Verticillium Wilt on Fiber Flax: Symptoms and Pathogen Development In Planta

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Abstract

Fiber flax (*Linum usitatissimum* L.), an important crop in Normandy (France), is increasingly affected by Verticillium wilt caused by the soilborne fungus *Verticillium dahliae*. This disease leads to nonnegligible yield losses and depreciated fibers that are consequently difficult to upgrade. Verticillium wilt is a major threat to a broad range of agriculture. In this study, susceptible fiber flax cultivar Adélie was infected by VdLu01 (isolated from fiber flax, this study) or green fluorescent protein-tagged VdLs17 (transformed and provided by the department of Plant Pathology, University of California, Davis). Between 3 and 4 weeks postinoculation, wilting symptoms on leaves were first observed, with acropetal growth during the following weeks. Pathogen development was tracked by confocal laser-scanning microscopy during the asymptomatic and symptomatic stages. First, conidia

germination led to the development of hyphae on root epidermis; more particularly, on the zone of cell differentiation and around emerging lateral roots, while the zone of cell division and the root tip were free of the pathogen. At 3 days postinoculation, the zone of cell differentiation and lateral roots were embedded into a fungal mass. Swelling structures such as appressoria were observed at 1 week postinoculation. At 2 weeks postinoculation and onward, the pathogen had colonized xylem vessels in roots, followed by the stem and, finally, leaves during the symptomatic stage. Additionally, observations of infected plants after retting in the field revealed microsclerotia embedded inside the bast fiber bundle, thus potentially contributing to weakening of fiber. All of these results provide a global account of *V. dahliae* development when infecting fiber flax.

Fiber flax (*Linum usitatissimum* L.) is an important source of fiber used for textile manufacture and composite materials (van Sumere 1992). Currently, France is the worldwide leader in fiber flax production, ahead of Belgium, Belarus, and the Russian federation (FAO 2016); more than half of this production is grown in the Normandy region (<http://www.chambre-agriculture-normandie.fr/panoramalin-normandie/>). Historically, most research on flax diseases has focused on Fusarium wilt (caused by *Fusarium oxysporum* f. sp. *lini*) and rust (caused by *Melampsora lini*), which have been the main limiting factors in flax production (Rashid 2003). However, among other harmful flax diseases, Verticillium wilt, a vascular disease caused by the soilborne fungus *Verticillium dahliae* Kleb. is becoming increasingly prevalent among fiber flax culture in France (Valade et al. 2015) (<http://www.fiches.arvalis-infos.fr/>).

Verticillium wilt is caused by species of the group *Verticillium* sensu stricto (Klosterman et al. 2009) and constitutes a major threat to a broad range of crops, leading to significant yield losses (Agrios 1997; Pegg and Brady 2002). Among the *Verticillium* spp., *V. dahliae* has the largest host range (Inderbitzin and Subbarao 2014) and the most significant economic impact, causing billion-dollar losses worldwide (Pegg and Brady 2002). The pathogen can form black-pigmented, multicellular resting structures called microsclerotia that can remain viable in the soil or in debris for 20 years (DeVay et al. 1974; Krikun and Bernier 1990; Wilhelm 1955). Verticillium wilt is difficult to control because of its inaccessibility during infection, long-term persistence in fields, broad host range, and scarcity of resistant plants. At present, no efficient solution exists, even if research

on varietal selection, agronomic methods, and antagonist microbes seems to be promising (Fradin and Thomma 2006; Klosterman et al. 2009; Yadeta and Thomma 2013). Several studies focused on race-specific resistance have highlighted an allelic form of *Ve1*, a gene encoding a cell-surface glycoprotein that leads to an incompatible reaction with *V. dahliae* race 1 pathotypes. *V. dahliae* race 1 strains are more aggressive than so-called race 2 strains on susceptible tomato cultivars (de Jonge et al. 2012; Diwan et al. 1999; Fradin et al. 2009; Grogan et al. 1979; Kawchuk et al. 2001; Rick et al. 1959; Schaible et al. 1951). To date, there is no fiber flax variety resistant to *V. dahliae* and no effective chemical control. In addition, the 6-to-7-year interval between two flax cultures in crop rotation recommended to fight against soilborne diseases is limited because the inoculum is present for up to 20 years in soil and could have a broad host range. Therefore, an understanding of pathogen infection, colonization, and subsequent symptoms produced on fiber flax must be the first step toward innovative solutions against this disease.

