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Pathogen Profile

Verticillium longisporum, the invisible threat to oilseed rape and other brassicaceous plant hosts

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SUMMARY

Introduction: The causal agents of Verticillium wilts are globally distributed pathogens that cause significant crop losses every year. Most Verticillium wilts are caused by *V. dahliae*, which is pathogenic on a broad range of plant hosts, whereas other pathogenic *Verticillium* species have more restricted host ranges. In contrast, *V. longisporum* appears to prefer brassicaceous plants and poses an increasing problem to oilseed rape production.

Taxonomy: Kingdom Fungi; Phylum Ascomycota; Class Sordariomycetes; Subclass Hypocreomycetida; Family Plectosphaerellaceae; genus *Verticillium*.

Disease symptoms: Dark unilateral stripes appear on the stems of apparently healthy looking oilseed rape plants at the end of the growing season. Microsclerotia are subsequently formed in the stem cortex beneath the epidermis.

Genome: *Verticillium longisporum* is the only non-haploid species in the *Verticillium* genus, as it is an amphidiploid hybrid that carries almost twice as much genetic material as the other *Verticillium* species as a result of interspecific hybridization.

Disease management: There is no effective fungicide treatment to control *Verticillium* diseases, and resistance breeding is the preferred strategy for disease management. However, only a few Verticillium wilt resistance genes have been identified, and monogenic resistance against *V. longisporum* has not yet been found. Quantitative resistance exists mainly in the *Brassica* C-genome of parental cabbage lines and may be introgressed in oilseed rape breeding lines.

Common name: Oilseed rape colonized by *V. longisporum* does not develop wilting symptoms, and therefore the common name of Verticillium wilt is unsuitable for this crop. Therefore, we propose 'Verticillium stem striping' as the common name for *Verticillium* infections of oilseed rape.

Keywords: amphidiploid, *Arabidopsis*, *Brassica*, host range, pathogenicity, disease management, vascular wilt.

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INTRODUCTION

Verticillium is a relatively small genus of ascomycete fungi that currently comprises 10 species (Inderbitzin *et al.*, 2011a). All presently recognized *Verticillium* species are soil-borne fungi, and several cause wilt disease on a variety of plant hosts across the world (Pegg and Brady, 2002). Although symptoms may vary considerably between plant hosts, the most frequently observed disease symptoms of Verticillium wilt include wilting, stunting, chlorosis, vascular discoloration and early senescence (Fradin and Thomma, 2006). The economic impact of *Verticillium* diseases can be severe, with an estimated annual loss of €3 billion worldwide in the 20 most affected hosts (M. Siebold and A. V. Tiedemann, unpublished data). *Verticillium dahliae* is the most economically important species of the *Verticillium* genus, and has the ability to infect more than 200 plant host species (Inderbitzin *et al.*, 2011a; Pegg and Brady, 2002). *Verticillium albo-atrum*, *V. alfalfae*, *V. non-alfalfae* and *V. longisporum* are also vascular pathogens, albeit with a more restricted host range. Members of the genus reproduce asexually and a sexual stage has not yet been described for any *Verticillium* species (Short *et al.*, 2014).

TAXONOMY AND MORPHOLOGY

Verticillium belongs to the family Plectosphaerellaceae (Zare *et al.*, 2007) in the subclass Hypocreomycetidae of the class Sordariomycetes, which is part of the phylum Ascomycota (Zhang *et al.*, 2006). *Verticillium* is subdivided into two major groups:

Table 1 Non-molecular criteria for the taxonomic discrimination of *Verticillium longisporum* and *V. dahliae*.

Parameter	*V. dahliae*	*V. longisporum*
Microsclerotial shape*,‡,**	Mostly rounded or spherical	Mostly elongate
Conidial size*,¶,**	Mostly short (3.5–5.5 μm)	Mostly long (7.1–8.8 μm)
Extracellular polyphenol oxidase activity*,‡,¶	Mostly strong	Mostly none
Culture filtrate fluorescence*	No	Yes
Host range*,†,§	Broad (vegetables, trees, legumes, ornamental crops)	Mainly restricted to Brassicaceae

*Karapapa *et al.* (1997).
†Bhat and Subbarao (1999).
‡Zeise and Tiedemann (2001).
§Zeise and Tiedemann (2002).
¶Steventon *et al.* (2002).
**Inderbitzin *et al.* (2011b).

Clade Flavexudans and Clade Flavnonexudans (Inderbitzin *et al.*, 2011a). *Verticillium longisporum* is a member of the Flavnonexudans lineage and thus lacks the ability to produce yellow hyphal pigmentation. The taxonomic history of *Verticillium*, including *V. longisporum*, is complicated as a result of name changes and taxonomic disagreements. *Verticillium longisporum* was first described as a variety of *V. dahliae*, as *V. dahliae* var. *longisporum* (Stark, 1961), and was then elevated to species rank 37 years later (Karapapa *et al.*, 1997). Although first contested, the name *V. longisporum* is now widely adopted (Inderbitzin and Subbarao, 2014). The evolutionary history of *V. longisporum* is unique among *Verticillium* species, as *V. longisporum* is an amphidiploid hybrid that evolved repeatedly by hybridization among four different ancestors (Inderbitzin *et al.*, 2011b; Ingram, 1968).

The differentiation of *V. longisporum* from related species may be based on morphological and cultural features (Table 1, Fig. 1), but these may not consistently discriminate *V. longisporum* from *V. dahliae*. In general, *V. longisporum* conidia are longer than those of its close relative *V. dahliae* (Karapapa *et al.*, 1997; Stark, 1961) and, with respect to *V. dahliae, V. longisporum* has been reported to have elongated microsclerotia and a tendency towards the presence of three phialides in each whorl (Karapapa *et al.*, 1997). However, for some *V. longisporum* strains, the conidial size ranges overlap with those of *V. dahliae*, microsclerotia are rounded and there are more than three phialides per whorl (Inderbitzin *et al.*, 2011a). In addition, no morphological characters allow for the differentiation of the different hybrid lineages in *V. longisporum*. Therefore, the identity of *V. longisporum* strains and other *Verticillium* species should be confirmed using molecular techniques, such as DNA amplification with species-specific primers (Inderbitzin *et al.*, 2013) or DNA sequence determination of species-specific gene regions (Inderbitzin *et al.*, 2011a).

