

Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques

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Abstract The differential interactions of *V. longisporum* (VL) and *V. dahliae* (VD) on the root surface and in the root and shoot vascular system of *Brassica napus* were studied by confocal laser scanning microscopy (CLSM), using GFP tagging and conventional fluorescence dyes, acid fuchsin and acridin orange. VL and VD transformants expressing sGFP were generated by *Agrobacterium*-mediated transformation. GFP signals were less homogenous and GFP tagging performed less satisfactory than the conventional fluorescence staining when both were studied with CLSM. Interactions of both pathogens were largely restricted to the root hair zone. At 24 h post-inoculation (hpi), hyphae of VL and VD were found intensely interwoven with the root hairs. Hyphae of VL followed the root hairs towards the root surface. At 36 hpi, VL hyphae started to cover the roots

with a hyphal net strictly following the grooves of the junctions of the epidermal cells. VL started to penetrate the root epidermal cells without any conspicuous infection structures. Subsequently, hyphae grew intracellularly and intercellularly through the root cortex towards the central cylinder, without inducing any visible plant responses. Colonisation of the xylem vessels in the shoot with VL was restricted to individual vessels entirely filled with mycelium and conidia, while adjacent vessels remained completely unaffected. This may explain why no wilt symptoms occur in *B. napus* infected with VL. Elevated amounts of fungal DNA were detectable in the hypocotyls 14 days post-inoculation (dpi) and in the leaves 35 dpi. Root penetration was also observed for VD, however, with no directed root surface growth and mainly an intercellular invasion of the root tissue. In contrast to VL, VD started ample formation of conidia on the roots, and was unable to spread systemically into the shoots. VD did not form microsclerotia in the root tissue as widely observed for VL. This study confirms that VD is non-pathogenic on *B. napus* and demonstrates that non-host resistance against this fungus materializes in restriction of systemic spread rather than inhibition of penetration.

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Introduction

Verticillium wilt on oilseed rape (*Brassica napus* spp. *oleifera*) is caused by the host-adapted, near-diploid fungus *Verticillium longisporum* (Karapapa et al. 1997b; Zeise and von Tiedemann 2001, 2002a, b). The growing area of rapeseed cultivation and the relatively intense crop rotation has rendered this disease an increasing threat to oilseed rape (OSR) production particularly in Europe (Krüger 1989; Zielenski and Sadowski 1995). In Sweden, Verticillium wilt has been known as the cause of economic losses of OSR since the 1960s (Svenson and Lerenius 1987) and has become a significant problem since the 1970s (Dixelius et al. 2005). In the main cropping areas for winter OSR in Germany, the occurrence of *V. longisporum* has increased rapidly since the mid 1980s (Daebeler et al. 1988; Zeise and Seidel 1990; Günzelmann and Paul 1990). For both winter and spring type OSR, breeding for resistance has been severely hampered by the absence of sufficient resistance in commercially available breeding material; however, recently, some promising genotypes of cabbage (*B. oleracea*) with enhanced resistance were identified (Happstadius et al. 2003; Dixelius et al. 2005).

A closely related species to *V. longisporum* is *V. dahliae*. This ubiquitous soilborne fungus causes wilt diseases on many economically important crops, including cotton, cucurbits, alfalfa, sunflower, eggplant, mint, strawberry, tomato and potato (Domsch et al. 1980; Schnathorst 1981; Pegg 1984; Subbarao et al. 1995; Bhat and Subbarao 1999). A differentiation of long-spored *Verticillium* isolates collected from horseradish, classified as *V. dahliae* var. *longisporum*, was first made in the early 1960s (Stark 1961), until the detailed description of distinct morphological, physiological and molecular traits led to the proposition of treating *V. longisporum* as a distinct species (Karapapa et al. 1997b). Nonetheless, there is still controversy concerning the taxonomy of *V. longisporum* as a separate host-specific species of *Verticillium* (Fahleson et al. 2003). Thus it has been reported that *Brassica* crops can occasionally host short-spored *Verticillium* isolates (Collins et al. 2003) and that *V. longisporum* is able to infect plant species outside the *Brassicaceae* family (Fahleson et al. 2003; Johannson et al. 2006). Additional confusion has been caused by some misidentification of the two

species. In several studies, *V. dahliae* has been regarded as the causal agent of Verticillium wilt in *Brassica* crops (Xiao and Subbarao 2000; Söchting and Verreet 2004) or on horseradish (Babadoost et al. 2004), without considering that long-spored isolates may have been involved.

The host range, epidemiology and infection process on various hosts of *V. dahliae* have been intensely investigated in previous works (Schnathorst 1981; Beckmann 1987; Gold et al. 1996; Rowe and Powelson 2002). After germination of microsclerotia, which is inducible by the root exudates of host plants (Mol and Scholte 1995), the fungal hyphae reach the root surface, penetrate the epidermal cells, transverse the root cortex and enter the immature xylem elements (Beckman 1987). Most of the time of its life cycle *V. dahliae* is constricted to the vascular system, which is a nutrient-limited environment containing low amounts of sugars, inorganic salts, and amino acids, to which the fungus is well adapted (Wood 1961; Dimond 1970; Dixon and Pegg 1972; Green 1981; Pegg 1981, 1985; Van Alfen 1989). In this liquid environment *V. dahliae* exhibits both hyphal and a 'yeast-like' growth, producing conidia budding directly from hyphae, or formed on short phialides (Buckley et al. 1969). With the transpiration stream the released conidia are carried upwards in the vascular elements where they are eventually trapped in bordered pits or at vessel end walls. Colonization proceeds by formation of germ tubes, which penetrate into the upstream vessel elements where new conidia are produced (Beckman 1987; Gold et al. 1996). In the later stages of the disease cycle, as the foliage begins to senesce, the pathogen enters a limited saprophytic growth phase in which microsclerotia are formed in the dying stem parenchyma, thus having a critical role as the primary source of disease in the field (Schnathorst 1981; Neumann and Dobinson 2003).

Currently, little is known about the infection patterns of both *V. longisporum* and *V. dahliae* on roots of *B. napus*. In order to visualize their colonization of the oilseed rape rhizosphere by confocal laser scanning microscopy (CLSM), we used the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie and Kain 1998; Tsien 1998) to label both *V. longisporum* and *V. dahliae*. Since the first report on the use of GFP as a vital marker in plant-pathogen-interaction studies

(Spellig et al. 1996), the GFP transformation has become a common tool in the analysis of various fungal/fungus-like plant pathogens (Lorang et al. 2001). A particular advantage of the method is that plant infection and colonization of GFP-expressing fungi can be followed by fluorescence microscopy in intact plant tissues or tissue sections without requiring cofactors or substrates (Horowitz et al. 2002; Shan and Godwin 2004; Bolwerk et al. 2005).