On various plants, infection by *V. dahliae* occurs at the root surface level; fungal hyphae later invade xylem vessels and progress slowly in acropetal direction (i.e., from the bottom to top of the plant). Upon germination, each microsclerotium has the capacity to produce one to several infection hyphae, thus increasing the chance to engender a successful infection (Fitzell et al. 1980). However few of the infections lead to xylem colonization: Gerik and Huisman (1988) have estimated that 0.02% of root infections lead to systemic colonization in cotton. Similarly, when eggplant roots were inoculated with *V. dahliae* microsclerotia, only 1 of 205 infections led to systemic colonization (Bejarano-Alcázar et al. 1999). Recent studies of *V. dahliae* on different hosts were carried out by green fluorescent protein (GFP) transformed strains (inoculum of conidia) and confocal laser-scanning microscopy (CLSM), thereby providing substantial knowledge about the infection process on root and the colonization within the plant (Maruthachalam et al. 2013; Vallad and Subbarao 2008; Zhang et al. 2012; Zhao et al. 2014). According to these studies, germination of conidia on inoculated plants was generally fast; in less than 24 h, one germ tube emerged from one or both ends of a conidium. Thereafter, the germinated conidia rapidly

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developed appressoria or evolved in a complex hyphal network that formed later appressoria between junctions of epidermal cells, points of entry for the further internal colonization (Vallad and Subbarao 2008). On *Arabidopsis*, spinach, or lettuce, hyphae growth followed the longitudinal axis of epidermal cells and was generally intercellular (Maruthachalam et al. 2013; Vallad and Subbarao 2008; Zhao et al. 2014). However, even if the infection occurs rapidly, the preferential root zones leading to a successful one vary according to the reports. For example, substantial colonization was limited to the tip of lateral roots and within the root elongation zone on lettuce (Vallad and Subbarao 2008). On *Arabidopsis*, only the root hair zone was colonized by germinated conidia (Zhao et al. 2014). In contrast, Zhang et al. (2012) observed that most of the conidia were on the meristematic and elongation zones of cotton roots but rarely on the root cap, and none were on the root hair. On spinach root, vascular colonization started from the root tip; in addition, a complex hyphal network leading to later infections was observed on the surface of the elongation zone (Maruthachalam et al. 2013). Internal colonization started within the elongation zone by entering the root cortical tissue intra- or intercellularly; subsequently, the pathogen hyphae reached the xylem vessels (Maruthachalam et al. 2013; Vallad and Subbarao 2008; Zhao et al. 2014). Once vascular colonization was established, the hyphae colonized neighboring vessels via pit pairs or perforation plates and pathogen development slowly extended to the lateral roots (Vallad and Subbarao 2008). The pathogen was either restricted to individual vessels (cotton or lettuce) or all of the xylem vessels were densely colonized (spinach). The hyphae progressed acropetally through root vessels toward the aboveground parts together with conidia production, accelerating the colonization by moving rapidly with the transpiration stream. Ultimately, the pathogen was also detected in the upper stem, inflorescence, and seed, leading to further contamination of the developing seedlings (Maruthachalam et al. 2013; Vallad and Subbarao 2008; Zhao et al. 2014).

Only a few scientific reports have dealt with Verticillium wilt on flax. Marchal (1940) reports wilting symptoms on plants and the presence of microsclerotia on roots in Belgium. Hoffmann and Rondonmiski (1959) mention that Verticillium wilt is responsible for fiber that is brittle and consequently nonmarketable, and Fitt et al. (1992) describe Verticillium wilt symptoms and assess its occurrence and severity in some U.K. and German fields. According to the French monitoring organizations, symptoms on fiber flax related to Verticillium wilt have a metallic blue appearance on stems during retting (a process employing the action of microorganisms to separate fiber from the other parts of the plant) that become brittle and fragile, thus causing damage. The damage causes considerable yield losses and, consequently, depreciated fibers are difficult to upgrade. However, before retting, Verticillium wilt is hardly diagnosable because of possible confusion with water stress or Fusarium wilt (Valade et al. 2015) (<http://www.fiches.arvalis-infos.fr/>). On various plants affected with Verticillium wilt, necrotic lesions at the root cap or vascular discolorations within the root in advance of the pathogen colonization may be the first symptoms to be observed (Eastburn and Chang 1994; Vallad and Subbarao 2008; Vallad et al. 2006). Wilting of leaves is triggered a few weeks after inoculation, usually in the oldest shoots; wilting starts on one half of an infected leaf and slowly progress acropetally. Plants affected with Verticillium wilt are usually stunted and their vascular tissues show characteristic brownish discoloration. Defoliation, gradual wilting and death of successive branches, or the abrupt collapse and death of the entire plant may all be consequences of Verticillium wilt (Agrios 2005). However, the consequences of a Verticillium infection can differ between hosts; there are no unique symptoms that exist in all plants (Fradin and Thomma 2006). For example, infection on tomato triggers yellowing, and necrosis of the tips and edges of lower leaves, causing typical V-shaped lesions. In some cases, the veins may turn purple or brown, and yellow blotches may appear on leaves that later become necrotic and brown (Fradin and Thomma 2006). Wilt symptoms on spinach first affect the bolting stem, then inflorescence, including bracts that develop a chlorosis and flaccid aspect (Maruthachalam et al. 2013). On lettuce, Verticillium wilt symptoms

first consist of angular chlorosis regions on leaves that become flaccid, then evolve into angular areas of necrosis (Pegg and Brady 2002; Vallad et al. 2006).

Considering that the symptoms and the resulting damage are unique in each host plant–*V. dahliae* interaction and the lack of reports on Verticillium wilt on fiber flax, we provide a complete description of observed symptoms and pathogen development, including spatial and temporal pathogen progress on the root, stem, and leaf, as monitored by CLSM.

