Verticillium Longisporum, Infection, Host Range, Prevalence and Plant Defence Responses

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Licentiate thesis
Swedish University of Agricultural Sciences
Uppsala 2006
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Abstract


Verticillium wilt is the major disease responsible for yield losses in oilseed crops in Sweden today. *V. longisporum* is the most prevalent Verticillium pathogen found in Swedish soils, but *V. dahliae* and *V. tricorpus* can also be found. Very little data exists concerning the infection pattern, host range and triggered defence responses in plants toward *V. longisporum*. This thesis aims to extend our knowledge about *V. longisporum* and its host.

The infection process of *V. longisporum* and *V. dahliae* in oilseed crops was compared. *V. longisporum* was found to colonize oilseed crops through lateral roots and root hairs, compared with *V. dahliae* that colonize via the primary roots. At plant maturation, *V. longisporum* was found throughout the plant, in roots as well as in stems and leaves. Whereas *V. dahliae* in 67% of the cases only was present in the root and lower stem.

To investigate the host specificity of *V. longisporum*, seven species, both crops and weeds were inoculated with three isolates (VD2, VD4 and G1-11). *Brassica napus, Sinapis arvensis* and *Matricaria inodora* were found to be highly susceptible to the pathogen, but microsclerotia could also be re-isolated from *Triticum aestivum* and *Avena sativa*.

In Swedish soils the density of microsclerotia ranged from 1 to 48 cfu/g soil, causing between 4 and 72% disease incidence in the fields. However, no direct correlation could be found between microsclerotia density and disease incidence. A regression analysis showed that the yield of the winter oilseed rape crop could be predicted by using three variables: percentage Verticillium wilt diseased plants, temperature in May and temperature in September.

The early plant defence response in *Arabidopsis thaliana* towards *V. longisporum* is dependent on ethylene and jasmonic acid. Increased ethylene production is also important for later symptom development, such as wilting and chlorosis. Six mutants were found to be susceptible to *V. longisporum: ein4-1, ein2-1, ein6-1, ndrl-1* and *npr1-1*. *RFO1* is a wall-associated-receptor-like protein, found to confer resistance to *V. longisporum* similar as earlier found to *Fusarium oxysporum*.

Key words: *Arabidopsis thaliana*, ethylene, host specificity, microsclerotia, oilseed crops, *RFO1*.

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The present thesis is based on the following papers, which will be referred to by their Roman numerals:


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>BBCH</td>
<td>BASF, Bayer, Ciba-Geigy and Hoechst</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>COI1</td>
<td>Coronatine-insensitive protein 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDS1</td>
<td>Enhanced disease susceptibility 1</td>
</tr>
<tr>
<td>EIN2</td>
<td>Ethylene insensitive 2</td>
</tr>
<tr>
<td>ERS1</td>
<td>Ethylene response sensor 1</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETR1</td>
<td>Ethylene resistant 1</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>IRX4</td>
<td>Irregular xylem 4</td>
</tr>
<tr>
<td>ISR</td>
<td>Induced systemic resistance</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>JAR</td>
<td>Jasmonic acid resistant</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NAHG</td>
<td>SA-degrading salicylate hydroxylate</td>
</tr>
<tr>
<td>NPR1</td>
<td>Nonexpressor of PR –genes 1</td>
</tr>
<tr>
<td>PAD4</td>
<td>Phytoalexin-deficient 4</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDF 1.2</td>
<td>Plant defensine 1.2</td>
</tr>
<tr>
<td>PEST</td>
<td>Pro-Glu-Ser-Thr –like sequence</td>
</tr>
<tr>
<td>PPO</td>
<td>Polyphenol oxidase</td>
</tr>
<tr>
<td>PR1</td>
<td>Pathogenesis-related 1</td>
</tr>
<tr>
<td>RFO1</td>
<td>Resistance to <em>Fusarium oxysporum</em> 1</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>SID2</td>
<td>Salicylic acid induction deficient 2</td>
</tr>
<tr>
<td>SSU-rRNA</td>
<td>Small subunit-ribonuclear RNA</td>
</tr>
<tr>
<td>TGA</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>VCG</td>
<td>Vegetative compatibility group</td>
</tr>
<tr>
<td>VDH1</td>
<td><em>Verticillium dahliae</em> hydrophobin gene 1</td>
</tr>
<tr>
<td>VMK1</td>
<td><em>Verticillium</em> MAP kinase 1</td>
</tr>
<tr>
<td>WAKL</td>
<td>Wall-associated receptor-like kinase</td>
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Introduction

Verticillium wilt

Verticillium spp. are soil-borne plant pathogens responsible for Verticillium wilt diseases in temperate and sub-tropical regions. Collectively, they affect close to 250 hosts, including many economically important crops and trees (Pegg & Brady 2002). Especially two of the species V. dahliae and V. albo-atrum attack a high number of host species. V. longisporum has a more narrow host range, but is the main contributor to Verticillium wilt in oilseed crops in Sweden (Dixelius et al., 2005).

The genus name Verticillium derives from the morphological structure of the conidiophore. The conidiophores are branched and occurs in several levels, in a “verticilliate” disposition (Figure 1a).

![Figure 1. Morphology of V. longisporum a) Conidiophore b) Conidia.](image)

Normally the Verticillium mycelium is hyaline, simple or branched, septate and multinucleate. The conidia are ovoid, to elongate and are produced on long phialides positioned in a spiral-like shape around the conidiophores. V. longisporum has, in most cases, a very distinct morphology compared to V. dahliae (Karapapa et al., 1997). They can be distinguished by conidia-shape, polyphenol oxidase (PPO) activity and the size of the conidia (Figure 1b; Table 1).