In the present study the GFP-labelling was compared with a technique combining conventional fluorescence staining with confocal microscopy. We describe (i) the *Agrobacterium tumefaciens* mediated transformation of both *V. longisporum* and *V. dahliae* resulting in stable expression of GFP, (ii) compare the suitability of the GFP reporter gene technique with classical fluorescence staining and (iii) describe new aspects in the early and advanced stages of *V. longisporum* and *V. dahliae* on the roots and in the vascular system of oilseed rape. The microscopical results are further supported by the documentation of disease development by real-time PCR analysis of fungal DNA.

Materials and methods

Plant material

The susceptible winter oilseed rape variety ‘Falcon’, supplied by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ), was used in this study. Seeds were double surface-sterilized by sequential immersion in 70% ethanol for 2 min and 1% sodium hypochlorite containing 0.1% Tween-20 for 15 min under constant shaking (Zou et al. 2004). Subsequently, seeds were washed two times with autoclaved tap water.

Fungal isolates

Verticillium longisporum isolate VL 43 from *Brassica napus* and *V. dahliae* isolate VD 73 (vegetative compatibility group 2B) from *Linum usitatissimum* were used throughout this work. Both isolates originated from hosts grown in the north of Germany (Zeise and von Tiedemann 2001, 2002a, b). Long-term storage with conidial suspensions in a concentration of $1\text{--}3 \times 10^6$ conidia ml^{-1} in Czapek Dox

medium supplemented with 25% glycerol at -80°C was used. For propagation, droplets of these suspensions were plated onto potato dextrose agar (PDA) and incubated for 14 days at 23°C in the dark. Spores were obtained by gently flooding the dishes with 0.9% NaCl solution (modified according to Melouk 1992). Inoculum for root dip inoculation was produced by adding 500 μl of the spore stock solution to 250 ml potato dextrose broth (PDB). The cultures were subsequently incubated for 7 days at 23°C on the rotary shaker. The resulting suspension was filtered through sterile gauze. Spore concentration was determined with a haemocytometer and diluted to 1×10^6 spores ml^{-1} .

Bacterial vector strain

Escherichia coli strain DH5 α (Hanahan 1983) was used during construction and maintenance of plasmids. *Agrobacterium tumefaciens* strain AGL-1 (Lazo et al. 1991) was kindly provided by Dr. Susanne Frick, Leibniz Institute of Plant Biochemistry, Halle/Saale. This strain carries the hypervirulent Ti helper plasmid pTiBo542 Δ T, which lacks one border sequence and thus an intact T-DNA region (Hood et al. 1986; Komari et al. 1986; Lazo et al. 1991). For the transformation of plants or fungi, this strain has to carry a binary vector in which the T-DNA is defined by both left and right border sequences (Bevan 1984). Short-term storage of the *Agrobacterium* cells was performed on solid LB (Luria Bertani) medium (Maniatis et al. 1982), supplemented with 25 $\mu\text{g ml}^{-1}$ rifampicin and 50 $\mu\text{g ml}^{-1}$ carbenicillin at 4°C . Cells were stored long term as electrocompetent cultures in the freezer at -80°C .

Binary vector construction

The binary vector used in this study was constructed on the backbone of pPK2 (Covert et al. 2001). A fragment of about 2.9 kb of gGFP (Maor et al. 1998), containing the *sgfp* gene driven by the *Aspergillus nidulans* promoter and terminated by the *A. nidulans* trpC terminator (Punt et al. 1987), was isolated by digestion with XbaI and EcoRI. sGFP is a variant of GFP carrying S65T mutation, which was constructed with the goal to improve optical properties of GFP expressed in plants and proved superior to wildtype

GFP when expressed in fungal mycelium (Maor et al. 1998).

In two different reactions the plasmid pBluescript-SK⁻ (Acc. No. X52330, Short et al. 1988) was digested on the one hand with the restriction enzymes AflIII and XbaI and on the other hand with AflIII and EcoRI, leading to fragments of 450 bp and 2.5 kb size, respectively. These fragments were ligated with the XbaI/EcoRI fragment from gGFP in a triparental ligation, resulting in the plasmid pBS::gpd::sgfp. Subsequently, the sGFP expression cassette was removed from pBS::gpd::sgfp again by digesting with XbaI and HindIII, which is located downstream to the EcoRI site. The resulting fragment was inserted into the corresponding XbaI/HindIII sites of pPK2. The final construct, pGV04 (Fig. 1), was introduced into *A. tumefaciens* by electroporation (Wen-jun and Forde 1989) at 2.5 kV, 400 ohms and 25 μ F using a cuvette with a 1 mm gap between the electrodes.

Agrobacterium-mediated transformation of *V. longisporum* and *V. dahliae*

The *A. tumefaciens* strain AGL1, containing the binary vector pGV04, was grown at 28°C for 48 h

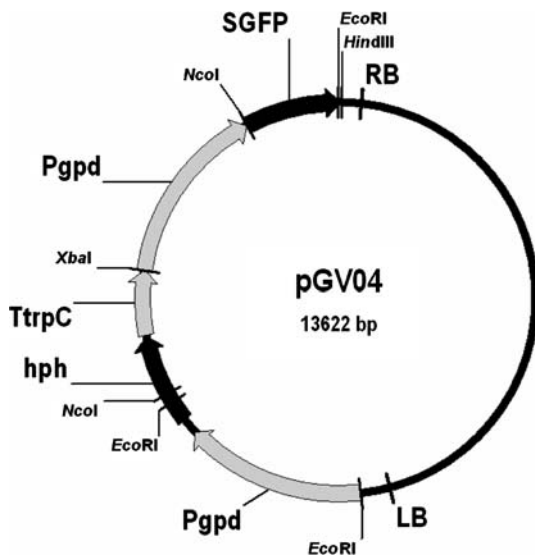


Fig. 1 Restriction enzyme map of pGV04, an *Agrobacterium* binary vector constructed on the backbone of pPK2 (Covert et al. 2001). The T-DNA is running clockwise from the left border (LB) to the right border (RB). Pgpd: *Aspergillus nidulans* glyceraldehydes 3-phosphate promoter; hph: hygromycin B resistance as a selection marker; TtrpC: *A. nidulans* transcriptional terminator

in LB medium supplemented with rifampicin (25 μ g ml⁻¹), carbenicillin (25 μ g ml⁻¹) and kanamycin (50 μ g ml⁻¹). After reaching an optical density of OD₆₆₀ = 0.6–0.9 bacterial cells were harvested and washed with induction medium (IM, Bundock et al. 1995) supplemented with 200 μ M acetosyringone (AS). Subsequently, the bacterial suspension was diluted with induction medium to OD₆₆₀ = 0.15. The cells were grown for an additional period of 6–12 h before being mixed with an equal volume of a spore suspension of either *V. longisporum* isolate VL 43 or *V. dahliae* isolate VD 73 (1–3 \times 10⁶ spores ml⁻¹). From this mixture aliquots of 200 μ l were plated on a cellophane membrane placed on solid cocultivation medium (same composition as IM except that it contains 5 mM glucose instead of 10 mM glucose) supplemented with 200 μ M acetosyringone. After cocultivation at 23°C for 60 h the growing fungal mycelium and the *Agrobacteria* were suspended in 0.9% NaCl solution supplemented with 200 μ M cefotaxim (for separation from *A. tumefaciens*) and plated on Czapek Dox medium containing hygromycin B (50 μ g ml⁻¹) as a selection agent for the transformed fungi and again cefotaxim (200 μ M). The plates were incubated at 23°C and after 8–10 days discrete colonies developed. Each colony was checked under the fluorescence microscope and those showing the typical GFP fluorescence were subcultured for further studies. To determine their mitotic stability, all transformants were successively cultured in PDB for at least seven generations without supplementation of hygromycin B and checked under the fluorescence microscope. Throughout this subcultivation, the transformants did not show any alterations in growth or colony morphology compared to the wild-type.

