Growth of *Verticillium longisporum* in Xylem Sap of *Brassica napus* is Independent from Cultivar Resistance but Promoted by Plant Aging

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ABSTRACT

As *Verticillium* stem striping of oilseed rape (OSR), a vascular disease caused by *Verticillium longisporum*, is extending into new geographic regions and no control with fungicides exists, the demand for understanding mechanisms of quantitative resistance increases. Because *V. longisporum* is strictly limited to the xylem and resistance is expressed in the systemic stage post root invasion, we investigated a potential antifungal role of soluble constituents and nutritional conditions in xylem sap as determinants of cultivar resistance of OSR to *V. longisporum*. Assessment of biometric and molecular genetic parameters applied to describe *V. longisporum* resistance (net area under disease progress curve, stunting, stem thickness, plant biomass, and *V. longisporum* DNA content) showed consistent susceptibility of cultivar ‘Falcon’ in contrast to two resistant genotypes, ‘SEM’ and ‘Aviso’. Spectrophotometric analysis revealed a consistently stronger *in vitro* growth of *V. longisporum* in xylem sap extracted from OSR compared with the water control. Further comparisons of fungal growth in xylem sap of different cultivars revealed the absence of constitutive or *V. longisporum*

induced antifungal activity in the xylem sap of resistant versus susceptible genotypes. The similar growth of *V. longisporum* in xylem sap, irrespective of cultivar, infection with *V. longisporum* and xylem sap filtration, was correlated with about equal amounts of total soluble proteins in xylem sap from these treatments. Interestingly, compared with younger plants, xylem sap from older plants induced significantly stronger fungal growth. Growth enhancement of *V. longisporum* in xylem sap of aging plants was reflected by increased contents of carbohydrates, which was consistent in mock or *V. longisporum*-infected plants and independent from cultivar resistance. The improved nutritional conditions in the xylem of more mature plants may explain the late appearance of disease symptoms, which are observed only in late maturity stages of plants in the field. While falsifying the presence of antifungal activity in xylem sap of resistant cultivars, this study strengthens previous findings that indicated a significant role of physical cell wall bound resistance factors involved in quantitative, cultivar-related resistance of *B. napus* to *V. longisporum*.

Among the *Verticillium* species typically inducing vascular diseases in crop plants, *Verticillium longisporum* is an only recently evolved host-specialized vascular pathogen of oilseed rape (OSR) (Inderbitzin et al. 2011; Zeise and von Tiedemann 2002), which has become a potential threat to OSR production in Europe (Carré and Pouzet 2014) and Canada (CFIA 2016). In contrast to other *Verticillium* diseases and in spite of its potential to colonize the plant vascular system, *V. longisporum* does not induce wilt in OSR (Lopisso et al. 2016). Typical symptoms of *V. longisporum* in OSR are leaf chlorosis, premature senescence, increased branching (Lopisso et al. 2016), and stem striping (Depotter et al. 2016), which may result in substantial yield losses (Dunker et al. 2008). Systemic infection and long term survival of this pathogen in the soil hamper control of this disease in OSR with fungicides. Hence, besides a few suggested preventive measures such as crop rotation (Bhat and Subbarao 1999; Zeise and von Tiedemann 2002), improvement of cultivar resistance remains the sole potential control strategy. Previous studies searching for resistant *Brassica* genotypes (Happstadius et al. 2003; Rygulla et al. 2007a, b) and studying their performance in the field (Eynck et al. 2009a) have identified sources of effective quantitative resistance to *V. longisporum* in the *Brassica* gene pool.

Generally, plants may respond to attack from vascular pathogens with physical or biochemical defense. The most common physical defense mechanisms that prevent or contain systemic spread of vascular pathogens in the xylem include increased synthesis of

lignins (Gayoso et al. 2010) and accumulation of gels and tyloses (Williams et al. 2002) in tomato as well as vascular coating, xylem wall swelling, and induction of vascular hypersensitive responses in pepper (Rahman et al. 1999). Mechanisms of resistance to *V. longisporum* in OSR have been insufficiently studied so far and are not yet fully understood. Existence of internal quantitative resistance in certain OSR genotypes and the potential role of vascular occlusions in hypocotyl tissue associated with accumulation of phenolics and lignin, supposedly blocking fungal linear spread at the interface between root and shoot have been reported previously (Eynck et al. 2009b).

