

Black root rot: A long known but little understood disease

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Abstract

Black root rot caused by the pathogen *Thielaviopsis basicola* has been known since the mid 1800's. The disease is important on many agricultural and ornamental plant species and has been found in at least 31 countries. Since its description, the pathogen has had a complex taxonomic history that has resulted in a confused literature. A recent revision of the *Ceratocystidaceae* following the advent of DNA sequencing technology has made it possible to resolve this confusion. Importantly, also to show that there are two pathogens in the *Ceratocystidaceae* that cause black root rot. They reside in the newly established genus *Berkeleyomyces* and are now known as *B. basicola* and *B. rouxiae*. This review considers the taxonomic history of the black root rot pathogens, and their global distribution. Prospects relating to the serious diseases that they cause and the likely impact that the era of genomics will have on our understanding of the pathogens are also highlighted.

Introduction

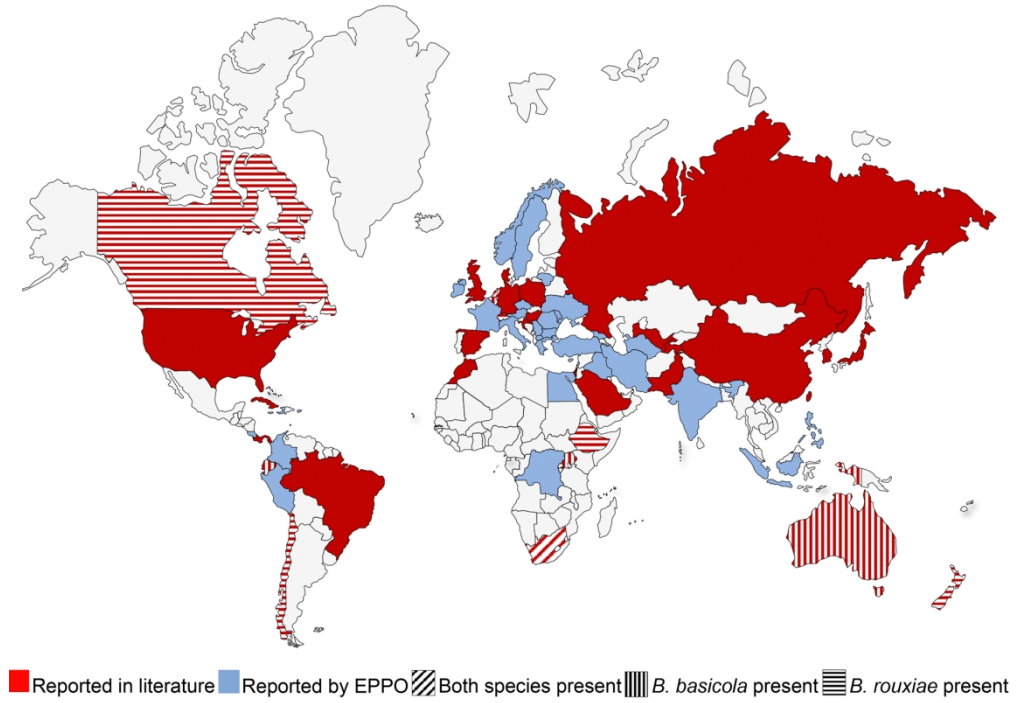


Figure 1. World-map illustrating the distribution of black root rot disease. Reports from published literature and the EPPO website are indicated in red and blue, respectively. Areas where *B. basicola*, *B. rouxiae*, or both have been detected are indicated with vertical, horizontal, and diagonal lines.

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The fungus best known as *Thielaviopsis (Tp.) basicola* is an important plant pathogen that causes the disease known as black root rot (Niu *et al.*, 2008, Pereg, 2013). The disease occurs on a variety of plants important to both agriculture and horticulture in many parts of the world (**Figure 1**). Since its first description in the genus *Torula (Ta.)* (Berkeley & Broome, 1850), the pathogen has had a complex taxonomic history, having been treated in several different genera and assigned numerous different species names. Recently, against a background of growing DNA sequence databases for the *Ceratocystidaceae* (De Beer *et al.*, 2014), *Tp. basicola* has been shown to represent two distinct fungal species in the newly described genus *Berkeleyomyces* (Nel *et al.*, 2018a).

