculation experiments in the absence of any other predisposing factor. Thirdly, *D. torresensis* but not the second most abundant group (*Cadophora* and *Leptodontidium* spp.) was obviously associated with developing root rot lesions in field material.

Dactylonectria (formerly *Ilyonectria*) *torresensis* was recently characterised as one of the constituent species of the *I. macrodidyma* complex (Cabral et al. 2012b; Lombard et al. 2014) and is prominently associated with black foot disease of grapevine (Cabral et al. 2012b; Reis et al. 2013). Although Cabral et al. (2012b) mentioned that *D. torresensis* had been isolated from strawberry roots, to the best of our knowledge it has not previously been implicated as a major cause of strawberry or raspberry black root rot. Similarly, *I. crassa* and *I. robusta*, both of which belong to the *I. radicicola* species complex (Cabral et al. 2012a), have not been specifically associated with strawberry or raspberry diseases. The safe identification of species of both *Dactylonectria* and *Ilyonectria* is currently possible only by DNA sequence analysis (Cabral et al. 2012a, b; Outram et al. 2014). Therefore, *'Cylindrocarpon' destructans* previously reported from strawberries (Yuen et al. 1991; Rigotti et al. 2003) and blackberries (Cedeño et al. 2004) cannot be correlated to any currently recognised *Ilyonectria* species with any degree of certainty (Cabral et al. 2012a; Jankowiak et al. 2016a). It is possible that any or all of our morphotype II isolates (*I. crassa, I. pseudodestructans, Cylindrocarpon* sp. 2) would have been identified as '*C.*' *destructans* on the basis of microscopic features (Booth 1966) in the past.

Irrespective of the uncertain current identity of '*C*'. *destructans*, our conclusion of *D. torresensis* being the main cause of black root rot of strawberries and raspberries in Northern Germany is at odds with previous publications which have described other genera and a complexity of factors in this disease. *Ceratobasidium* sp. and especially its