Table 1. Morphological and physiological differences between V. dahliae and V. longisporum (Karapapa et al., 1997; Steventon et al., 2002).

<table>
<thead>
<tr>
<th></th>
<th>Verticillium dahliae</th>
<th>Verticillium longisporum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape of conidia</td>
<td>Small, spherical</td>
<td>Elongate</td>
</tr>
<tr>
<td>PPO-activity</td>
<td>High activity(^1)</td>
<td>No activity</td>
</tr>
<tr>
<td>Size of conidia</td>
<td>3.5-6 µm</td>
<td>6.5-12 µm</td>
</tr>
<tr>
<td>Nit mutants</td>
<td>Do form</td>
<td>Do not form</td>
</tr>
<tr>
<td>DNA content/spore</td>
<td>0.028-0.042 pg</td>
<td>0.035-0.076 pg</td>
</tr>
</tbody>
</table>

\(^1\) Can become lost during long time of in vitro culture.

The DNA content in V. longisporum is usually twice as high as that found in other non-longispored Verticillium isolates (Steventon et al., 2002).
**Taxonomy**

The *Verticillium* genus was a large and heterogeneous group and has rather recently experienced a major revision (Zare *et al*., 2000, 2001; Gams & Zare, 2001; Sung *et al*., 2001; Zare & Gams, 2001). Fungi earlier classified as *Verticillium* have now been classified as three new genus: *Pochonia* (nematode parasites and myxomycetes), *Lecanicillium* (insects and fungi used as hosts) and *Haptocillium* (free-living nematode parasites). Remaining in the original genus of *Verticillium* are seven species: *V. albo-atrum*, *V. dahliae*, *V. longisporum*, *V. nigrescens*, *V. nubilum*, *V. tricorpus* and *V. theobromae*. The species *V. albo-atrum*, *V. dahliae*, *V. longisporum*, *V. nigrescens* and *V. tricorpus* form three distinct clusters when conserved regions of nuclear and mitochondrial genes were sequenced (Fahleson *et al*., 2004). Three species, *V. albo-atrum*, *V. longisporum* and *V. dahliae* clustered together, where *V. longisporum* showed the closest relationship to *V. albo-atrum*. Of the two other species, *V. tricorpus* is the closest related fungus to the first cluster. *V. longisporum* was first reported as a variant to *V. dahliae* by Stark (1961) and proposed to be an individual species by Karapapa *et al.* (1997) based on molecular and morphological differences. This division is supported by the presence of a large intron in the SSU-rRNA gene with a size of 839 bp in *V. longisporum*, which is absent in *V. dahliae* and *V. albo-atrum* (Karapapa & Typas, 2001). The species *V. dahliae* can be divided into three subgroups based on their complementation between nitrate-non-utilizing (*nit*) mutants, so called vegetative compatibility groups (VCG) (Joaquim & Rowe, 1990). *V. longisporum* do not form *nit* mutants and are not classified in any of the present VCG groups (Zeise & von Tiedemann, 2001). These classifications are based on American isolates. Two Dutch isolates have e.g been classified into two separate vegetative groups, where the NL1 is compatible with VCG 3 and 4 and NL2 is compatible with VCG 1 and 2 (Goud & Termorshuizen, 2002). The species concept of *V. longisporum* has however been questioned. It was argued by Collins *et al.* (2003) and Barbara & Clewes (2003) that the use of *V. longisporum* for long-spored *Verticillium* isolates found on cruciferous host might be premature, especially since not all *Verticillium* ssp. found on crucifers were long-spored and that only European isolates were used in the analysis. However, Californian isolates have later been found to cluster together with European isolates as a distinct phylogenetic group (Fahleson *et al*., 2004). It was concluded that the name *V. longisporum* is misleading, since some short-spored isolates clustered together with long-spored isolates on a molecular level (Fahleson *et al*., 2004). Therefore, it is very difficult to, with certainty, distinguish between the two species on a morphological level. For accurate classification, molecular analysis is needed.

**Disease cycle and symptom development**

The monocyclic disease cycle of *Verticillium* can be divided into two phases: inside the vascular tissue as an endophyte during the earlier stages of the disease and a semi-necrotrophic phase at the later stages of disease, where microsclerotia are formed due to the increased senescence of the plant. The germination of microsclerotia in the soil is stimulated by root exudates (Mol & Vanriessen, 1995),
secreted from the root tip and the root hair. No species specificity seems to exist for this step, since germination also occurs in the presence of exudates from non-hosts (Mol & Vanriessen, 1995). *V. dahliae* infection of a host takes place either by penetration of primary roots or via wounds. Further colonization occurs by penetration and invasion of the cortex, followed by growth in the vascular system. Within the vascular system, conidia are spread throughout the plant via the vascular stream (Beckman, 1987). At later stages of the disease, the pathogen starts colonizing the non-vascular tissue and forms microsclerotia on the senescing and dead tissue.

Early symptoms of Verticillium wilt are chlorosis of lateral branches or leaves (Figure 2a) of the plant. These symptoms can easily be mistaken for early senescence symptoms and are therefore often overlooked. Later symptoms include further chlorosis, usually one-sided, and stunting of the plant. Internal symptoms arise from the protective plugging of the vascular system containing a phenol-pectin mix, giving rise to the dark-brown colour (Figure 2b). If an infected stem is cut laterally, a brown-dark circle can be observed in many species (Beckman & Talboys, 1981). The phenol symptoms can be seen fairly early in most species infected with *Verticillium*, but is hard to detect early in oilseed crops. Towards the end of the growth season, a bronze colorization of the stem is commonly seen before the microsclerotia development is initiated below the epidermal cell-layer (Figure 2c).