EVOLUTIONARY HISTORY, GENOMICS AND PATHOGENICITY

The typical life cycle of ascomycete fungi is dominated by the haploid state, whereas *V. longisporum* is amphidiploid as a result of hybridization between two haploid ancestors. Phylogenetic analysis separates *V. longisporum* isolates into three lineages with different ancestors (Inderbitzin *et al.*, 2011b). Four parental lines are known that belong to three different *Verticillium* species. The

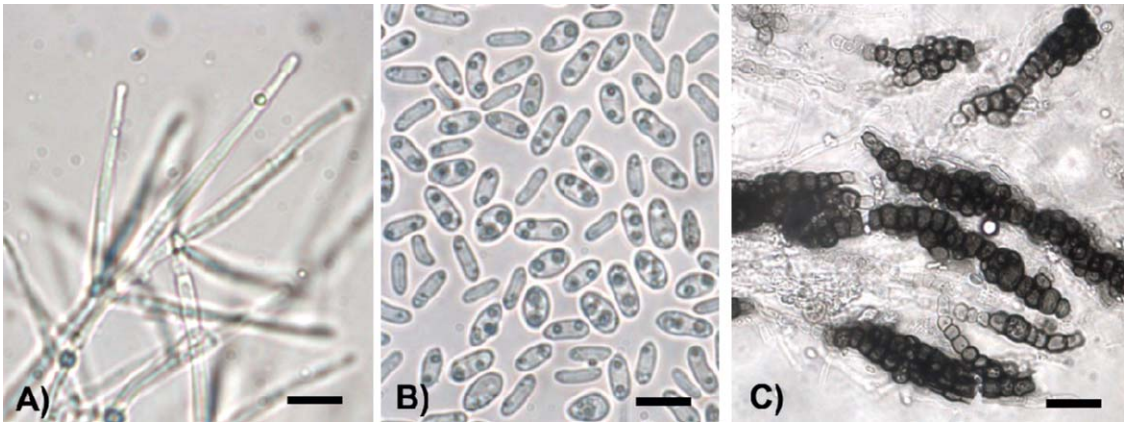

Fig. 1 Microscopic appearance of *Verticillium longisporum in vitro*. (A) Verticillate conidiophores (bar, 20 μm). (B) Conidia (bar, 10 μm). (C) Young microsclerotia (bar, 25 μm).

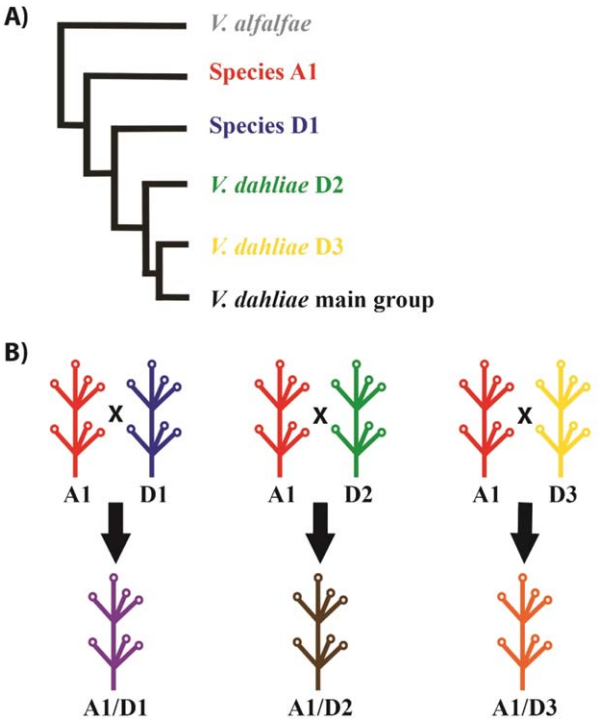

Fig. 2 The genetic constitution of the three lineages of *Verticillium longisporum*. (A) Phylogenetic relationship between the parents of *V. longisporum* (adjusted from Inderbitzin and Subbarao, 2014). (B) The three hybridization events that resulted in the hybrid species *V. longisporum*. A1 and D1 progenitors are unknown and provisionally named haploid *Verticillium* species, whereas progenitors D2 and D3 are both *V. dahliae* lineages. A1 is a parent of all three *V. longisporum* lineages, as it hybridized with D1, D2 and D3, resulting in the three *V. longisporum* lineages A1/D1, A1/D2 and A1/D3, respectively.

parents include two *V. dahliae* genotypes, the *V. dahliae* lineage D2 and *V. dahliae* lineage D3, and two unknown species that were provisionally called Species A1 and Species D1. Based on ribosomal internal transcribed spacer (ITS) sequences and intron-rich portions of the five protein-encoding genes *actin* (*ACT*), *elongation factor 1-alpha* (*EF*), *glyceraldehyde-3-phosphate dehydrogenase* (*GPD*), *mitochondrial oxaloacetate transport protein* (*OX*) and *tryptophan synthase* (*TS*), it was determined that all characterized *V. longisporum* isolates contain alleles derived from the Species A1 parent, in combination with Species D1, *V. dahliae* lineage D2 or *V. dahliae* lineage D3 alleles, to form the A1 × D1, A1 × D2 and A1 × D3 hybrids, respectively (Fig. 2).

Verticillium longisporum has also been referred to as a 'near-diploid' as its nuclear DNA content is ±1.7–1.8 times that of *V. dahliae*, depending on the isolate, although the amount may vary considerably between isolates (Collins *et al.*, 2003; Karapapa *et al.*, 1997; Steventon *et al.*, 2002). The DNA content of some isolates can be more than double the amount of others (Steventon *et al.*, 2002). This difference in genome size may be a result of variation in DNA content between ancestors, may reflect the genomic plasticity of fungi (Zolan, 1995) or may indicate DNA loss associated with hybridization, as in the endophyte *Neotyphodium uncinatum* (Craven *et al.*, 2001; Moon *et al.*, 2004). However, two copies have so far been found for all nuclear genes examined in *V. longisporum* (Inderbitzin *et al.*, 2011b; Tran *et al.*, 2013), with the exception of the nuclear ribosomal region (rDNA), for which only one type was detected in each lineage (Inderbitzin *et al.*, 2011b, Tran *et al.*, 2013). The *V. longisporum* lineage A1/D3 rDNA region was derived from *V. dahliae*, whereas the *V. longisporum* lineage A1/D1 and A1/D2 rDNA regions were derived from Species A1. In addition to DNA loss, concerted evolution could also account for the loss of one rDNA type in each of the *V. longisporum* lineages.

Several suggestions have been made with regard to the origin of *V. longisporum*. Parasexual recombination has been proposed as the underlying mechanism (Karapapa *et al.*, 1997), although parasexual processes generally end with chromosome loss to regain a haploid state after fusion of hyphae and nuclei (Caten, 1981). Thus, the stability of *V. longisporum* as a hybrid makes the hypothesis of interspecific hyphal fusion followed by nuclear fusion more plausible than parasexual processes (Inderbitzin *et al.*, 2011b).