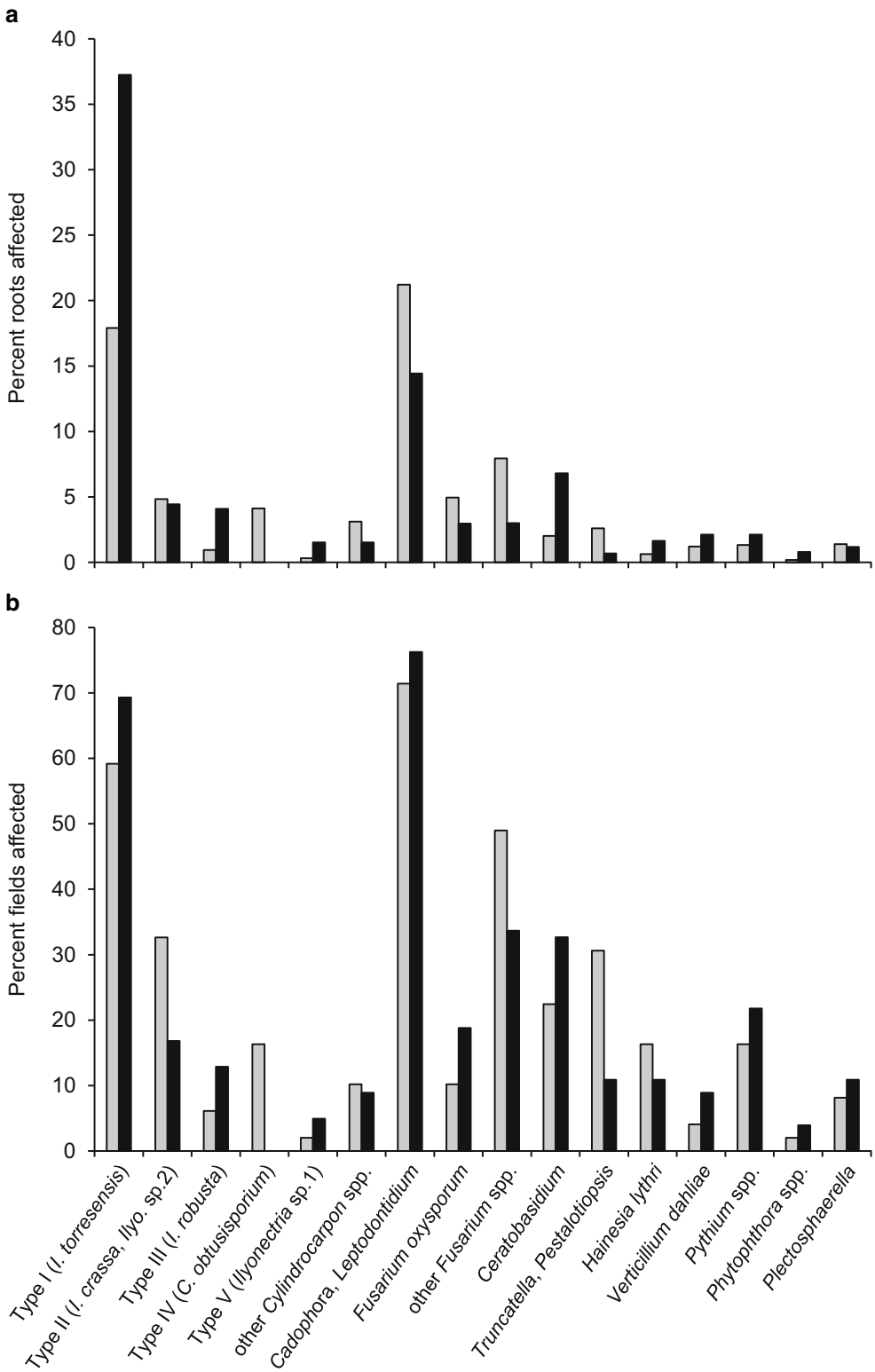

Fig. 6 Frequency of fungal isolates in the roots of strawberry plants from nurseries (*grey*) and production fields (*black*). **a** Percentage of roots affected; **b** percentage of batches or fields affected

anamorph *R. fragariae* have been implicated as a common cause of black root rot symptoms (Wilhelm et al. 1972), and the anastomosis group I to which two Northern German isolates belonged is also known to be pathogenic on strawberries especially at cooler temperatures (Martin 1988; LaMondia and Martin 1989; Botha et al. 2003). However, at up to 70% of all roots examined the frequency of *R. fragariae* in these cited reports was much higher than in the present study from Northern Germany where this fungus was sporadic in occurrence.