![Figure 2](image)

**Figure 2.** a) One sided chlorosis formed as a result of Verticillium wilt b) Dark-brown colouring of the vascular stream, caused by phenolic vascular plugging. c) Later symptoms of Verticillium wilt, dark-brown, black microsclerotia emerging from the epidermal cell-layer.

**Host range**

Verticillium wilt is a disease where the hosts include potatoes, strawberry, alfalfa, oilseed crops and several trees like maple and olive trees (Hiemstra, 1998; Bhat & Subbarao, 1999; Goud, 2003; Ligoxigakis *et al.*, 2002; Zeise & von Tiedeman, 2002). The main hosts for *V. dahliae* are dicotyledonous species, however reports of the colonisation of the roots in monocotyledonous plants such as barley, wheat
and oat exists (Malic & Milton, 1980; Mathre, 1986; Mathre, 1989). *V. dahliae* do infect *Brassicaceae* species, but oilseed crops are generally more susceptible to *V. longisporum* than to *V. dahliae*. The main hosts of *V. longisporum* are therefore reported to be mainly cruciferous species (Zeise & von Tiedemann, 2002).

**Disease control**

Verticillium wilt disease can in severe cases cause a disease incidence of up to 70% (Koike *et al.*, 1994). The rate of colonization of the plant determines if the plant is susceptible or not. The numbers of conidia do not seem to be of importance (Veronese *et al.*, 2003). At the early stages of the disease, the pathogen grows and propagates inside the vascular tissues of the plants (Schnathorst, 1981), which makes any chemical control aimed towards the pathogen impossible without killing the plants. The main focus of disease control has therefore been on limiting the amount of inoculum in the soil and to develop tools to predict the future disease incidence based on the level of soil inoculum. Alternative strategies are soil amendments like lignin (Debode *et al.*, 2005), biological soil disinfestations (Goud *et al.*, 2004), and several kinds of biological control agents such as rhizobacteria like *Burkholderia cepacia* and the non-pathogenic bacteria *Paenibacillus alvei* K165 (Berg, 1996; Mannanov, 2001; Tjamos *et al.*, 2005).

Methods to reduce the amount of microsclerotia have been several: use of methyle bromide (today banned), use of elemental sulphur as an antifungal component (Cooper & Williams, 2004), mulching (Goud, *et al.*, 2004) and the growth of crops not functioning as hosts for the pathogen in the crop rotation, all aimed to minimize the increase of propagules. Several attempts have also been made to breed for disease resistance in different crops against Verticillium wilt. The *Ve1* and *Ve2* genes in tomato confer a race-specific resistance against *V. albo-atrum* (Kawchuk *et al.*, 1998; 2001). The *Ve2*-genes express a class of cell-surface glycoproteins with receptor-mediated endocytosis-like signals and leucine zipper or Pro-Glu-Ser-Thr (PEST) sequences. Cloning has been made of *Ve*-homologues in *Solanum torvum* Swartz, (Fei *et al.*, 2004) and *S. lycopersicoides* (Chai *et al.*, 2003). The *Arabidopsis* genome contains several leucin rich repeats (LRR) linked to plant defence, also found in the *Ve*-genes, but no further homology is seen. However, a large family of *Cf2/Clavatal*-like genes that are similar to the *Ve*-gene family can be found in *Arabidopsis*. *Cf2* is known to induce resistance to *Cladosporium fulvum* in tomato, but do not require SA to do so (Brading *et al.*, 2000). Since there is no known sexual reproduction stage for *V. longisporum* and the gene flow is low, the pathogen can be considered to be a “low risk” pathogen, when considering resistance breeding (McDonald & Linde, 2002). The use of major gene resistance, like that achieved by *Ve1/2* may thus be the solution.

The germplasm of *Brassica oleracea* and some wild plants from *B. cretica*, *B. incana*, *B. insularis* and *B. villosa* together with few *B. rapa* accessions with enhanced resistance against Verticillium wilt have been identified which now are incorporated in breeding programmes of oilseed rape (Happstadius *et al.*, 2003; Dixelius *et al.*, 2005).
Microsclerotia

Microsclerotia growth is initiated by V. dahliae, V. longisporum and V. tricorpus when the Verticillum diseased plant starts to senesce. Microsclerotia consist of grapelike clusters of thick-walled and heavily melanized cells. These resting bodies are formed within the vascular strands (Figure 3), but will further protrude through cortex and outer epidermal cell layer (Schnathorst, 1981).

The resting structures are returned to the soil via the decay of dead plant material. In the soil the microsclerotia are viable for many years (Heale & Karapapa, 1999). The exposure of microsclerotia to root exudates in combination with irrigation or rainfall can cause the resting bodies to germinate and start another infection cycle.

Several studies have been performed to elucidate the cellular processes of microsclerotia formation. MAPK kinases have been found to play an important role in the developmental process. Orthologs to the MAPK kinase FUS3 gene in Saccaromyces cerevisae have been found in both Magnaporthe grisea (Xu & Hamer, 1996) and in V. dahliae (Rauyaree et al., 2005). Mutation of the VMK1 (Verticillum map kinase) gene had a major effect on both microsclerotia formation and the pathogenicity of V. dahliae. Another gene important for microsclerotia development is VDH1 (Klimes & Dobinson, 2006). VDH1 expresses a protein similar to hydrophobins II. Mutation of VDH1 does not seem to influence the growth rate of the fungus or pathogenicity of V. dahliae, but the production of microsclerotia is greatly reduced. VHD1 is also important for enhanced desiccation and cold tolerance of the conidia.