Materials and Methods

Plant material. Fiber flax seed (*L. usitatissimum* ‘Adélie’) were provided by the cooperative Terre de Lin (Saint-Pierre-le-Vigier, France). Seed were sterilized by immersion in 70% ethanol for 2 min, rinsed in sterile water for 5 min, immersed in sodium hypochlorite 2.6% (vol/vol) for 10 min, and finally rinsed three times in sterile water before sowing. In July 2014, a 12.5-ha fiber flax plot (*L. usitatissimum* ‘Aretha’) was investigated in La Haye Aubrée, France (49°39′ 73.0″ N, 0°67′50.1″ E) approximately 3 months after planting (capsule stage). Within the plot, an infectious site containing wilting plants was clearly visible and, thus, susceptible to be affected by Verticillium wilt. Twenty samples of infected plants (C1 to C20) and 20 of noninfected plants were collected. Samples of flax straws, brittle or not, were collected in September 2014 (post retting) from the same plot.

Pathogen strains. *V. dahliae* strain VdLu01 was previously sampled from straw of diseased fiber flax during retting in August 2005 in Gueures (Upper Normandy, France) by Terre de Lin. This strain has the capacity to produce enough inoculum for an experimental assay. The Department of Plant Pathology (University of California, Davis) provided *V. dahliae* strains VdLs16 and VdLs17, isolated on lettuce and previously described as race 1 and race 2, respectively (Vallad et al. 2006), both of which were transformed with the GFP gene. Fluorescence was checked using an epifluorescent microscope (DM1000; Leica, Ketzlar, Germany) fitted with a 50-W Hg lamp (Leica). To obtain pathogen inoculum, each strain was grown in potato dextrose broth (PDB; 26.5 g liter⁻¹) with sterile water in a 500-ml Erlenmeyer flask, and placed on an agitator (150 rpm) for 15 days at 20°C in the dark. Conidia were subsequently isolated in phosphate-buffered saline (PBS) by crushing fungal masses on a 100-μm-pore sieve. Recovered conidia were counted on a Malassez cell and adjusted to 10⁶ conidia/ml before inoculation. Microsclerotia were obtained by using malt extract (MA; 10 g liter⁻¹) with sterile water in a 500-ml Erlenmeyer flask for 1 month at 20°C in the dark.

Inoculation procedure and plant cultivation. Two systems, a hydroponic/root-dipping method and a peat system/soil-drench method, were used for plant cultivation and inoculation. In both systems, plants were inoculated with VdLu01 and VdLs17 at a concentration of 10⁶ conidia/ml. All experiments were performed in a growth chamber at 23°C, with a photoperiod of 16 h of light and 8 h of darkness. The hydroponic/root-dipping method was used for symptomatic observations and CLSM analysis (roots and above-ground parts). Sterilized seed were sown in sterile Murashige and Skoog medium (4.3 g liter⁻¹) solidified with agar (15 g liter⁻¹). Ten-day-old seedlings were gently sampled. Roots were immersed for 1 h in a conidia solution or in PBS as a control. The root seedlings were subsequently transferred into sterile hydroponic Hoagland medium (Hoagland and Arnon 1950). The peat system/soil-drench method was used for symptomatic observations and CLSM analysis (aboveground parts). Sterilized seed were sown in Jiffy-7 pots (Jiffy Products International BV, Moerdijk, The Netherlands) with 1 seed/pot. The bottoms of the Jiffy pots containing 10-day-old seedlings were drenched for 1 h with conidia solution or with PBS for controls. Jiffy pots were next transplanted into larger peat pots and plant stakes were set up after 3 weeks.

Light microscopy. Plant symptoms were imaged on a digital microscope (VHX-5000; Keyence, Osaka, Japan) with VH-Z20R and VH-Z100R (fitted with an OP-72405 polarization illumination attachment) objectives (Keyence), and *V. dahliae* fungal structures were imaged on a transmitted light microscope (DM1000; Leica) with ×10 and ×100 objectives at UniLaSalle (Mont-Saint-Aignan, France).

Fresh and fixed samples in methanol, according to Zelko et al. (2012), were observed. Cross-sections of stems and roots were hand-cut under a binocular microscope, then mounted on a glass slide with glycerol 80% (vol/vol). Lactophenol blue solution (Sigma-Aldrich, St. Louis)

was used to stain fungal structure. Briefly, mycelium was stained 5 min on a glass slide, then rinsed with distilled water before observation.

