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 soil-borne fungus *Verticillium dahliae* Kleb. This disease leads to non-negligible yield losses and depreciated fibers that are consequently difficult to upgrade. Verticillium wilt is a major threat to a large broad range of agriculture. In this study, fiber flax susceptible cv. 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, UC Davis, USA). Between three and four weeks post inoculation, wilting symptoms on leaves were first observed, with acropetal growth during the following weeks. Pathogen development was tracked by confocal laser scanning microscopy (CLSM) during the asymptomatic and symptomatic stages. First, conidia germination led to

the development of hyphae on root epidermis more particularly on 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 three days post inoculation, the zone of cell differentiation and lateral roots were embedded into a fungal mass. Swelling structures such as appressoria, were observed at one week post inoculation. At two weeks post inoculation and onwards, 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 field revealed microsclerotia embedded inside the bast fiber bundle, thus contributing, potentially to weakening of fiber. All of these results provide a global account of the *V. dahliae* development when infecting fiber flax.

Introduction

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 Russian federation (FAOSTAT data 2016); more than half of this production is grown in the Normandy region (http://www.chambre-agriculture-normandie.fr/panorama-lin-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 soil borne 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 crop, leading to significant yield losses (Agrios, 1997; Pegg and Brady, 2002). Among the *Verticillium* species, *V. dahliae* has the largest host range (Inderbitzin and Subbarao, 2014) and the most significant economic impact, causing billion dollars losses worldwide (Pegg and Brady, 2002). The pathogen can form black pigmented, multicellular resting structures, so-called, microsclerotia that can remain viable in the soil or in debris for 20 years (Wilhelm, 1955; Devay *et al*., 1974; Krikun *et al*., 1990). 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 (Schaible *et al*., 1951; Rick *et al*., 1959; Grogan *et al*., 1979; Diwan *et al*., 1999; Kawchuk *et al*. 2001; Fradin *et al*., 2009; de Jonge *et al*., 2012). To date, there is no fiber flax variety resistant to *V. dahliae* and no effective chemical control. In addition, the six-seven year interval between two flax cultures in crop rotation recommended to fight against soil-borne diseases is limited because the inoculum is present for up to 20 years in soil and could have a broad host range. Therefore, the understanding of the pathogen infection, colonization and subsequent symptoms produced on fiber flax must be the first step towards 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 out of 205 infections led to systemic colonization (Bejarano-Alcazar *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), therefore providing substantial knowledge about the infection process on root and the colonization within the plant (Vallad and Subbarao, 2008; Zhang *et al*., 2012; Maruthachalam *et al*. 2013; Zhao *et al*., 2014). According to these studies, germination of conidia on inoculated plants was generally fast, in less than 24 hours one germ tube emerged from one or both ends of a conidium. Thereafter, the germinated conidia developed rapidly 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 (Vallad and Subbarao, 2008; Maruthachalam *et al*. 2013; 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 conidia were on the meristematic and the elongation zones of cotton roots, but rarely on the root cap and none 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-intercellularly, subsequently the pathogen hyphae reached the xylem vessels (Vallad and Subbarao, 2008; Maruthachalam *et al*. 2013; Zhao *et al*., 2014). Once vascular colonization established, the hyphae colonized neighboring vessels via pit pairs or perforation plates and the 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 are densely colonized (spinach). The hyphae progressed acropetally through root vessels towards the above-ground parts together with conidia production, accelerating the colonization by moving rapidly with the transpiration stream. Ultimately, the pathogen was also detected in upper stem, inflorescence and seed, leading to further contamination to the developing seedlings (Vallad and Subbarao, 2008; Maruthachalam *et al*. 2013; 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 Rondomanski (1959) mention that Verticillium wilt is responsible for fiber that is brittle and consequently non-marketable; Fitt *et al*., (1992) describe Verticillium wilt symptoms and assess its occurrence and severity in some UK 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 (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 depreciated fibers are consequently 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 *et al*. 2006; Vallad and Subbarao, 2008). Leaves wilting are triggered a few weeks after inoculation usually in the oldest shoots, wilting starts at 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 consequence of a Verticillium infection can be different 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 bolting stem, then inflorescence including bracts that develop a chlorosis and flaccid aspect (Maruthachalam *et al*. 2013). On lettuce, Verticillium wilt symptoms first consists of angular chlorosis regions on leaves that become flaccid, then evolving into angular areas of necrosis (Pegg and Brady, 2002; Vallad *et al*. 2006).