Plant defence response

Molecular plant defence

Plant pathogens can be divided roughly into two main categories, necrotrophs or biotrophs, dependent on their life-cycles. Necrotrophic pathogens like Botrytis cinerea thrive on dead tissues and kill the host when attacking. Biotrophic pathogens, like Peronospora parasitica need living tissue to survive. Plants have developed different ways to cope with this type of pathogen. An incompatible biotrophic pathogen induces a hypersensitive response (HR) and cell death at the site of infection. The HR triggers a systemic response known as SAR (reviewed by Durrant & Dong, 2004; Glazebrook, 2005). SAR is dependent on SA production
and the expression of NPR1 (non-expressor of pathogenesis related protein 1) (Figure 4). The NPR1 proteins found downstream of SA, triggers the expression of several PR (pathogenesis related) genes. What exactly the different PR-genes do is unclear, but roles in the defence responses have been suggested (Kitajima & Sato, 1999, van Loon et al., 2006). Other genes important for SAR are: SNI1 (a negative repressor of PR1) (Li et al., 1999), EDS1 and PAD4 (also required for R-gene signalling) (Wiermer et al., 2005) and SID2 (important for the production of SA) (Nawrath & Métraux, 1999). SA-dependent triggering of PR-proteins that are not NPR1 dependent have also recently been reported (Shah, 2003). Necrotrophic pathogens live on dead tissue, and related defence signalling can roughly be divided in two sub-groups: those strictly jasmonic acid (JA) dependent (Figure 4) and those requiring both JA and ethylene (ET). For example, resistance to Botrytis cinerea and Erwinia carotovora is both JA/ET dependent, whereas Alternaria brassicicola is strictly JA-dependent (Glazebrook, 2001). Important genes for JA and ET dependent resistance are ETR1, EIN2, FAD3/7/8, JAR1, PAD1 and COI1. There is also a complex synergy –antagonistic relationship between SA and JA, where SA can either be a positive or negative regulator of JA, or JA can inhibit SA (Schenk et al., 2003; Spoel et al., 2003; Traw et al., 2003; Pieterse & van Loon, 2004).

![Plant defence diagram](image)

Figure 4. General overview of the molecular plant defence. (Adapted from van Loon et al., 2006; Kunkel & Brooks, 2002). The biotroph P. syringae triggers a SA and NPR1 dependent defence signalling pathway. The necrotroph A. brassicicola triggers a JA dependent defence pathway. The expression of NPR1 inhibits the production of JA. An expression of both ET and JA dependent genes at the same time like EIN2 and COI1 triggers the expression on several defence related genes (PDF1.2, HEL, THI2.1 and CHIB) and the defence of the necrotroph B. cinerea. The non-pathogenic P. fluorescens primes the plants to be more resistant towards several different kinds of pathogen. This is called induced systemic resistance (ISR) and is ETR1, JAR1 and NPR1 dependent.
Not all microorganisms found in the close vicinity of plants are pathogenic. Several bacteria from the genus *Pseudomonas* have been documented to have a favourable protective effect called induced systemic resistance (ISR). ISR induces resistance within the plant against several different pathogens: *Alternaria brassicicola, Botrytis cinerea, Fusarium oxysporum, Pseudomonas syringae* cv. tomato, (van Loon *et al.*, 1998) and *V. dahliae* (Tjamos *et al.*, 2005). The induction of ISR is very similar to SAR and ISR is *ETR1* and *JAR1* dependent as well as *NPR1* dependent, but not SA-dependent (van Loon *et al.*, 1998). In comparison to the JA/ET pathway that induces the expression *PDF 1.2* and SAR that induces *PR-1*, no known PR-proteins are expressed during ISR (van Loon *et al.*, 1998).

**Aims of the study**

The aim of this work is to get an enhanced understanding of how *V. longisporum* infects the plant and the plant defence mechanisms to it. Investigations were focused on the interactions between *V. longisporum* and *B. napus* together with *Arabidopsis thaliana*. Compared to *V. dahliae* (Fradin & Thomma, 2006), very little of basic properties of *V. longisporum* are available, when it comes to issues like infection biology, host range and defence responses. Parts of this knowledge have been considered in this project. The work was divided in following parts.

- To investigate the host range of *V. longisporum* and the influence of microsclerotia density in soil.
- To study the infection process of *V. longisporum* in *Brassica napus*.
- To utilize *Arabidopsis* mutants and gene expression studies to get an understanding of defence signalling mechanisms.

**Results and Discussion**

**Differences and similarities in infection process between *V. longisporum* and *V. dahliae* (I)**

In this study, we compared the infection process of *V. dahliae* and *V. longisporum* in *Brassica napus*. The infection process was studied from seedling to mature plant stage. A clear difference could be observed in the area of entrance of the two pathogens. *V. dahliae* mainly entered oilseed rape via the primary roots, while *V. longisporum* entered via the lateral roots. By staining sections of inoculated plants with tryphan blue together with the PCR-technique *V. longisporum* was detected in roots, basal stem, lower stems, leaves and upper stem pieces in more than 90% of the plants. *V. dahliae* was detected in 90% of the plants, but *V. dahliae* was mainly
detected in the roots and basal stems (67%). *V. dahliae* do not manage to transport itself further than to the lower parts of the stem and therefore the plant only develops milder disease symptoms.