CLSM. Samples were fixed in methanol and then stored at 4°C as described by Zelko et al. (2012) for further analyses. Root

Fig. 1. Identification of VdLu01, a *Verticillium dahliae* strain isolated on fiber flax straw. **A** and **B**, **E** to **H**, morphological and **C** and **D**, molecular traits. Macroscopic aspect of VdLu01 after growing **A**, 1 week on potato dextrose broth agar medium and **B**, 1 month on malt extract liquid medium. Polymerase chain reaction amplification using primers targeting **C**, internal transcribed spacer-specific regions of *V. dahliae* or **D**, *Ave1* on *V. dahliae* strains VdLu01, VdLs16, and VdLs17; expected amplicon size 334 bp (**C**) or 216 bp (**D**). Microscopic observation of hyphal structure: **E**, apex of a conidiophore; **F**, torulose cells surrounding a microscerotium blue lactophenol staining; **G**, heap of microscerotia; and **H**, isolated microscerotium. Abbreviations: co = conidium, cp = conidiophore, ph = phialide, ms = microscerotia, and tc = torulose cell. Bars = 20 μ m (**E**), 40 μ m (**F** and **H**), and 200 μ m (**G**).

samples were sectioned because of their large size and longitudinal and cross-sections of stems were hand-cut under a binocular microscope. All samples were mounted on a glass slide with distilled water. Images were acquired by CLSM (TCS SP2 AOBS and TCS SP8 MP; Leica) at Primacem, Mont-Saint-Aignan, France. Observations were performed using $\times 10$, $\times 25$, $\times 40$, and $\times 100$ oil-immersion objectives. GFP fluorescence was detected by a 488-nm ray line of an argon laser and emissions were collected in the range of 498 to 570 nm. Fluorescent and transmitted light captures were performed; overlay images and scales were obtained by ImageJ software (Rasband WS, 1997–2015; <https://imagej.nih.gov/ij/>).

Plant and fungal DNA extraction and quantification. Up to 50 mg of plant (stem or straw, noninfected or infected) or fungus (pure culture) samples were transferred to -20°C before use. Plant samples were previously finely cut before DNA extraction. Total DNA extraction was performed using a PowerPlant DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Concerning fungal samples, a preliminary step was added. Fungal tissues were crushed with a sterile pestle and subsequently digested at 30°C overnight in 450 μl of PowerPlant bead solution, with the addition of 400 U of a lyticase solution (Sigma-Aldrich). Total DNA concentrations were assessed using a Fluorescent DNA quantification kit (Bio-Rad, Hercules, CA, USA) and a Varioskan Flash Multimode Reader (ThermoScientific, Waltham, MA, USA) following the manufacturer's instructions. All extracted DNA samples were stored at -20°C .

Verticillium race determination by specific polymerase chain reaction assay. Race-specific determination was performed using the primers Vd_Ave1_F1 (5'-ACTCGCCAGAAGAAGTCCAA-

3') and Vd_Ave1_R1 (5'-CCACAACAGGGAGACAGGTT-3'). The primers were designed by Primer3 software (Koressaar and Remm 2007; Untergasser et al. 2012). The *Ave1* sequence obtained from *V. dahliae* strain St14.01 (accession number JQ625341) was used as template. In this experiment, strains that lack *Ave1* could belong to race 2. The strains VdLs16 race 1 and VdLs17 race 2 DNA were used as controls. Primers targeting the internal transcribed spacer (ITS) region (Vd-ITS1-45-F and Vd-ITS2-379-R) (Bressan et al. 2016) were used as a positive control to guarantee the presence of the DNA samples. End-point polymerase chain reaction (PCR) was performed in a 25- μl reaction volume: 50 ng of fungal DNA (pure culture), 0.1 μM each primer (Vd_Ave1_F1 and Vd_Ave1_R1), and 12.5 μl of GoTaq Green Master Mix (Promega Corp., Madison, WI, USA). PCR cycling was achieved using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) following a program of 95°C for 2 min; then, 35 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 30 s; and, finally, 72°C for 7 min for final extension. The PCR products, with an expected amplicon size of 216 bp, were visualized by electrophoresis on a 1% agarose gel stained by ethidium bromide.

***V. dahliae* detection on plant samples by PCR assay.** *V. dahliae* detection was performed using the primers Vd-ITS1-45-F and Vd-ITS2-379-R (Bressan et al. 2016). End-point PCR was performed in a 25- μl reaction volume: 50 ng of plant DNA (stem or straw, non-infected or infected), 0.1 μM each primer (Vd-ITS1-45-F and Vd-ITS2-379-R), and 12.5 μl of GoTaq Green Master Mix (Promega Corp.). PCR cycling was achieved using a GeneAmp PCR System 9700 (Applied Biosystems) following a program of 95°C for 2 min; then, 35 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 30 s; and, finally 72°C for 7 min for final extension. The PCR products, with an expected amplicon size of 334 bp, were visualized by electrophoresis on 1% agarose gel stained by ethidium bromide.

Results

Identification of VdLu01, a *V. dahliae* isolate from fiber flax.

After growing for 1 week on PDB-agar medium, the strain VdLu01 produced a white colony (Fig. 1A); an abundant and floccose aerial mycelium was observed. According to morphological traits described by Inderbitzin et al. (2011), conidiophores were erect and branched, narrowing toward the apex, hyaline and septate. Phialides were flask shaped, arranged in whorls along conidiophores, pointed, and carrying ovoid and hyaline terminal conidia (Fig. 1E). Furthermore, when cultivated in MA-liquid medium for 1 month, VdLu01 formed microsclerotia composed of rounded, brown-pigmented cells (Fig. 1B and H) embedded in a mycelial matrix (Fig. 1G). Occasionally, hyphae formed by torulose cells were attached to a microsclerotium (Fig. 1F). The ITS region specific to *V. dahliae* (Bressan et al. 2016) was detected on VdLu01 and the lettuce isolates VdLs16 and VdLs17 (Fig. 1C), whereas the *Ave1* sequence was detected on neither VdLu01 nor VdLs17; thus, both could be assigned to race 2 (Fig. 1D).