More than 500 research articles have been published with a focus on *Tp. basicola*. At least 110 of these papers have appeared in the past 15 years, illustrating the importance of the causal agents of the disease. This review is primarily focussed on the taxonomic history of the black root rot pathogens, but also provides a perspective regarding a current understanding of their biology.

Pathogen background

Berkeleyomyces basicola and *B. rouxiae* are hemibiotrophic plant pathogens (**Figure 2**). They penetrate and colonize living root tissue during the short biotrophic phase of their life cycles. As infection progresses, these fungi enter a necrotrophic phase during which cells are lysed and consumed by the fungi (Mims *et al.*, 2000, Pereg, 2013). Necrosis induced during this phase leads to characteristic black discolouration of the plant roots (Nehl *et al.*, 2004b). This necrosis also results in brittleness of the root tissues and insufficient water and nutrient uptake by the plants leading to stunting and reduced crop yield (Nehl *et al.*, 2000). In cases of very severe infection, the vessels of the roots can become completely blocked by penetrating

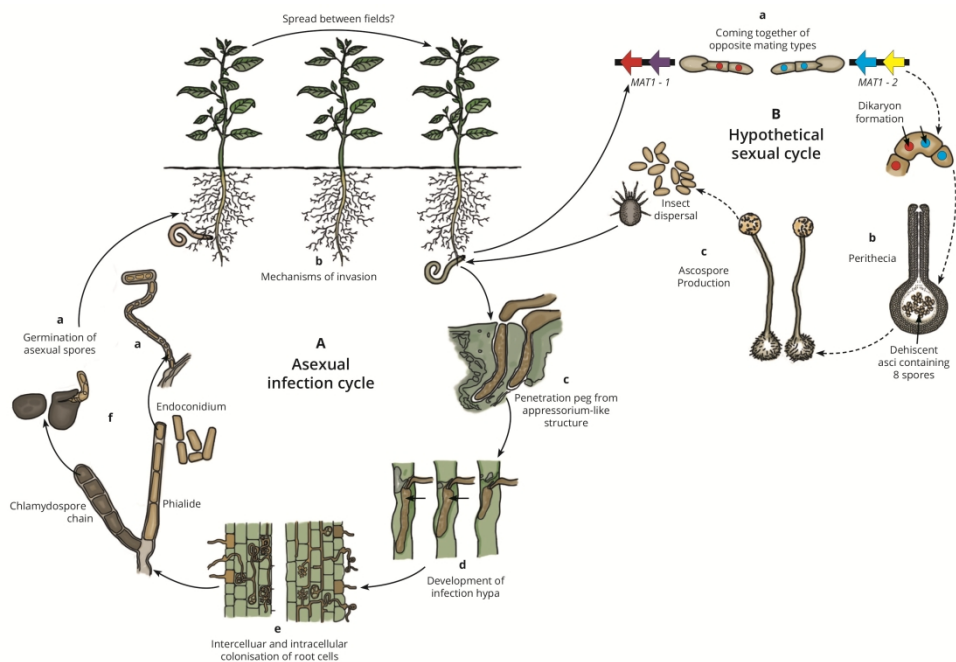


Figure 2. Putative life cycles of *Berkeleyomyces basicola* and *B. rouxiae* (A) Putative infection and asexual reproductive cycle: *Berkeleyomyces basicola* and *B. rouxiae* are hemibiotrophic plant pathogens.