The commonly recorded symptoms of the disease include, stunting, wilting and chlorosis, which are all primary signs of the defence response of the plant. Both susceptible and resistant plant species exhibit similar non-host physical plant defence against Verticillium wilt (Mol & Vanriessen, 1995). Lignification of the cell wall, formation of lignitubers, and the restriction of vascular spread by occlusion are all common physiological responses in plants against Verticillium infection (Beckman & Talboys, 1981). Cell wall thickening or lignification have been reported for several plants and is reported to occur primarily at the time for conidia germination in the root (Beckman & Talboys, 1981). Lignitubers consist of the 1-3-ß-glucan callose and lignin. Callose deposition and lignification of cell walls restrict the pathogen from entering the host. To further separate and restrict the spread of Verticillium already in the vascular system, vascular plugs are formed. Vascular plugs consist of a mixture of several phenol compounds and pectins. The paradox in this system is that vascular plugs not only restrict the pathogen for further spread, they also restrict the water transportation within the vascular system. The water deficiency causes the wilting appearance that is considered one of the main symptoms of vascular wilt. The plugging is only temporary, eventually conidia will break through the phenol matrix for further colonisation of the vascular strands. The outcome of this arms race is dependent on several factors. If the Verticillium infection occurs in a relatively young developmental stage of the plant and the break of the phenol plugging occurs faster than a new plugging can occur, the plant will show severe wilting symptoms as a result of water deprivation. When, on the other hand, infection occurs in older plants, plugging of one or several strands is not as essential for survival of the plant, since several other non-affected vascular strands still exists and only mild symptoms will be seen. Visual symptoms may be lacking, but conidia and microsclerotia can still be isolated from the plant. In *Brassica napus*, the *V. longisporum* can break through the vascular plugging and further spread throughout the host, while *V. dahliae* cannot. This could probably be the link to the difference in symptom development in plants infected by *V. dahliae* or *V. longisporum*.

**Host specificity (II)**

The aim with this study was to investigate the host specificity of *V. longisporum* on Swedish common crops and weeds. Seven crop species, 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 five weed species, barren brome (*Bromus sterilis*), black-grass (*Alopecurus myosuroides*), charlock (*Sinapis arvensis*), cleavers (*Galium aparine*) and scentless mayweed (*Matricaria inodora*) were studied under greenhouse conditions. Visual symptoms as well as the distribution of the pathogen in inoculated plants were studied. To verify the pathogen in inoculated plants, *V.
specific primers were used (Steventon et al., 2001). For visual scoring, the length of the plants was measured regularly over time together with re-isolation of microsclerotia. Three species, oilseed rape, charlock and scentless mayweed were all highly affected by the pathogen. The plants were highly stunted and microsclerotia could be re-isolated. Wheat and oat were also slightly stunted, but had recovered at harvest time. A high quantity of microsclerotia was re-isolated from inoculated plants of oilseed rape, charlock, scentless mayweed, oat and wheat. None of the other species showed any symptoms of disease nor was it possible to re-isolate the fungus. Three different isolates of V. longisporum (VD2, VD4 and G1-11) with different pathogenicity (Steventon et al., 2001) were assessed. Species within the Brassicaceae family were equally affected by all three isolates, while other species only were affected by the most aggressive isolate. Similarly, plants inoculated with VD2 achieved a significantly lower yield, while the yield in oilseed rape inoculated with VD4 and G1-11 was unaffected. Several wild weedy relatives to Brassica oilcrops exist in the close vicinity of the fields. To reduce disease incidence of Verticillium wilt, the most important task is to reduce the inoculum in the field.

In conclusion, oilseed rape, charlock and scentless mayweed were found to be susceptible to V. longisporum and the fungus could colonize several other species. For a reduction of disease inoculum in the soil, a good crop rotation combined with weed control is important. The cleansing of the field from older plant debris and spilled grain is important to restrict the return of inoculum to the soil. A wide range of plants that can be colonized due to non-host plants may reduce inoculum in the soil, since further development of microsclerotia does not occur.

Density of microsclerotia in Swedish soils (II)

Very little data exists about the density of Verticillium longisporum in Swedish soils. The aim with this study was to evaluate the density of microsclerotia in Swedish soils regularly planted with B. napus. Nine soils were tested over three time periods. To calculate the microsclerotia density, a wet sieving method was used. To perform species differentiation, visual scoring was used together with PCR-analysis. The density of colony forming units (cfu) ranged from 1 to 48 cfu/g soil and the disease incidence in 2003 ranged from 4 to 72% in the different fields. This can be interpreted that even at such a low density as 1 cfu/g soil of V. longisporum microsclerotia can cause considerable damage. No correlation could be seen between disease incidence and microsclerotia density. Studies have shown that the statistical variation within a method is larger than between different methods (Goud et al., 2003). Thus, it is important when using either of these methods to have a well-standardized protocol, which allows replications. Similarly, the way of calculating the disease incidence is very sensitive for several reasons. First, different persons doing the observations in field may come to different conclusions. Second, timing of the disease is also equally important. Within four days a field has been shown to go from showing almost no symptoms to be 100% completely diseased.
Molecular analyses of re-isolated microsclerotia from several fields, together with visual scoring, showed that the majority of microsclerotia found in Swedish soils derive from *V. longisporum*. *V. dahliae* is prevalent only in a few fields, while *V. tricorpus* has only been very scarcely found in Swedish fields (Dixelius *et al.* 2005). Visual scoring was based on re-isolated microsclerotia using the wet-sieving method (Goud & Termorshuizen, 2003). *V. longisporum* microsclerotia had a more irregular shape than either *V. dahliae* or *V. tricorpus*. For molecular scoring, PCR was used with species-specific primers (Karapapa & Typas, 2001; Steventon *et al*., 2002) on DNA extracted from diseased plant material and re-isolated microsclerotia. The density of microsclerotia in Swedish soils is much higher than any other reports found on *V. dahliae*. This is most probably correlated to the high extent of oilseed monoculture in Sweden during the 1950’s.