Considering that the symptoms and the resulting damage is unique in each host plant-*V. dahliae* interactions and the lack of reports on Verticillium wilt on fiber flax, we provide a complete description of observed symptoms and of the 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 seeds (*Linum usitatissimum* L.) cv. Adélie were provided by the cooperative Terre de Lin (Saint-Pierre-le-Vigier, France). Seeds were sterilized by immersion in 70% ethanol for 2 min, rinsed in sterile water for 5 min, immersed in sodium hypochlorite 2.6% (v/v) for 10 min and finally rinsed three times in sterile water before sowing. In July 2014, a 12.5 hectare fiber flax plot (*Linum usitatissimum* L., cv. 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, thus susceptible to be affected by Verticillium wilt. Twenty samples of infected plants (C1 to C20) and 20 of non-infected were collected (Fig. 7A). Samples of flax straws, brittle or not were collected in September 2014 (post retting) from the same plot.

Pathogen strains

The *V. dahliae* strain VdLu01was previously sampled from straw of diseased fiber flax during retting on 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, CA USA) 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 epi fluorescent microscope (DM1000, Leica, Germany) fitted with a 50 W Hg lamp (Leica, Germany). To obtain pathogen inoculum, each strain was grown in potato dextrose broth (PDA - 26.5 $g.l^{-1}$) with sterile water in 500 ml Erlenmeyer flask, placed on an agitator (150 rpm) for fifteen days at 20°C in the dark. Conidia were subsequently isolated in PBS (phosphate buffered saline) by crushing fungal masse on a 100 µm pore sieve. Recovered conidia were counted on a Malassez cell and adjusted to 10^6 conidia per mL before inoculation. Microsclerotia were obtained by using malt extract (MA - 10 $g.l^{-1}$) with sterile water in 500 ml Erlenmeyer flask for one month at 20°C in the dark.

Inoculation procedure and plants 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 10^6 conidia per mL. All experiments were performed in a growth chamber at 23°C, with a 16h light and 8h dark photoperiod. The hydroponic/root-dipping method was used for symptomatic observations and CLSM analysis (roots and above-ground parts). Sterilized seeds were sowed in sterile Murashige & Skoog medium (4.3 $g.L^{-1}$) solidified with agar (15 $g.L^{-1}$). Ten-day seedlings were gently sampled. Roots were immersed for 1 hour 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 (above-ground parts). Sterilized seeds were sowed in Jiffy-7® pots (Jiffy Products International BV, Moerdijk, The Netherlands) with 1 seed per pot.

The bottom of the Jiffy pots containing ten days seedlings were drenched 1 hour into conidia solution or in 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, Japan) with VH-Z20R and VH-Z100R (fitted with an OP-72405 polarization illumination attachment) objectives (Keyence, Japan) and *V. dahliae* fungal structures were imaged on a transmitted light microscope (DM1000, Leica, Germany) with x10 and x100 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% (v/v). Lactophenol blue solution (Sigma Aldrich, Saint Louis MO, USA) was used to stain fungal structure. Briefly, mycelium was stained 5 min on glass slide then rinsed with distilled water before observation.

Confocal laser scanning microscopy (CLSM)

Samples were fixed in methanol and then stored at 4°C as described Zelko *et al*., 2012 for further analyses. Root 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, Germany) at Primacen, Mont-Saint-Aignan France. Observations were performed using x10, x25, x40 and x100 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 and 570 nm. Fluorescent and transmitted light captures were performed then; overlay images and scales were obtained by ImageJ software (Rasband WS, 1997-2015, http://imagej.nih.gov/ij/, 1997-2015).