The relative karyotypic stability of *V. longisporum* is unusual within *Verticillium*, as artificially induced hybrids between *Verticillium* species tend to be unstable (Fordyce and Green, 1964; Hastie, 1973; Typas and Heale, 1976) and undergo parasexual processes. In contrast, in a *V. longisporum* strain maintained in culture for 51 years, no gene loss was observed (Inderbitzin *et al.*, 2011b). The amphidiploid nature of *V. longisporum* also explains why auxotrophic mutants for the study of vegetative compatibility groups could not be generated (Bhat and Subbarao, 1999; Joaquim and Rowe, 1990; Puhalla, 1979; Nagao *et al.*, 1994; Subbarao *et al.*, 1995; Zeise and Tiedemann, 2001).

Individual *V. longisporum* lineages are genetically homogeneous, as only a single substitution was found across seven nuclear loci. This suggests a recent origin of *V. longisporum* (Inderbitzin *et al.*, 2011b). However, there are differences in pathogenicity and virulence between the lineages. Lineage A1/D1 is the most pathogenic lineage on oilseed rape, whereas lineage A1/D3 isolates are generally not pathogenic on this crop (Novakazi *et al.*, 2015; Tran *et al.*, 2013). Lineage A1/D2 is known only from horseradish in Illinois (USA) (Eastburn and Chang, 1994; Inderbitzin *et al.*, 2011b), and was the most virulent lineage on this crop (Novakazi *et al.*, 2015).

DISEASE CYCLE

As far as details are available, the infection process of *V. longisporum* is highly similar to that of *V. dahliae*. Verticillium wilts are monocyclic diseases (Klosterman *et al.*, 2011) (Fig. 3). Like *V. dahliae*, *V. longisporum* produces melanized microsclerotia (Stark, 1961) for survival to bridge the gap between hosts. Microsclerotia are clusters of melanized, thick-walled fungal cells, which are

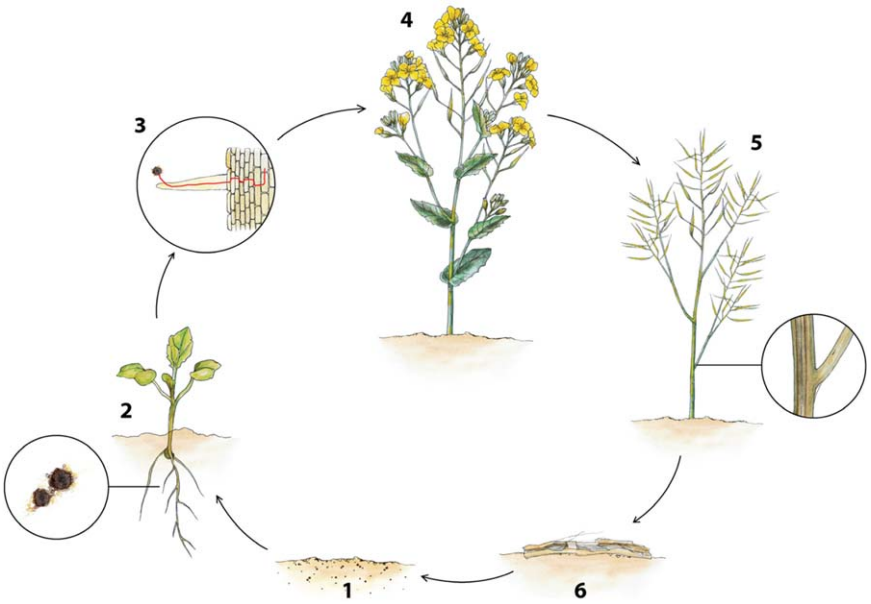

Fig. 3 Disease cycle of *Verticillium longisporum* on oilseed rape. 1. Microsclerotia are persistent resting structures that reside in the soil and bridge the gap between hosts. 2. Triggered by root exudates, microsclerotia start to germinate and hyphae grow towards the root of the plant. 3. The fungus enters the root through wounds, or by direct penetration of epidermal cells of lateral roots or root hairs. In the root, hyphae grow intercellularly and intracellularly towards the central cylinder and enter the xylem. 4. No disease symptoms are observed during the major part of the growing season. 5. Dark unilateral striping develops on the stem of oilseed rape during the ripening of the crop. Ultimately, black microsclerotia are formed in the stem cortex. 6. Microsclerotia are released into the soil on decomposition of plant debris.

derived from hyphal cells through lateral budding of the hyaline mycelium (Klebahn, 1913). In the absence of a host, *V. dahliae* microsclerotia remain dormant and viable in the soil for more than 10 years (Wilhelm, 1955), which may be similar for *V. longisporum*. Root exudates stimulate the germination of *V. longisporum* microsclerotia, after which hyphae grow towards the root of the plant (Berlanger and Powelson, 2000; Leino, 2006). Subsequently, hyphae colonize the surface of the root hairs and grow towards the root surface (Eynck *et al.*, 2007; Zhou *et al.*, 2006). On oilseed rape (*Brassica napus*), the fungus enters the root by direct penetration of rhizodermal cells of lateral roots or root hairs. Once inside the root, hyphae initially grow both intercellularly and intracellularly in the root cortex towards the central cylinder, where the pathogen enters the xylem (Eynck *et al.*, 2007). Next, conidia may be produced that are carried upwards with the transpiration stream. Conidia that become trapped in pit membranes or at vessel end walls may germinate and penetrate into adjacent vessels (Garber and Houston, 1966). The colonization induces occlusions of the vessels, which may disturb the sap stream in the xylem (Kamble *et al.*, 2013). Only during senescence does the pathogen grow out of the xylem vessels, invades the stem parenchyma and forms microsclerotia beneath the stem epidermis and in the stem pith. The microsclerotia are released into the soil during tissue decomposition (Heale and Karapapa, 1999). Clear evidence for a transmission of *V. longisporum* by seeds has not been provided so far.