Thick-walled chlamydo spores (a) in the soil germinate in the presence of host root exudates. Germ tubes grow to make contact with the host where they penetrate the living tissue (b) through the epidermal cells (Wick & Moore, 1983, Mims et al., 2000); root hairs (Linderman & Tousson, 1968, Hood & Shew, 1997); occasionally via stomata (Pierre & Wilkinson, 1970); or through wounds caused by nematode (Wheeler et al., 2000) and arthropod feeding or mechanical damage (Baard & Laubscher, 1985). Tissue penetration occurs either via simple penetration pegs (Nan et al., 1992, Hood & Shew, 1997) or (c) penetration hyphae arising from appressoria-like structures (Baard & Laubscher, 1985, Mauk & Hine, 1988). Within the host tissue, penetration hyphae colonize the root cells either directly by characteristic "beaded" (d) infection hyphae (Baard & Laubscher, 1985) or by development of "spear-head" shaped terminal infection vesicles that later give rise to the infection hyphae (Mims et al., 2000). Colonization of the root tissue continues via cell-to-cell contact by a combination of (e) intra- and intercellular growth (Punja et al., 1992). As lesions develop on the tissue surface, (f) endoconidia are produced within the root cells leading to continuous secondary infection (Mosma & Struck, 2013). As these tissues start to die off and the pathogens enter the necrotrophic phase of their life cycles, chlamydo spores are produced abundantly on the dead tissues resulting in increased inoculum load in the soil (Linderman & Tousson, 1968, Pierre & Wilkinson, 1970, Mosma & Struck, 2013). Further spread then occurs via infected plant tissue, contaminated soil or possibly mite or arthropod activity.

(B) Hypothetical sexual cycle: Sexual reproduction has not been observed for either of the two *Berkeleyomyces* species, but their mating type loci suggest a heterothallic sexual cycle (Nel et al., 2018b). Thus, if sexual reproduction were to take place in these species (a) two isolates, of opposite mating type would come into contact. Once genetic exchange occurs between the opposite mating type isolates (b) the fungi would produce sexual structures which for the Ceratocystidaceae would be ascogonia probably with deliquescent asci and sheathed (c) sexual spores.

hyphae, impairing water and nutrient uptake, ultimately resulting in plant death (Noshad *et al.* 2006).

Within and upon the diseased tissues, very distinctive dark-coloured and muriform chlamydospores are produced in chains and contained within a sheath until maturation (**Figure 3**) (Pereg, 2013). These spores are sufficiently characteristic of the two *Berkeleyomyces* species that they have been utilised as primary means of black root rot identification (King & Presley, 1942, Nel *et al.*, 2018a). Identification of these pathogens utilising sequence data for the ITS region is not informative and other regions of the genome such as Actin, MCM7 and RPBII are recommended.

Thus far, black root rot symptoms have been reported on more than 170 different agricultural and ornamental plant species (**Supplementary Table 1**). Some of the more common species affected are cotton (Pereg, 2013), tobacco (Stover, 1950), carrot (AbdAllah *et al.*, 2011), groundnut (Baard & Laubscher, 1985) and chicory (Prinsloo *et al.*, 1991).

Taxonomic history

The causal agent of black root rot disease was initially described as *Torula basicola* (Berkeley & Broome, 1850), *Helminthosporium fragile* (Sorokin, 1876) and *Milowia nivea* (Masse, 1884). These names were applied to different host plants with an assumption that three different diseases were involved. However, over time, each of these species were subjected to taxonomic scrutiny and revision (Cooke, 1885, Masse, 1893, 1912, Saccardo, 1886a, 1886b).

The initial confusion surrounding the identity of these species was resolved in 1912 when all names were reduced to synonymy with the name *Thielaviopsis basicola*. This resulted from Masse (1912) recognising that *Milowia nivea*, the species that he had described, and *Torula basicola* described by Berkeley & Broome (1850), represented the same fungus. Later the same



Figure 3. Morphological characteristics of *B. basicola* and *B. rouxiae*. (A & B) Phialides; (C & D) Chlamydospores; (E-H) Secondary chlamydospores; (I & J) Endoconidia; (K – N) Four different cultural variants of *B. basicola*; (O – R) Black root rot symptoms induced on carrot discs using different isolates of *B. basicola* and *B. rouxiae*. Scale Bars = 25µm.

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year, Ferraris (1912) recognised that *Helminthosporium* (= *Clasterosporium*) *fragile* and *Torula basicola* represented a single fungus. However, he also recognized that the structures produced by these species resembled those of species in *Thielaviopsis* more than those of *Torula*. Consequently he synonymized *Torula basicola* (syn = *Milowia nivea*) and *Helminthosporium* (= *Clasterosporium*) *fragile* under the new name *Thielaviopsis basicola*.