206 **Plant and fungal DNA extraction and quantification**

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

218 **Verticillium race determination by specific PCR assay**

219 Race-specific determination was performed using the primers Vd_Ave1_F1 (5'-
220 ACTCGCCAGAAGAAGTCCAA- 3') and Vd_Ave1_R1 (5'-
221 CCACAACAGGGAGACAGGTT- 3'). The primers were designed by Primer3 software
222 (Koressaar and Remm, 2007; Untergasser *et al*., 2012). The *Ave1* sequence obtained from *V.*
223 *dahliae* strain St14.01 (accession no. JQ625341) was used as template. In this experiment,
224 strains that lack *Ave1* could belong to race 2. The strains VdLs16 race 1 and VdLs17 race 2
225 DNA were used as controls. Primers targeting the ITS region Vd-ITS1-45-F and Vd-ITS2-
226 379-R (Bressan *et al*., 2016) were used as a positive control to guarantee the presence of the
227 DNA samples. End-point PCR was performed in a 25 µL reaction volume: 50 ng of fungal
228 DNA (pure culture), 0.1 µM of each primer (Vd_Ave1_F1 and Vd_Ave1_R1) and 12.5 µL of
229 GoTaq® Green Master Mix (Promega, Madison WI, USA). PCR cycling was achieved using

a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City CA, USA) following the program: 95°C, 2 minutes then 35 cycles of 95°C, 45 seconds; 60°C; 1 minute; 72°C; 30 seconds; and finally 72°C, 7 minutes for final extension. The PCR products, with an expected amplicon size of 216 bp, were visualized by electrophoresis on 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 of each primer (Vd-ITS1-45-F and Vd-ITS2-379-R) and 12.5 µL of GoTaq® Green Master Mix (Promega, Madison WI, USA). PCR cycling was achieved using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City CA, USA) following the program: 95°C, 2 minutes then 35 cycles of 95°C, 45 seconds; 60°C; 1 minute; 72°C; 30 seconds; and finally 72°C, 7 minutes 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 one week growing on PDA-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 towards 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 one month, VdLu01 formed microsclerotia composed of rounded brown-pigmented cells (Fig. 1B and

1H) 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 *Ave1* sequence was detected neither on VdLu01 nor on 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 triggered rapidly. Lateral roots exhibited dryness and a brown discoloration on surface was observed (Fig. 2B). Occasionally, these roots were tightly hanging on 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, 3D and 3E). By 2 hours post inoculation (hpi), germination of conidia was observed; only one germ tube emerged from one end per cell (Fig. 3A and 3B). One day post inoculation (dpi), hyphae had grown parallel to the longitudinal axis of the root or not, occasionally developing secondary structures along the main hyphae (Fig. 3F, 3G and 3H). The growth pattern of most hyphae followed a random path, 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 also, a large fungal mass extended on the zone of cell differentiation and lateral roots, encompassing the entire area, sometimes developing in 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 in correlation to the observations performed by the light reflection microscope. By 1 week post inoculation (wpi), the zone of cell differentiation was entirely colonized by the fungus. Most hyphae were oriented parallel with the longitudinal axis of the root (Fig. 3K and 3L). 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 4B). 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 4D): a set of several hyphae followed the path of the vascular tissues (Fig. 4E and 4F). 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, 4F and 4G). Cross-sections of the main root at 4 wpi confirmed the pathogen colonization within the xylem vessels (Fig. 4G, 4H and 4I). Not all vessels were filled by the pathogen (Fig. 4G, 4H and 4I), and those contaminated were filled with different amounts of hyphae (Fig. 4H).

Pathogen development and symptoms on stem and leaves

In VdLu01infections, some early symptoms occasionally appear 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 VdLs17), wilting symptoms on leaves started between three and four wpi (Fig. 5B). Necrosis was displayed first at the leaf tips, which turn yellow and roll 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. 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 6B). More, neither symptomatic leaves nor apical part of the stem revealed the presence of the pathogen. Therefore, symptoms, including the 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 tenth 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. Similarly to what was observed in root, xylem vessels were partially colonized by various quantity of pathogen hyphae (Fig. 6F and 6G), and several neighboring vessels, or only a single vessel, were occasionally colonized (Fig. 6C and 6E). Finally at this time, the pathogen could disturb the hydraulic system but the fiber bundles were not invaded (Fig. 6D and 6E). Symptomatic