SUSCEPTIBLE CROPS

The currently known *V. longisporum* isolates are mainly found on brassicaceous hosts, whereas *V. dahliae* infects this plant family relatively infrequently (Inderbitzin and Subbarao, 2014). The most likely first description of a *V. longisporum* infection on a brassicaceous host was from Brussels sprouts in England (Isaac, 1957). Oilseed rape was first reported as a host of *V. longisporum* in the west and south of Scania, southern Sweden, in 1969 (Kroeker, 1970). The hybridization events that resulted in the novel species *V. longisporum* may have facilitated this shift in host preference (Inderbitzin *et al.*, 2011b; Mallet, 2007), as *V. longisporum* is more pathogenic on brassicaceous hosts than *V. dahliae* (Novakazi *et al.*, 2015). However, clear host range segregation is not found between *V. dahliae* and *V. longisporum*, as examples of hosts to both species include oilseed rape (Steventon *et al.*, 2002), horseradish (*Armoracia rusticana*) (Babadoost *et al.*, 2004), sugar beet (*Beta vulgaris*) (Jackson and Heale, 1985) and the model plant *Arabidopsis thaliana* (Fradin *et al.*, 2011; Yadeta *et al.*, 2011).

Pathogenicity tests confirm that *V. longisporum* has the capacity to infect non-brassicaceous plants (Bhat and Subbarao, 1999; Novakazi *et al.*, 2015; Qin *et al.*, 2006; Zeise and Tiedemann, 2002). Moreover, *V. longisporum* lineages can be more or equally virulent to *V. dahliae* on non-brassicaceous hosts, such as eggplant, tomato, lettuce and watermelon (Novakazi *et al.*, 2015). This suggests that either the natural host range of particular *V. longisporum* lineages comprises non-brassicaceous plant species, or that natural infection of *V. longisporum* encounters a barrier that is not encountered in pathogenicity tests. The pathogenicity tests performed in these studies involved the inoculation of plants by root dipping in a conidial suspension, which may differ from natural infections which originate from microsclerotia.

Oilseed rape is economically the most important crop affected by *V. longisporum*. The oil from the seeds is used for human

Fig. 4 Typical disease symptoms caused by *Verticillium longisporum* on oilseed rape. Dark unilateral striping appears on the stems of apparently healthy looking plants at the end of the growing season (A), indicating the necrosis of cortical tissue. The necrosis develops further in a later stage of the disease, which may lead to stem cracks (B). Finally, microsclerotia are formed in the stem cortex beneath the epidermis (C).

consumption and biodiesel, whereas byproducts become a protein source used in animal feed (Berry *et al.*, 2012). Oilseed rape is the second most important arable oilseed crop, after soybean, and comprises spring and winter cultivars (Berry *et al.*, 2012; Diepenbrock, 2000). Winter oilseed rape is sown between mid-August and mid-September in Northwest Europe and has a higher yield than spring oilseed rape, which is sown between March and April (Christen *et al.*, 1999). In 2013, the worldwide annual production of oilseed rape was over 72 megatons, with China, Canada, India and Germany as the leading producers (FAOSTAT, 2015). *Verticillium longisporum* is one of the major pathogens of oilseed rape and is found in Europe (Gladders *et al.*, 2011; Karapapa *et al.*, 1997; Steventon *et al.*, 2002; Zeise and Tiedemann, 2002), Russia (Pantou *et al.*, 2005) and, recently, in Canada (CFIA, 2015).

Verticillium longisporum is also pathogenic on a broad range of brassicaceous horticultural crops. The pathogen has been found on several diseased *Brassica oleracea* species, including cauliflower (Debode *et al.*, 2005a; Koike *et al.*, 1994), cabbage (Inderbitzin *et al.*, 2011b; Subbarao *et al.*, 1995) and Brussels sprouts (Isaac, 1957; Karapapa and Typas, 2001; Karapapa *et al.*, 1997). *Verticillium longisporum* has also been reported in Japan on other brassicaceous vegetables: Chinese cabbage (*Brassica rapa* var. *pekinensis*) (Narisawa *et al.*, 2004; Watanabe *et al.*, 1973), turnip (*B. rapa* var. *rapa*) (Carder and Barbara, 1994) and wild radish (*Raphanus sativus* var. *hortensis* f. *raphinistroides*) (Okoli *et al.*, 1994).

Interestingly, not all *Brassica* crops are susceptible to *V. longisporum*. Typical Verticillium wilt symptoms are not observed on broccoli grown in infested soil. *Verticillium longisporum* is able to colonize the cortical surface of the roots of this crop, but does not progress into the vascular system (Njoroge *et al.*, 2011; Shetty *et al.*, 2000). However, resistance of broccoli to Verticillium wilt is not consistently observed if the plants are inoculated by root dipping in a conidiospore suspension (Zeise and Tiedemann, 2002). Nevertheless, cultivars from the USA demonstrated resistance against 15 isolates from different hosts using this root dipping inoculation method (Bhat and Subbarao, 2001).

DEVELOPMENT OF DISEASE SYMPTOMS AND IMPACT

Most field research on *V. longisporum* has been conducted on oilseed rape and cauliflower. Although both crops are *Brassica* species, the disease symptoms on these crops differ. Infected oilseed rape develops dark, unilateral striping on the stem late in the growing season, indicating the necrosis of cortical tissue (Heale and Karapapa, 1999) (Fig. 4). Symptom development coincides with increased pathogen colonization of root and shoot tissues (Dunker *et al.*, 2008). In the final stages of the disease, the fungus forms black microsclerotia in the stem cortex. In contrast with the disease caused by this pathogen on other crops, conventional wilting symptoms are typically not observed on oilseed rape. Rather, the crop ripens prematurely, making disease symptoms difficult to distinguish from natural senescence. Strikingly, not much is known about the impact of *Verticillium* infection on the yield of oilseed rape under field conditions. *Verticillium longisporum* symptoms can be omnipresent with a disease incidence of up to 80% (Dixelius *et al.*, 2005). Under practical conditions, yield losses caused by *V. longisporum* have been suggested to range between 10% and 50%, but this has not yet been experimentally verified (Dunker *et al.*, 2008). One study showed no significant effect on the 'thousand-seed-weight' (TSW) yield or oil content of the crop after artificial inoculation of the soil (Dunker *et al.*, 2008). Although there was no effect on the whole-plot yield, disease symptoms of single plants in the field negatively correlated with yield.

Interestingly, disease development in oilseed rape on artificial inoculation differs from disease development under field conditions. Whereas symptoms in the field involve dark unilateral striping on the stem late in the growing season, on root dip inoculation in the seedling stage, oilseed rape plants exhibit chlorosis, vascular discoloration and stunting at an early stage (Eynck *et al.*, 2007, 2009b; Floerl *et al.*, 2008; Zeise and Tiedemann, 2002). Moreover, clear biomass reduction is observed, whereby roots are significantly more affected than shoots (Keunecke, 2009). Recent studies have demonstrated that extensively increased branching of the shoot occurs on inoculated plants (D. Lopisso and A. V. Tiedemann, unpublished data). It is currently not understood why these differences in disease development occur.