It is not yet known to what extent the microsclerotia density in Swedish soils actual influences the actual harvest of oilseed rape. Several factors influence the yield of oilseed crops: temperature over several months, humidity and disease pressure in the soil. The most important variables contributing to the yield of winter oilseed rape are *Verticillium* wilt and the temperature in May and September. A regression analysis using these three variables gives a very high correspondence between predicted yield and actual yield (R²=0.839, Q²=0.832) (Staal *et al*., unpublished). The data were collected over a time period of sixteen years from SMHI and SJV. Data collected over such a long time period, contributes to the high predictive value (Q²=0.832), which confirms that these three variables are the major components influencing yield of winter oilseed rape.

![Figure 5. Regression analysis of winter oilseed rape yield, using the variables of temperature in May and September and *Verticillium* wilt field scoring. R²=0.839, Q²=0.832.](image)

In conclusion, a very high density of *V. longisporum* microsclerotia was found in Swedish soils, but no correlation could be found between microsclerotia density and disease incidence. The yield of the oilseed rape crop, however depended on mainly three things: temperature in May and September and the disease incidence of *Verticillium* wilt.
Defence responses against *V. longisporum* in Arabiopsis (III)

*Arabidopsis* and *Brassica napus* belongs to the same plant family, *Brassicaceae* and both species can be diseased by *Verticillium* spp. (Portenko, 2000; Steventon et al., 2001; Zeise & von Tiedemann, 2002; Veronese et al., 2003). The sequence similarity between *Arabidopsis* and *B. napus* is very high, with an average of coding domain sequence similarity of 86% (Parkin et al., 2005). *Arabidopsis* is a widely used model organism for elucidating several different processes within plants, like plant metabolomics, defence responses and developmental studies (Alonso-Blanco & Koornneef, 2000; Baulcombe, 2004; Bevan & Walsh, 2005; Bhalla et al., 2005; Fukusaki & Kobayashi, 2005; Glazebrook, 2005). Hence, *Arabidopsis* is a usable tool with a short generation time and multiple mutants generated in defined pathways, to study the genetic bases of defence against *Verticillium longisporum*. A natural variance in resistance exists within the *Arabidopsis* population towards *V. longisporum*. A screen of 169 accessions resulted in the findings of 115 ecotypes with an elevated degree of susceptibility towards the pathogen. Two QTL’s controlling chlorosis were also found on chromosome 2 and 3 in the Bay-0 X Shahdara RI-lines developed by Loudet et al. (2002).

*Ethylene and jasmonate dependency*

Ethylene is a versatile hormone, implicated in many plant functions throughout the growth cycle of the plant. It contributes to the seedling growth response, maturation of fruit and several defence responses towards several pathogens (Chen et al., 2005). Ethylene signalling in *Arabidopsis* is perceived by five ethylene receptors (ETR1, ERS1, ETR2, EIN4, ERS2). These receptors can further be divided into two sub-groups: Group I (ETR1 and ERS1) and group II (ETR2, EIN4 and ERS2) (Urao et al., 2001; Guo & Ecker, 2004). ETR1 and ERS1 contain three transmembrane domains and a conserved histidine kinase domain. Ethylene receptors in Group II harbour four transmembrane domains and a degenerate histidine kinase. In order to understand the role of two ethylene receptors (ETR1 and EIN4) and the downstream factors to these regulators (EIN2, EIN3 and EIN6) in plant defence corresponding mutants were evaluated in their response to *V. longisporum*.

By screening several ET or JA-deficient *Arabidopsis* mutants it was found that both plant hormones are involved in the plant defence signalling against *V. longisporum*. The mutants ein2-1, ein4-1, ein6-1 and pad1-1 were significantly more susceptible compared to wild-type. When ACC (a pre-cursor to ET) and MeJA (methyl jasmonate) was separately added three days prior inoculation, ACC and MeJA enhanced the resistance against *V. longisporum*. A positive relationship between symptom development and ethylene production was established by measuring the ethylene production for up to 11 days.

The group II receptor EIN4 seems to be of greater importance than ETR1 (group I), since fresh weight in ein4-1 is significantly decreased 10 dpi. The mutant etr1-1 on the other hand showed an increase in tolerance, which also was found in the *Arabidopsis* – *V. dahliae* interaction (Veronese et al., 2003). Similarly, an addition of ACC increased tolerance significantly in the wild-type upon *V. longisporum* challenge. However, no difference could be seen when ACC was added to etr1-1.
The main investigations on the involvement of ethylene receptors in plant defence have been focused on the role of the group I receptor ETR1 (Knoester et al., 1998; van Loon et al., 1998; Veronese et al., 2003; Tjamos et al., 2005). Very little data exists about the role of receptors from group II in plant defence. The mutant ein4-1 (Group II) is susceptible but etr1-1 (Group I) is not, suggesting that the defence response in Arabidopsis against V. longisporum is dependent on sub-family II ethylene receptors. Data furthermore showed a simultaneous acceleration in ethylene production and symptom development. This has also been reported to be the case in other plants infected with V. dahliae (Robinson et al., 2001). The result suggests that ethylene both has an important role as part of the defence signalling pathway at earlier stages and a role in the later stages of disease. What exactly controls the transition of ethylene being involved in defence response versus being involved in symptom development is not clear.

Links between ET and JA production to both increased lignification and increased pathogen defence have been reported (Ellis & Turner, 2001) and a similar association between ethylene and lignification of the cell walls can be proposed as an early defence response in our pathosystem.

The lignin-production in Ler-0 increased 10 dpi after inoculation with V. longisporum (Figure 6). The lignin-production in irx4-1 is half of the production in wild-type. But the mutant still was moderately tolerant to V. longisporum. Increased thickness of the cell wall, may be the reason behind this increased tolerance. A moderately increased resistance against V. longisporum could be seen in mutants containing 50% more lignin than the wild type Arabidopsis.