lower and upper leaves 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, fewer capsules were observed as compared to the non-infected plants (Fig. 7C and 7D). 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), thus potentially involved in brittleness of infected fibers. PCR amplification confirmed presence of *V. dahliae* both on 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 above-ground 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 VdLu01isolated from fiber flax. Fiber flax exhibits wilting symptoms after three or four weeks similarly when inoculated by VdLs17 or VdLu01in 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 benthamina* 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 suspension were preferentially selected to initiate infection because conidia are quite uniform, making them easier to prepare and quantify relative to microsclerotia (Vallad *et al*., 2008). In our report, 3 different main steps are distinguishable: development on the root epidermis broadly asymptomatic, colonization within the root xylems then, those of the stem and leaves leading to symptoms. During the first steps of infection, conidia germinate probably due to root exudates that overcome fungistasis (Schreiber and Green, 1963), giving rise to mycelia hyphae that extend over a maximum area of 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 the 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 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 tip after 1 dpi, contrary to reports on spinach, cotton and lettuce (Vallad and Subbarao, 2008; Zhang *et al*. 2012; Maruthachalam *et al*. 2013), 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 notes that MAMPs (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, cells that 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 *Arabidopsi*s 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 root 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 taproot, 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 two 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 CWDEs (Cell wall degrading enzymes), which are implicated in cell walls degradation to release sugar as a source of nutrition (Klosterman *et*

al., 2011). Once entering xylem vessels, *V. dahliae* is faced with a set 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 three and four weeks after *V. dahliae* inoculation and grew acropetally. Wilting symptoms are also a consequence of *Fusarium 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 symptoms development is one 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 seeds (Karajeh *et al*., 2006; Maruthachalam *et al*. 2013), environmental samples (Banno *et al*., 2011; Bilodeau *et al*., 2012) and symptomatic plants from the field (Mercado-Blanco *et al*., 2003; Atallah *et al*., 2007) because it is accurate and sensitive. Necrotic leaves at four 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, an although recombinant NLP1 and NLP2 cause symptoms on tobacco only the NLP1 knockout mutant stops symptoms on tobacco (Wang *et al*., 2004; Wang *et al*., 2012; Santhanam *et al*., 2013). 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 six and eight wpi, the pathogen was sequestered within stem xylem vessels. However, pathogen progression had yet not reached the bast fiber bundle. Generally, fiber flax cultivation requires at least 120 days obtaining 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 field revealed that *V. dahliae* has reached the fiber after retting, numerous microsclerotia were embedded inside the bast fiber bundle, consequently contribute potentially to weakening of fiber. Cellulose is the major compound of 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 (Morvan *et al*., 1989; Focher, 1992; Baley *et al*., 2002). Analysis of the *V. dahliae* genome shows genes encoding CWDEs, 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, it 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.

Acknowledgments

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Recommended internet resources

Chambre régionale d'agriculture de Normandie : Le lin en Normandie. http://www.chambre-agriculture-normandie.fr/panorama-lin-normandie/ (accessed 2017.11.07).

Arvalis institut du végétal : Fiche accident, la verticilliose sur lin fibre. http://www.fiches.arvalis-infos.fr/fiche_accident/fiches_accidents.php?mode=fa&type_cul=10&type_acc=4&id_acc=381 (accessed 2017.11.07).