In vitro root inoculation assay

Sterilized seeds of *B. napus* cv. ‘Falcon’ were sown on a cellophane membrane placed on water agar in Petri dishes, preventing the roots from growing into the medium and therefore allowing for an undisturbed microscopic analysis of the interaction between plant roots and fungus. The Petri dishes were sealed with Parafilm and subjected to a light regime of 14/10 h (light/dark) and a temperature of 23/20°C (day/night) in a climate cabinet. This system kept the intact

plants free from contaminations by other microorganisms. After the plants had developed a well-defined root system, droplets of a spore suspension of either the transformed *V. longisporum* or the transformed *V. dahliae* strain were placed on the cellophane membrane close to the roots.

Gnotobiotic sand inoculation systems

Sterilized seeds of *B. napus* cv. ‘Falcon’ were sown in pots (7 × 7 cm) containing double-autoclaved silica sand resulting in two plants per pot. This system was used in order to provide conditions for roots approximating to the unsterile situation in natural soils, but nevertheless enabling the harvest of complete clean roots without any adhering soil or organic particles. The pots were watered daily and fed two times a week with a full nutrient solution (‘Flory Basisdünger’, EUFLOR) throughout the experiments. Plants were kept in a controlled environment chamber at 23/20°C (day/night) and 14-h day length (Philips TL5 HO lamps).

Inoculations were either made by direct application of spore suspensions to the sand or by root-dipping. Direct inoculation was conducted one week after germination. The plantlets were inoculated by pouring 8 ml of a spore suspension of transformed strains of *V. longisporum* or *V. dahliae* per plant on the sand surface. After inoculation, all plants were gently watered in order to wash the inoculum into the soil and to evenly spread the spores in the rhizosphere of the plants.

Root-dipping inoculation was used in the experiments for real-time PCR analysis and the documentation of symptom development in the *B. napus*/*V. longisporum* and *B. napus*/*V. dahliae* interaction. After growing for 10 days, seedlings were carefully removed from the sand and the roots washed gently under tap water. Inoculation was performed by dipping the intact roots in a spore suspension of either *V. longisporum* isolate VL 43 or *V. dahliae* isolate VD 73 for 30 min. Control plants were dipped in sterilized tap water for the same time. Subsequently, 60 plantlets of each variant (non-inoculated control, VL, VD) were transferred into pots with a sand:soil (1:1) mixture and grown in a climate chamber under standardised environmental conditions (see above).

Assessment of disease development

Plants were scored weekly for disease symptoms using an assessment key with nine classes (Table 1; following Zeise 1992, modified). Scoring was conducted weekly over five weeks. AUDPC values (area under the disease progress curve) were calculated from the disease severity values according to the following formula (Campbell and Madden 1990):

$$\text{AUDPC} = \sum (y_i + y_{i+1} / 2) * (t_{i+1} - t_i)$$

where y_i is the disease severity value for observation number i , t_i is the corresponding number of days post-inoculation, and n the number of observations. Statistical analyses were conducted with the computer software StatGraphics. Differences among means were tested using Fisher’s least significant difference (LSD).

Staining and microscopy of inoculated plant tissue

All microscopic investigations were conducted with GFP tagged strains. Examinations of the in vitro infection were started 11 h post-inoculation (hpi) and continued at 12 h intervals for the first 4 days using different plants for each time point. Additional observations were made after 3 weeks in order to examine the development of microsclerotia in the root tissue.

Examinations of plants grown in the sand system were started 24 hpi and continued at 24 h intervals for 7 days and at 48 h intervals in the following week.

Table 1 Assessment key for scoring disease symptoms induced by *Verticillium sp.* on young *B. napus* plants inoculated in the greenhouse (following Zeise 1992, modified)

Score	Symptom description
1	no symptoms
2	slight symptoms on oldest leaf (yellowing, black veins)
3	slight symptoms on next younger leaves
4	about 50% of leaves show symptoms
5	>50% of leaves show symptoms
6	up to 50% of leaves dead
7	>50% of leaves dead
8	only apical meristem still alive
9	plant dead

Furthermore, plants were investigated after 3, 4, and 5 weeks for the extent of colonization of the xylem vessels. For interaction studies with the sGFP tagged strains, whole roots were placed directly on glass slides in drops of water, covered with a cover glass, and examined. For the classical histological studies, whole roots or free-hand made sections of roots and stems were stained either with 0.05% acid fuchsin (C.I. 42685, Merck; 1 g 100 ml⁻¹ A. dest.) dissolved 1:10 in lactophenol or in a 1% acridine orange (C.I. 46005, Merck; 0,1 g 100 ml⁻¹ A. dest.) solution in water. After rinsing the samples either with lactophenol or water they were mounted on glass slides in drops of lactophenol or water, respectively, closed with a cover slip and immediately examined. Observations were carried out in at least four independent experiments.

Microscopic analyses were performed with a Leica TCS SP2 Confocal Laser Scanning Microscope (CLSM; Leica, Mannheim, Germany). Digital images of GFP tagged strains were acquired by scanning with 488 nm excitation and 520–540 nm emission filters. Settings for acid fuchsin fluorescence were 543 nm for excitation and 560–620 nm for emission. Digital images of acridine orange-stained specimens were acquired by two-channel-analysis with subsequent drafting of an overlay (488 nm for excitation/500–530 nm for emission and 543 nm for excitation/560–650 nm for emission). Stacks of optical sections were processed to maximum projections.

DNA extraction and real-time PCR analysis

Ten plants from each treatment were harvested for real-time PCR analysis at weekly intervals from 7 dpi (days post-inoculation) until 35 dpi. Hypocotyls and leaves were separated resulting in twenty tissue samples and PCR analyses for each treatment. Roots were not included in the analysis as a proper discrimination between fungal biomass in the roots from fungus merely attached to the root surface through inoculation was not possible.

First, the plant tissue was ground in liquid nitrogen using a mortar and a pestle resulting in a fine powder. DNA extraction was conducted using the DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Fungal biomass was quantified by determination of

fungal DNA in infected plant extracts with real-time PCR. Primers OLG 70 (CAGCGAAACGCGATATGTAG) and OLG 71 (GGCTTGTAGGGGGTTT-AGA) (P. Karlovsky, unpublished) served to multiply a fragment specific for both *V. longisporum* and *V. dahliae*. For amplification and melting curve analysis the iCycler System (BioRad, Hercules, CA, USA) was used.