In contrast with oilseed rape, cauliflower displays typical wilting symptoms on infection with *V. longisporum*, which starts with chlorosis and necrosis of the lower leaves (Koike *et al.*, 1994). At maturity, stunting of the plants and wilting can be observed. Furthermore, *V. longisporum* infection may lead to an increase in the number of cauliflower leaves (Subbarao *et al.*, 1995), although this is not universally observed across cultivars (Debode *et al.*, 2005b; Xiao and Subbarao, 1998).

The symptom development of the disease caused by *V. longisporum* in cauliflower and oilseed rape seems to be temperature dependent. Cauliflower grown as a winter crop in infested fields remains unaffected, whereas more disease is observed at higher temperatures (Koike *et al.*, 1994). At the same time, increased temperatures may also be unconducive to *V. longisporum* infection, as infected cauliflower did not display any symptoms when grown in a glasshouse at temperatures in the range 27–35°C. More recently, studies in a soil heating facility demonstrated a significant increase in *V. longisporum* colonization of winter oilseed rape when soil temperatures were elevated by 1.6 or 3.2°C with respect to ambient temperature, indicating a higher vulnerability of spring-sown crops growing into the warmer season (Siebold and Tiedemann, 2013).

The incidence and severity of disease caused by *V. longisporum* on cauliflower and oilseed rape is not always correlated with inoculum density, although symptoms on cauliflower can occur earlier at higher inoculum densities (França *et al.*, 2013; Johansson *et al.*, 2006a; Xiao and Subbarao, 1998). In contrast, in a study on oilseed rape, the disease incidence and severity were positively correlated to inoculum level (Dunker *et al.*, 2008).

PLANT RESPONSES

Chemical and mechanical responses

The influence of plant secondary metabolites on the interaction between *V. longisporum* and its host plants has not been extensively explored to date, but glucosinolate concentrations in infected hosts have been investigated. Glucosinolates are constitutively expressed sulfur-containing phytochemicals that are predominantly found in brassicaceous plants (Wittstock and Halkier, 2002). In general, they are grouped into aliphatic, aromatic and indole glucosinolates, depending on whether they originate from aliphatic amino acids, aromatic amino acids or tryptophan. Upon tissue damage, glucosinolates are hydrolysed with the formation of biologically active and sometimes toxic compounds. Interestingly, concentrations of aliphatic glucosinolates are generally higher in the roots of infected broccoli than in the shoots, whereas the opposite is observed in cauliflower, which may be implicated in the resistance of broccoli towards *V. longisporum* (Njoroge *et al.*, 2011). Levels of glucosinolates in the roots of *V. longisporum*-infected *Arabidopsis* plants are higher than in non-inoculated plants. However, the increase in glucosinolates is not accompanied by an increase in glucosinolate breakdown products in the roots (Witzel *et al.*, 2015). *Verticillium longisporum* infection induces the transcriptional activation of genes involved in tryptophan biosynthesis and tryptophan-derived secondary metabolism. Furthermore, genetic disruption of tryptophan-derived secondary metabolism leads to enhanced susceptibility. However, no increase in antifungal indole glucosinolate breakdown products is observed and the tryptophan-derived phytoalexin, camalexin, does not contribute significantly to defence against *V. longisporum* in roots (Iven *et al.*, 2012). This indicates that other, as yet unidentified, tryptophan-derived metabolites play an important role in fungal defence in roots. In contrast with the protective role of the tryptophan-derived secondary metabolites, monoterpenes produced by the monoterpene synthase TPS23/27 stimulate *in vitro* conidial germination and subsequent invasion of *V. longisporum* in *Arabidopsis* roots (Roos *et al.*, 2015).

In leaf tissue of *A. thaliana*, soluble phenylpropanoids, rather than tryptophan-derived metabolites, have been found to accumulate in response to *V. longisporum* infection. Mutant analysis and *in vitro* growth assays have revealed that sinapate glucose and coniferin are involved in restriction of the pathogen (König *et al.*, 2014). In addition, the phenylpropanoid pathway is important for the defence of *B. napus* against *V. longisporum*, as more phenolic compounds were produced by a resistant line of *B. napus* on infection with *V. longisporum* when compared with a susceptible line (Eynck *et al.*, 2009a). Moreover, concentrations of phenylpropanoids were correlated with *V. longisporum* resistance in *B. napus* (Obermeier *et al.*, 2013).

The ability of *V. longisporum* to synthesize aromatic amino acids and the cross-pathway control of amino acid biosynthesis are required for pathogenicity. Silencing mutants impaired in chorismate synthase or CPC1, the conserved transcription factor of cross-pathway control, caused less disease and showed reduced growth in the hypocotyl of *B. napus* and *Arabidopsis*. Chorismate is essential for the biosynthesis of tryptophan, phenylalanine and tyrosine, whereas cross-pathway control allows fungi to increase

amino acid biosynthesis on amino acid starvation (Singh *et al.*, 2010; Timpner *et al.*, 2013). *Brassica napus* xylem sap contains only low concentrations of amino acids, and aromatic amino acids are especially scarce (Singh *et al.*, 2010). An increased production of plant secondary metabolites in response to *V. longisporum* infection probably further depletes amino acid concentrations in the xylem. Hence, the fungus requires a functional cross-pathway control to overcome the imbalance in amino acid supply in the xylem.

Drought stress tolerance increases when *Arabidopsis* plants are challenged with *V. longisporum*, which may be the result of pathogen-induced reduction of stomatal apertures or *de novo* xylem formation (Reusche *et al.*, 2012; Roos *et al.*, 2014). Reduction in stomatal apertures may be linked to increased abscisic acid (ABA) levels in *Arabidopsis* leaves in response to *V. longisporum*, as ABA is a known central regulator of the stomatal apparatus (Acharya and Assmann, 2009; Roos *et al.*, 2014). Furthermore, *V. longisporum* infection induces transdifferentiation of bundle sheath cells into functional xylem elements in *A. thaliana* and *B. napus*. *Verticillium longisporum* also causes the reinitiation of cambial activity and the transdifferentiation of xylem parenchyma in *A. thaliana*, resulting in xylem hyperplasia (Reusche *et al.*, 2012, 2014).