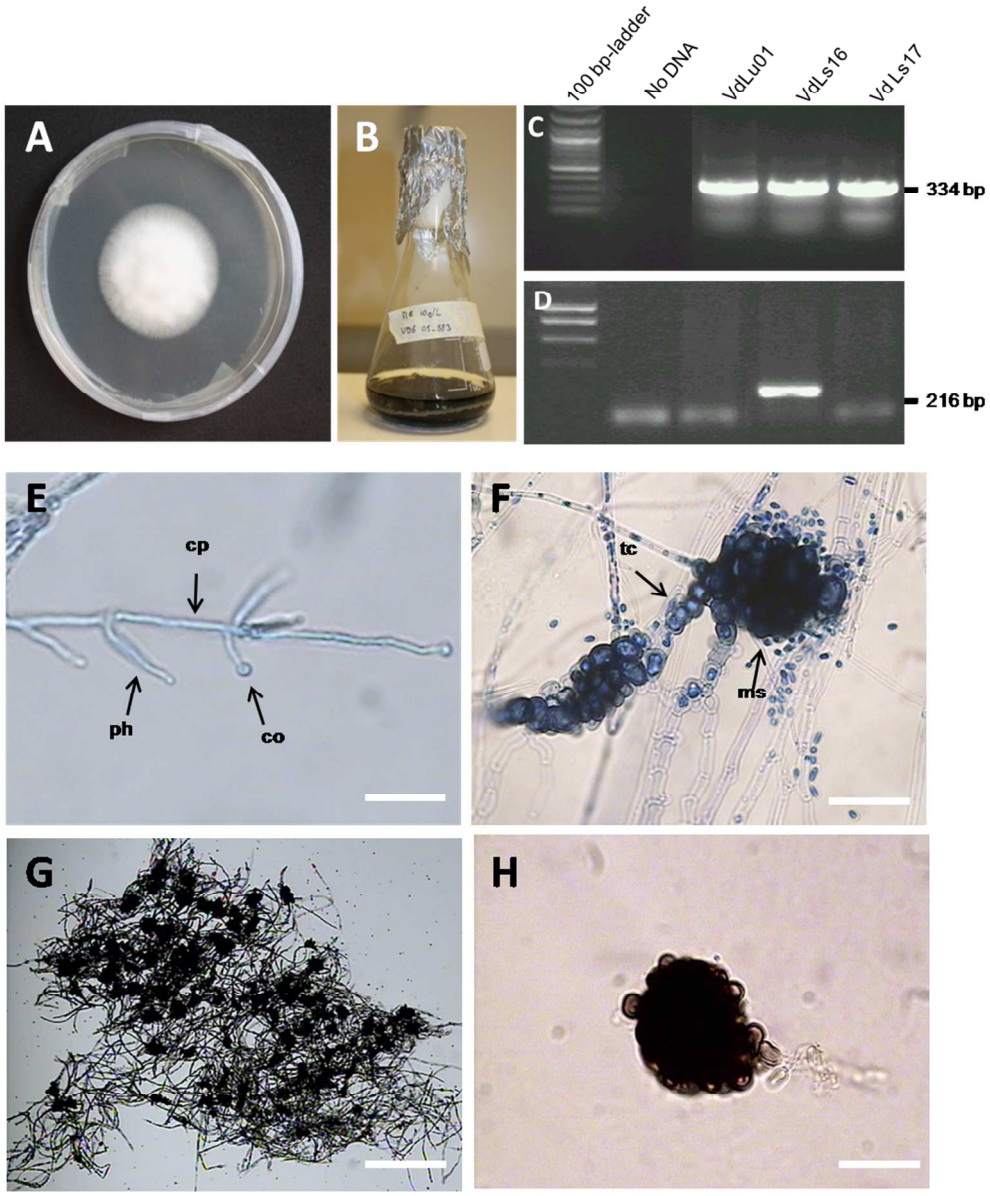

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Fig. 1. Identification of VdLu01, a *V. dahliae* strain isolated on fiber flax straw. Morphological (A and B, E to H) and molecular (C and D) traits. Macroscopic aspect of VdLu01 after growing one week on PDA-agar medium (A) and one month on MA-liquid medium (B). PCR amplification using primers targeting ITS specific regions of *V. dahliae* (C) or *Ave1* (D) on *V. dahliae* strains VdLu01, VdLs16 and VdLs17. Expected amplicon size 334 bp (C) or 216 bp (D). Microscopic observation of hyphal structure: apex of a conidiophore (E), torulose cells surrounding a microsclerotium (F) blue lactophenol staining; heap of microsclerotia (G) and isolated microsclerotium (H). co= conidium, cp= conidiophore, ph= phialide, ms= microsclerotia, tc= torulose cell. Bars= 20 µm (E), 40 µm (F and H) and 200 µm (G).

