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Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils

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Abstract

Verticillium longisporum is a soil-borne fungal pathogen causing vascular wilt of *Brassica* crops. This study was conducted to enhance our knowledge on the host range of *V. longisporum*. Seven crop species (barley, oat, oilseed rape, pea, red clover, sugar beet and wheat) and five weed species (barren brome, black-grass, charlock, cleavers and scentless mayweed) all common in southern Sweden were evaluated for infection by response to *V. longisporum*. Oat, spring wheat, oilseed rape, scentless mayweed and charlock inoculated with *V. longisporum* in a greenhouse showed stunting to various degrees close to the fully ripe stage. Based on the extent of microsclerotia formation, explants were separated into four groups: for pea and wheat, $<5\%$ of the samples had formed microsclerotia; for scentless mayweed, 5–10%; for oat, 10–20%; and for charlock and oilseed rape $>80\%$. The results suggest that plant species outside the *Brassicaceae* can act as reservoirs of *V. longisporum* inoculum. Soil inoculum densities in nine fields were monitored over a period of 12 months, which ranged from 1 to 48 cfu g^{-1} soil. Density of microsclerotia was lowest just after harvest, reaching its maximum six months later. No significant correlation between inoculum density in soil and disease incidence on oilseed rape plants was found. However, the data suggest that a threshold of 1 cfu g^{-1} soil is needed to cause disease on oilseed rape. Species identification based on microsclerotia morphology and PCR analysis showed that *V. longisporum* dominated in soil of seven, and *V. dahliae* in two of the nine fields studied.

Introduction

The first report of Verticillium wilt in *Brassica* oil crops in Sweden derives from an inventory of oilseed rape and turnip rape fields in Skåne in 1969 (Kroeker, 1970). A few years later, severe outbreaks of Verticillium wilt on *Brassica* oil crops were reported further north in Östergötland (Björkman and Sigvald, 1980). The level of disease incidence of Verticillium wilt has been high in Skåne the last 35 years, often ranging from 50% to 80% (Dixelius et al., 2005). The severe disease incidence in these two regions is most likely to be due to very intensive cultivation, in some places monoculture, of *Brassica* oilseed crops between 1945 and 1955, influenced by strong governmental subsidizes of oil crops at that period.

In Sweden and northern Europe, Verticillium wilt on *Brassica* crops is caused by the soil-borne fungus *Verticillium longisporum* (Karapapa et al., 1997; Steventon et al., 2002; Fahleson et al., 2003; Fahleson et al., 2004). Both oilseed rape and turnip rape are attacked without significant difference between spring or winter types. Common

symptoms are chlorosis of the lateral branches or leaves, and bronze colouring of stems before microsclerotia protrude from the epidermis causing a blackening appearance of the stem (Dixelius et al., 2005). External symptoms are often ambiguous, appearing late in oilseed rape fields and are therefore very often considered as normal plant senescence phenomena. Many of these symptoms, except microsclerotia formation, resemble those recently reported for *Fusarium oxysporum* and *F. avenaceaum* which are most prevalent in western Canada (Bailey et al., 2003). Fusarium wilt appears to be favoured by warm and dry conditions, especially warm soil temperatures, which might be one of the reasons why it does not constitute a problem on *Brassica* oil crops anywhere in Europe.

Verticillium dahliae is a well studied fungus, including the wilting disease it causes on a vast number of plant hosts (Pegg and Brady, 2002). Microsclerotia are stimulated to germinate in response to root exudates (Mol, 1995). The mycelia colonise the root cortex and gain entry to the immature xylem elements. This process is followed by hyphal proliferation and conidial formation. Conidia are dispersed within the xylem elements and spread in the vascular system by the transpiration stream (Beckman, 1987). In this manner, rapid and systemic infection of the plant follows, and foliar symptoms of wilting, like chlorosis and necrosis, appear. As the foliage begins to senesce, the non-vascular tissue becomes colonised and microsclerotia are formed in the dying plant tissue. Following incorporation of dead plant tissue into the soil, the microsclerotia are released as the tissue decays. In the soil, the microsclerotia can remain viable for longer than 10 years (Heale and Karapapa, 1999). The long persistence of these resting structures is one of the main causes of the control problems related to this disease. The infection and plant colonisation pattern of *V. longisporum* seems, to a large extent, to resemble those described for *V. dahliae* (Dixelius, et al., 2005) but *V. longisporum* is reported to preferentially attack *Brassica* species (Karapapa et al., 1997; Zeise and Tiedemann, 2002).