Zopf (1876) described what he believed to be the sexual state of *Ta. basicola* as *Thielavia* (*Tl.*) *basicola*. This was based on the perceived presence of perithecial structures intermingled with the chlamydo-spores of *Ta. basicola*. Zopf (1876) thus proposed that a teleomorph-anamorph connection existed between the two states he observed. Although this connection was supported in several subsequent publications (Ferraris, 1912, Masee, 1912, Brierley, 1915, Johnson, 1916, Johnson & Hartman, 1919), McCormick (1925) disproved the existence of a sexual state in the fungus. Her experiments involving single spore cultures of *Tl. basicola* and *Tp. basicola*, as well as those of others (Lucas, 1949, Stover 1950), showed that the sexual and asexual structures represented different fungi. However, prior to this discovery, the sexual name *Thielavia basicola* was commonly used in literature, resulting in considerable confusion for later researchers.

The name *Tp. basicola* remained in use until 1975 when Nag Raj & Kendrick (1975) described the endoconidial state of the fungus with a different name to that of the chlamydo-spore-producing state. In their monograph, the authors stated that *Tp. basicola* was applicable only to the chlamydo-spore state because this was the form treated in the original description by Berkeley & Broome (1850). Nag Raj & Kendrick (1975) subsequently described the endoconidial state in the genus *Chalara* (*Ch.*) (Corda, 1838, Corda, 1842, Rabenhorst, 1844). However, because the epithet “*basicola*” had been connected to the chlamydo-spore state, these authors proposed the new combination *Ch. elegans*.

With the dual nomenclature of fungi in place, the two asexual names, *Thielaviopsis basicola* and *Chalara elegans*, were used interchangeably in literature. This proceeded mostly without dispute until DNA sequence-based phylogenetic inference became common place in the 1990's. However, Carmichael *et al.* (1980) suggested that the *Thielaviopsis* state of the pathogen was best accommodated in the genus *Trichocladium* (*Tr.*) and proposed the name *Tr. basicola*. But this name was never widely accepted and appeared in published literature only once with reference to *Tp. basicola* (Nan *et al.*, 1992).

A phylogenetic revolution

The first phylogenetic study to consider the taxonomic placement of the black root rot pathogen was that of Paulin & Harrington (2000). Their analyses grouped six species of *Chalara*, including *Ch. elegans* (syn = *Tp. basicola*), in the *Ceratocystidaceae*. Although no taxonomic changes were proposed at that time, the authors suggested that the name *Tp. basicola* should be used rather than *Ch. elegans*. This was supported by the fact that all six *Chalara* species that grouped in the *Ceratocystidaceae* were morphologically different to *Ch. fusidioides*, which is the type species of *Chalara*.

Paulin-Mahady *et al.* (2002) amended the generic description of *Thielaviopsis* (Went, 1893) to incorporate all *Chalara* spp. that were shown to have *Ceratocystis* affinities. This was despite the fact that neither investigation included data for *Ch. fusidioides*. However, the fact that *Ch. fusidioides* does not reside in the *Ceratocystidaceae* was later confirmed in the phylogenetic analyses of Réblová *et al.* (2011), which rendered *Tp. basicola* the only valid name for the pathogen.

De Beer *et al.* (2014) provided an extensive revision of the *Ceratocystidaceae* applying the one fungus one name principle (Hawksworth *et al.*, 2011, Wingfield *et al.*, 2012). These authors showed that *Tp. basicola* was not appropriately accommodated in *Thielaviopsis* or any of the

other genera of the *Ceratocystidaceae*. Although the description of a new genus for the monophyletic lineage defined by *Tp. basicola* was justifiable based on their data, De Beer *et al.* (2014) chose not to take this step. This was largely because an ex-type isolate representing *Tp. basicola* was not available for study. However, this decision left the species taxonomically unresolved.