Besides such putative physical and cell wall-bound resistance factors, various soluble antifungal defense compounds accumulating in the xylem sap have been identified and suggested to be potential resistance factors against vascular pathogens. Among these, the fungitoxic elemental sulfur (Williams et al. 2002), H₂O₂ (Gayoso et al. 2010), and PR proteins (Rep et al. 2002) in tomato as well as peroxidases in rice (Hilaire et al. 2001), grapevine (Basha et al. 2010), and tomato (Gayoso et al. 2010) have been reported to be involved in resistance. In addition, the possible involvement of pre-existing soluble proteins in the xylem sap (Singh et al. 2012) and soluble and cell wall-bound phenylpropanoids (Kamble et al. 2013; Obermeier et al. 2013) has been suggested. Floerl et al. (2008) identified *V. longisporum* induced accumulation of defense related antifungal proteins (endochitinase, peroxidase, and glucanase) in the leaf apoplast washing fluid of a *V. longisporum* susceptible *B. napus* genotype. Another study aiming at the general analysis of xylem sap proteins in *B. napus* identified more than 60 different proteins, some of which were associated with plant defense against pathogens (Kehr et al. 2005).

An interesting finding from previous studies is the induction of elevated levels of salicylic acid (SA) in xylem sap or hypocotyl tissue extracts from OSR infected with *V. longisporum*, but not of

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary tables are published online.

jasmonic acid (JA) and abscisic acid (ABA) (Kamble et al. 2013; Ratzinger et al. 2009). This is in contrast to *Arabidopsis thaliana* responses to *V. longisporum*, which were associated with JA and ABA signals but did not involve SA (Johansson et al. 2006; Ralhan et al. 2012). Similarly, Siebold (2012) showed significantly higher levels of SA induced by *V. longisporum* in stem extracts of a susceptible *B. napus* cultivar compared with a resistant genotype. It is obvious that these plant hormones, at in situ concentrations, have no fungicidal or growth inhibitory effect on pathogens but rather act as potential triggers of resistance responses. Moreover, the fact that SA levels in infected OSR correlated with susceptibility makes it unlikely that SA is a soluble resistance factor in the xylem.

As *V. longisporum* strictly remains in the xylem vessels after entering the roots and fungal systemic growth is significantly reduced in resistant cultivars, the question arises whether soluble inhibitory metabolites (either preformed or induced by infection) exist in the xylem acting as resistance factors against *V. longisporum* systemic growth. The present study therefore aimed at investigating whether cultivar-related quantitative resistance of OSR to *V. longisporum* is caused by or related to soluble antifungal compounds and/or the nutritional conditions in the xylem sap. More specifically, we aimed at (i) detecting antifungal activity in xylem sap of OSR plants infected and noninfected with *V. longisporum*, (ii) describing the suitability of xylem sap as growth medium for *V. longisporum*, and (iii) assigning xylem sap nutritional properties to cultivar resistance and plant age.

MATERIALS AND METHODS

Experimental design, plant material, and conditions of plant cultivation. Two independent studies each consisting of greenhouse and in vitro assays were conducted. In both studies, experiments were laid out in a completely randomized design with four replications and repeated at least twice. Three *B. napus* genotypes, *V. longisporum* susceptible cultivar ‘Falcon’ (NPZ, Germany) (Rygulla et al. 2007b) and *V. longisporum* resistant cultivars ‘Aviso’ (Danisco Seed, Sweden) (Keunecke 2009) and ‘SEM-05-500256’ hereafter called ‘SEM’ (SW and Lantmännen, Sweden) (Eynck et al. 2009b), were used. Greenhouse experiments were conducted to verify cultivar responses to *V. longisporum*, collect xylem sap, and analyze xylem sap constituents. Likewise, in vitro bioassays were carried out to investigate the presence of growth promoting or inhibiting factors in OSR xylem sap.

In the first study (experiment 1), 20 mock and 20 *V. longisporum*-inoculated plants (representing four replicates from five plants merged to one sample) of cultivars Falcon and SEM were used for phenotypic and fungal DNA-based disease assessments. Since collecting xylem sap required destructive sampling, at least 45 extra plants were included per treatment. Surface-sterilized seeds (two times treated with 70% ethanol for 2 min and rinsed with autoclaved tap water) were sown on autoclaved silica sand and grown in a climate-controlled chamber (24°C, 14 h light, and 70% relative humidity) for 2 weeks. After inoculation with *V. longisporum*, seedlings were transferred to plastic pots (200 ml) filled with a 3:1 (vol/vol) mixture of commercial soil and sand and grown for 4 weeks (until 28 days postinoculation [DPI]) in the greenhouse under similar conditions as described above.