MicroRNAs (miRNAs)

It has been demonstrated recently that *V. longisporum* interferes with plant miRNAs to reprogram plant gene expression. Sixty-two miRNAs were responsive to *V. longisporum* infection in *B. napus*, the majority of which were down-regulated. Important targets of down-regulated miRNAs include auxin response factors (ARFs), which control the transcription of genes in response to auxin. The resulting increase in ARFs may suppress plant defence responses by enhancing auxin signalling. Another down-regulated miRNA targets a positive regulator of leaf senescence. At early infection stages, the greatest suppression was observed for miR168, which interferes with Argonaute 1 (AGO1) (Shen *et al.*, 2014). AGO1 is an RNA-binding protein involved in RNA silencing that regulates diverse physiological processes, including a number of pathogen-associated molecular pattern (PAMP)-triggered immune responses (Li *et al.*, 2010). AGO1 mutants were clearly more resistant to *V. longisporum*, suggesting a key role of AGO1 in the compatible interaction with *V. longisporum* (Shen *et al.*, 2014).

Plant hormones

To date, the involvement of typical plant hormone signalling pathways in the interaction with *V. longisporum* remains unclear, and the role of the various plant hormones in the defence of *A. thaliana* and *B. napus* against *V. longisporum* appears to be different (Ratzinger *et al.*, 2009). *Verticillium longisporum* infection increases the level of jasmonic acid (JA) in *A. thaliana* and activates the corresponding marker genes *VSP2* and *PDF1.2*, but biosynthesis and signalling mutants do not show major differences in disease susceptibility when compared with wild-type plants (Johansson *et al.*, 2006b; Ralhan *et al.*, 2012). This suggests that JA does not contribute to *V. longisporum* resistance in *A. thaliana*. However, the treatment of *Arabidopsis* plants with methyl jasmonate (MeJA) results in enhanced resistance towards *V. longisporum* (Johansson *et al.*, 2006b). Moreover, *V. longisporum* requires JA-independent CORONATINE INSENSITIVE1 (COI1) function in the roots to elicit disease symptoms in *A. thaliana* shoots (Ralhan *et al.*, 2012). In oilseed rape, JA concentrations increase over time in both healthy and infected plants, which is probably caused by aging-related processes, as JA acts in senescence (He *et al.*, 2002; Ratzinger *et al.*, 2009).

In *Arabidopsis*, metabolites of the salicylic acid (SA) pathway, salicylic acid glucoside (SAG) and dihydroxybenzoic acid, increase after *V. longisporum* infection, and the SA marker genes *PR1* and *PR2* are activated. However, mutants in the SA pathway (*eds1-1*, *NahG*, *npr1-3*, *pad4-1* and *sid2-1*) do not exhibit enhanced susceptibility, indicating that SA signalling may not contribute to *V. longisporum* resistance in *Arabidopsis* (Johansson *et al.*, 2006b; Ralhan *et al.*, 2012). In contrast, SA appears to play a role in *B. napus* susceptibility to *V. longisporum* infection (Ratzinger *et al.*, 2009). Concentrations of SA and SAG in the xylem sap of *B. napus* plants increase on *V. longisporum* infection and correlate with disease severity; a strong correlation between SAG levels in the shoot and the amount of *V. longisporum* DNA in hypocotyls was found. However, the exact role of the enhanced levels of SA and SAG in xylem sap after infection with *V. longisporum* is not clear (Ratzinger *et al.*, 2009).

Ethylene (ET) production and the expression of ET-dependent plant defences have been shown to be induced by *V. longisporum* in *Arabidopsis*. Moreover, pretreatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) enhances host resistance to *V. longisporum*. The *Arabidopsis* mutants impaired in ET signalling, *ein4-1*, *ein2-1* and *ein6-1*, were more susceptible than the wild-type to *V. longisporum*. In contrast, the *Arabidopsis* mutant *etr1-1* showed enhanced resistance and a higher chlorophyll content compared with the wild-type, indicating that prolonged ET perception via ETR1 enhances susceptibility via the induction of senescence (Johansson *et al.*, 2006b; Veronese *et al.*, 2003). Reusche *et al.* (2013) found that *V. longisporum* triggers early senescence in *Arabidopsis* by actively decreasing cytokinin levels in the leaves. Senescing tissue may provide easy access to nutrients for the development of microsclerotia during the last phase of the life cycle of *V. longisporum*. Stabilization of cytokinin levels inhibits fungal growth and reduces disease symptom development (Reusche *et al.*, 2013).

ABA levels increase after infection with *V. longisporum* in *Arabidopsis* (Ralhan *et al.*, 2012; Roos *et al.*, 2014). The ABA-

deficient mutant *aba2-1* is susceptible to *V. longisporum* and accumulates less anthocyanin than wild-type plants, whereas ABA-insensitive mutants do not show enhanced susceptibility (Johansson *et al.*, 2006b; Veronese *et al.*, 2003). In *B. napus*, however, ABA concentrations in xylem sap are not affected by *V. longisporum* infection (Ratzinger *et al.*, 2009).

DISEASE MANAGEMENT

Chemical control, heat treatments and solarization

The management of Verticillium wilts is challenging, as current disease control strategies do not provide appropriate protection. Consequently, a combination of management techniques is necessary to contain the disease. Protective or curative control by conventional fungicides is not an option for *V. longisporum*. Soil fumigation is a successful strategy to reduce the inoculum density of *V. dahliae* in the soil (Powelson and Carter, 1973), but is no longer available for agricultural use because of its detrimental effects on stratospheric ozone (Subbarao, 2002). Although heat treatment of the soil can similarly reduce the viability of microsclerotia, steam-mediated heat treatment and most other heat treatment methods are energy consuming and not cost-effective in most commercial field production systems (Pullman *et al.*, 1981). However, soil solarization by heating the soil under a tarpaulin may be economically feasible and effective, depending on the temperature and duration of treatment (Pullman *et al.*, 1981), and is currently only commercially practised in Mediterranean, desert and tropical climates (Stapleton, 2000), because these climates allow the accumulation of adequate heat units to neutralize the pathogen.

Weed management and crop rotation

The more confined host range of *V. longisporum*, in comparison with *V. dahliae*, theoretically facilitates the use of crop rotation as a disease management strategy. However, the persistence of microsclerotia, potential non-brassicaceous reservoir plants (Johansson *et al.*, 2006a) and inadequate weed management may jeopardize the effects of crop rotation. Brassicaceous weeds may act as a reservoir for *V. longisporum*, as *Verticillium* isolates have been recovered from *B. rapa* spp. *campestris*, shepherd's purse (*Capsella bursa-pastoris*), annual wall-rocket (*Diplotaxis muralis*), clasping pepperweed (*Lepidium perfoliatum*), tumble mustard (*Sisymbrium altissimum*), *Descurainia hartwegiana*, field pennycress (*Thlaspi arvense*) and charlock (*Sinapis arvensis*) (Johansson *et al.*, 2006a; Vargas-Machuca *et al.*, 1987; Woolliams, 1966). In addition to weed management, the prevention of *Brassica* volunteers in subsequent crops is also important; oilseed rape is particularly prone to volunteers as a result of high seed losses before and during harvest (Price *et al.*, 1996).

