Verticillium longisporum – identifying resistance to a new threat in winter oilseed rape

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Abstract

Verticillium in oilseed rape (OSR) is known since the 1930s in Sweden and the 1980s in Germany. The pathogen, *Verticillium longisporum* (VL), is host-specific to the *Brassica* species and particularly threatens the production of OSR in intense crop rotations. In contrast to diseases induced by other Verticillium species, VL does not induce wilt symptoms in OSR but stunted growth and premature bloom and maturity. The colonization of the plant by VL in the field is characterized by an extended latent phase until the beginning of maturity after which the pathogen bursts out of the xylem vessels to produce ample masses of microsclerotia underneath the stem epidermis, in the stem pith and roots. We transformed VL with the green fluorescent protein (GFP) or used conventional fluorescence dyes in interaction studies on roots of *Brassica napus* with the confocal laser scanning microscope. The pathogen infects the roots in the root hair zone by direct penetration of the rhizodermis without formation of appressoria-like structures and invades the vascular system, where it spreads by hyphal growth and the production of conidia. In the field, detection of VL in infected plants by ELISA (or PCR) revealed the spread of infection being retarded until early maturity stages of the crop (> GS 85). As chemical control of VL is not possible, a broad search for sources of resistance in *B. napus* lines and parent *B. oleracea* and *B. rapa* genotypes has been started. In this screen based on a greenhouse bioassay a considerable variation in disease severity among *Brassica* genotypes was found. Resistance predominantly originated from *B. oleracea* accessions, but also from a few *B. rapa* and *B. napus* lines. Resistance was due to inhibition of fungal spread in the vascular system rather than through the prevention of root penetration. Reduced pathogen spread in less susceptible genotypes was associated with distinct phenolic responses in the adjacent xylem parenchyma of the hypocotyl. Resistant genotypes detected in the greenhouse were evaluated under field conditions at several locations in Germany in 2006. The final objective of these studies is to characterize and evaluate resistance factors against *V. longisporum* and to identify sources of resistance potentially useful in breeding programmes.

Key words: oilseed rape, *Brassica* spp., Verticillium, confocal laser scanning microscopy, gfp, vascular diseases

Introduction

Verticillium wilt on oilseed rape (*Brassica napus* L. spp. *oleifera*) is caused by the host-adapted fungal pathogen *Verticillium longisporum* (Karapapa et al., 1997b; Zeise & von Tiedemann, 2001, 2002). Because of an increasing area under rapeseed cultivation and an accumulated crop rotation this disease has become a major threat to oilseed rape (OSR) production particularly in Northern Europe (Krüger, 1989). In Sweden, Verticillium wilt is known since the 1930s (Svenson, & 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 & Seidel, 1990). Because *V. longisporum* is a soil-borne pathogen, chemical plant protection by fungicides is ineffective. This problem is aggravated due to the fact that the fungus is able to survive in the soil for decades by means of microsclerotia that are produced in senescing plant tissue. Finally, for both winter and spring rapeseed, breeding for resistance has been severely hampered by the absence of sufficient resistance in commercially available breeding material.

Against this background the aim of these studies is on the one hand the identification of resistance sources potentially useful in breeding programmes. For these purposes a comprehensive screening based on a greenhouse bioassay as well as downstream tests for field resistance at different locations in Germany were conducted. A further objective is to characterize and evaluate potential resistance factors in Brassica species against *V. longisporum*. In order to elucidate at which interaction state defence reactions may occur, we conducted interaction studies particularly on and in roots of *B. napus* by confocal laser scanning microscopy comparing the use of a GFP-labelled strain with a technique combining conventional fluorescence staining with confocal microscopy. Finally we investigated the occurrence of different phenolic compounds and their role in disease resistance.

Materials and Methods

Greenhouse screening: Different Brassica species were tested for resistance towards *V. longisporum* under standardised environmental conditions, among them mainly *B. napus* varieties but also some *B. oleracea*- and *B. rapa*-accessions as well as resynthesised oilseed rape forms. Inoculation of 10 day-old plantlets of the control varieties ´Express` (tolerant, Happstadius et al., 2003) and ´Falcon` (susceptible) along with those of the particular accessions was performed by a root-dip-method (Zeise, 1992). Subsequently, 24 inoculated and 24 control plants of each accession were transferred into a sand-peat-compost (1:1:2)

mixture in pots containing two plants each. Each plant was scored weekly for disease symptoms over a time period of four weeks using an assessment key slightly modified from Zeise (1992). Area under the disease progress curve (AUDPC) values were calculated from the disease severity values according to Campbell and Madden (1990).