Against the background of these previous studies, Nel *et al.* (2018a) utilised a robust multi-gene phylogenetic approach that included six different gene regions, to show that *Tp. basicola* forms a discrete lineage in the *Ceratocystidaceae*. Their results also revealed the existence of a cryptic sister lineage to *Tp. basicola*. Consequently, these authors introduced the new genus *Berkeleyomyces* (**Figure 3**) to accommodate the two species. Isolates representing the cryptic sister lineage to *B. basicola* were described as the new species *B. rouxiae*.

Population genetics

Even though *Tp. basicola* has been known for more than a Century, very little work has been done regarding its global population structure. In fact, there are apparently only four investigations involving this pathogen that include data relating to this subject. Three of these studies employed RAPD analyses to investigate isolate variation in collections of North American (Punja & Sun 2000) and Australian isolates (Harvey *et al.*, 2002, Nehl *et al.*, 2004a). The remaining study (Geldenhuis *et al.*, 2006) utilized microsatellite markers, developed two years earlier (Geldenhuis *et al.*, 2004), to consider the diversity and possible origin of a global collection of isolates.

Although focussing on two very different geographic regions, the results emerging from the investigations by Punja & Sun (2000) and Nehl *et al.* (2004a) were similar. In both cases, the included isolates formed genetically distinct groups based primarily on the geographic areas from which they were collected. Both investigations also reported these primary groups

separating into smaller secondary groups, usually based on the host from which they had been isolated. Upon closer inspection of their results, the RAPD banding patterns of Punja & Sun (2000) and Harvey *et al.* (2002) showed that isolates of *Tp. basicola* separate in two distinct clades. Although the banding patterns of the RAPD analyses was not shown by Nehl *et al.* (2004a), the phylogeny derived from their data also suggested the presence of two clades among their isolates. However, because these investigations focussed on isolate variability rather than population diversity, the authors seemingly placed no significant relevance on these clades.

The two distinct clades emerging from these earlier studies also emerged in the microsatellite investigation of Geldenhuis *et al.* (2006). These authors defined the clades as Clade A and Clade B. Although their study did not consider generic boundaries, it is clear that these authors had inadvertently recognised the two different species of *Berkeleyomyces*. This becomes evident when isolates common to the studies of Geldenhuis *et al.* (2006) and Nel *et al.* (2018a) are considered. Such a comparison suggests that isolates in Clade A of Geldenhuis *et al.* (2006) represent *B. rouxiae* and those in Clade B represents *B. basicola*.

Inspection of the phylogeny of Geldenhuis *et al.* (2006) also provides some insights into the population structure of *B. basicola* and *B. rouxiae*. Even though Geldenhuis *et al.* (2006) had a relatively limited population of *B. rouxiae*, their data revealed at least three distinct genetic groups for their isolates, with two of these occurring in South Africa. Their much more geographically diverse population of *B. basicola* isolates illustrated a substantial degree of genetic variation, even among isolates originating from the same country. However, the origin of this genetic variation remains to be determined. This is because a sexual state, which would influence genetic diversity, has not been discovered for either species

Reproductive biology

During the mid 1920's, *Thielavia basicola* was conclusively shown not to be the sexual state of *Tp. basicola* (McCormick, 1925). However, subsequent to the work of McCormick (1925) who disproved the relationship of these two states, very little effort has been made to induce sexual reproduction in *Tp. basicola*. This is other than a study by Johnson & Valleau (1935) who crossed a large collection of *Tp. basicola* in an attempt to induce a sexual state. The crosses were incubated for up to one year, but no sexual structures were ever observed. Various authors have suggested that the pathogen could exist as an exclusively asexual fungus (Tabachnik & DeVay, 1980, Paulin & Harrington, 2000, Paulin-Mahady *et al.*, 2002) although no clear justification for this notion has been provided.

The contemporary availability of whole genome sequences for fungi has led to the discovery that various plant pathogens, originally thought to be asexual, harbour the genes required for sexual reproduction. Examples are readily found in species of the *Ceratocystidaceae*, including species of *Thielaviopsis* (Wilken *et al.*, 2018), *Huntia* (Wilson *et al.*, 2015a), and *Ceratocystis* (Wilken *et al.*, 2014). However, many of these species do not conform the conventional homothallic or heterothallic mating system, but utilize secondary homothallic strategies such as mating type switching (Witthuhn *et al.*, 2000, Wilken *et al.*, 2014) and unisexuality (Wilson *et al.*, 2015a, 2015b).