Several control measures have been suggested to decrease the yield losses caused by Verticillium wilt including resistance breeding. The limiting factor to further raising the resistance level in cultivars of *B. napus* is the narrow genetic variation for resistance traits to Verticillium wilt within the gene-pool of *B. napus*. Thus, germplasm within *B. oleracea* and *B. rapa* have been collected and extensive screening work has been performed (Happstadius et al., 2003; Dixelius et al., 2005). Promising genotypes have now been identified and these novel genotypes are now being incorporated in *B. napus* breeding programmes. However, it will take an additional 10–15 years before a new variety with improved resistance to Verticillium wilt reaches the market. Many reports have noted a reduction in inoculum levels when including non-host plants in crop rotation schemes (Pegg and Brady, 2002). This approach has now gained attention in northern Europe where *V. longisporum* is prevalent. Hence, the objectives for this study were: (1) to evaluate the infection capacity of *V. longisporum* on non-cruciferous crops and common Swedish weeds *in Brassica* fields, under greenhouse conditions (2) to determine to what level microsclerotia are present in agricultural soils in Skåne, the main region for *Brassica* oil crops in Sweden, and (3) to distinguish Verticillium species which can form microsclerotia from soil samples of agricultural fields.

Materials and methods

Fungal isolates

Three isolates, VD2 (CBS110272), VD4 (CBS1 10219) and G 1-1, all determined as *Verticillium longisporum* by RFLP or AFLP analysis (Steventon et al., 2002; Fahleson et al., 2003), were used in host interaction studies. All the isolates derived from *Brassica napus* and were cultured and stored as described earlier (Steventon et al., 2002). For long-term storage at −70 °C, a bead cryo-preservation system was used according to the manufacturers' instruction (STC Technical Service Consultants Ltd, Lancashire, UK).

Seven crop species important for Swedish agriculture and five common weeds were assessed: barley (*Hordeum vulgare*) cv. Cecilia, oat (*Avena sativa*) cv. Belinda, oilseed rape (*Brassica napus*) cv. Maskot, pea (*Pisum sativum*) cv. Carneval, red clover (*Trifolium pratense*) cv. Fanny, sugar beet (*Beta vulgaris*) line 97080034, wheat (*Triticum aestivum*) cv. Vinjett, and the weeds barren brome (*Bromus sterilis*), black-grass

(*Alopecurus myosuroides*), charlock (*Sinapis arvensis*), cleavers (*Galium aparine*) and scentless mayweed (*Matricaria inodora*). Seeds of barley, oat, oilseed rape, pea, red clover, sugar beet, wheat, charlock, and scentless mayweed were sown in vermiculite and grown in a greenhouse with a 18 h photoperiod and a temperature of 15 °C (dark) and 23 °C (light). For vernalisation, seeds of barren brome and cleavers were sown in vermiculite, and kept at 4 °C for 3 weeks before transfer to greenhouse conditions.

Inoculation and disease assessment

From each species, 50 seedlings, with cotyledons completely unfolded or when the first true leaf emerged from coleoptiles, were inoculated separately with the three fungal isolates, respectively, using a root-dip method (Koike et al., 1994). Plants were gently separated from the vermiculite and the roots were rinsed with tap water. The roots of the plants were dipped in a conidial solution (1.6×10^6 conidia ml^{-1}) for 30 min and replanted in trays with sterile potting soil (80% peat, 20% clay). Fifty seedlings of each species were dipped in water, re-planted in trays with sterile potting soil and used as control plants.

Disease development was recorded weekly and was linked to the different plant growth stages according to the BBCH-scale, jointly developed by the *B*ASF, *B*ayer, *C*iba-Geigy and *H*oechst companies (Lancashire et al., 1991). Oilseed rape, pea, scentless mayweed and charlock were assessed at BBCH 25 (5 side shoots visible), BBCH 45 (9 or more visibly extended internodes), BBCH 65 (full flowering) and at BBCH 85 (seed pods fully ripe). The cereals and the two weed grasses were assessed at BBCH 25 (5 tillers visible), BBCH 45 (late boot stage), BBCH 65 (full flowering) and at BBCH 85 (dough stage). Visible symptoms, such as stunting, discolouration of stem and leaves and/or chlorosis of leaves were observed as described earlier (Happstadius et al., 2003).

The height of 16 plants per isolate was recorded at BBCH 85 and seeds were collected for weight measurements. Root, stem (8 cm above the soil) and leaf samples from the lower part of the plant were collected from 24 plants of each species and fungal inoculation set, for fungal re-isolation assessments. To induce the growth of microsclerotia on the collected plant samples, all the explants were rinsed with sterile water, surface-sterilised with 0.25% hypochlorite for 1 min, followed by three rinses in sterile water and treatment in a diquat solution of 0.3% (wt/vol) Reglone (Syngenta, Copenhagen, Denmark), for 1 min (Henriksson, 1995; Gunnarsson, 2002). The tissue samples were incubated on Petri dishes with 95% humidity for 24 h and left to dry for up to a month at room temperature. In addition, ten samples per species and isolate were taken from the roots and the lower part of the stems for vertical and horizontal dissection to assess the discolouration of the vascular system.