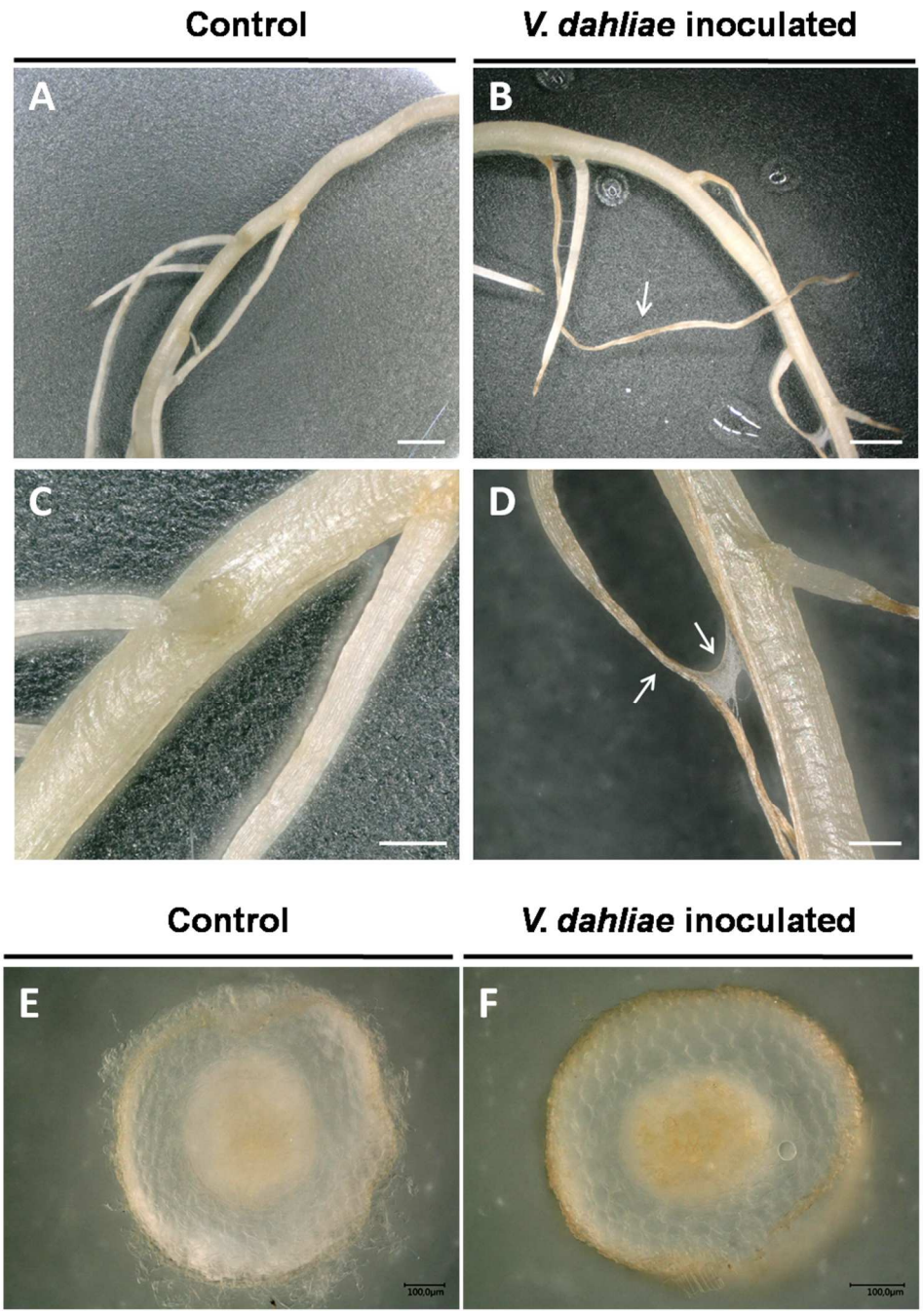

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Fig. 2. Root symptoms on the zone of cell differentiation and lateral roots infected by the strains VdLu01 or VdLs17 in (B) and (D), ten dpi. (A) and (C), roots of control plants for comparison. Bars= 600 µm for (A)(B) or 200 µm for (C)(D). Cross-sections of control (E) and inoculated (F) roots four wpi. Bars= 100 µm.