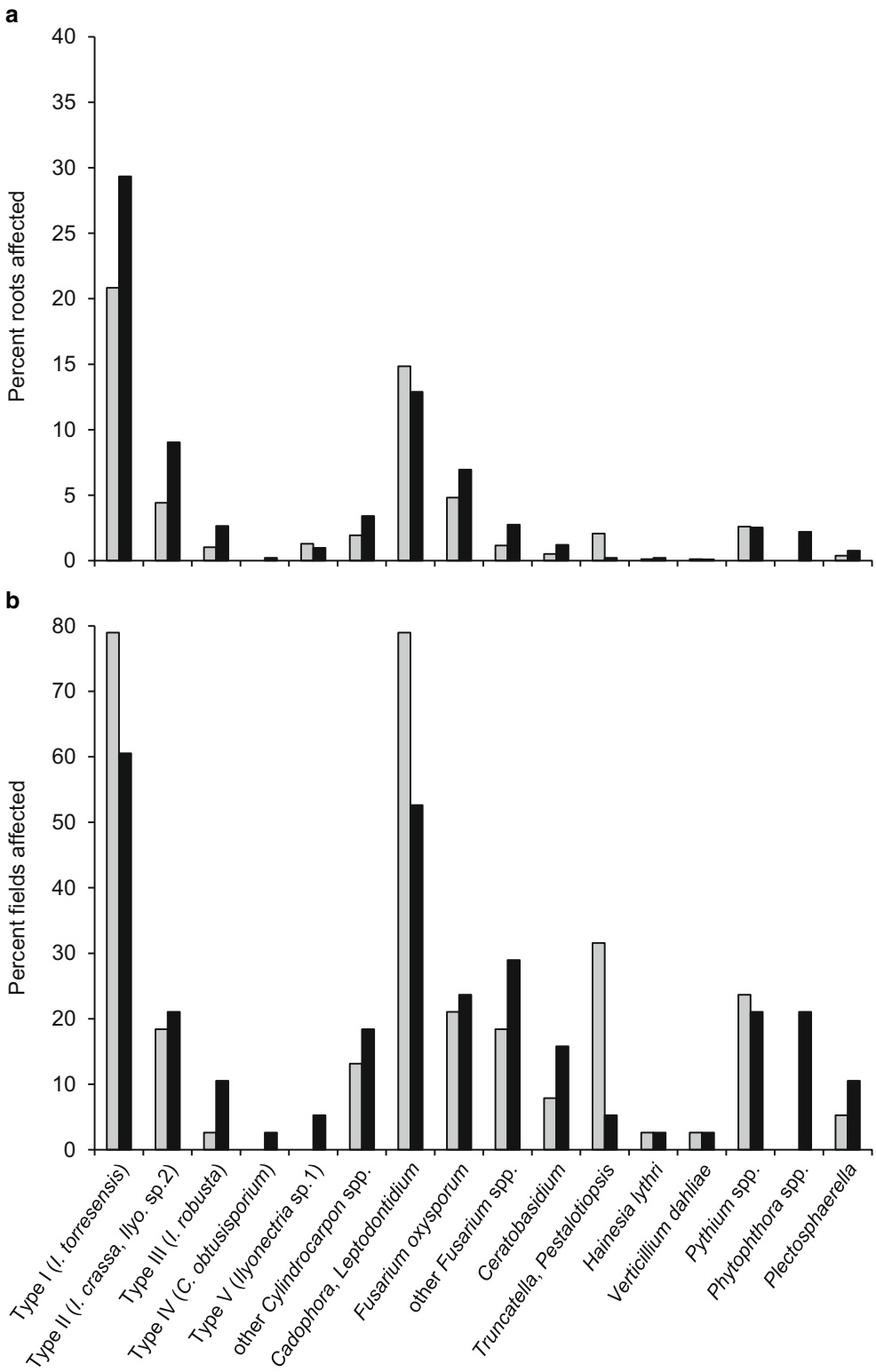

Fig. 7 Frequency of fungal isolates in the roots of raspberry plants from nurseries (*grey*) and production fields (*black*). **a** Percentage of roots affected; **b** percentage of batches or fields affected

Among *Fusarium* spp. isolated from strawberries, *F. oxysporum* f. sp. *fragariae*, cause of a wilt disease, is the most prominent in the literature. It is reported as being distinct in pathogenicity and genetic identity from other *formae speciales* of *F. oxysporum* (Maas 1998). None of our three *F. oxysporum* sequences was identical to any ITS sequence of *F. oxysporum* f. sp. *fragariae* available in GenBank (DQ452448, AF162889, KT833080). However, a similar sequence non-match was observed for pathogenic *F. oxysporum* isolates from strawberries in Cal-

ifornia (Koike et al. 2009). Therefore, we cannot formally rule out a (minor) contribution of *F. oxysporum* to the Northern German black root rot complex.

Several further species known or suspected to be involved in the strawberry or raspberry black root rot complex were isolated in the present study. These include *Hainesia lythri* (Sutton and Gibson 1977), *Pythium* spp. (Nemec and Sanders 1970) and *Gnomoniopsis fructicola* (formerly *Gnomonia fragariae*; Morocko et al. 2006). Several species of *Pestalotiopsis* have been described as strawberry pathogens (Lieten 2015; Chamorro et al. 2016; Grantina-Ievina and Kalniņa 2016), although *P. guepinii* identified in the present work is not among them. *Plectosphaerella* spp. which we isolated in the present study are further potential root rot pathogens of other plants (Carlucci et al. 2012). All these species as well as *Verticillium dahliae* and *Phytophthora* spp. grew well in either of the two isolation media used in our study. However, they were present on strawberry and raspberry roots in only a minority of fields and on less than 5% of all roots examined. Therefore, we consider their contribution to be marginal in the Northern German context. All in all, our report represents one of the strongest cases for a principal causal role of *Cylindrocarpon*-like fungi in black root rot on strawberries and raspberries published to date.

The abundance of *D. torresensis* in nursery material of both strawberries and raspberries deserves comment. Rigotti et al. (2003) have recorded the occurrence of several putative black root rot pathogens in strawberry nursery plants certified to be free of them, and Seemüller and Riedel (1980) detected the widespread presence of the red root rot pathogen *P. fragariae* in strawberry nursery plants. Clearly, therefore, certification schemes need to be revised in the light of new pathogens such as *D. torresensis* which has not previously been reported from strawberry nursery plants, even if it can be very common on grapevine nursery stock (Reis et al. 2013). An effective routine diagnostic test for the full set of black root rot pathogens in initial (symptomless) stages of infection would be desirable for strawberries but is unavailable at present. Meanwhile, farmers are advised visually to inspect their planting material, and to reject batches with obvious signs of black root rot. Nurseries should practise crop rotation in order to avoid the build-up of *Cylindrocarpon*-like fungi, as has been recommended for grapevines in Portugal where these fungi are troublesome (Rego et al. 2009).

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Conflict of interest R.W.S. Weber and A.-P. Entrop declare that they have no competing interests.

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