Pathogen development and symptoms on root. Symptom observations were performed using the strains VdLu01 and VdLs17. In both infections, first root symptoms were triggered rapidly. Lateral roots exhibited dryness, and a brown discoloration on the surface was observed (Fig. 2B). Occasionally, these roots were tightly hanging on to the main root by fungal mycelium (Fig. 2D). Additionally, a brown discoloration of epidermis and vascular tissues in the main root was noticed (Fig. 2F).

Time-course tracking of pathogen development by CLSM was undertaken using the GFP-tagged VdLs17 strain. Two hours after inoculation performed by the root-dipping method, the entire root was covered by conidia, including the zone of cell differentiation, lateral roots, root hairs, zone of cell elongation, and root tips. Conidia were randomly distributed, isolated or in heaps and spotted on the root epidermis (Fig. 3C, D, and E). By 2 h postinoculation (hpi), germination of conidia was observed; only one germ tube emerged from one end per cell (Fig. 3A and B). At 1 day postinoculation (dpi), hyphae had grown parallel to the longitudinal axis of the root or not, occasionally developing secondary structures along the main hyphae (Fig. 3F, G, and H). The growth pattern of most hyphae followed a random path

Fig. 2. B and D, Root symptoms on the zone of cell differentiation and lateral roots infected by the strains VdLu01 or VdLs17, 10 days postinoculation. A and C, Roots of control plants for comparison. Bars= 600 μm (A and B) or 200 μm (C and D). Cross-sections of E, control and F, inoculated roots 4 weeks postinoculation. Bars = 100 μm .

but it is likely that the hyphae extended over a maximum area along the root surface (Fig. 3H). At the same time, some conidia germinated and developed a germ tube; others remained in a quiescent state on the root epidermis (Fig. 3F). Interestingly, whereas successful germination of conidia and hyphal development was found on the zone of cell differentiation and lateral roots, the zone of cell division and root tip were free of the pathogen. By 3 dpi, the pathogen developed from the tip of lateral roots (Fig. 3I) but, in addition, a large fungal mass extended into the zone of cell differentiation and lateral roots, encompassing the entire area, sometimes developing on the periphery (Fig. 3J). Consequently, these sections of roots were covered by the pathogen, causing dryness of lateral roots, collapse of tissues, and cessation of meristematic activity in some cases. These symptoms were correlated with the observations performed by the light reflection microscope. By 1 week postinoculation (wpi), the zone of cell differentiation was entirely colonized by the fungus. Most hyphae were oriented parallel to the longitudinal axis of the root (Fig. 3K and L). Simultaneously, on some areas of the zone of cell differentiation, each oriented mycelial hyphae developed several swelling structures similar to appressoria, in contact with the junction of two epidermal cells (Fig. 4A and B). It is likely that these weakly protected areas are the point of entry of intercellular colonization into the cortical tissues

leading to vascular colonization. However, no cortical intercellular colonization was observed. By 2 wpi, colonization of the root vascular system by the pathogen was clearly observed (Fig. 4C and C); a set of several hyphae followed the path of the vascular tissues (Fig. 4E and F). As in the main root, vascular colonization occurred in lateral roots. The pathogen was always detected on the basal area, whereas it was still absent from apical areas of the root. Interestingly, once vascular colonization was established, no additional pathogen was observed on the root surface (Fig. 4C, F, and G). Cross-sections of the main root at 4 wpi confirmed the pathogen colonization within the xylem vessels (Fig. 4G, H, and I). Not all vessels were filled by the pathogen (Fig. 4G, H, and I), and those contaminated were filled with different amounts of hyphae (Fig. 4H).

Pathogen development and symptoms on stem and leaves. In VdLu01 infections, some early symptoms occasionally appeared on seedlings or plants with growth retardation after 1 wpi: apical leaves exhibited chlorosis and an altered morphology (data not shown). In both infections (VdLu01 or VdLu17), wilting symptoms on leaves started between 3 and 4 wpi (Fig. 5B). Necrosis was displayed first at the leaf tips, which turned yellow and rolled up (Fig. 5A). Over the following weeks, necrosis extended to the whole leaf but did not reach or affect the stem, and falling leaves could be observed (Fig.