Soil sampling and analysis of inoculum density

Soil samples were collected from nine Swedish farms located in the southern part of Sweden, where winter oilseed rape (cv. Banjo) was grown and harvested in 2003 (Figure 1). Winter oilseed rape was followed by winter wheat in autumn 2003. Each soil sample (1 kg) comprised of 50 sub-samples taken using a randomised design scheme for each field. The soil samples were mixed and sieved before particles smaller than 2 mm were air-dried at room temperature for 2 weeks. From the air-dried soil, ten 50 g sub-samples were collected and thoroughly mixed and 12.5 g of each sub-sample was used for wet plating and incubation on semi-selective agar media (Goud et al., 2003). The soil was sieved, using tap water, through 106 and 20 μm nested sieves. Particles from the 20 μm sieve were suspended in 0.08% sterile water agar (Difco) reaching a total volume of 55 ml. About 800 μl of the suspension, representing 0.18 g soil equivalent, was subsequently distributed on each MSEA plate (Harris et al., 1993). Ten plates per soil sample were prepared. The plates were left to dry for 15 min and maintained at 20 °C, in the dark for 4 weeks before examination. In order to facilitate species discrimination, MSEA plates were inoculated using reference isolates of *V. dahliae* (CBS38449), *V. tricorpus* (CBS38484) and *V. longisporum* (VD4 deposit 8 no CBS110219) previously characterised by AFLP analysis (Fahleson et al., 2003).

DNA extraction and PCR analysis

Fungal DNA was extracted from the plant material taken at different growth stages and treated with diquat to trigger the development and growth of microsclerotia. All 72 samples were ground in

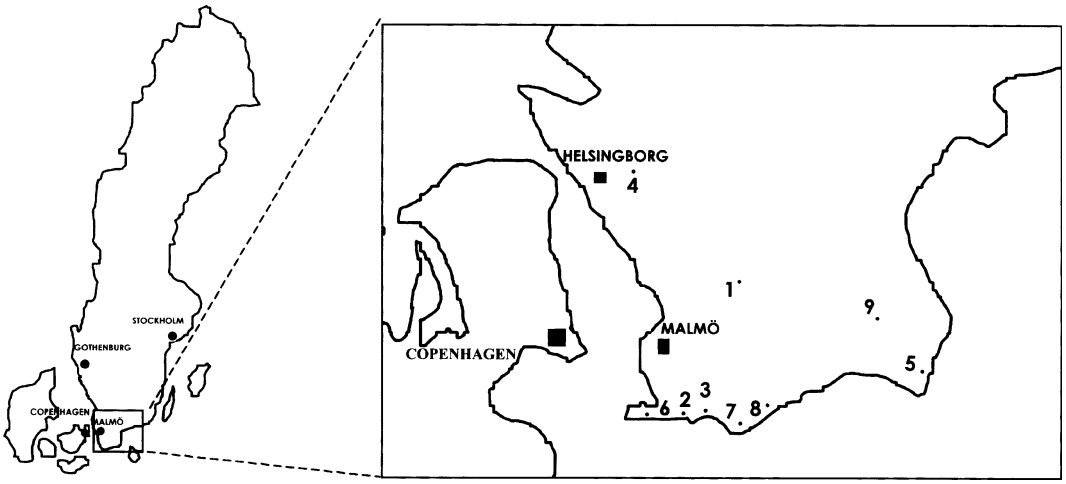

Figure 1. Soil sampling sites (1–9) in Skåne, southern Sweden.

liquid nitrogen together with glass beads (150–212 microns, Sigma-Aldrich, St. Louis, MO). Total DNA was isolated according to Bernatzky and Tanksley (1986) but 20% sarkosyl was substituted for 10% SDS in the extraction buffer.

From the soil samples microsclerotia were isolated from MSEA plates (Harris et al., 1993) by collecting them with a syringe needle. To obtain individual colonies, each microsclerotium was rolled on water agar plates before transfer to either fresh MSEA or ethanol (0.01% v/v) agar (Difco, BD, Sparks, MD) plates. Before DNA extraction, the colonies were grown for 1 week in 50 ml liquid PDB-media (Difco) on a shaker (150 rpm) in the dark at 25 °C.