The role of salicylic acid

The dependency of SA was examined by the use of several SA-mutants and by the addition of exogenous SA, as was previously done with ACC and MeJA. Only the npr1-1 mutant was significantly more susceptible than the wild-type Arabidopsis. Neither of the other SA-dependent mutants tested (NahG, sid2-1, eds1-1 and pad4-1) were susceptible. A rapid increase of disease and symptom development in wild-type plants inoculated with V. longisporum was seen 5 dpi. This was accompanied by a rapid increase of ethylene production and the expression of the SA-dependent genes PR-1 and PR-2.

Figure 6. Lignification in Arabidopsis. Samples were taken 10 days after mock-inoculation or inoculation with V. longisporum.

a) Ler-0 – mock-inoculated. b) Ler-0 – inoculated with V. longisporum. c) Leaf of irx4 inoculated with V. longisporum. d) Leaf of Ler-0 inoculated with V. longisporum.
The expression of SAR upon plant defence is both SA and NPR1 dependent and the Arabidopsis npr1-1 mutant was originally found in a screen for plants that did not express PR genes after SAR induction (reviewed by Dong, 2004; Durrant & Dong, 2004; Pieterse & van Loon, 2004). In its inactive form, NPR1 can be found in the cytoplasm as an oligomeric complex bound together with several sulphide bounds. Upon pathogen infection SA accumulates and causes a redox change in the cytosol breaking the disulfide bonds, thereby causing dimerization of NPR1. The monomers are then translocated into the nucleus. In the nucleus the NPR1 binds to TGA-transcription factors like TGA2. TGA transcription factors are basic leuzin zippers that inhibit the PR-1 activation. In non-induced cells intramolecular disulfide bonds in TGA prevent the interaction with NPR1. When the redox-change occurs due to SA accumulation, the bonds are reduced, and a conformational change enables TGA to interact with NPR1 causing an activation of PR-1. In a microarray study, npr1-1 and npr1-3 were found in different clusters, suggesting two different roles of NPR1 (Glazebrook et al., 2003). The change of a histidine to a tyrosin causes a destabilization of the protein in npr1-1 (Cao et al., 1997). This destabilization disables the NPR1-binding to the TGA-transcription factor, thus preventing the expression of PR-1. The npr1-3 mutant is allelic to npr1-1. The mutation in npr1-3 is caused by the formation of a premature stop-codon, causing an obstruction in the C-terminal, which abolishes the nuclear-localization signal in NPR1 (Dong, 2004). The lack of increased susceptibility in npr1-3 when V. longisporum was introduced may be due to the fact that a small amount of NPR1 can be present in the cytosol and thus partially can be involved with SA/JA-crosstalk (Beckers & Spoel, 2006). However, the exact role of NPR1 in V. longisporum – defence in Arabidopsis is still not known and needs to be further elucidated.

There are several similarities between the genes involved in defence against V. longisporum and the response genes triggered by the non-pathogenic Pseudomonas fluorescens causing induced systemic resistance. Both are ethylene and JA dependent and the mutant npr1-1 inhibits both the ISR response as well as the defence response to V. longisporum in Arabidopsis (Tjamos et al., 2005) but is not SA dependent. For a thorough review concerning ISR, see Pieterse et al. (1998). ISR is not connected with any kind of expression of PR-gen (van Loon et al., 1998). However, significant expression of SA-dependent PR-gen (PR-1 and PR-2) was not found until at the earliest 7 dpi with V. longisporum challenge. This suggests that SA-dependent PR-gen do not play a primary role in early plant defence against V. longisporum.

Links between Fusarium oxysporum and Verticillium longisporum
In a parallel study, the gene RFO1 was found to cause increased resistance in Arabidopsis to Fusarium oxysporum (Diener & Ausubel, 2005). Several similarities between V. longisporum and F. oxysporum exist. They are both soil-borne fungi and colonize the roots of their respective hosts. The continuation of pathogen spread is maintained via the vascular system. The defence response in plants infected by F. oxysporum (Berrocal-Lobo & Molina, 2004) and symptoms of Fusarium wilt are also very similar, like wilting and chlorosis of leaves, stunting of young plants and ultimately death. Thus, it was hypothesized that rfo1 also
would be susceptible towards \textit{V. longisporum}. A significant decrease in fresh weight compared to wild-type was also found (III). Application of ACC or MeJA did not restore the tolerance in \textit{rfo1} to wild-type level indicating that the induced plant defence regulated by \textit{RFO1} is ET and JA independent. \textit{RFO1} encodes a wall-associated receptor-like kinase and is identical to \textit{WAKL22}. Elevated expression of several \textit{WAKL} genes due to pathogen attack or wounding has been documented (He \textit{et al.}, 1998; Verica \textit{et al.}, 2003). \textit{WAKL22} belongs to the same family as the \textit{Arabidopsis} cell wall-associated receptor kinase, \textit{WAK1} (He \textit{et al.}, 1998). The induction of \textit{WAK1} in \textit{Arabidopsis} is NPR1-dependent and \textit{WAK/WAKL} genes are expressed due to pathogen infection, wounding and/or SA production (Verica \textit{et al.}, 2003). The lack of ET and JA dependency indicates that two separate pathways are active in \textit{Arabidopsis} towards \textit{V. longisporum} and \textit{F. oxysporum} concerning the defence response.

In conclusion, extensive cross-talk between several signalling pathways is present in the \textit{Arabidopsis-V. longisporum} pathosystem. Plant defence response in \textit{Arabidopsis} is especially dependent on ET and JA signalling. \textit{RFO1}, a cell-wall associated kinase-like protein is important for resistance in \textit{Arabidopsis} to both \textit{F. oxysporum} and \textit{V. longisporum}. 