Few crop rotation studies have been conducted with *V. longisporum*, and more long-term research is needed to determine whether crop rotation could be an effective management strategy. Hitherto, only studies on the impact of fallow treatment in cauliflower fields have been conducted. These suggest that fallow treatment does not reduce microsclerotial accumulation in the soil (França *et al.*, 2013; Subbarao and Hubbard, 1999). The microsclerotial density in the soil after 2 years of consecutive cauliflower crops is not significantly higher than when a cauliflower crop is followed by a fallow treatment the year after (Subbarao and Hubbard, 1999). Moreover, even a 4-year fallow period after a long history of cauliflower cropping did not reduce the microsclerotial density in the soil (França *et al.*, 2013). The long-term release of microsclerotia from the plant debris of the previous crop may be the reason why fallow did not lead to a significant reduction in the study of Subbarao and Hubbard (1999), as the microsclerotial density continued to increase during the fallow period. Therefore, a more extended fallow period may be more effective in decreasing microsclerotial densities in the soil. In contrast, França *et al.* (2013) did not report an increase in microsclerotial density in the soil of fallow-treated plots, but reported a fluctuation with a seasonal pattern. Interestingly, similar patterns and inoculum levels occur in the soil of plots with continuous cauliflower cropping. Possibly, the amount of microsclerotia formed in the cauliflower debris may be affected by the incomplete inflorescence development of cauliflower as the generative phase of the plant is interrupted by the harvest of the curd (França *et al.*, 2013). However, this hypothesis is not in line with the increasing microsclerotial density observed previously (Subbarao and Hubbard, 1999; Xiao *et al.*, 1998).

Bio-control agents and organic soil amendments

Several microorganisms, including bacteria and fungi, have the ability to reduce the colonization by, and deleterious effects of, *V. longisporum*, and can thus potentially serve as biological control agents (BCAs), provided that an ecologically fit and effective agent is developed. Specific, non-pathogenic *Verticillium* isolates, such as the *V. isaacii* isolate Vt305, are able to suppress disease symptoms caused by pathogenic isolates. The strain Vt305 was isolated from a Verticillium wilt-suppressive cauliflower field in Belgium (França *et al.*, 2013). Vt305 appears to be an endophyte of cauliflower and shows effective biological control capacities under controlled conditions (Tyvaert *et al.*, 2014). Inoculation of Vt305, 1 week prior to *V. longisporum* inoculation, reduced symptom development and the colonization of plant tissue by *V. longisporum*. However, the mechanism by which Vt305 protects cauliflower against Verticillium wilt is unknown, although it has been suggested that competition for infection sites and induced resistance responses are the two most likely possibilities (Tyvaert *et al.*, 2014).

Microsphaeropsis ochracea is another ascomycete and BCA of *V. longisporum* (Stadler and Tiedemann, 2014). The effectiveness of *M. ochracea* as a BCA has been proven *in vitro* and under sterile soil conditions, as it causes high rates of microsclerotial mortality. Nevertheless, *M. ochracea* appears not to have sufficient microbial competitiveness to control *V. longisporum* under field conditions (Stadler and Tiedemann, 2014).

A large-scale screening for root-colonizing and endophytic fungi with BCA capacities has led to the isolation of two *Phialocephala fortinii* isolates, one *Heteroconium chaetospira* isolate and one *Meliniomyces variabilis* isolate (Narisawa *et al*., 1998, 2000, 2004; Ohtaka and Narisawa, 2008). All isolates reduced the symptoms of *V. longisporum* on *in vitro*-grown Chinese cabbage when the colonization of the plant by the BCA preceded *V. longisporum* infection. Only the *H. chaetospira* and *M. variabilis* isolates were able to reduce the disease severity and incidence of Verticillium wilt in Chinese cabbage under field conditions.

In addition to fungal BCAs, the plant beneficial bacterium *Serratia plymuthica* HRO-C48 also reduces *Verticillium* symptoms in oilseed rape (Müller and Berg, 2008). A biological product based on *Serratia plymuthica* HRO-C48 has been developed, called RhizoStar® (E-nema, Raisdorf, Germany). Furthermore, certain *Bacillus amyloliquefaciens* strains are BCAs towards several fungal pathogens of oilseed rape, including *V. longisporum* (Danielsson *et al*., 2007). *Bacillus amyloliquefaciens* ssp. *plantarum* UCMB5113 is the most effective strain against *V. longisporum* and also shows plant growth-promoting activity. UCMB5113 produces antibiotic compounds and bio-surfactants, which are probably involved in the bio-control properties of the bacterium (Danielsson *et al*., 2007; Niazi *et al*., 2014.). However, its BCA capacities can also act indirectly and be caused by plant defence priming (Sarosh *et al*., 2009). The latter hypothesis is supported by the observation that the soil-borne isolate UCMB5113 confers resistance to airborne pathogens, where the spatial separation of BCA and the pathogen prevents direct interaction between the two (Sarosh *et al*., 2009).

Amendments of organic material to soils can suppress soil-borne fungal diseases (Bonanomi *et al*., 2007). Several crop residues are able to reduce microsclerotium viability in naturally infested cauliflower fields (Debode *et al*., 2005a; França *et al*., 2013). The incorporation of ryegrass and corn residues is more effective than brassicaceous plant material. However, the reduction of primary inoculum does not reduce the incidence or severity of the disease. Other publications have reported a Verticillium wilt-suppressive effect by broccoli residues (Subbarao and Hubbard, 1996, 1999). The broccoli amendments reduce disease abundance and microsclerotium viability in naturally infested soils (Xiao *et al*., 1998). Moreover, broccoli residues may even inhibit the cauliflower root colonization ability of surviving microsclerotia (Njoroge *et al*., 2011; Shetty *et al*., 2000). The lignin content of the incorporated crop residues appears to be a key determinant for the effectiveness of *Verticillium* control. The 'lignin–melanin hypothesis' proposes that enzymes involved in lignin biodegradation also degrade fungal melanin (Butler and Day, 1998; Debode *et al*., 2005a; Shetty *et al*., 2000). Melanin protects microsclerotia against biotic and abiotic stress during the period between hosts (Bell and Wheeler, 1986). Therefore, soil amendments with relatively high lignin content may stimulate microbial organisms that decompose lignin and that simultaneously reduce microsclerotial viability.