In order to distinguish between *V. longisporum, V. dahliae* and *V. tricorpus*, a set of PCR-primer combinations were used; *Verticillium longisporum*-specific primers VL 1: 5′-TCT CCT CTC TAC GAG AAC GA-3′, VL2: 5′-CAC TTT CTA AGT ATC CTT CCT AT-3′ which amplify a 340 bp fragment (Steventon et al., 2002), nuclear SSU-rRNA primers 18SVDF: 5′-GCG AAA CTG CGA ATG GCT-3′, 18SVDR: 5′-GTA ATG ATC CCT CCG CTG-3′ that amplify a 2.5 kb fragment in *V. longisporum*, and a 1.65 kb fragment in both *V. dahliae* and *V. tricorpus* (Karapapa and Typas, 2001), and *V. tricorpus*-specific primers VT1 5′-CGC CGG TAC ATC AGT CTC TT-3′, VT2 5′-ACT CCG ATG CGA GCG AA-3′ (Moukhamedov et al., 1994). The PCR reactions for the *V. longisporum* and *V. tricorpus* specific primers were set up with 0.2 U *Taq* Gold polymerase (Applied Biosystem, Foster City, Canada) in 25 µl reaction mixtures, containing 0.4 µM of each primer, 120 µM of each of four dNTPs (Applied Biosystems), and 1 µl (approximately 10 ng) of template DNA. For the primer set 18SVDF/18SVDR the PCR reactions were set up with 0.02U Phusion^{TM} High-Fidelity DNA Polymerase (Finnzyme, Espoo, Finland) in 25 µl reaction mixtures, containing 1 µM of each primer, 200 µM of dNTPs (Applied Biosystem), and 1 µl (approximately 10 ng) of template DNA and run on a PCR Express Thermal Cyclar (Hybaid Limited, Middlesex, UK). The following conditions were used: VL1/VL2: denaturation for 5 min at 95 °C, 35 × (95 °C for 2 min, 2 min at 48 °C, 3 min at 72 °C) followed by an elongation step of 10 min in 72 °C. VT1/VT2: denaturation for 2 min at 95 °C, 35 × (95 °C for 1 min, 1 min at 60 °C, 1 min at 72 °C) followed by an elongation step of 10 min in 72 °C. 18SVDF/18AVDR: denaturation for 5 min at 95 °C, 35 × (95 °C for 1 min, 1 min at 60 °C, 3 min at 72 °C) followed by an elongation step of 10 min at 72 °C. Electrophoresis of the amplified DNA products was performed using 1% agarose (Invitrogen, Carlsbad, CA, USA) gels.

Data analysis

Data for plant height and seed weight were analysed with a One-sided Student's *t*-test, assuming equal variances in the inoculated and the control plants (Sokal and Rolf, 1981). The relationship

between soil inoculum density and disease incidence was calculated using linear regression of arcsine-transformed percentages of diseased oilseed rape plants on untransformed soil inoculum densities observed in the spring of 2003.

Results

Host range to V. longisporum

Oilseed rape and charlock inoculated with *V. longisporum* were severely stunted. Oilseed rape showed, on average, a 20% height reduction ($P < 0.001$) (Table 1, Figure 2) compared to control plants at full maturation and stunting could be seen as early as at BBCH 25. A 41% reduction in plant height was observed for inoculated plants of charlock at BBCH 45. Later measurements of charlock plants were not feasible due to its growth habits. Discolouration of the vascular system in the lower part of the stem and roots was observed at BBCH 45 in 90% of the inoculated plants of oilseed rape and charlock, while chlorosis of leaves of all plants were observed at BBCH 65 in both species.

The extent of microsclerotia development on the plants from each species was assessed. The plants were grouped as: for pea and wheat, < 5% of the samples developed microsclerotia; scentless mayweed, 5–10%; oat, 10–20%; and charlock and oilseed rape > 80%. This differentiation was obtained independently of the fungal isolate used. Microsclerotia were isolated from the roots at BBCH 45, and from the lower stem and leaves at BBCH 65 of both oilseed rape and charlock plants. Inoculated wheat and oat plants were 5% ($P < 0.01$) and 10% ($P < 0.01$), respectively, shorter than the control plants (Table 1, Figure 2) at harvest (BBCH 85). Microsclerotia were re-isolated from the lower leaves at the late booting stage, but not from the roots from either of the two species (Figure 2). Scentless mayweed inoculated with CBS110272 resulted in approximately 50% height reduction and microsclerotia were isolated from the leaves at full flowering, but no other disease symptoms were observed. The presence of *V. longisporum* in oilseed rape, charlock, wheat, oat and scentless mayweed and pea was confirmed by PCR analysis (Figure 3).