Fig. 3. Germination of conidia and early steps of hyphae growth of VdLu17 strain on flax roots. Overlay of a brightfield image and a fluorescent image. **A** and **B**, Germinated and nongerminated conidia on the zone of cell differentiation, 2 h postinoculation (hpi). **C** and **E**, Conidia on the zone of cell differentiation, including lateral root junction (only **C**), 2 hpi. **D**, Conidia on the zone of cell elongation, 2 hpi. Growth of mycelial hyphae on the zone of cell differentiation **F** and **G**, 1 day postinoculation (dpi) and **H**, 3 dpi. **I**, Hyphae caused dryness at the tip of a lateral root, 3 dpi. **J**, Fungal mass wrapped the zone of cell differentiation, including a lateral root, 3 dpi. **K** and **L**, Growth of mycelial hyphae on the zone of cell differentiation, 1 week postinoculation. **L**, Only fluorescent image. Abbreviations: co = conidium, gc = germinated conidium, gt = germ tube, mh = main hypha, ss = secondary structure, lr = lateral root, and mr = main root.

Fig. 4. Vascular colonization of VdLu17 strain on flax roots. Overlay of a brightfield image and a fluorescent image. **A** and **B**, Mycelial hyphae developing appressoria-like structures on intercellular junction of the zone of cell differentiation, 1 week postinoculation (wpi). **B**, Only fluorescent image. **C** to **I**, Vascular colonization of the pathogen in a main root, 2 wpi. Cross-sections, 4 wpi (**G** to **I**). **D**, **E**, and **F**, Only fluorescent images. **G**, Image stacking (10 images), depth = 60 μ m. Abbreviations: a = appressorium-like and h = hyphae.

5A). These symptoms were always observed first at the base and then spread to the apex, clearly showing the growing symptoms to be acropetal (Fig. 5B). However, this growth of symptoms appeared to be very slow. Furthermore, a brown vascular discoloration was observed within the basal part of the stem (hypocotyl) at 4 wpi (Fig. 5D).

Further CLSM investigations at 4 wpi confirmed extensive pathogen colonization within the root whereas a weak fluorescent signal was detected within the hypocotyl (Fig. 6A and B). Moreover, neither symptomatic leaves nor the apical part of the stem revealed the presence of the pathogen. Therefore, symptoms, including brown discoloration and wilting leaves, appeared well in advance of the presence of the pathogen. Nevertheless, growth into vascular tissues was slow. Between 6 and 8 wpi, observations on the stem were then performed between the cotyledon and 10th leaf. Longitudinal sectioning of the stem was necessary to detect the GFP signal; actually, it revealed a high signal around the pith showing the acropetal growth of pathogen hyphae (Fig. 6H). Nevertheless, the pathogen did not colonize the entire area around the pith. No hyphal progression was observed toward the leaves. Further information was obtained by observation of the stem cross-sections. Similar to what was observed in the root, xylem vessels were partially colonized by various quantities of pathogen hyphae (Fig. 6F and G), and several neighboring vessels, or only a single vessel, were occasionally colonized (Fig. 6C and E). Finally, at this time, the pathogen could disturb the hydraulic system but the fiber bundles were not invaded (Fig. 6D and E). Symptomatic lower and upper leaf samples were collected and did not exhibit macroscopic differences. The pathogen invaded vascular tissues of the midrib and veins in lower leaves only (Fig. 6I). The pathogen did not colonize the entire leaf, only the basal area toward the apical area (Fig. 6J).

Observations of Verticillium wilt in field. At the capsule stage, wilted plants were randomly distributed within an area of the plot (Fig. 7A). On the infected plants sampled, the global aspect of infected plants exhibited typical wilting symptoms and fewer capsules were observed as compared with the noninfected plants (Fig. 7C and D). Stems were dried and chlorotic on apical parts, containing small black spots

(Fig. 7E). Leaves were rolled up and contained black fungal mycelium (Fig. 7D). On the brittle straws sampled after retting, reflection microscopy revealed numerous black microsclerotia on the surface of brittle straws (Fig. 7F). Several dry fungal structures similar to brown-pigmented torulose cells were observed on the internal parts of the epidermal tissues, seemingly forming a microsclerotium (Fig. 7G). After retting, the bast fiber bundle was easily removable from the straw and also contained numerous black microsclerotia (Fig. 7H). Closest investigations revealed that microsclerotia were embedded inside the bast fiber bundle (Fig. 7I) and, thus, potentially involved in brittleness of infected fibers. PCR amplification confirmed the presence of *V. dahliae* on both infected plants and brittle straws (Fig. 7B).

Discussion

Here, we provide the first report of how the pathogen *V. dahliae* develops in fiber flax roots and aboveground parts using VdLs17 isolated from lettuce and previously transformed with the GFP gene. The strain VdLs17 belongs to race 2 (Vallad et al. 2006) and lacks the *Ave1* sequence, as does the strain VdLu01 isolated from fiber flax. Fiber flax exhibits similar wilting symptoms after 3 or 4 weeks when inoculated by VdLs17 or VdLu01 in both inoculation systems. Therefore, VdLs17 can be considered to be a flax pathogen. Bhat and Subbarao (1999) have reported cross-pathogenicity of isolates from specific crops on a set of host plants (e.g., bell pepper, cabbage, cauliflower, cotton, eggplant, and mint isolates). In addition, *Nicotiana benthamiana* exhibits wilting symptoms after 12 days when inoculated by the lettuce strain VdLs17 (Klosterman et al. 2011). These observations support the idea that *V. dahliae* rapidly adapts to new niche hosts.