The second study (experiment 2) was conducted in order to investigate the impact of increasing plant age on inhibitory or nutritional properties of xylem sap. Here, two genotypes expressing contrasting levels of resistance to *V. longisporum*, namely Aviso (resistant) and Falcon (susceptible) were used. Experimental design and greenhouse conditions were similar to experiment 1.

Fungal isolate, inoculation procedure, and disease assessment. VL43, a highly virulent isolate of *V. longisporum* (Zeise and von Tiedemann 2002), was used throughout this study. For both the greenhouse and the bioassay studies, VL43 was cultivated in potato extract glucose broth (Carl Roth GmbH + Co. KG, Karlsruhe,

Germany) at 23°C in the dark with constant shaking (100 rpm). After 7 days, mycelium was filtered through a sterile cheese cloth and spore density was adjusted to 1×10^6 conidia/ml with a hemocytometer. Roots of 2-week-old seedlings (BBCH 12) were carefully washed under running tap water and inoculation was performed by submerging roots in VL43 conidia suspension for 1 h. Mock-inoculated plants were treated the same way with autoclaved tap water. Disease severity evaluation was done in weekly intervals from 7 to 28 DPI using a nine-class assessment key, modified from Zeise (1992). Disease severity scores were used to calculate area under disease progress curve (AUDPC) values from which net AUDPC was calculated according to Eynck et al. (2009b). Stunting was measured by recording plant height (from the base of the hypocotyl to the tip of the main plant axis) at 28 DPI. At the same time, stem thickness was measured above the hypocotyl with a digital caliper. Quantification of fungal DNA by quantitative real-time PCR was performed according to the method developed recently (Knüfer et al. 2016). Cultivar responses to infection with *V. longisporum* were further determined by comparing the shoot biomass determined after oven drying at 70°C for 24 h.

Xylem sap collection and bioassay. The in vitro bioassay consisted of 10 treatments derived from variation of genotype (two cultivars), inoculation (mock/*V. longisporum*), and filtration (with/without). Two controls, conidia in sterile bidistilled water and sterile bidistilled water alone, were also included. In experiment 1, xylem sap was collected at two time points (21 and 28 DPI) from the lower part of the hypocotyl using the pressure bomb technique (Scholander et al. 1965). Briefly, plants were uprooted and adhering soil washed off the roots under running tap water. The shoot was removed by cutting the stem shortly above the hypocotyl. The remaining hypocotyl with the roots was immediately inserted into the pressure chamber of the Scholander bomb (Model 600; PMS Instrument Company, Albany). Subsequently, xylem sap was collected by gradually increasing the chamber pressure until xylem sap appeared at the cut surface. In order to get sufficient amounts of xylem sap in repeated experiments, collection was done by cutting plants at the tip of the hypocotyl and sucking the exuding xylem sap with a hand-held pipette (Kehr et al. 2005). Aliquots of approximately 1 ml of xylem sap were obtained by merging samples of 15 plants from one treatment and considered one replicate. Each treatment had three biological replications. Xylem sap samples were immediately stored at –20°C until further use. In experiment 2, xylem sap collection was performed at 2- to 3-day intervals from 13 to 28 DPI using the same pressure bomb technique as described above.

To analyze the growth of VL43 in OSR xylem sap, an in vitro bioassay was performed following the method of Broekaert et al. (1990). Briefly, mycelium of VL43 (cultivated as described above) was removed by filtration with sterile cheesecloth. From the filtered culture, conidia were harvested by centrifugation for 10 min at $1,046 \times g$ followed by threefold washing in sterile bidistilled water. The final conidial density was adjusted to 5×10^3 conidia/ml with a hemocytometer and supplemented with filter sterilized streptomycin (20 ppm) to eliminate bacterial growth during the in vitro assay. Xylem sap was filtered through a 0.2 µm Anotop 10 plus sterile syringe filter (Whatman GmbH, Dassel, Germany). The procedure was as follows: 50 µl of VL43 spore suspension (see above) and 200 µl of xylem sap were loaded in a well on a 96-well microtiter plate in three technical replicates. Prior to incubation, spores were allowed to settle for 30 min and the initial absorbance was taken at 580 nm in a microplate spectrophotometer (µQuant, Bio-Tek Instruments, Bad Friedrichshall, Germany). Plates were placed in a moist chamber and incubated at 23°C in the dark. Fungal growth was monitored daily by recording the mean absorbance value of 25 (5 × 5 grid pattern) readings taken from a single well. Fungal growth at different days after start of incubation (DAI) in xylem sap was obtained by subtracting the initial absorbance (0 DAI) from the daily absorbance readings.