The weight of seeds, both as bulked seeds of each experiment and 1000-kernel weights revealed reduced weight of seeds from inoculated pea, oat and wheat plants compared to control plants, but the differences, irrespectively of the fungal isolate used, were not significant. In contrast, oilseed rape inoculated with VD2 revealed significantly lower 1000-kernel weight ($P = 0.004$) compared to the uninoculated control plants. No significant differences in 1000-kernel weight were observed when uninoculated oilseed rape was compared to oilseed rape inoculated, with VD4 or G1-1, respectively.

Table 1. Disease symptoms caused by *Verticillium longisporum* root dipping inoculations on seven crop and five weed species common in Sweden

Species	Disease symptoms	
	Stunting[a]	Chlorosis of leaves
Barley (*Hordeum vulgare*)	0%	No
Oilseed rape (*Brassica napus*)	20%	Yes
Oat (*Avena sativa*)	10%	No
Pea (*Pisum sativum*)	0%	No
Red clover (*Trifolium pratense*)	0%	No
Sugar beet (*Beta vulgaris*)	0%	No
Wheat (*Triticum aestivum*)	5%	No
Barren brome (*Bromus sterilis*)	0%	No
Black-grass (*Alopecurus myosuroides*)	0%	No
Charlock (*Sinapis arvensis*)	59%	Yes
Cleavers (*Galium aparine*)	0%	No
Scentless mayweed (*Matricaria inodora*)	~50%	No

[a]Stunting was measured as the percentage of the average height lost of inoculated plants compared to control plants at BBCH 85 except for *S. arvensis* and *M. inodora* where the height was measured at BBCH 45.

Microsclerotia density in Swedish fields

Soil inoculum densities at different locations varied to a large extent. In samples taken during the spring of 2003, in fields where winter oilseed rape was grown, values ranged from 1 to 24 colony forming units per gram soil (Table 2). In soil samples taken in the same fields just after sowing of winter wheat, which followed oilseed rape, the inoculum densities were lower and ranged from 1 to 12 cfu g^{-1}. Six months later (spring 2004), samples from the same winter wheat fields showed values from 0 to 48 cfu g^{-1} (Table 2). The numbers of microsclerotia in the colonies on the scanned plates varied widely from 1–3 to over 200, suggesting considerable variation in the size or