The reaction mixture consisted of NH₄-reaction buffer (16 mM (NH₄)₂SO₄, 67 mM Tris-HCl, 0.01% (v/v) Tween-20, pH 8.8 at 25°C, Bioline, Luckenwalde, Germany), 3 mM MgCl₂ (Bioline, Luckenwalde, Germany), 0.2 mM of each dATP, dTTP, dCTP and dGTP (Bioline, Luckenwalde, Germany), 0.3 μM of each primer, 0.25 u BIOTaq DNA polymerase (Bioline, Luckenwalde, Germany), 10 nM fluorescein (BioRad, Hercules, CA, USA), 100,000 times diluted SYBR Green I solution (Invitrogen, Karlsruhe, Germany), 2 μl of template DNA and ddH₂O up to 25 μl. The quantification of PCR products was performed using filters with optimal settings for SYBR Green I which are 490 ± 10 nm for excitation and 530 ± 15 nm for emission.

PCR amplification was carried out using an initial denaturation step for 2 min at 94°C, which is followed by 36 reaction cycles consisting of a 20 s denaturation step at 94°C, an annealing step for 30 s at 59°C and 40 s at 72°C. The final elongation was performed for 5 min at 72°C. During the amplification process, the detection of fluorescence was carried out in the annealing step of each cycle. To verify amplification of the specific target DNA, a melting curve analysis was included. Melting curves were acquired by heating the samples to 95°C for 1 min, cooling to 55°C for another min and then slowly increasing the temperature from 65 to 95°C at the rate of 0.5°C s⁻¹, with a continuous measurement of the fluorescence. The amount of DNA of *V. longisporum* and *V. dahliae*, respectively, was estimated from a calibration curve using increasing amounts of genomic *V. longisporum* DNA from 0.5 to 64.0 pg. Preliminary studies confirmed that the amplification is not hampered by the plant matrix. The concentration of *V. longisporum* DNA used for the construction of the calibration curve was estimated by densitometry of agarose gels stained with ethidium bromide, using Lambda Phage DNA as a standard.

Results

Disease development

Fourteen dpi, first disease symptoms appeared on *V. longisporum* infected plants, in the form of chlorosis and dark-coloured veins especially on older leaves (Table 2). At 21 dpi, all plants inoculated with *V. longisporum* showed stunted growth and moderate to less severe disease symptoms. Thus, 20% of the plants had symptoms on 50% or more of the leaves. This corresponds to disease scores from 4 to 8 (see Table 1). In the following weeks the disease developed until 35 dpi when nearly 50% of the inoculated plants showed severe disease symptoms. Plants inoculated with *V. dahliae* did not express any symptoms significantly different from the control and occasionally, they even looked healthier than the control plants. The corresponding AUDPC values were 46.6 for the control, 45.9 for *V. dahliae* and 71.1 for plants inoculated with *V. longisporum*.

GFP expression in *V. longisporum* and *V. dahliae*

Overall, 33 transgenic *V. longisporum* and 20 *V. dahliae* isolates were obtained. Eighty percent of the hygromycin B-resistant *V. dahliae* and 60% of the obtained *V. longisporum* isolates expressed the GFP, which was a success rate similar to reports either on *Agrobacterium*-mediated transformation (Lagopodi et al. 2001; Oren et al. 2003) or electroporation (Robinson and Sharon 1999). GFP expression was generally high and uniform in conidia and hyphae with the exception of vacuoles that did not show any fluorescence and appeared as dark areas in the fungal cytoplasm (Fig. 2A–D). In contrast to this, microsclerotia did not fluoresce at all, probably because of

the melanin deposited in the cell walls. GFP expression remained stable after successive transfers on Czapek Dox medium with and without hygromycin B.

Suitability of GFP-tagged strains vs. classical staining methods

In this study, we compared the suitability of tagging fungi with GFP with conventional fluorescence staining for plant-fungus interaction studies. Due to the fact that GFP is constitutively expressed in the cytoplasm of the transformed fungi, only younger hyphae showed bright fluorescence and are thus well visible under the fluorescence microscope. This hampers a comprehensive analysis of all stages of colonization and infection at one particular time point. In order to localize the fungal structures in the plant tissue we used the overlay feature of the CLSM in order to generate compound images of normal transmission underneath the corresponding fluorescence image. The result, however, was not satisfactory and allowed only for a rough localization of the fungus in the plant tissue (Fig. 2E, F).

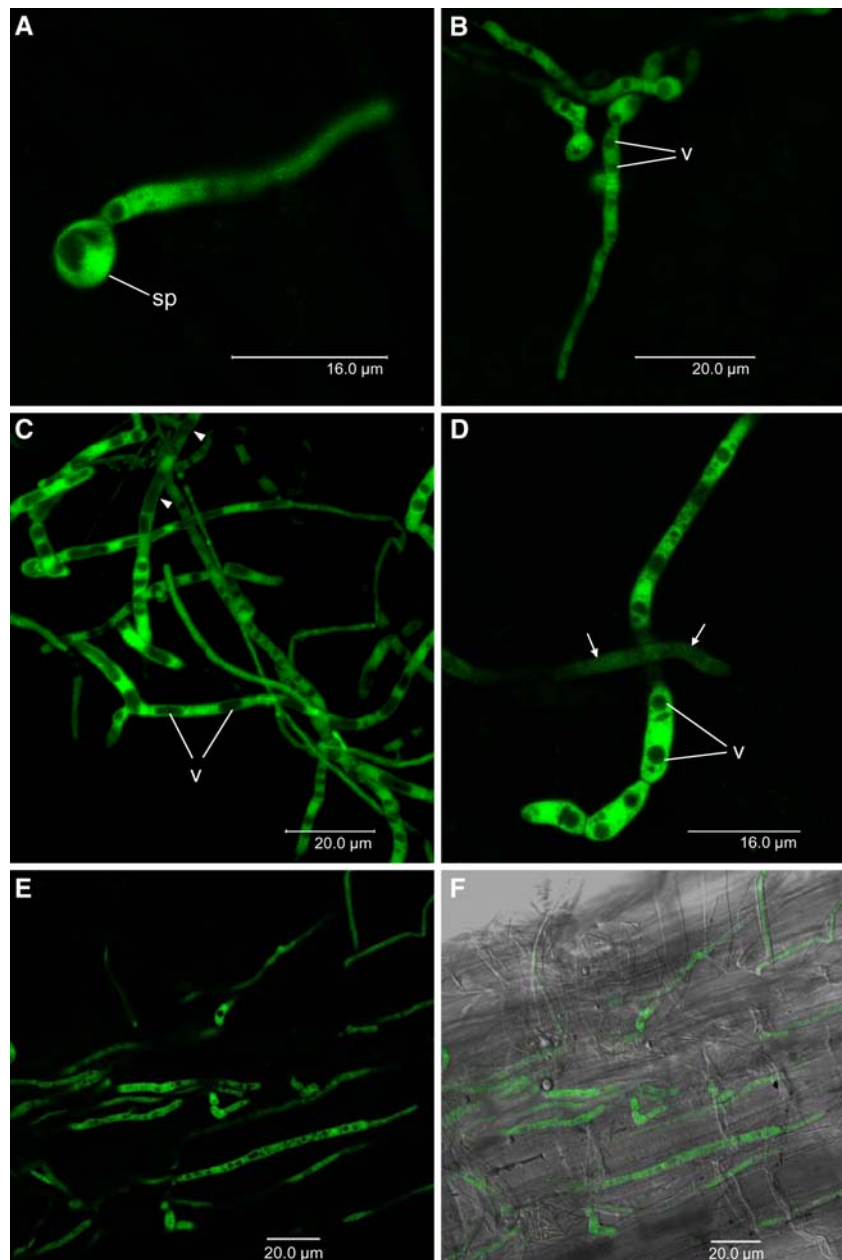
By applying acid fuchsin or acridine orange as conventional dyes we obtained a strong staining of all fungal structures but also a faint but distinct staining of cell walls and the plasma membranes allowing for accurate studies of the pathogen invading the plant tissue. As a further problem with the GFP samples, photobleaching occurred after repeated and extended scanning on the same sample sites. In general, this problem can be overcome by reducing the energy level of the laser. However, this option is limited by the fluorescence intensity of the referring object. Regarding all these facts, we concluded that the conventional staining techniques combined with