Quantification of carbohydrate and protein contents in xylem sap. The total protein content in xylem sap was measured with the Bradford method (Bradford, 1976). Xylem sap or double bidistilled water (used as blank) was loaded on the microtiter plate wells in triplicates. Absorbance was measured at 595 nm with a microplate spectrophotometer. Total protein content in xylem sap was determined by referring to the standard curve produced from known concentrations of bovine serum albumin. Total carbohydrate (CHO) content was determined following the microplate format phenol-sulphuric acid protocol (Masuko et al. 2005). Aliquots of standard glucose (Glc) solutions were prepared in double bidistilled water. One hundred microliters of the standard solution or xylem sap samples was mixed with 300 μ l of concentrated sulphuric acid. This solution was immediately amended with 60 μ l of 5% phenol and incubated for 5 min at 90°C in a static water bath. Subsequently, the solution was cooled for 5 min in a water bath at room temperature and 230 μ l of the reaction mixture was loaded on 96-well plates in triplicates. Absorbance was measured at 490 nm, reflecting the absorption maximum of most sugars including mannose, xylose, fructose, galactose, and glucose (Masuko et al. 2005). To calculate total CHO contents in xylem sap, mean absorbance values of xylem sap samples were referred to the standard curve produced from known concentrations of Glc.

Data analysis. Unless otherwise specified, all statistical analyses were performed using STATISTICA 12 (StatSoft, Inc., OK). Since disease data (net AUDPC, relative stunting, and qPCR) were relative values derived from comparison of *V. longisporum*-inoculated treatments with the mock-inoculated treatments which resulted in only one factor (cultivar), these data were analyzed using one-way analysis of variance (ANOVA). For the biometric data related to cultivar and inoculation as experimental factors, a factorial ANOVA was applied. Unlike the xylem sap treatments, the negative (sterile bidistilled water) and positive (sterile bidistilled water plus VL43) control treatments in the in vitro bioassays varied only by one factor (i.e., without mock, *V. longisporum*, or filtration factors); therefore, these data were analyzed with one-way ANOVA. For further analysis

of the effect of cultivar, plant age, xylem sap filtration, and interactions between these factors on the in vitro growth of VL43 in xylem sap, a factorial analysis was performed without the negative (sterile bidistilled water) and positive (sterile bidistilled water plus VL43) control treatments. All ANOVA outputs for combined analyses are provided as supplementary tables. In both one-way and factorial ANOVA, Fisher's least significant difference (LSD) test was used to separate means obtained from four or three biological replicates of greenhouse and in vitro fungal growth experiments. Differences were considered statistically significant when *P* values were less than 0.05. Before ANOVA, all data sets were tested for normal distribution using the Shapiro-Wilk test and, whenever required, ANOVA was performed on transformed data. When assumptions for ANOVA were not met, a nonparametric test (Kruskal-Wallis test with Dunn's posthoc) was performed in XLSTAT 2016. Relationships among disease parameters or between in vitro fungal growth and concentrations of xylem sap constituents were determined by Pearson correlation analysis performed in STATISTICA 12.

RESULTS

Characterization of cultivar responses to *V. longisporum*.

Four weeks postinoculation, infected SEM plants remained green, vigorous, and healthy while diseased Falcon plants showed extremely stunted growth with shriveled, yellowing, and black-veined leaves, which are typical symptoms of *V. longisporum* infection in *B. napus* (Fig. 1A). ANOVA showed a significant cultivar effect ($P = 0.000$) on disease severity and interactive effects of cultivar and *V. longisporum* ($P = 0.000$ to 0.029) on biometric parameters (Supplementary Table S1). Disease levels measured by net AUDPC showed five times higher disease severity in Falcon than SEM (Fig. 1B). Similarly, qPCR analysis (Fig. 1C) revealed a 74-fold accumulation of *V. longisporum* DNA in hypocotyl tissue of Falcon (1,419.2 ng/g) compared with SEM (19.3 ng/g). In contrast, infection with *V. longisporum* induced significant stunting ($P = 0.000$) in both genotypes (Fig. 1D). However, this effect was more severe in Falcon.