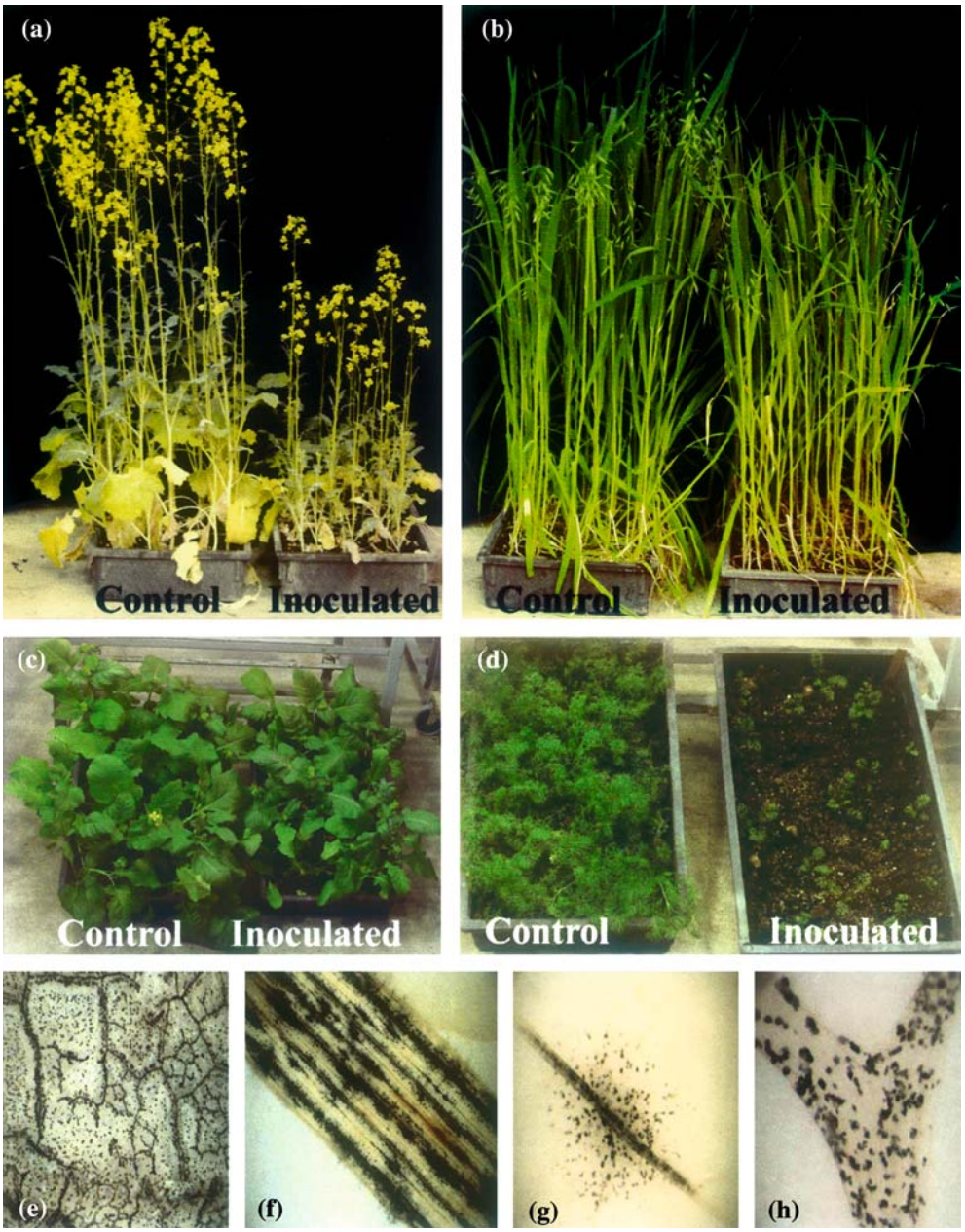

Figure 2. Disease symptoms caused by *Verticillium longisporum* inoculated plants. Stunting of plants (a) *Brassica napus* at BBCH 65, (b) *Avena sativa* at BBCH 65, (c) *Sinapis arvensis* at BBCH 45 and (d) *Matricaria inodora* at BBCH 45. Formation of microsclerotia after treatment with diquat on (e) leaf of *Brassica napus* (f) leaf of *Avena sativa*, (g) stem of *Sinapis arvensis*, and (h) leaf of *Matricaria inodora*.

potency of the propagules from which they originated. This variation was found at all three timepoints and was independent of the species determined in the soil samples taken in the spring of 2004.

Verticillium wilt was observed on oilseed rape plants grown in all fields where soil samples were collected, regardless the inoculum density. The disease incidence scored in July 2004 ranged from 4% to 72%. However, no significant correlation

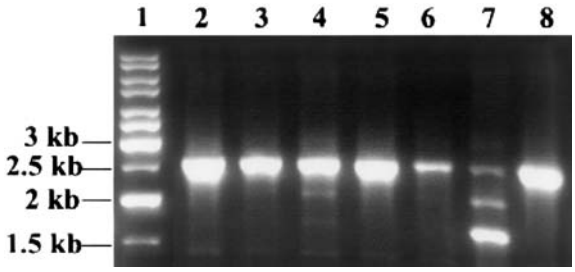

Figure 3. Electrophoresis of the PCR amplification product from either fungal DNA (VD2) or DNA extacted from inoculated plants using a *Verticillium longisporum* specific nuclear SSU-rRNA primer. Expected band size 2.5 kb. Lane 1: 1 kb ladder (Fermenta, Germany); lane 2: *Verticillium longisporum* (VD2); lane 3: *Matricaria inodora;* lane 4: *Sinapis arvensis;* lane 5: *Pisum sativum;* lane 7: *Triticum aestivum* (Non-specificity due to a low fungi:plant DNA ratio) lane 8: *Avena sativa;* lane 9: *Brassica napus*.

between soil inoculum density in spring 2003 and disease incidence ($P = 0.13$, $r^2 = 0.008$) was observed.

Verticillium *species in soils*

Morphological discrepancies between the two *Verticillium* species could be established concerning microsclerotia grown on MSEA plates. Careful scanning of samples taken during spring 2004 revealed the following morphological criteria under the microscope: Microsclerotia of *V. longisporum* are irregular, often with dark hyphae, whereas microsclerotia of *V. dahliae* are globose to elongate, with smooth edges, dark hyphae are usually absent. In both species microsclerotia have an average area of length × width < 1275 μm^2. Differentiation between the different *Verticillium* species was compared to reference isolates and confirmed via PCR analysis using *Verticillium* specific primers (Figure 4). In six of the nine soil sampling sites, *V. longisporum* accounted for the majority of colonies found, whereas *V. dahliae* dominated in two locations (Table 3). No colonies of *V. tricorpus* were identified in the soil samples.

Table 2. Fluctuation of microsclerotia colony forming units per gram soil (cfu g^{-1}) between 2003–2004 in nine Swedish fields sown with winter oilseed rape autumn 2003 and with winter wheat autumn 2004

Location[a]	Soil inoculum (cfu g^{-1})			Disease incidence[b] (%)
	2003 Spring	2003 Autumn	2004 Spring	2003
1	8	4	2	72
2	19	7	14	66
3	24	4	4	58
4	3	2	0	48
5	1	1	1	48
6	7	12	48	26
7	7	6	6	22
8	1	7	37	18
9	3	2	4	4

[a]The location of different sampling sites is visualised in Figure 1.
[b]Disease incidence was calculated as % diseased plants in each field.