Field trials 2005/06: Some of the *B. napus* accessions that had shown a certain level of resistance in the greenhouse were grown in the field at four different locations in Germany, one being artificially inoculated with Verticillium-infested straw. Immediately after harvest, at each site a total of fifty stubbles per accession was collected and scored for the occurrence and amount of microsclerotia produced under the epidermis, in the stem pith and in the root tissue. At one location, samples of root and the lower stem part were collected throughout the vegetation period in order to follow the spread of the fungus in the plant by ELISA analysis.

Interaction studies: Plants of the susceptible winter oilseed rape *cv.* ´Falcon` were grown either in double-autoclaved silica sand or *in vitro* on solid MS medium and inoculated with spores of *V. longisporum*. In order to visualize the colonization of the oilseed rape rhizosphere by confocal laser scanning microscopy (CLSM), we used a GFP-labelled strain or applied the conventional fluorescence dyes acid fuchsin and acridine orange. *Histochemical and biochemical analysis of phenolics*: For the histochemical detection of phenolics, cross sections of hypocotyls of 3 Brassica genotypes with different resistance levels were stained at certain time points after infection either with toluidine-blue (Gerlach, 1984) or Folin-Ciocalteau reagent (Singleton et al., 1974). Lignin was detected with phloroclucinol-HCl (Johannsen, 1940; Siegel, 1953). The total amounts of free polyphenols as well as cell-wall-bound polyphenols (released after alkaline hydrolysis) were determined with Folin-Ciocalteau reagent using tannic acid as a standard. Total amount of lignin was determined using thioglycolysis (Lange, et al., 1995).

Results

Greenhouse screening: A total of about 1.400 Brassica genotypes were tested for resistance to Verticillium wilt in a greenhouse test. There was large variation in response to Verticillium infection among the used accessions. Thus, we could observe an enhanced level of resistance in several accessions from the group of *B. oleracea* genotypes and a predominantly low resistance level in the *B. rapa* pool. Accessions from *B. napus* also showed a high degree of variation. Nevertheless, some *B. napus*-types with resistance levels higher than that of ´Express` were found. Screening of resynthesised *B. napus* material indicated a resistance level significantly superior to the cv. ´Express` in most of the tested hybrid genotypes.

Field trials: In total 36 *B. napus* accessions, including five reference varieties – ranging from tolerant (cv. ´Lion`) to highly susceptible (cv. ´Laser`) – were tested under field conditions at four different sites. Although there was a certain degree of variation concerning the disease values at the different sites, accessions that had shown good tolerance in the greenhouse also showed low disease levels in the field in most locations. The detection of fungal biomass by ELISA revealed that the spread of the fungus into the upper parts of the plant is retarded until early maturity stages of the crop (> GS 85).

Interaction studies: Based on the comparative application of the two approaches we concluded that the conventional staining techniques combined with CLSM were superior to the GFP tagging for *in situ* studies of pathogens at least in the present pathosystem. Attachment of *V. longisporum* to the root was largely restricted to the root hair zone. Thus, at 24 hpi, hyphae were found intensely interwoven with the root hairs. At 36 hpi, the fungus started to cover the roots with a hyphal net strictly following the grooves of the junctions of the epidermal cells. *V. longisporum* started to penetrate the root epidermal cells without any specific infection structures. Subsequently, hyphae grew intracellularly as well as intercellularly through the root cortex towards the central cylinder, without inducing any visible plant responses. Colonisation of the xylem vessels in the shoot was restricted to individual vessels entirely filled with mycelium and conidia, while adjacent vessels remained completely unaffected.

Histochemical and biochemical analysis of phenolics: Using the Folin-Ciocalteau method, the total concentration of free and bound polyphenols in hypocotyls of inoculated plants was substantially greater than that in control plants at 21 and 28 dpi, except for the susceptible *B. napus* type at 21 dpi. Biochemical analysis of the total lignin content also revealed a substantially higher content in infected plants compared to control plants, especially in the highly tolerant *B. oleracea* accession. At first glance surprisingly, the total amount of all polyphenolic compounds was lower in the tolerant *B. napus* genotype than in the susceptible one in control as well as in infected plants. However, using histochemical methods, a clear difference in the distribution of polyphenols was found between control and inoculated plants of both tolerant and susceptible genotypes.

Discussion

In the screening of different Brassica genotypes, several accessions with a high level of resistance to *V. longisporum* were identified, particularly in the group of *B. oleracea*. This finding is consistent with earlier studies of Zeise & Tiedemann (2002). These observations indicate that the Brassica C genome contains resistance trait(s) to the fungus, as was already suggested by Happstadius et al. (2003). Screening of resynthesised oilseed rape material revealed a resistance level higher than in the *B. rapa* parent and also superior to the tolerant cv. ´Express`. Thus, it is concluded that the resistance derived from the *B. oleracea* parent is expressed in most of the resynthesised genotypes.