Resistance breeding

Resistance breeding is the most favoured means of *Verticillium* disease management, and several crops with polygenic *V. longisporum* resistance have been reported (Fradin and Thomma, 2006; Kemmochi *et al*., 2000; Rygulla *et al*., 2008). Unfortunately, a genuine resistance (*R*) gene against *V. longisporum* has not yet been found, and *Ve1* presently remains the only *R* gene that has been described against Verticillium wilts (Fradin *et al*., 2009, 2011). Although *Ve1* was initially identified in tomato (Kawchuk *et al*., 2001), functional *Ve1* homologues have also been identified in other plant species, such as *Nicotiana glutinosa* (Zhang *et al*., 2013), lettuce (Hayes *et al*., 2011) and cotton (Zhang *et al*., 2011, 2012). Tomato *Ve1* confers resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* (presently *V. alfalfae*) which contain the *Ave1* gene (de Jonge *et al*., 2012). *Ave1* encodes an effector protein that activates *Ve1*-mediated resistance, but *Ave1* contributes to fungal virulence in susceptible plants that lack *Ve1*. Thus far, functionality of *Ve1*-mediated resistance has not been demonstrated against *V. longisporum*, which has been attributed to the observation that the currently investigated isolates do not carry the *Ave1* gene (Fradin *et al*., 2011). However, there are genetic resources that may be used to reduce the susceptibility of brassicaceous plants to *V. longisporum*. For instance, constitutive expression of the *Enhancer of vascular Wilt Resistance 1* (*EWR1*) gene enhances the resistance against Verticillium wilt caused by *V. albo-atrum* (presently *V. alfalfae*), *V. dahliae* and *V. longisporum* in *Arabidopsis* (Yadeta *et al*., 2011, 2014). *EWR1* encodes a putatively secreted protein of unknown function and has homologues that are only found within the Brassicaceae family (Yadeta *et al*., 2014). *EWR1* homologues facilitate enhanced *Verticillium* resistance in transformed *A. thaliana*. Interestingly, the brassicaceous-specific *EWR1* homologues can also be used to increase resistance against Verticillium wilts in non-brassicaceous plants, as *Nicotiana benthamiana* displays resistance against *V. dahliae* when Brassicaceae *EWR1* homologues are overexpressed.

Current European oilseed rape cultivars possess a low level of *Verticillium* resistance, and the availability of novel germplasm for resistance breeding is limited because of the narrow genetic basis of currently used cultivars (Cowling, 2007; Seyis *et al*., 2003).

However, three quantitative trait loci (QTLs) that significantly correlate with *V. longisporum* resistance have been identified, one on the C1 and two on the C5 chromosome, of the partly resistant oilseed rape cultivar Express 617 (Obermeier *et al.*, 2013). These QTLs indicate sources of quantitative resistance available in the C-genome derived from oilseed rape parental cabbage lines. Interestingly, the QTLs co-localize with loci for two soluble phenylpropanoids that are negatively correlated with disease severity during *V. longisporum* infection, whereas a positive correlation exists with precursors of cell wall-bound phenols related to lignin. This is in agreement with the higher constitutive and induced levels of cell wall-bound phenols in roots and hypocotyls of resistant rapeseed genotypes. The resistance genotypes were identified in *V. longisporum* resistance screenings of *B. napus* accessions (Eynck *et al.*, 2009b). One of the identified accessions with quantitative resistance to *V. longisporum* tolerates root invasion, but hinders the pathogen from colonizing the shoot by means of vascular occlusions, and strongly enhances the accumulation of phenols in the xylem parenchyma at the hypocotyl interface (Eynck *et al.*, 2009a). In spite of these specific observations, the genuine mechanism of quantitative resistance is not entirely clear.

Resistance traits in *B. oleracea* and *B. rapa* can be applied in oilseed rape resistance breeding, as *B. napus* is an interspecific hybrid between these two plant species (Eynck *et al.*, 2009b; Happstadius *et al.*, 2003; Obermeier *et al.*, 2013; Rygulla *et al.*, 2007a, b,2008). Cultivars of *B. oleracea* crops have been screened for *V. longisporum* susceptibility, with differences in susceptibility found among cauliflower cultivars (Debode *et al.*, 2005b) and dominant polygenic resistance occurring in cabbage (Kemmochi *et al.*, 2000).

In addition to breeding, resistance sources from outside the Brassicaceae may improve the resistance of current *V. longisporum* hosts. These include sugar beet, whose *BvGLP-1* gene reduces *V. longisporum* disease symptoms in *Arabidopsis*. *BvGLP-1* has high sequence homology to a set of plant germin-like proteins, and is highly induced after nematode (*Heterodera schachtii*) infection of resistant sugar beet plants containing the single dominant resistance gene $Hs1^{pro\text{-}1}$ (Knecht *et al.*, 2010).

CONCLUSION

Verticillium longisporum is becoming a global problem in oilseed rape production. Recently, the disease has been reported outside continental Europe in two important oilseed rape production areas: the UK in 2011 (Gladders *et al.*, 2011) and Canada in 2015 (CFIA, 2015). To improve the management of the *V. longisporum* disease of oilseed rape and other crops, several steps could be implemented. First, the search for sources of resistance should be intensified. Host resistance is generally considered to be the most desirable control strategy, but *R* genes, such as *Ve1* in tomato against *V. dahliae*, are unknown for *V. longisporum*. Therefore, more *B. napus*, *B. oleracea* and *B. rapa* germplasm should be screened for resistance traits for introgression into new, improved cultivars. *R* genes should be deployed cautiously and combined with other resistance traits and management measures to improve the durability of resistance. Second, phytosanitary measures should be expanded to prevent the spread of *V. longisporum* with contaminated soil and equipment to new areas. *Verticillium longisporum* is a soil-borne pathogen and cannot move autonomously over great distances. Therefore, international trade and travel are likely to be responsible for *V. longisporum*'s continually expanding geographical range. Finally, accurate assessments of the impact of *V. longisporum* infections on crop quality and yield under field conditions are required. These would determine the economic relevance of the pathogen, and provide a solid economic basis for disease management decisions.

Verticillium longisporum on oilseed rape is not a causal agent of wilt, and therefore the use of 'Verticillium wilt' as the common name of *V. longisporum* on oilseed rape is incorrect. Perhaps, *V. longisporum* mainly has an endophytic lifestyle in oilseed rape, which causes symptoms on the stem that do not result in reduced crop quality or yield losses. Therefore, we propose 'Verticillium stem striping' as the common name to describe *V. longisporum* on oilseed rape.

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