Discussion

Verticillium wilts occur worldwide but are most important in temperate regions where a vast range of dicotyledonous plant species and trees can be attacked and become diseased (Hiemstra, 1998). The growing importance of Verticillium wilt on *Brassica* oilseed crops in Sweden incited this study on potential hosts, and the presence of *Verticillium* species and microsclerotia density in the soil are important in order to obtain some further understanding of this plant disease.

Stunting was generally observed among all the plant species evaluated in this study as an early response to the infection. Seven species recovered

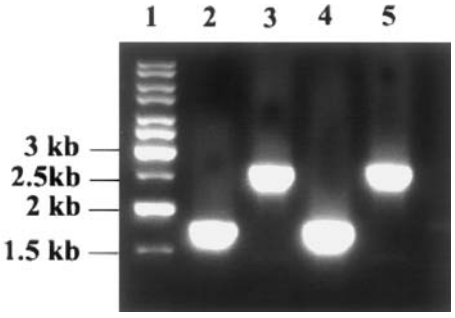

Figure 4. Electrophoresis of the amplification products from PCR of DNA from microsclerotia re-isolated and purified from either soil samples or fungal DNA using nuclear SSU-rRNA primers to detect *V. dahliae* (Expected band size 1.65 kb) and *V. longisporum* (Expected band size 2.5 kb), respectively. Lane 1:1 kb ladder (Fermentas, Germany); lane 2: Microsclerotia morphologically classified as *Verticillium dahliae*; lane 3: Microsclerotia morphologically classified as *V. longisporum;* lane 4: V. *dahliae* (CBS 38449); lane 5: V. *longisporum* (VD4).

Table 3. Verticillium species identified in soil samples taken in spring 2004

Location[a]	No. of microsclerotia forming colonies	
	V. longisporum	*V. dahliae*
1	1	3
2	24	2
3	5	2
4	1	0
5	0	0
6	35	53
7	8	2
8	63	5
9	7	0

[a]The location of different sampling sites are described in Figure 1.

except oat, spring wheat, oilseed rape, scentless mayweed and charlock which all remained stunted to various degrees when the plants were close to being fully ripe. Recovery is a common phenomenon reported from a range of *V. dahliae* infected crops and many tree species (Goud, 2003). Microsclerotia were re-isolated infrequently from peas and spring wheat, whereas they were more common on scentless mayweed, rather frequent on oat explants and retrieved from oilseed rape and charlock to a large extent.

Monocotyledonous plants very occasionally have been cited as true hosts for *Verticillium*. Wheat, barley and oats have earlier been shown to harbour *V. dahliae* in the superficial tissues of their roots (Malic and Milton, 1980; Mathre, 1986; Mathre, 1989). Oats and barley were found to be susceptible while wheat exhibited rather weak symptoms. Isaac and Levy (1971) observed that *V. dahliae* could penetrate barley roots followed by rapid microsclerotia formation, leading to no further colonisation in the vascular system. Systemic infections have, nevertheless, been found where *V. dahliae* was isolated from the leaves of wheat, oats and barley (Krikun and Bernier, 1987, Mathre, 1989). Field studies in the USA revealed recovery of *V. dahliae* from roots, stubble and foliar portions of oats (Davis et al., 2000). No reports exist about any Verticillium wilt disease on small-grain cereals in Sweden. However, in a restricted survey of discoloured (very dark) barley seeds, harvested in autumn 2004 in southern Sweden, PCR analysis resulted in the positive detection of *V. dahliae* in one sample (Dixelius C, unpublished). Despite the small numbers analysed, this observation suggest that barley in Sweden can function as a host to this fungal pathogen. Taken together, this implies that oats and perhaps other cereals have been overlooked as possible bridging hosts for several *Verticillium* species. That common weeds, such as charlock and scentless mayweed, can become infected and microsclerotia can be formed on their aerial plant parts is also alarming. The infection rate of charlock resembled very much that observed on *B. napus*, further suggesting that members of the *Brassicaceae* are the preferred hosts of *V. longisporum*. These findings suggest that other plant species than those already known can act as reservoirs of inoculum, and control of weeds and volunteer *Brassica* plants is of importance to reduce inoculum levels in the soil. Furthermore, the observed reduction in the 1000-kernel weight of inoculated oilseed rape plants implies that, besides the variation in pathogenicity, *V. longisporum* infections have the potential to impact on yield. In addition to the reduced total yields, there is also a reduction in quality, since small seeds contain a higher proportion of seed coats and thereby a lower percentage of total lipid content (Appelkvist, 1976).