Fig. 1. Resistant and susceptible responses of *Brassica napus* genotypes to infection with *Verticillium longisporum* as verified by phenotypic and molecular disease severity assessments 28 days after inoculation (experiment 1). **A**, Phenotypes of resistant (SEM) and susceptible (Falcon) plants. **B**, Net area under disease progress curve values generated from weekly disease index measurements. **C**, qPCR quantification of fungal DNA in the hypocotyl. **D**, Plant height showing stunting effect. Inoculation was done by submerging noncut roots of 2-week-old seedlings in conidia suspension for 1 h. Mock: water-inoculated control. VL43: inoculated with *V. longisporum*. Bars indicate standard deviations. Values with different letters indicate significant differences (Fisher's least significant difference test; $P = 0.05$).

Assessment of biometric traits, such as hypocotyl thickness and biomass yield, further proved the contrasting responses of the two genotypes to infection with *V. longisporum* (Fig. 2). Likewise, in experiment 2, similar resistance of cultivar Aviso to *V. longisporum* was verified by assessment of plant height and disease index (data not shown). The consistent resistant or susceptible responses of the genotypes used in this study were confirmed by the strong positive correlation of phenotypic and molecular disease assessment data and by the strong negative correlations between disease and biometric variables (Table 1).

Analysis of *V. longisporum* growth in xylem sap of *B. napus*. To investigate the presence of constitutively expressed or *V. longisporum*-induced cultivar-specific antifungal resistance factors in native xylem sap, growth of *V. longisporum* in xylem sap collected from resistant and susceptible plants was investigated in an in vitro bioassay. Spectrophotometric measurements of *V. longisporum* growth in xylem sap collected 28 DPI showed ample germination and fungal growth within 2 days after incubation. There was a general enhancement of fungal growth in xylem sap compared with the water control, irrespective of cultivar, infection with *V. longisporum* or xylem sap filtration (Fig. 3; Supplementary Table S2). Interestingly, results of the factorial ANOVA showed that the rate and level of *V. longisporum* growth in xylem sap of resistant or susceptible plants did not significantly differ, regardless of plant infection state or xylem sap filtration (Supplementary Table S3). A slightly stronger fungal growth was observed in unfiltered versus filtered xylem sap and in xylem sap of *V. longisporum*-inoculated compared with noninoculated plants (Fig. 3). Again, these effects were similar in both genotypes. Analysis of fungal growth in xylem sap collected at 21 DPI showed similar results (data not shown).

Xylem sap soluble protein content. The potential impact of cultivar resistance or *V. longisporum* on soluble proteins in xylem sap and, more importantly, the putative effect of changes in xylem sap protein concentration on fungal growth were determined by analysis of the total soluble protein content of xylem sap samples. Results revealed the presence of considerable amounts of soluble proteins in *B. napus* xylem sap. In mock inoculated plants, the quantities of total soluble proteins were 104.9 and 101.8 µg/ml in SEM and Falcon, respectively. In plants infected with *V. longisporum*, a slight but insignificant increase in xylem sap protein contents was found (Table 2). Factorial ANOVA showed that filtration of xylem sap to remove microbial contamination can significantly ($P = 0.043$) reduce protein concentrations.

Effect of plant aging on *V. longisporum* growth in xylem sap. In addition to the impact of cultivar resistance, the role of xylem sap constituents in different-age *B. napus* plants was investigated. Two genotypes expressing contrasting levels of resistance to *V. longisporum*, Aviso (resistant) and Falcon (susceptible), were used. Xylem sap was collected from mock and *V. longisporum*-infected plants in 2- to 3-day intervals between 13 and 28 DPI. Results of

the in vitro bioassay were consistent with the independently conducted experiments on cultivar effects. Accordingly, xylem sap collected from mock or *V. longisporum* inoculated Aviso or Falcon plants at 28 DPI provided equally suitable conditions for fungal growth. Compared with the control (sterile bidistilled water), a significantly enhanced fungal growth was recorded starting from 2 DAI (Fig. 4). However, in general, neither infection with *V. longisporum* nor cultivar resistance significantly altered in vitro growth of the pathogen in xylem sap (Supplementary Table S4). Interestingly, fungal growth was significantly ($P = 0.003$) enhanced in xylem sap from plants at increasing age, an effect that occurred similarly in both cultivars (Fig. 4; Supplementary Table S5).