Table 2 Means of disease scores on *B. napus* plants inoculated with *V. longisporum* (+VL) or *V. dahliae* (+VD) compared to control plants treated with water

Treatment	Days post-inoculation (dpi)				
	7	14	21	28	35
Control	1.00 (±0.00) a	1.00 (±0.00) a	2.00 (±0.00) a	2.40 (±0.15) a	2.50 (±0.17) a
+VD	1.00 (±0.00) a	1.00 (±0.00) a	2.00 (±0.00) a	2.31 (±0.15) a	2.50 (±0.16) a
+VL	1.00 (±0.00) a	1.42 (±0.00) a	3.31 (±0.00) a	3.35 (±0.31) b	4.58 (±0.79) b

Standard error is shown in brackets. Values followed by the same letter do not differ on a significance level of $P \leq 0.05$ (LSD). For disease assessment key see Table 1

Fig. 2 Confocal microscopy image of *Verticillium longisporum* and *V. dahliae* expressing GFP. **(A)** Germinating spore of *V. longisporum*. **(B)** Hyphae of *V. dahliae*, vacuoles of fungal cells are visible as dark areas in the fluorescing cytoplasm. **(C)** Mycelium of *V. longisporum* showing hyphal dimorphism with very thin and normal, vacuolated hyphae. Arrow heads mark areas of weak fluorescence. **(D)** Hyphae of *V. longisporum*. Arrows point to a very weak glowing hypha. sp = conidium, v = vacuole. **(E)** Fluorescence image of GFP-tagged mycelium of *V. longisporum* on a root of *B. napus* (48 hpi). **(F)** Compound micrograph of bright field transmission and corresponding fluorescence images (same view as E)



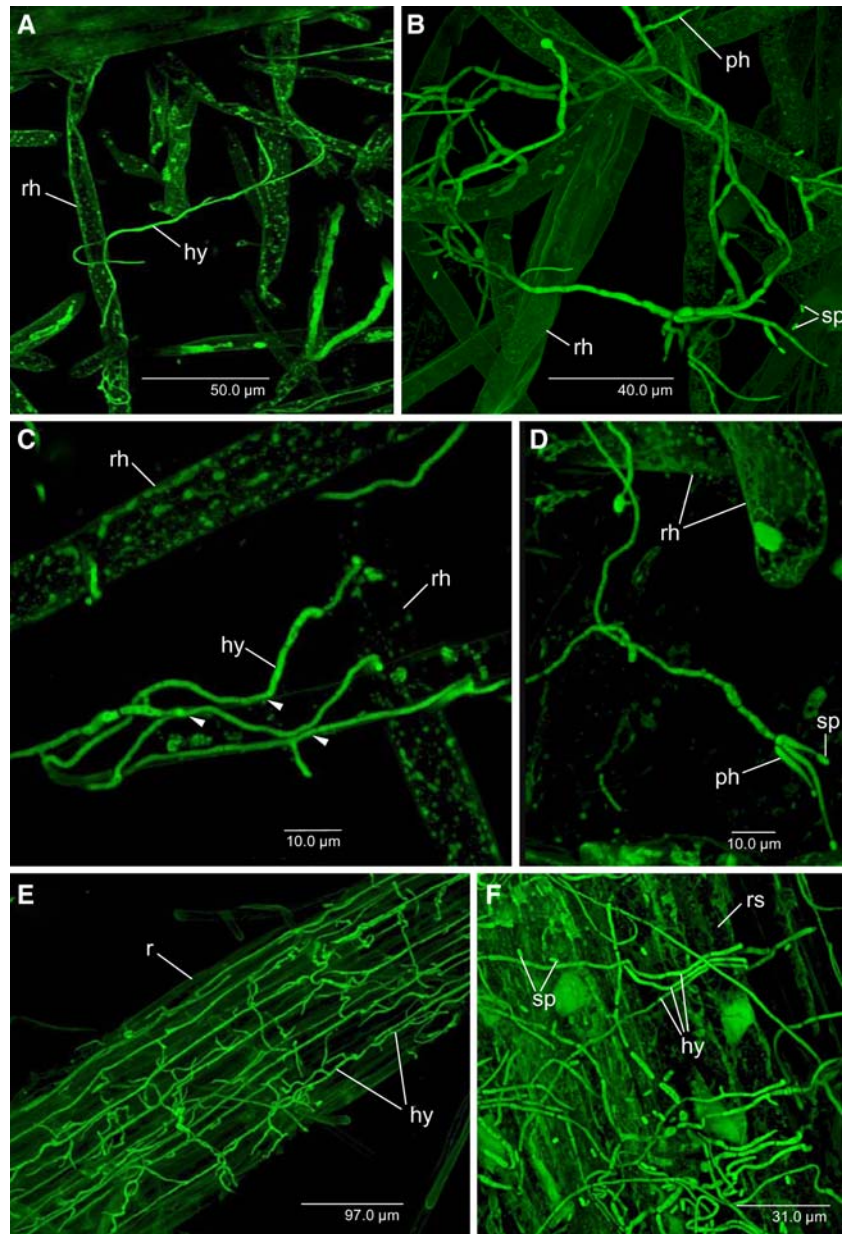
CLSM were superior to the GFP tagging for in situ studies of pathogens at least in the present pathosystems and we therefore decided to continue further studies with fuchsin and acridinorange.