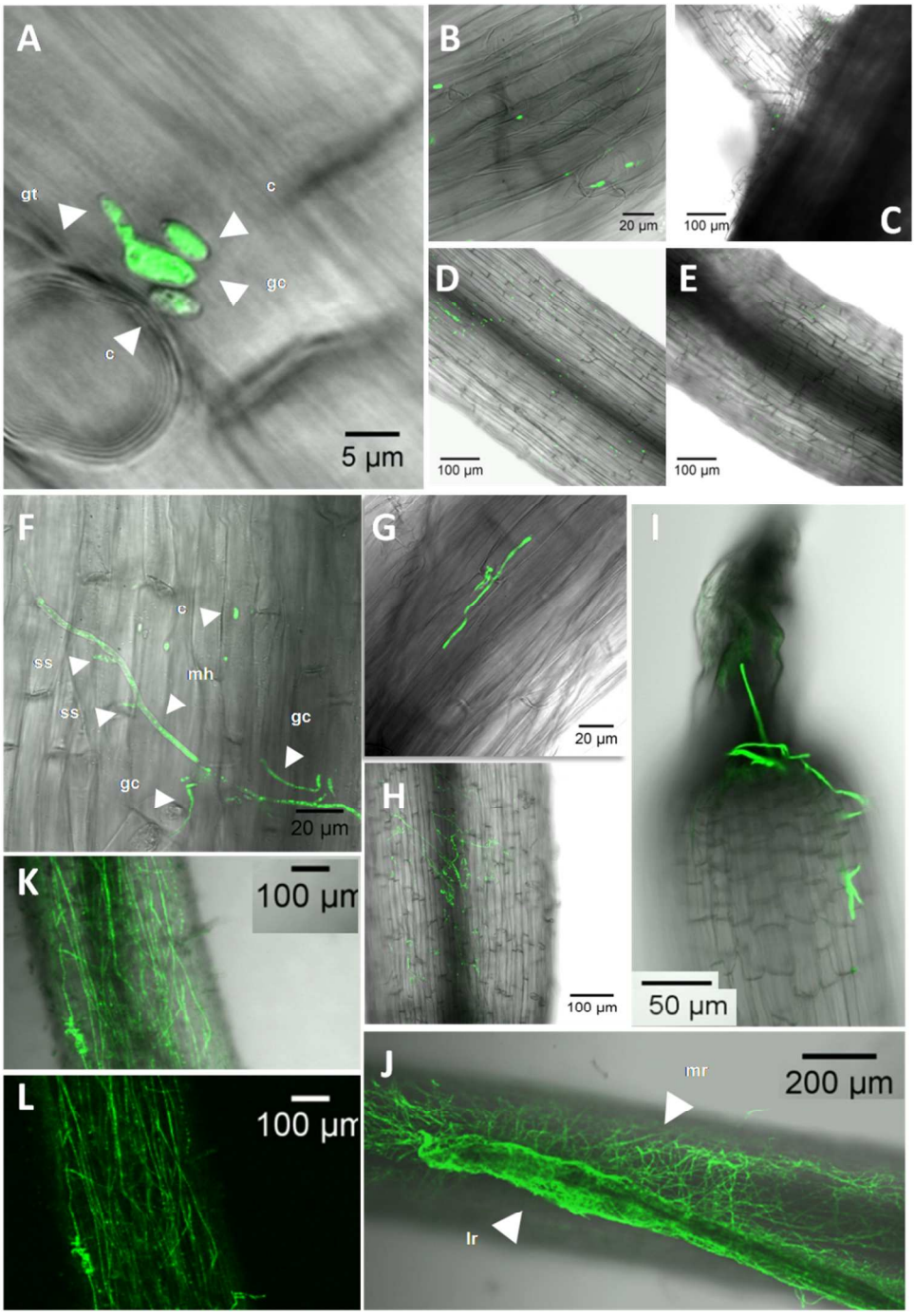

Fig. 3. Germination of conidia and early steps of hyphae growth of VdLs17 strain on flax roots. Overlay of a bright field image and a fluorescent image. (A) and (B) Germinated and non germinated conidia on the zone of cell differentiation, two hpi. (C) and (E) Conidia on the zone of cell differentiation including lateral root junction (only C), two hpi. (D) Conidia on the zone of cell elongation, two hpi. (F), (G) and (H) Growth of mycelia hyphae on the zone of cell differentiation, one dpi (F) and (G); three days dpi (H). (I) Hyphae caused dryness at the tip of a lateral root, three dpi. (J) Fungal mass wrapped the zone of cell differentiation including a lateral root, three dpi. (K) and (L) Growth of mycelia hyphae on the zone of cell differentiation, one wpi. (L) Only fluorescent image.Co= Conidium, Gc= Germinated conidium Gt= Germ tube, Mh= Main hypha, Ss= Secondary structure, Lr= Lateral root, Mr= Main root.