A downstream test for field-resistance showed that accessions with enhanced tolerance under greenhouse conditions also showed low disease levels in the field in most of the cases. Thus, despite a certain degree of variation among the different screening runs and also among the disease values of particular accessions in the field trials, this screening method proofed to be a fast and reliable method for identifying new, robust sources of resistance for further breeding efforts.

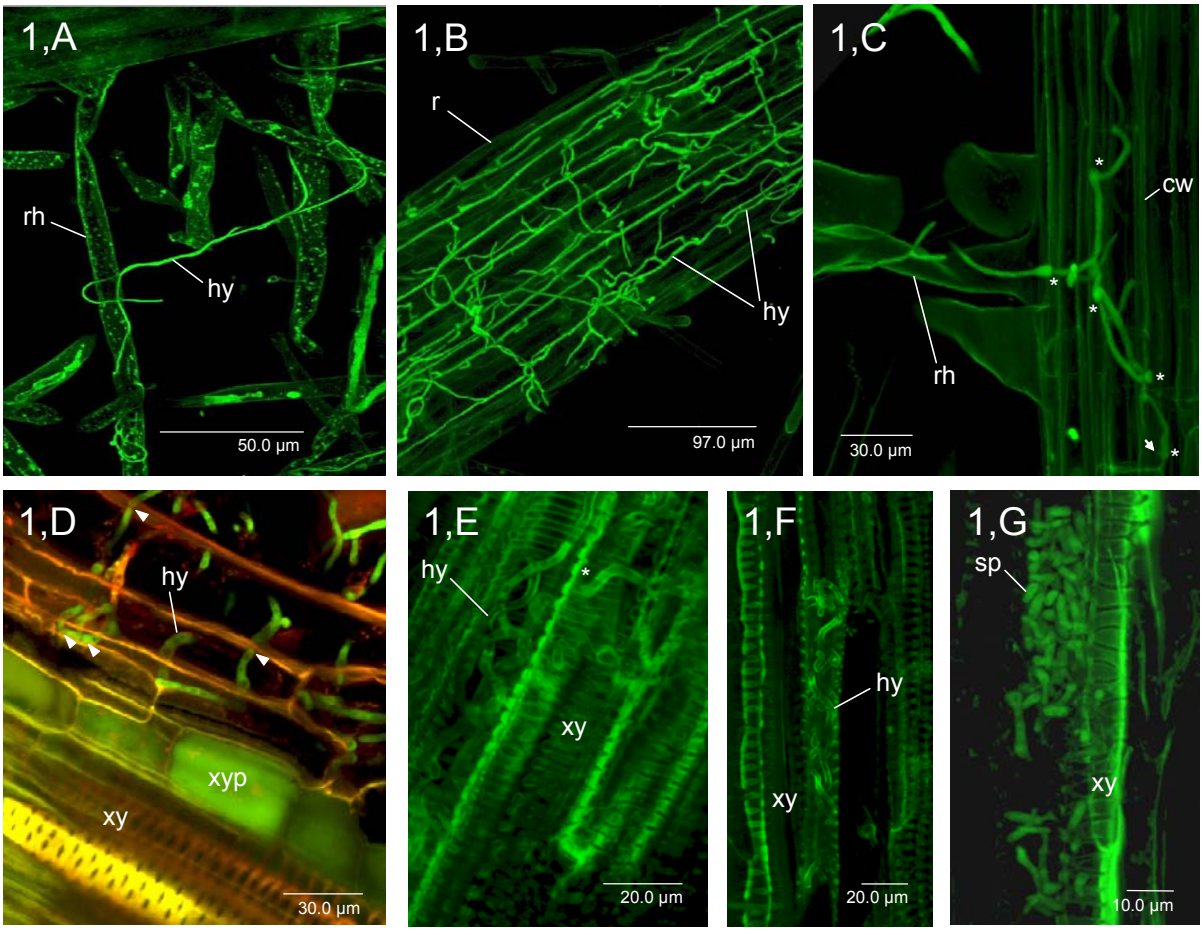

Fig. 1: **A –D**: Early stages of root colonization by *V. longisporum* on oilseed rape, as observed by confocal laser scanning microscopy after staining with acid fuchsin. **A**. Contact of hyphae of *V. longisporum* with root hairs (24 hpi). **B**. Hyphae of *V. longisporum* growing along the junctions of the epidermal cells forming a network (48 hpi). **C**. 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 (60 hpi). **D**. Directed growth of *V. longisporum* in the root cortex towards the xylem. Arrow heads assign points of penetration (60 hpi). **E-G**: Colonization of the shoot, 21 dpi. **E**. Hyphae of *V. longisporum* in xylem vessels of *B. napus*. Proliferation of mycelium into adjacent vessels through plasmodesmata (asterisk). **F**. Colonization of a single vessel element which is filled with mycelium. **G**. Conidiospores clumped together at the end of a vessel. hy = hypha, xy = xylem elements, xyp = xylem parenchyma, sp = conidiospores, ph = phialide, r = root, rh = root hair.