Plant-pathogen interaction studies

Spores of *V. longisporum* and *V. dahliae* started to germinate after *in vitro* infection 11 hpi. Usually, a

secondary germ tube developed in opposite position to the emergence site of the primary germ tube. In the sand culture system, hyphae of both *V. longisporum* and *V. dahliae* were found 24 hpi intensely interwoven with the root hairs close to the main and lateral roots. A tight attachment of hyphae to the root hairs was observed at random positions along the root hair zone for both fungi (Fig. 3A–C). The root tip was the only part of the root, which was not colonised. At

Fig. 3 Early stages of root colonization by *V. longisporum* and *V. dahliae* on oilseed rape, as observed by confocal laser scanning microscopy after staining with acid fuchsin. The classical staining method results in a stable and homogenous bright green fluorescence of the entire fungal biomass and a faint background staining of the plant tissue. A–D: 24 hpi; E, F: 48 hpi. (A) Contact of hyphae of *V. longisporum* with root hairs. (B) Intermingling of hyphae of *V. dahliae* with root hairs. Already at this stage the fungus produces spores. (C) Attachment (arrow heads) of fungal hyphae of *V. longisporum* to root hairs. (D) *V. dahliae*, production of conidia inbetween root hairs. (E) Hyphae of *V. longisporum* growing along the junctions of the epidermal cells forming a network. (F) *V. dahliae* growing in an undirected manner on the root surface of *B. napus*. hy = hypha, ph = phialide, r = root, rh = root hair, sp = conidium



24 hpi, *V. dahliae* already showed colonization of the root to a much lesser extent than *V. longisporum* and started to produce ample masses of conidia in the vicinity of root hairs (Fig. 3B, D). In contrast, the formation of conidia by *V. longisporum* was never observed outside the roots throughout the investigations.

Hyphae of *V. longisporum* showed growth on and along root hairs towards the root surface. At 36 hpi, the first contacts between hyphae and the root surface

were observed. After attachment to the root surface, hyphae of *V. longisporum* grew strictly following the grooves of the junctions of the epidermal cells. At 48 hpi, the root surface was covered with a closely attached hyphal net depicting the cellular structure of the root epidermis (Fig. 3E). In contrast, growth of *V. dahliae* hyphae on the root surface was random not following any such pattern (Fig. 3F).

At 60 hpi, first penetrations of *V. longisporum* into the roots were observed. The fungus entered the root

tissue by direct penetration of the epidermal cells without forming any conspicuous infection structures like appressoria or hyphopodia. Only slight hyphal swellings were formed before entering epidermal cells (Fig. 4A), probably due to the accumulation of cytoplasm in the hyphal tip as a response to the mechanical resistance of the plant tissue. Plant cell walls were perforated by a thin penetration peg. In the lumen of the epidermal cells, the hyphae regained their regular diameter (Fig. 4B). Subsequently, hyphae grew intracellularly and intercellularly in the root cortex, in a more or less directed manner towards the central cylinder (Fig. 4C). Whenever growing through cell walls, hyphae showed the typical sequence of swelling, constriction to a narrow infection peg and regaining the regular size after penetration (Fig. 4D). Remarkably, the roots, although intensely colonised by *V. longisporum*, did not show any responses such as discolouration or necrotic lesions. Even host cells invaded by intracellular hyphae showed an intact structure of the cytoplasm.

Verticillium dahliae was also able to penetrate the root tissue, but this occurred much less frequently (Fig. 4E). In the root cortex, *V. dahliae* was preferentially found in the intercellular rather than the intracellular space (Fig. 4F). Also, in the in vitro inoculation system *V. dahliae* started to produce microsclerotia after 2 weeks. Interestingly, microsclerotia of *V. dahliae* were not formed in the roots or on the root surface but were scattered on the surrounding medium. In contrast, microsclerotia of *V. longisporum* were consistently formed in the root cells, which were filled with microsclerotia 3 weeks after inoculation.

Colonisation of the xylem vessels of the shoot with *V. longisporum* started three weeks after inoculation. However, the fungus never managed to colonise the entire vascular system. Moreover, it was restricted to individual vessels which, however, were entirely filled up with mycelium (Fig. 5A, B). Adjacent xylem vessels were easily invaded through plasmodesmata (Fig. 5A). Conidia were formed either by budding (Fig. 5C, D) or on phialides arranged in a typical verticillate manner (not shown).

Real time-PCR analysis

Seven days post-inoculation both *V. longisporum* and *V. dahliae* were detectable in the hypocotyls of

infected plants (Table 3). The amount of *V. longisporum* DNA was substantially higher than that of *V. dahliae*, corroborating the histological studies. The amount of *V. longisporum* continued to increase in the hypocotyl until 35 dpi, whereas *V. dahliae* remained at a very low level. In leaves, *V. longisporum* was first detectable in one single plant at 14 dpi, then slightly increased until 28 dpi, and sharply spread at 35 dpi. In contrast, no significant colonisation of *B. napus* leaves with *V. dahliae* was detected throughout the time of observation.

Discussion

This study reports on the *A. tumefaciens*-mediated transformation of the phytopathogenic fungi *V. longisporum* and *V. dahliae*, resulting in the stable expression of GFP. This transformation method proved to be an effective tool to introduce foreign genes into the genome of *Verticillium* species, as indicated by the relatively high number of gained transformants. In comparison, attempts of a DNA-mediated transformation of *V. dahliae* spheroplasts resulted in a low transformation efficiency (Dobinson 1994). The transformants obtained in this study were indistinguishable from the wild-type strain concerning colony morphology, growth rate and pathogenicity/aggressiveness (data not shown). However, the transformants showed differences in the fluorescence intensity due to different levels of GFP expression. This may be caused by positional effects resulting from different sites of integration into the fungal genome. The phenomenon of older hyphae showing a reduced or no expression of GFP has been described earlier in studies on *Leptosphaeria* spp. and *Oculimacula* spp. (Eckert et al. 2005). This may be due to the fact that the cytoplasm in senescing mycelium is translocated from the older into younger hyphae.

In our studies we directly compared GFP fluorescence with conventional staining using a fluorescence dye. This analysis clearly revealed a superior performance of the applied fluorochromes as the entire mycelium was made visible regardless of its physiologic state. In addition, the faint non-specific staining of the plant tissue enabled a proper localization of the fungus in the host. Nevertheless, there are still several advantages in the GFP labelling of plant pathogens. First, GFP fluorescence requires no

Fig. 4 Advanced stages of the interaction of *V. longisporum* and *V. dahliae* with roots of *B. napus*. Confocal laser scanning microscopy analysis after staining with acid fuchsin and acridine orange (C.). A–C, E, F = 60 hpi; D = 96 hpi. (A) Hyphae of *V. longisporum* growing along a root hair towards the root surface, penetrating an epidermal cell and growing further into the root cortex. Asterisks mark the points of intracellular penetration through plant cell walls. (B) Intracellular growth of *V. longisporum* in the root cortex. Arrow heads mark the plant cell wall which is penetrated twice (asterisks). After penetration, hyphae regain their regular diameter. (C) Directed growth of *V. longisporum* in the root cortex towards the xylem. Arrow heads assign points of penetration. (D) Magnified view of the penetration of a plant cell wall (arrow heads) by *V. longisporum* in the root cortex. Asterisk marks the swelling of the hypha before penetrating with a thin penetration peg. (E) Growth of *V. dahliae* in the root cortex of *B. napus*. Asterisk marks a primary penetration event. (F) Mainly intercellular growth of *V. dahliae* in the root cortex. cw = cell wall, hy = hypha, rh = root hair, xy = xylem, xyp = xylem parenchyma