Although microsclerotia are the primary source of inoculum in infested soils, conidia suspensions were preferentially selected to

Fig. 5. Symptoms of Verticillium wilt on stem and leaf of flax. **A**, Wilting symptom on a basal leaf at 3 and 5 weeks postinoculation (wpi) and control leaf. Bars = 5 mm. **B**, Wilting symptoms at the plant level infected by VdLu01 or VdLs17, apical and basal level, 5 wpi, and control plant for comparison. Cross-sections of **C**, control and **D**, inoculated roots, 4 wpi. Bars = 100 μ m.

Fig. 6. Vascular colonization of VdLs17 strain within the aboveground parts. Overlay of a brightfield image and a fluorescent image. **A** and **B**, Comparison of pathogen fluorescence within xylem cross-sections of root and stem (hypocotyl) on the same plant, only fluorescent images, 4 weeks postinoculation (wpi). **C** to **J**, Between 6 and 8 wpi. **C** to **G**, Growth of mycelia hyphae within stem cross-section. **H**, Growth of mycelia hyphae within stem transversal section. **I** and **J**, Growth of mycelia hyphae within basal part of a symptomatic leaf. Abbreviations: fb = fiber bundle, P = pith, and X = xylems.

initiate infection because conidia are quite uniform, making them easier to prepare and quantify relative to microsclerotia (Vallad and Subbarao 2008). In our report, three different main steps are distinguishable: development on the root epidermis was broadly

asymptomatic, followed by colonization within the root xylems and then of the stem and leaves, leading to symptoms. During the first steps of infection, conidia probably germinated due to root exudates that overcome fungistasis (Schreiber and Green 1963), giving rise to

Fig. 7. Verticillium wilt caused by *Verticillium dahliae* on flax at the capsule stage and after retting. **A**, Delimitation of the fiber flax plot investigated (red lines), area where the infectious site was detected (green circle), and C1 to C20 (yellow lines) = points where the infected plants were sampled. **B**, Polymerase chain reaction amplification using primers targeting internal transcribed spacer-specific regions of *V. dahliae* on *V. dahliae* strain VdLu01, noninfected flax, infected flax, noninfected flax straw, and infected flax straw. Expected amplicon size = 334 bp. **C** to **E**, Wilting symptoms on aboveground parts (nonaffected plant on left). Close-up view: **D**, apical parts and **E**, basal parts. **F** to **I**, Fungal structures of *V. dahliae* on fiber flax stem after retting. **F**, Microsclerotia observed on a straw surface. **G**, Brown-pigmented torulose hyphal cells attached on the internal parts of the epidermal tissues. **H**, Microsclerotia on bast fiber bundles. **I**, Close-up view showing microsclerotia embedded in bast fiber bundle. Abbreviations: ms = microsclerotium and tc = torulose hyphal cells. Bars = 50 µm (F), 20 µm (G and I), and 100 µm (H).

mycelia hyphae that extend over a maximum area between 2 hpi and 1 dpi. Subsequently, the pathogen develops at 3 dpi only on the zone of cell differentiation and lateral roots, causing dryness, brown discoloration, and collapse of tissues as a consequence of massive surface colonization. This fungal mass tends to organize itself along the longitudinal axis of the epidermal cells at 7 days. In contrast, Zhang et al. (2012) observed pathogen development on the meristematic and elongation zones of cotton roots. Nevertheless, colonization of lateral roots leading to a successful infection was reported on *Arabidopsis* and lettuce (Vallad and Subbarao 2008; Zhao et al. 2014). Zhao et al. (2014) reported in *Arabidopsis* that, despite massive hyphae encompassing the root surface, only a few hyphae successfully invaded the root internal tissues. The same observation was previously reported for cotton roots (Zhang et al. 2012). Observations at 7 dpi revealed that several swelling structures similar to appressoria developed along mycelia hyphae on the root surface. The pathogen tends to colonize inner tissues through epidermal cell junctions. These observations confirm the presence of appressoria as structures of penetration. However, pathogen colonization did not spread onto the zone of cell elongation and root tips after 1 dpi, contrary to reports on spinach, cotton, and lettuce (Maruthachalam et al. 2013; Vallad and Subbarao 2008; Zhang et al. 2012), suggesting that these zones are particularly distant from the infection in this study. Indeed, the zone of cell elongation is generally considered to be a major site for the exudation of secondary metabolites (McDougall and Rovira 1970). A previous study noted that microbe-associated molecular patterns such as the flagellar peptide (Flg22) and peptidoglycan (PGN) trigger a defense response in the epidermal layer of the cell elongation zone in *Arabidopsis* (Millet et al. 2010). Border cells, which are separated from the root tip of higher plants and produce metabolites in the rhizosphere (Hawes et al. 2000), protect the pea root meristem when infected by *Nectria haematococca* (Gunawardena and Hawes 2002; Gunawardena et al. 2005). Border-like cells of *Arabidopsis* and flax respond to Flg22 and PGN by triggering an oxidative burst, the expression of defense-related genes, and cell wall redistribution of extensins (Plancot et al. 2013).