The recently released genome of *B. basicola* (Wingfield *et al.*, 2018) made it possible for Nel *et al.* (2018b) to show that both *B. basicola* and *B. rouxiae* possess the genes that are required for a heterothallic mating system. Thus, the *MATI-1* and *MATI-2* idiomorphs of the *MAT* locus were found in different isolates of both species (**Figure 4**). However, despite considerable effort in crossing isolates of different mating idiomorphs, these authors failed to induce sexual reproduction in either species.

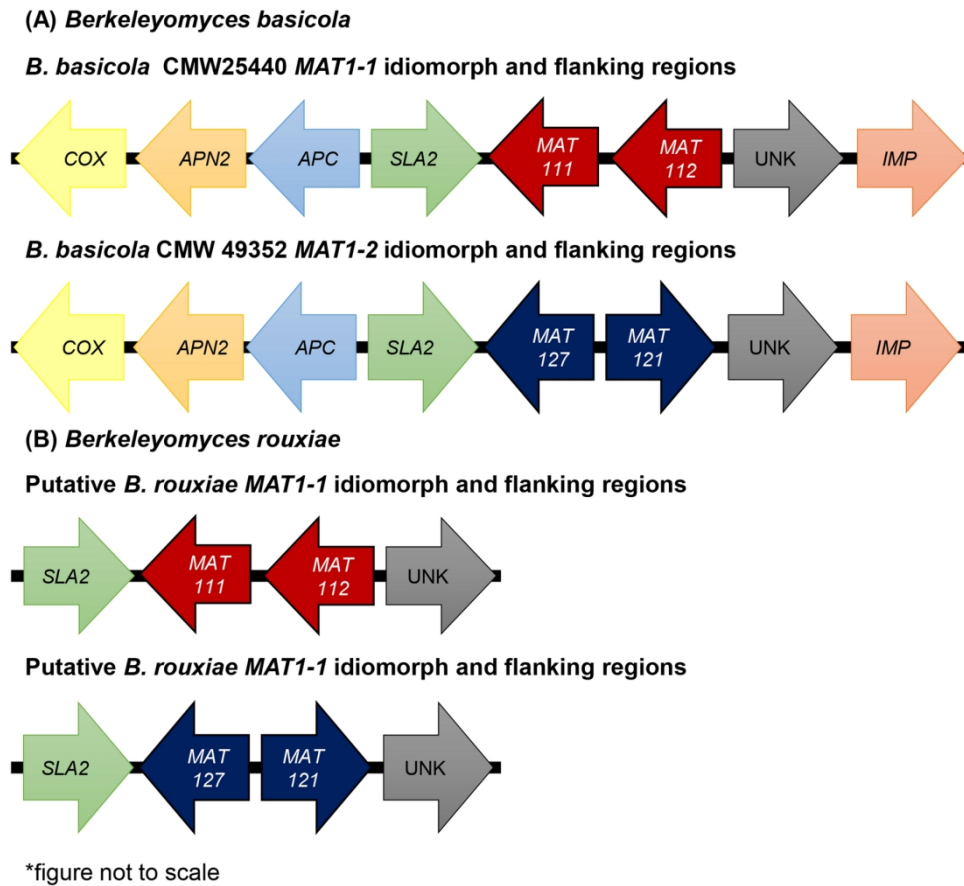


Figure 4. (A) Diagram showing the structure of the *MAT-1* locus of *B. basicola*. (B) Diagram showing the putative structure of the *MAT-1* locus of *B. rouxiae* elucidated using PCR amplification.

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The inability to induce sexual reproduction in heterothallic species is not uncommon for many reasons, including the fact that the conditions required for mating to take place can be very complex. This has for example been found in *Aspergillus fumigatus*, with its *MAT*-locus being characterized many years before sexual reproduction was successfully induced (Paoletti *et al.*, 2005, O'Gorman *et al.*, 2009). The availability of the *B. basicola* genome will hopefully allow future research efforts to unravel more of the complex biology of these two pathogens and possibly result in the discovery of a sexual state for them.