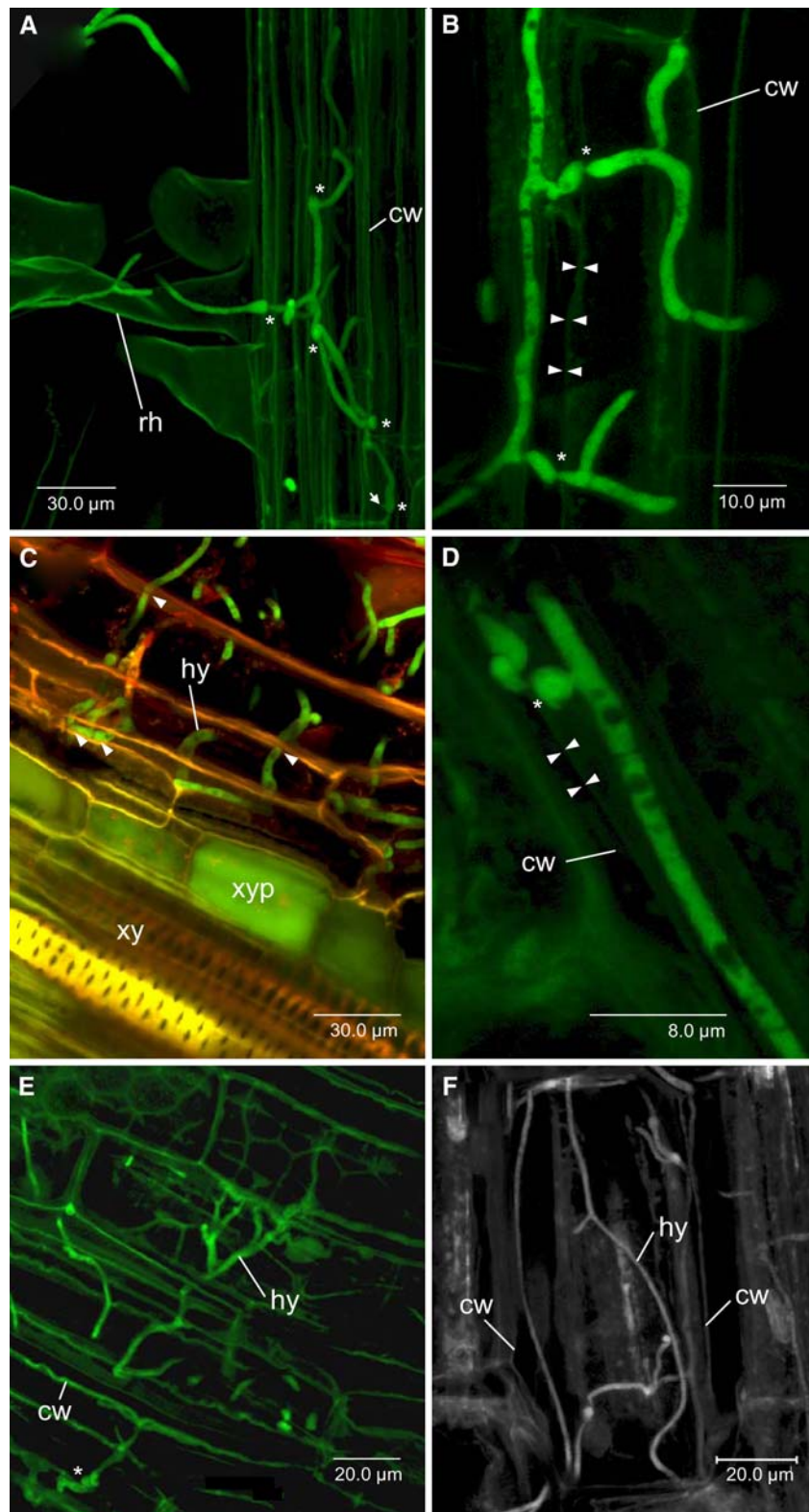


Fig. 5 Colonization of the xylem of *B. napus* by *V. longisporum*. Confocal laser scanning microscopy analysis after staining with acid fuchsin (21 dpi). (A) Hyphae of *V. longisporum* in xylem vessels of *B. napus*. Proliferation of mycelium into adjacent vessels through plasmodesmata (asterisk). (B) Colonization of a single vessel element filled with mycelium. (C) Hyphal growth and production of conidia in a xylem element. (D) Conidia clumped together at the end of a tracheid. hy = hypha, xy = xylem elements, sp = conidia

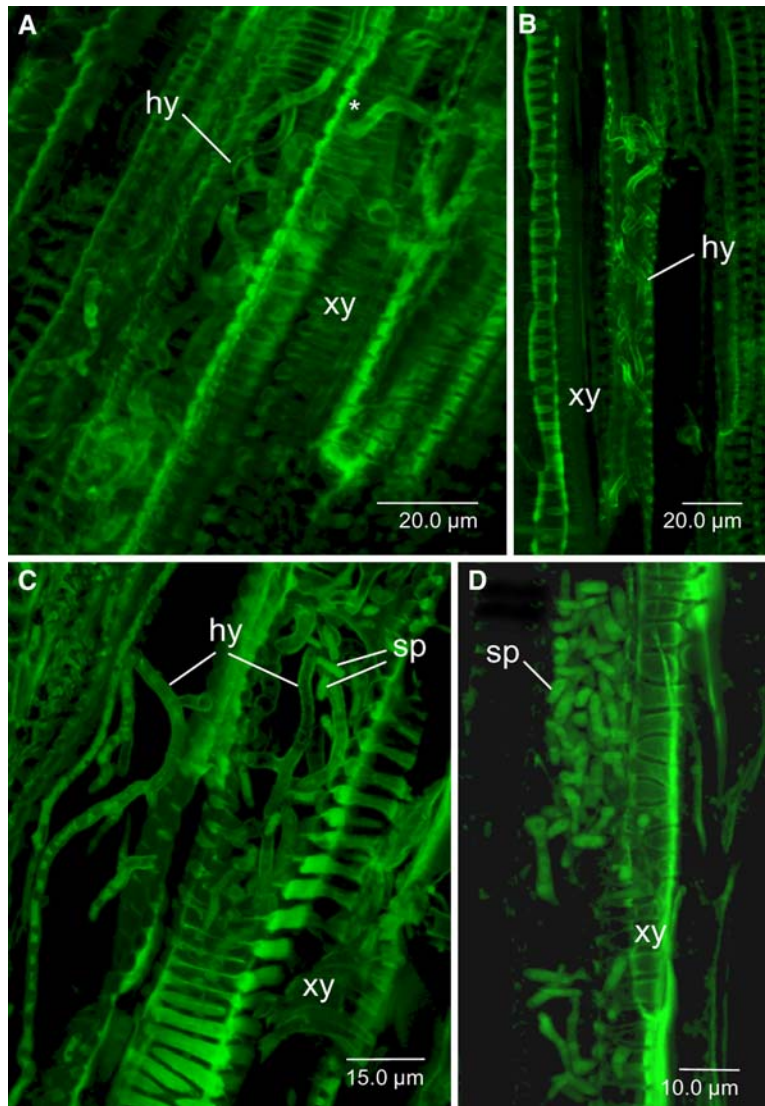


Table 3 Detection of *Verticillium*-DNA with real-time PCR in tissue samples of *B. napus* seedlings inoculated with *V. longisporum* (VL) or *V. dahliae* (VD)