Vascular colonization into roots was observed for the first time at 2 wpi in the main root as well as in lateral roots but the colonization of xylems was incomplete. In susceptible lettuce taproots, restricted advancement of mycelia through xylem vessels has also been observed (Vallad and Subbarao 2008). This is in contrast to observations in spinach, where all xylem vessels are heavily colonized (Maruthachalam et al. 2013). The pathogen remains in roots during the following 2 weeks. It was suggested that wilt pathogens exploit this niche to avoid competition with other microbes (McCully 2001). The xylem is a nutritionally poor environment; nevertheless, analysis of whole-genome sequences reveals that the *V. dahliae* genome is enriched in genes encoding cell-wall-degrading enzymes (CWDE), which are implicated in cell wall degradation to release sugar as a source of nutrition (Klosterman et al. 2011). Once entering xylem vessels, *V. dahliae* is faced with a set of plant defense reactions combining tyloses, pectin gels and gums, phenolic compounds, and inorganic sulfur (Yadeta and Thomma 2013).

In our study, common wilting symptoms emerged between 3 and 4 weeks after *V. dahliae* inoculation and grew acropetally. Wilting symptoms are also a consequence of *F. oxysporum* f. sp. *lini* infection (Kroes et al. 1999); reports in the field show that Fusarium wilt can occur at any time of the growing season, and symptoms generally start on upper leaves (Rashid 2003). Therefore, basipetal development of foliar symptoms is a feature of Fusarium wilt whereas acropetal symptom development is a feature of Verticillium wilt. Based on this difference, symptom diagnosis in the field could distinguish Fusarium wilt from Verticillium wilt. In addition, as recommended for an early diagnostic on lettuce (Vallad et al. 2006), the onset of the root vascular brown discoloration in advance of the pathogen could be an early diagnostic on fiber flax as well. Because of a lack of a reliable diagnosis for Verticillium wilt in early steps of cultivation, combining PCR with symptomatology of plant samples could be an efficient approach to detecting cases of Verticillium wilt in the field. In recent years, real-time PCR has gained in popularity for Verticillium wilt detection and quantification in seed (Karajeh 2006; Maruthachalam et al. 2013), environmental samples (Banno et al. 2011; Bilodeau et al.

2012), and symptomatic plants from the field (Atallah et al. 2007; Mercado-Blanco et al. 2003) because it is accurate and sensitive. Necrotic leaves at 4 weeks did not display the presence of the pathogen; it takes two additional weeks to detect hyphae within the midrib and veins of leaves. On infected lettuce, Vallad and Subbarao (2008) reported the presence of hyphae moving through the midrib and veins within chlorotic regions of leaves occurring after symptoms appear. Actually, there is no consensus about the physiological and biochemical basis of Verticillium wilt symptoms. Several studies demonstrated *V. dahliae* secretome activity that is detrimental to plant tissues such as Nep or NEP protein elicitors. Recombinant VdNEP causes symptoms on tobacco and cotton and, although recombinant NLP1 and NLP2 cause symptoms on tobacco, only the NLP1 knockout mutant stops symptoms on tobacco (Santhanam et al. 2013; Wang et al. 2004; Wang et al. 2012). Other studies note the symptoms to be an unfortunate secondary consequence of the host response; among them, the TMP1 protein, a defense response to *V. dahliae*, contributes to one aspect of symptom development: namely, water restriction and wilting of the host (Robb et al. 1983, 1989).

Finally, between 6 and 8 wpi, the pathogen was sequestered within stem xylem vessels. However, pathogen progression had not yet reached the bast fiber bundle. Generally, fiber flax cultivation requires at least 120 days to obtain long fibers, followed by a retting process to dissociate the fibers from the other parts of the stem. Damage caused by Verticillium wilt to fiber flax differs from other crops because the inner tissues (bast fiber bundles) are affected and, therefore, unmarketable. It could be suggested that fiber deterioration can occur later, once the plant is no longer capable of protecting itself (i.e., during retting). Our report in the field revealed that *V. dahliae* had reached the fiber after retting and numerous microsclerotia were embedded inside the bast fiber bundle, consequently potentially contributing to weakening of the fiber. Cellulose is the major compound of the fiber's secondary cell wall, ranging from 65 to 80% of dry weight at maturity. Cellulose is encompassed into a hemicellulose and pectin matrix that ensures cohesion (Baley 2002; Focher et al. 1992; Morvan et al. 1989). Analysis of the *V. dahliae* genome shows genes encoding CWDE, including polysaccharide lyases, glycoside hydrolases, carbohydrate binding modules, glycosyl transferases, and carbohydrate esterases. Polysaccharide lyases are well represented in the *V. dahliae* genome in comparison with other Ascomycota; therefore, *V. dahliae* is able to cleave different forms of pectin (Klosterman et al. 2011). Accordingly, the process of how and when the fiber is degraded by the pathogen remains an interesting question.

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