The existence of two species: Considerations regarding current knowledge

Discovery of the fact that there are two cryptic species that cause black root rot raises many intriguing and important questions. The most obvious and important of these is how this discovery influences our views on distribution and host range of each species and the disease that they cause.

Black root rot symptoms have been reported on more than 170 different plant species globally. Effectively, all reports from these different hosts have utilised the distinct morphology of the fungus formally known as *Tp. basicola* to identify the causal agent of the disease, rather than relying on DNA sequence data. Now, knowing the disease is caused by two different species will mandate that DNA sequence data must be used to identify the causal agents of black root rot. This for example has significant implications for quarantine regulations.

A similar challenge faces future studies considering the global distribution of black root rot. Using geographical data included in the studies of Geldenhuis *et al.* (2006) and Nel *et al.* (2018a), we were able to plot a putative distribution for both *Berkelyomyces* species and found that both species are present in the Netherlands and South Africa. Using these data, we also found that *B. basicola* occurs in Australia, Belgium, Ecuador, Indonesia and Uganda and that *B. rouxiae* occurs in Canada, Chile, Ethiopia, Israel, Switzerland, and New Zealand. However,

because these two studies include only a small number of global reports, a comprehensive assessment of the global distribution of *B. basicola* and *B. rouxiae* remains to be resolved.

Another challenge regarding the recognition that two different species cause black root rot relates to the host range of these fungi. There have been many studies focussed on determining the susceptibility of various plant species to black root rot disease when it was considered to be caused by a single pathogen (**Supplementary Table 2**). It is now conceivable that large variation in the results of those studies could be attributed to *B. basicola* and *B. rouxiae* having different host specificities. An apt example of this is found in the experiments carried out by Keller & Shanks (1955) where an isolate of *Bp. basicola* acquired from *Poinsettia* was unable to induce black root rot in tobacco plants and *vice versa*. This might suggest that one of the species of *Berkeleyomyces* is pathogenic on *Poinsettia* and the other on tobacco. Future research regarding these pathogens should incorporate both host and geographic data in an effort to resolve these intriguing questions

Questions relating to the host range and geographic distribution of *B. basicola* and *B. rouxiae* raise concerns regarding the quarantine status and regulations relating to the movement of these pathogens. With studies suggesting differences in the ability of isolates to infect certain plant species and against a background of their distinct distributions, a need to control the spread of *B. basicola* and *B. rouxiae* between countries or regions becomes relevant. This also illustrates a need for contemporary DNA-based technologies to be incorporated into quarantine regulations (McTaggart *et al.*, 2016). Furthermore, with many quarantine resources outdated in terms of fungal names (CABI 2018, EPPO 2018), and with some still considering *Chalara elegans* the causal agent of black root rot (CABI 2018), serious efforts should be made to prevent additional spread of these pathogens into new areas.

Concluding remarks

The black root rot pathogens, *B. basicola* and *B. rouxiae*, have had a long and complicated taxonomic history that dates back to the description of *Torula basicola* more than 150 years ago. Although the disease has been subjected to thorough study, the biology of these pathogens is far from fully understood. Indeed, this question has become more complex given the fact that two different species must now be considered.

A new technological era is rapidly emerging where genomes of fungi can be interrogated to better understand the biology of plant pathogens (Mardis, 2007, Schuster, 2008, Shendure & Ji 2008). Functional and comparative genomics are now being used to investigate niche and host adaptations, pathogenicity and evolution of various groups of fungi (Klimes *et al.*, 2015, Kohler *et al.*, 2015). The availability of rapidly increasing numbers of fungal genomes provides researchers with a new tool investigate complex biological questions. In future these resources will clearly also make it possible to address some of the newly emerging questions relating to black root rot, a disease that has been known for many years but that remains relatively poorly understood.

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Supporting Information Legends

Supplementary Table 1. Hosts reported to be susceptible to black root rot infection.

Supplementary Table 2. Variation in host susceptibility to black root rot infection by the fungus formally known as *Thielaviopsis basicola*