In a preliminary investigation of 13 fields in Sweden, as high as 165 colony-forming units per gram of soil were found in a sample taken in the autumn of 2002 from a field where oilseed rape had been grown (Andersson, 2003). This unexpectedly high amount prompted us to extend the soil survey and to include analyses of fluctuating inoculum densities over time and the classification of *Verticillium* species. In the nine fields analysed, the cfu g^{-1} values ranged from 1 to 24 in spring of 2003, 1–12 in autumn 2003, and 0–48 in spring 2004. Similar levels of inoculum densities were found in soil samples from neighbouring oilseed rape fields collected in the spring and autumnof 2004 (Algotsson, 2004). A seasonal pattern with the lowest values in September could be distinguished from our data. During the growing season, when large numbers of infection events take place, the amount of microsclerotia in the soil is expected to decrease. A gradual release of microsclerotia from plant debris after harvest are expected, resulting in higher values in spring 2004 compared to autumn 2003.

Rather low correlations were found between the inoculum density in the soils and disease incidence in plants. The lack of correlation between disease

incidence in oilseed rape fields and number of cfu has been documented for several seasons (Andersson 2003; Algotsson, 2004). Similar results have also been found by Paplomatas et al. (1992) that showed low correlation between the inoculum density of *V. dahliae* and disease incidence in cotton. The disease incidence component is very dependent on the timing of the field observations. We have observed that the level of disease incidence can increase more than threefold within 4 days close to harvest time (Algotsson, 2004), making the interpretation of field observations rather unreliable. Inconsistent results for the relationships between plant infection or yield loss and inoculum densities are reported by a number of researchers (Termorshuizen and Mol, 1995). These circumstances have led to a range of threshold levels for *V. dahliae*, for example in potatoes from 0.6 to 46 cfu g^{-1} lead to more than 70% of infection of potato stems. Inoculum density, interactions with other soil microorganisms, the effects of abiotic factors, and cropping history are factors suggested as being important for this fluctuation (Termorshuizen and Mol, 1995). However, our data indicate that a threshold of 1 cfu g^{-1} soil is enough to cause a disease incidence in a field of up to 48% in oilseed rape.

Verticillium dahliae and *V. longisporum* form microsclerotia, which are black melanised clumps, formed by the budding of mycelial cells (Isaac, 1967; Karapapa et al., 1997). Among all the other *Verticillium* species the less plant pathogenic *V. tricorpus* (Hiemstra, 1998) also has the capacity to form microsclerotia, besides resting mycelium and chlamydospores. These three species differ in morphology concerning the size of the conidia and the number of phialides on the conidiophores. However, in analyses of soil samples, which are based on microsclerotia formation on semi-selective media, the species can easily be missidentified resulting in inadequate values and erroneous conclusions. Thus, major efforts were spent on species differentiation, both molecular and morphological, from the samples collected. The majority of the microsclerotia studied were either of *V. longisporum* or *V. dahliae* origin, but with a predominance of *V. longisporum*. Similar numbers and distribution of *V. longisporum* and *V. dahliae* were found in ten soil samples collected in spring and autumn 2004 from additional fields in the same geographical region (Algotsson, 2004). However, in three spring samples, 1–5 colonies of *V. tricorpus* were identified. There is no obvious link between the crop rotation history of all these 19 fields and the presence of any of the Verticillium species, except oilseed rape and *V. longisporum*. We presume that a low level of soil inoculum may exist of either species and a population increase is imposed by a wide array of factors such as soil type, host plants, temperature and precipitation.

It can be concluded from this study that several plant species have the potential to act as bridging hosts for *V. longisporum* and thus have the capacity to maintain fungal structures in the soil between susceptible crops, similar to the situation found for *V. dahliae* (Davis et al., 2000). Low levels of soil inoculum seem, however, to be enough to cause disease on oilseed rape. In this study the use of selective media or molecular analysis has been helpful to evaluate soil samples, but a rapid, and easy way of scoring different *Verticillium* species, without excessive time spent on re-culturing or expensive equipment, is still lacking. The use of soil plating methods, however, still gives variable estimates (Goud and Termorshuizen, 2003) and cannot be seen as the main single option. A development of a strategy combining plating methods with basic molecular tools may be the option to obtain an accurate value of the number of Verticillium microsclerotia in the soil as well as their influence on disease incidence. To control this disease is a complex challenge; integrated management strategies are required to decrease the yield losses caused by Verticillium wilt on *Brassica* oil crops.

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