In interaction studies we directly compared GFP fluorescence with conventional staining with fluorescence dyes. This analysis clearly indicated a superior performance of the applied fluorochromes as – in contrast to the GFP-tagged strain - the entire mycelium was visualised regardless of its physiologic state. In addition, the faint unspecific staining of the plant tissue enabled a proper localization of the fungus in the host (Eynck et al. 2007). 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 (for example Schnathorst, 1981). However, our studies also provide significant novel information about colonization and infection. This particularly applies to the early interaction, including recognition and first contact between host and pathogen (Eynck et al., 2007).

The observation that even massive inoculation with *V. longisporum* results in a colonisation merely restricted to individual xylem vessels, while the other veins remain entirely free of the fungus, has not been reported so far. 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. The fact that the infection process of *V. longisporum* in the field is characterised by a pronounced latent phase may further explain why stunting is not observed in the field, in contrast to standardised conditions in the greenhouse.

Biochemical analysis as well as histochemical detection of changes in polyphenol compounds in different *Brassica* spp. due to infection revealed that the distribution of these compounds in certain tissue parts seems to play a bigger role than the total content. Because of their accumulation in both susceptible and tolerant genotypes, the relative contribution of any group of polyphenols to expression of resistance or the restriction of pathogen development remains to be elucidated. Besides their timing, localization and concentration the relationship of polyphenols to other putative defense responses, like the occlusion of vessels, shall be addressed in further studies allowing for a better understanding of resistance mechanisms in Brassica species to *V. longisporum*.

References

Campbell CL, Madden LV (1990) Introduction to Plant Disease Epidemiology. New York, USA. John Wiley.

Daebeler F, Amelung D, Zeise K (1988) Verticillium-Welke an Winterraps – Auftreten und Bedeutung. Nachrichtenblatt Pflanzenschutzdienst DDR 42: 71-73

Dixelius C, Happstadius I, Berg G (2005) Verticillium wilt on *Brassica* oil crops – a Swedish perspective. Journal of the Swedish Seed Association 115: 36-48

Eynck C, Koopmann B, Grunewaldt-Stöcker G, Karlovsky P, von Tiedemann A (2007) Differential interactions of *Verticillium longisporum* and *Verticillium dahliae* with *Brassica napus* detected with molecular and histological techniques. European Journal of Plant Pathology (accepted)

Gerlach D (1984) Botanische Mikrotechnik. Thieme Verlag

Happstadius I, Ljungberg A, Kristiannsson B, Dixelius C (2003) Identification of *Brassica oleracea* germplasm with improved resistance to Verticillium wilt. Plant Breeding 122, 30-34

Johannsen DA. (1940) Plant Microtechnique. McGraw-Hill, New York

Karapapa VK, Bainbridge BW, Heale JB (1997b) Morphological and molecular characterisation of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. Mycological Research 101: 1281- 1294

Krüger W (1989) Untersuchungen zur Verbreitung von *Verticillium dahliae* Kleb. und anderen Krankheits- und Schaderregern bei Raps in der Bundesrepublik Deutschland. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 41: 49-56

Lange BM, Lapierre C, Sandermann H Jr (1995) Elicitor-induced spruce stress lignin – structural similarity to early developmental lignins. Plant Physiology 108: 1277-1287

Schnathorst WC (1981) Life cycle and epidemiology of *Verticillium*. In: Mace ME, Bell AA, Beckmann CH (eds.) Fungal Wilt Diseases of Plants. Academic Press, New York, 81-111

Siegel SM (1953) On the biosynthesis of lignin. Physiol. Plantarum 6: 134-139

Singleton V.L., Orthofer R, Lamuela-Raventos RM 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology 299, 152-178

Svenson CH, Lerenius C (1987) An investigation on the effect of Verticillium wilt (*Verticillium dahliae* Kleb.) on oilseed rape. Working group integrated control in oilseed rape. IOBC/WPRS Bulletin X/4: 30-34

Zeise K (1992) Gewächshaustest zur Resistenzprüfung von Winterraps (*Brassica napus* L. var. *oleifera* Metzger) gegen den Erreger der Rapswelke *Verticillium dahliae* Kleb. Nachrichtenblatt Deutscher Pflanzenschutzdienst 44: 125-128

Zeise K, Seidel D (1990) Zur Entwicklung und Schadwirkung der Verticillium- Welkekrankheit am Winterraps. Raps 8: 20-22

Zeise K, von Tiedemann A (2001) Morphological and physiological differentiation among vegetative compatibility groups of *Verticillium dahliae* in relation to *V. longisporum*. Journal of Phytopathology 149: 469-475

Zeise K, von Tiedemann A (2002) Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. Journal of Phytopathology 150: 112-119