Xylem sap carbohydrate content. Due to the conspicuous enhancement of fungal growth in xylem sap of older plants, we investigated whether this effect was related to the carbohydrate content in xylem sap. Analysis of xylem sap samples used in the bioassays showed similar concentrations of total CHO in xylem sap of susceptible and resistant genotypes. However, xylem sap CHO concentrations were significantly ($P = 0.000$) affected by plant age (Fig. 5). Compared with earlier time points, total xylem sap CHO concentrations in 28 DPI mock or *V. longisporum*-infected Aviso and Falcon plants was significantly higher than in younger plants. In contrast to 28 DPI xylem sap samples, *V. longisporum* induced slight increases of CHO levels at earlier time points (14, 18, and 21 DPI) (Fig. 5).

Correlation of fungal growth with plant age, xylem sap CHO, and protein contents. There was a significant positive correlation ($r = 0.58$) between xylem sap CHO concentration and fungal growth in xylem sap (Fig. 6B). Similarly, a strong positive correlation ($r = 0.72$) was found between fungal growth and plant age at the time of collecting xylem sap (Fig. 6C). In contrast, the rate of fungal growth and concentration of total soluble xylem sap proteins were unrelated (Fig. 6A), which is in agreement with the bioassay results.

TABLE 1. Correlation coefficients of relationships among disease and biometric parameters under greenhouse conditions recorded 28 days after inoculation with *Verticillium longisporum* (experiment 1)^a

Parameters	Net AUDPC	Plant height	Hypocotyl diameter	Shoot DM
<i>V. longisporum</i> DNA	0.89***	−0.79***	−0.73**	−0.69*
Net AUDPC		−0.92***	−0.77***	−0.81**
Plant height			0.49 ^{ns}	0.78**
Hypocotyl diameter				0.61*

^a Analysis was performed using Pearson (product-moment) correlation (STATISTICA version 12). Minus signs indicate negative correlations. ns, not significant. *, significant at $P = 0.05$. **, significant at $P = 0.01$. ***, significant at $P = 0.001$. Net AUDPC: net area under disease progress curve. *V. longisporum* DNA: *V. longisporum* DNA in hypocotyl. DM: dry matter.

Fig. 2. Biometric responses of resistant (SEM) and susceptible (Falcon) *Brassica napus* genotypes to infection with *Verticillium longisporum* (VL) at 28 days postinoculation (experiment 1). **A**, Hypocotyl thickness. **B**, Shoot dry biomass. Mock: water-inoculated control. VL43: infected with *V. longisporum*. DM: dry matter. Bars indicate standard deviations. Values with different letters indicate significant differences (Fisher's least significant difference test; $P = 0.05$).

DISCUSSION

The main result from this study is a lack of impact of cultivar resistance on the growth conditions for *V. longisporum* in xylem sap of OSR, while plant aging had a significant effect on the growth of the pathogen in its typical environment. Different degrees of resistance against *V. longisporum* in the three *B. napus* cultivars used in this study were verified in a series of experimental approaches involving biometric parameters and fungal DNA measurements in the plants. Cultivars significantly differed in all parameters applied to describe resistance, including Net AUDPC, stunting, stem thickness, plant biomass and *V. longisporum* DNA content in plants. These results from greenhouse disease evaluations confirmed previous data from field and greenhouse resistance studies that showed resistance to *V. longisporum* in Aviso (Keunecke 2009) and SEM (Eynck et al. 2009b) and susceptibility in Falcon (Rygulla et al. 2007b).

The time points of xylem sap sampling at 21 and 28 DPI were chosen based on previous studies that identified this period as critical for expression of resistance in *B. napus* (Eynck et al. 2009b; Obermeier et al. 2013). Results showed that *B. napus* xylem sap provides a suitable medium for growth of *V. longisporum*. Surprisingly, fungal growth in xylem sap was unrelated to cultivar resistance and thus to the level of disease. Moreover, plant infection with *V. longisporum* did not significantly alter fungal growth conditions in none of the cultivars used compared with healthy plants, suggesting that *V. longisporum* does not induce synthesis of antifungal compounds in the xylem sap. On the contrary, fungal growth was slightly enhanced in xylem sap from infected plants compared with control plants and this effect was independent from cultivar resistance. Such enhanced growth has been previously reported for *V. dahliae* in vascular fluid from nematode infected cotton cultivars (Katsantonis et al. 2005), indicating improved nutritional conditions in the xylem of plants under biotic stress. A slight reduction of fungal growth was noticed in sterile filtered xylem sap, but this effect of filtration on the rate and level of *V. longisporum* growth was not significant.