Tissue	Leaves		Hypocotyl	
	ng VL-DNA/g FW	ng VD-DNA/g FW	ng VL-DNA/g FW	ng VD-DNA/g FW
7 dpi	0.00 (± 0.00)	0.00 (± 0.00)	2.88 (± 0.89)	0.53 (± 0.18)
14 dpi	0.01 (± 0.01)	0.00 (± 0.00)	9.94 (± 2.79)	0.18 (± 0.09)
21 dpi	0.51 (± 0.29)	0.02 (± 0.02)	3.95 (± 1.65)	0.05 (± 0.02)
28 dpi	0.59 (± 0.26)	0.01 (± 0.01)	10.41 (± 1.52)	2.62 (± 1.18)
35 dpi	6.89 (± 5.37)	0.06 (± 0.06)	25.58 (± 6.79)	0.24 (± 0.12)

Values are means of the amount of fungal DNA in leaves and hypocotyls; \pm standard error

co-factors or substrates and allows for a fast processing of the plant material. Further, the risk of potential artefacts is negligible due to the absence of additional chemicals. This enables time-lapse observations in vivo (Lagopodi et al. 2001). Also, studies of pathogens in non-sterile conditions would make GFP tagging the preferential approach.

There are several similarities from our microscopic studies of *V. longisporum* on and in *B. napus* roots with earlier reports on the infection process of *V. dahliae* on a wide range of host plants (Schnathorst 1981; Beckmann 1987; Gold et al. 1996). However, our studies also provide significant novel information about colonization and infection. This particularly applies to the early interaction, including recognition and first contacts between host and pathogen. We observed that *V. longisporum* initially approaches the roots by following the root hairs. This may be due to a stronger chemical attraction of the fungus to the root hairs than the root surface, as suggested in an earlier study with *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato roots (Lagopodi et al. 2001). Thereafter, hyphae of *V. longisporum* attached to the root surface where they preferably spread in a typical pattern of superficial hyphae. Primary infection occurs either at the junctions of epidermal root cells or directly into epidermal cells. We never observed penetration at the very root tip or into root hairs. This is in contrast to a former study (Zhou et al. 2006) where infection by *V. longisporum* was primarily localized in lateral roots or root hairs. Further, there was no indication that natural wounds from emerging lateral roots are necessary for infection, which is in agreement with previous studies on GFP-expressing *F. oxysporum* f. sp. *radicis-lycopersici* on tomato roots (Lagopodi et al. 2001) and *Fusarium verticillioides* on maize roots (Oren et al. 2003). It may therefore be speculated whether there exists a common mode of root colonization and infection by vascular pathogens, in which the pathogens are chemically directed to the root hair zone where they first attach and then penetrate directly into the epidermal cells (Oren et al. 2003).

The observation that even massive inoculation with *V. longisporum* results in a colonisation merely restricted to individual xylem vessels, while others remain entirely free of the fungus, has not so far been reported. Nonetheless, *V. longisporum* was able

to penetrate into adjacent vessels after being trapped at vessel end walls, which was similar to recent studies of *V. longisporum* (Zhou et al. 2006) and *V. albo-atrum* (Heinz et al. 1998), but has not been shown so far for *V. dahliae*. This partial colonisation may be an explanation for the absence of wilting symptoms in *V. longisporum*-infected oilseed rape, as observed both in the greenhouse and in the field. However, wilting is not only caused by the physical blocking of vessels but may also be induced by wilting toxins, which, however, are yet unknown for *V. longisporum*.

The interaction of *V. dahliae* with *B. napus* roots completely differs that of from *V. longisporum*. The undirected growth of hyphae which are only loosely attached to the root surface and the early, massive production of conidia and microsclerotia outside the root tissue strongly suggest that *B. napus* is not a suitable host plant for *V. dahliae*. This colonization pattern might be the result of stress or a deficiency situation derived from a non-host interaction. Nevertheless, *V. dahliae* was infrequently able to penetrate and colonize the root tissue, however, it rarely reached the shoots or leaves as indicated by histological and real-time PCR analyses. In conclusion, the present results demonstrate that the poor susceptibility of *B. napus* to *V. dahliae* is only partly due to the restriction of penetration but mainly related to inhibition of systemic growth into the shoot, which is in agreement with earlier observations (Zhou et al. 2006). While *V. longisporum*, upon penetration, readily spreads into the vascular system, the systemic growth of *V. dahliae* is strongly inhibited due to yet unknown factors. This kind of restricted invasion of a vascular pathogen represents an interesting intermediate case of parasitism, as root penetration is not followed by invasive spread in the host. As disease symptoms are lacking, the lifestyle of *V. dahliae* after infection resembles an endophytic state and implies a systemic non-host resistance. Similarly, a restriction to the basal plant parts has been found with certain other wilt pathogens in plants with enhanced host plant resistance (Beckman 1987).

The mechanisms governing the expression of this specific type of non-host resistance are not known. Increased synthesis of the aromatic glucosinolate gluconasturtiin in the roots appeared to be a major factor in the active resistance response of oilseed rape against a non-pathogenic *V. dahliae* strain which

failed to colonize the plant (Karapapa et al. 1997a). The pathogenic *V. longisporum* strain was either capable of suppressing gluconasturtiin synthesis in roots, or failed to trigger its synthesis by avoiding recognition. This implies an adaptation of *V. longisporum* to oilseed rape as its host, potentially by being less sensitive to this kind of host-specific defence, as compared to *V. dahliae*. This is corroborated by studies in which the pathogenicity of *V. longisporum* on high and low glucosinolate-producing genotypes of *B. napus* did not differ (Heale and Karapapa 1999). Similarly, Zhou et al. (2006) did not find any differences in the growth of *V. longisporum* on agar containing plant extracts from high or low glucosinolate oilseed rape varieties.

Finally, the present study provides cytological evidence that infrequent infection of OSR with *V. dahliae* may occur, but vascular colonization and disease development can be excluded. This finding confirms earlier studies on host range (Zeise and von Tiedemann 2002b) and is of importance in fields where oilseed rape is grown in rotation with susceptible hosts of *V. dahliae* such as potato, strawberry or sugar beet (Pegg and Brady 2002), and where enhanced soil infestation with microsclerotia from both *Verticillium* species may occur.

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