**Conclusions**

- *Verticillium longisporum* enters the plant host mainly via the lateral roots, not the primary roots as in the case of *V. dahliae* (I).

- Only the roots and lower stem is colonized by *V. dahliae*, while *V. longisporum* colonizes upper stem and leaves as well (I).

- Oilseed rape, charlock and scentless mayweed can be infected with *V. longisporum*, while the pathogen can colonize both oat and wheat without any visible symptoms. Microsclerotia could be isolated from oilseed rape, charlock, scentless mayweed, oat and wheat (II).

- The density of Verticillium microsclerotia in soils from southern Sweden ranged from 1- 48 cfu/g soil (II).

- In Swedish soils, the majority of *Verticillium* found is *Verticillium longisporum*. *V. dahliae* can be found only in a few of the soils analysed (II).

- No correlation can be found between the microsclerotia density in soils and disease incidence, but it is possible to predict yield of winter oilseed rape based on Verticillium wilt incidence, and the temperature in May and September (II).

- ET and JA are of importance for early plant defence in *Arabidopsis thaliana* against *Verticillium longisporum* (III). Ethylene is also important for the symptom expression of Verticillium wilt in the later stages of disease development (III).

- The expression of SA dependent genes *PR-1* and *PR-2* can be seen 7 dpi. Indicating an importance of SA in later stages of disease development (III).

- The genes *EIN4* and *NPR1* are equally important for induced defence response against *Verticillium* (III).

- *RFO1* encodes a wall-associated receptor-like kinase. The *rfo1* (Resistance to *Fusarium oxysporum*) mutant is both susceptible to *V. longisporum* and *F. oxysporum*, both being vascular fungal pathogens (III).
Future perspectives

- To detect *V. longisporum* resistance genes in Arabidopsis more knowledge is needed. One way to achieve this would be to produce a recombinant inbred line between the two most extreme susceptible and resistant ecotypes.

- The exact role of *NDR1* as a resistance gene toward *V. longisporum* is not clear. Microarray, comparing the difference expression pattern of genes in mock-inoculated and inoculated *ndr1*, and compare that with wild-type would give a clue about what genes are up – respectively down-regulated. Especially in relationship to MeJA, that enhances the tolerance in *ndr1-1* towards *V. longisporum*.

- It is not completely clear what part of the defence signalling pathway *RFO1* is involved in. A wall-associated receptor-like kinase might work as a guarding protein or as to enhance the tolerance in the plant against toxic level of SA. Either way, over-expression of *RFO1* in plants, might stimulate increased resistance towards *V. longisporum* as well as *F. oxysproum*. However, map kinases are involved on a vast variety of plant functions, thus investigations would be needed to study the viability of a plant over-expressing *RFO1*. 
References


during an incompatible interaction with *Alternaria brassiciola*. *Plant Physiology* 132, 999-1010.


Acknowledgments

Here comes the hardest part of all to write, but the most rewarding part too. Because I realise how many people I have around me to be grateful to!

I firstly and foremost want to thank the people in my group and mainly my supervisor Prof. Christina Dixellius, for introducing me to how real scientists work and always keeping me on my toes and pushing me to my limits. I have become a better scientist because of her. To Jan Fahleson, my deepest gratitude, for always being there to answer my odd questions and to giving so good inputs. You are my photoshop-guru! Jens Staal for all the good ideas about my projects, being wild cards or not. I will miss our discussions! Maria, for keeping my “sloppy-ness” in order and for all the nice coffee-breaks and conversations about everything else but science. Mattias P, your kind soul and kind remarks always made my time here easier! To Patrick Okori, thank you for answering all my questions I miss our conversations about science and life. Jan-Kees Goud, your time here was short, but you taught me a great deal about Verticillium. Gunilla, utan dig som tog hand om de mer praktiska sakerna och som påminde mig ibland vad som skulle göras skulle jag överhuvudtaget inte kommit så här långt! Tack!

I also want to thank all the technical personel, without your support I would not have made it as far as I have done. Gun, Ingrid. E, Ingrid S, Kristine-Sophie and Urban, ni har min djupaste tacksamhet!

To all my friends and colleagues in the lab and in the corridors: Matti, Jenny, Jennie, Carina, Monika, Johan, Andreas, Emma, Joel, Jens.Su, Mattias M., Magnus, Jon, Liv, Elena, Jesper and Derek. I do not know you half as much as you deserve, but I am glad I have met you and you will be greatly missed, because you all contributed to making my time here easier!

I want to thank the Nilsson-Ehle Foundation, Swedish Seeds and Oilseed Growers Association (SSO), Carl Tryggers Foundation, Swedish Research Council for Environmental, Agricultural Sciences and Spatial Planning (FORMAS) and the Swedish Farmers Foundation for Agricultural Research (SLF) for financial support.

I also want to send my warmest gratitude to people contributing with pictures in this thesis: Carina Andersson and Johanna Holmblad.

Finally, for my friends and family outside the scientific world: Till gänget i CoE, ni är de som höll om mig när jag behövde det och skapade balans i mitt liv! Tack! To Anna.S for being there to show me that there is more around than science and for nice lajv-discussions! Mamma och Bertil, för att ni alltid har ställt upp och visat på att allt är möjligt! Pappa och Barbro - för att ni trott på mig och låtit mig veta att: “Vill man så kan man!”

Till sist mitt djupaste tack till de två männen i mitt liv: Tommy och Pär. Ni är och förblir ljuset i mitt liv! Älskar er!!!!