The similar growth of *V. longisporum* in xylem sap independent of the cultivar may be partly explained by the similar concentrations of total soluble proteins which were found in xylem sap of resistant and susceptible genotypes and which were in a similar range as reported in previous studies with *B. napus* (Buhtz et al. 2004), cotton (Singh et al. 2013), and olive (Neumann et al. 2010). On the other hand, the higher protein concentrations found in infected plants indicate a slight, cultivar-independent and infection-induced

increase of protein levels in *B. napus* xylem sap. Since fungi use proteins as a source of carbon, nitrogen, and sulfur (Jennings 1995), it is not surprising that enhanced fungal growth was observed in xylem sap samples from infected plants. A further finding was a significant reduction in total soluble protein concentrations in filtered xylem sap. This might be due to a medium to high protein binding property of the syringe filter (Whatman, Anotop 10 plus) or the removal of microbes as a result of the filter sterilization of xylem sap samples, but this had no significant effect on the fungal growth bioassays.

The bioassay results of the present study are only partly in agreement with observations reported by Floerl et al. (2008) that showed *B. napus* xylem sap to be a suitable medium for the growth of *V. longisporum* and no alteration of total xylem sap protein concentration due to infection with *V. longisporum*. The previously described inhibitory effects of xylem sap upon vascular infection of a susceptible *B. napus* genotype with this pathogen were not confirmed in our study. The comprehensive data presented here do not indicate any inhibitory activity in xylem sap of *B. napus* toward the vascular growth of *V. longisporum*, neither in a susceptible, nor a resistant interaction. More than 69 pre-existing proteins with various potential functions related to plant defense and cell wall modification are known to exist in xylem sap of *B. napus* (Kehr et al. 2005). However, whether their presence and concentration varies among *B. napus* varieties and is related to infection and/or vascular disease resistance is not known. In tomato, infection of resistant and susceptible plants with virulent and avirulent strains of *V. albo-atrum* caused a general increase of xylem sap amino acid concentration (Dixon and Pegg 1972). In contrast, infection

TABLE 2. Total soluble protein content in xylem sap of resistant (SEM) and susceptible (Falcon) *Brassica napus* cultivars 28 days after inoculation with *Verticillium longisporum* (experiment 1)^a

Treatments		Total soluble protein (µg/ml)	
		Unfiltered xylem sap	Filtered xylem sap
SEM	Mock-inoculated	104.9 ± 17	99.1 ± 11
	<i>V. longisporum</i> -inoculated	109.4 ± 14	98.5 ± 12
Falcon	Mock-inoculated	101.8 ± 14	99.0 ± 12
	<i>V. longisporum</i> -inoculated	106.0 ± 12	100.5 ± 14

^a Mock: water-inoculated control. Protein content was determined by the Bradford method using bovine serum albumin as a standard. Means ± standard deviation values obtained from three biological replicates are shown. No statistically significant difference was found (Kruskal-Wallis test; $P = 0.05$).

Fig. 3. Spectrophotometric analysis of *Verticillium longisporum* growth in xylem sap collected from *Brassica napus* genotypes at 28 days postinoculation (experiment 1). M: mock-inoculated control; f: filtered; VL: *V. longisporum*-inoculated; BDWVL: fungal growth in sterile bidistilled water. BDW: sterile bidistilled water without *V. longisporum* inoculum.

Fig. 4. Spectrophotometric analysis of *Verticillium longisporum* growth in xylem sap of resistant (Aviso) and susceptible (Falcon) *Brassica napus* cultivars collected at increasing plant age (experiment 2). **A**, Fungal growth in xylem sap of water-inoculated Aviso. **B**, Fungal growth in xylem sap of *V. longisporum*-inoculated Aviso. **C**, Fungal growth in xylem sap of water-inoculated Falcon. **D**, Fungal growth in xylem sap of *V. longisporum*-inoculated Falcon. Treatment codes: A and AVL: fungal growth in xylem sap of mock- and *V. longisporum*-inoculated Aviso, respectively. F and FVL: fungal growth in xylem sap of mock- and *V. longisporum*-inoculated Falcon, respectively. Numbers after treatment codes refer to days postinoculation at which xylem sap was collected. BDWVL: fungal growth in sterile bidistilled water. BDW: sterile bidistilled water without *V. longisporum* inoculum.

Fig. 5. Total xylem sap carbohydrate content in resistant (Aviso) and susceptible (Falcon) *Brassica napus* plants at increasing plant age (experiment 2). Mock: water-inoculated control. VL43: infected with *Verticillium longisporum*. CHO: total carbohydrates. Mean values ($n = 3$) with different letters indicate significant differences (Kruskal-Wallis test; $P = 0.05$; SE, standard error; SD, standard deviation).

with the symbiotic bacterium *Bradyrhizobium japonica*, unlike the elicitor of *Phytophthora sojae*, caused no significant increase in concentrations of xylem sap proteins in soybean (Subramanian et al. 2009).

Fig. 6. Relationship between in vitro xylem sap growth of *Verticillium longisporum* and concentration of xylem sap constituents or plant age at the time of xylem sap sampling. *P* values (indicated in brackets) less than 0.05 indicate significant correlations. DPI: days postinoculation with *V. longisporum*. DAI: days after incubation in xylem sap. CHO: total carbohydrates. **A**, Pearson's correlation between xylem sap total protein content and fungal growth in xylem sap collected at 28 DPI. **B**, Pearson's correlation between total xylem sap CHO and fungal growth in xylem sap of plants at different ages (weekly interval; 14, 21, and 28 DPI). **C**, Pearson's correlation between plant age (weekly interval; 13, 20, and 28 DPI) at the time of xylem sap sampling and fungal growth at 5 DAI (asterisk) or daily fungal growth rate (diamond).

The second important observation in the present study was the enhanced growth of *V. longisporum* in xylem sap due to aging of plants (from 13 to 28 DPI). This finding may offer a plausible explanation of the unique pattern of *V. longisporum* disease development in the field. During juvenile growth stages of *B. napus* in the field, *V. longisporum* undergoes an extended period of latency, which, in winter OSR, may extend up to 8 months after sowing of the crop. When the crop reaches maturity stages, the typical brown stripes develop along the stem (Knüfer 2013) and subsequently, the fungus proliferates into the stem parenchyma to produce microsclerotia (Leino 2006).

Based on the results of this study, we speculate that improved supply with nutrients in the xylem sap of aging plants may serve as the signal to the pathogen to induce its final reproductive life cycle stage. The improved nutritional conditions may trigger enhanced vascular growth enabling *V. longisporum* to proliferate into the nonvascular stem tissues and produce microsclerotia in the stem pith and beneath the stem epidermis. A crucial driver of this late but sudden growth enhancement of *V. longisporum* in *B. napus* may be sugars known to be present and transported in low quantities in the xylem (Iwai et al. 2003; Loescher et al. 1990) and shown in this study to significantly increase in concentration with the plant aging. Similarly, increased sugar levels in shoots of *Arabidopsis* were suggested to be an indigenous signal for transition from juvenile to adult plant stages (Yu et al. 2013). In tomato, xylem sap sugar concentration in healthy and nematode infected plants strongly increased with plant age (Wang and Bergeson 1974). In *Arabidopsis*, levels of sugars (Glc, Suc, and Fruc) significantly increased in shoot extracts of plants between 15 and 60 days old (Yu et al. 2013). In the present study, the presence of about equal amounts of sugars irrespective of plant genotype is one possible explanation for the equal growth of *V. longisporum* in xylem sap of resistant and susceptible plants, since apparently, no inhibitory compounds are present or induced in the xylem of resistant cultivars. Similar results have been reported for Dutch elm disease and *Ulmus* genotypes showing no correlation between xylem sap sugar content and disease resistance (Singh and Smalley 1969).

Overall, our results support previous studies that indicated a significant role of physical or cell wall bound resistance factors (Eynck et al. 2009b), and suggest that soluble compounds in the xylem are not likely to be involved in cultivar-related resistance of *B. napus* to *V. longisporum*. This type of internal quantitative resistance which was first identified in the hypocotyl tissue as the major battlefield of the interaction between *B. napus* and *V. longisporum* demonstrated the significance of physical defense structures by de novo formation and synthesis of lignin as well as reinforcement of tracheary elements with cell wall bound phenolics and lignin (Eynck et al. 2009b). In another recent study focusing on the identification of QTLs involved in *V. longisporum* resistance in *B. napus*, a significant correlation between phenylpropanoids and *V. longisporum* resistance has been shown. More importantly, the study showed lower concentrations of the major lignin precursor (caffeic acid) in resistant lines compared with susceptible plants, suggesting substantial incorporation of this compound into the cell wall (Obermeier et al. 2013), thus again indicating an essential role of physical barriers in cultivar-related resistance of *B. napus* to *V. longisporum*. In conclusion, the findings of the present study suggest that soluble xylem sap constituents are not involved in cultivar-related resistance to *V. longisporum* in winter OSR, while the nutritional conditions in xylem sap improve as plant age increases and thus may explain the late fungal proliferation and appearance of disease symptoms in field-grown plants.

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