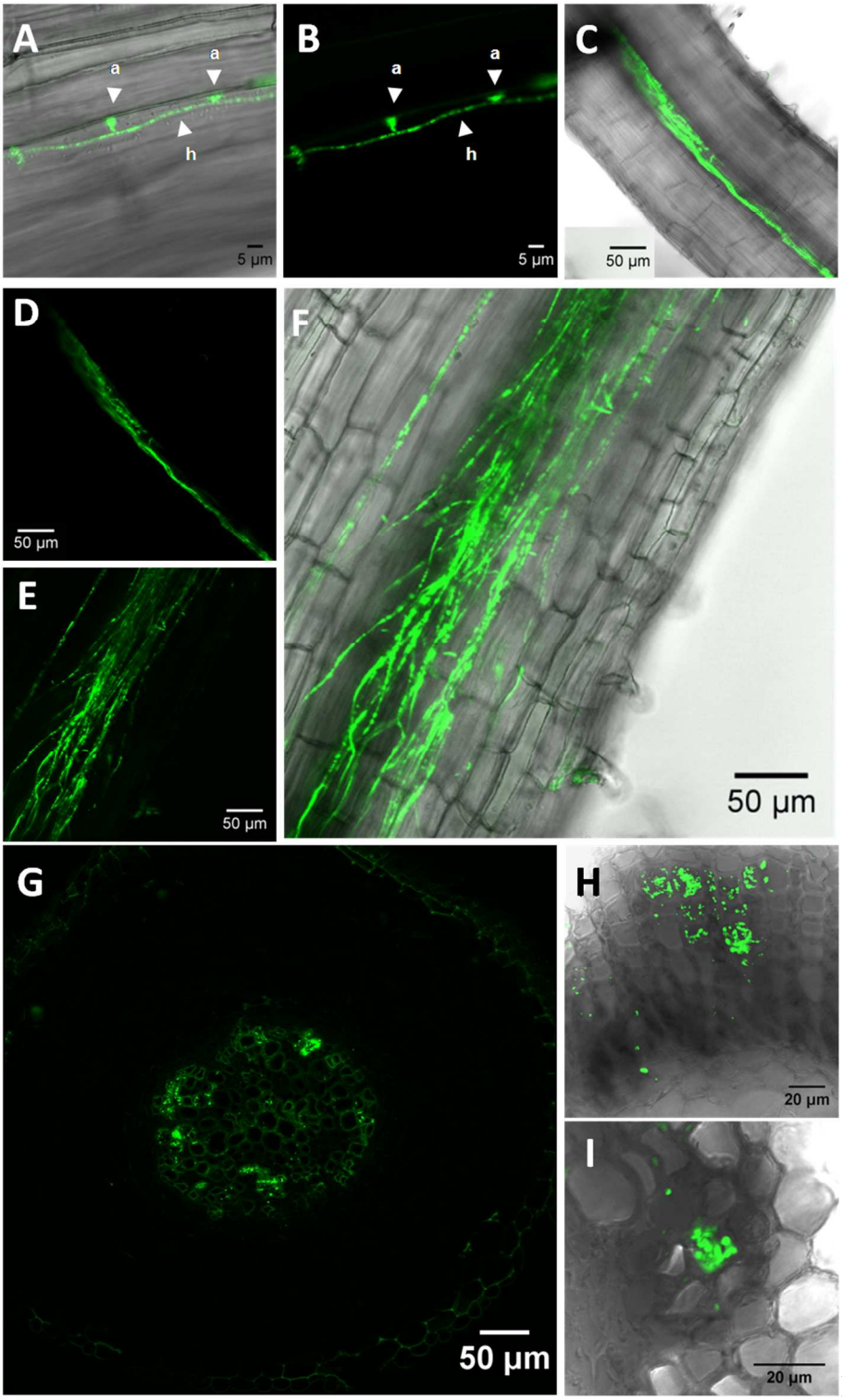

Fig.

4. Vascular colonization of VdLs17 strain on flax roots. Overlay of a bright field image and a fluorescent image. (A) and (B) Mycelia hyphae developing appressoria-like structures on intercellular junction of the zone of cell differentiation, one wpi. (B) only fluorescent image. (C) to (I) Vascular colonization of the pathogen in a main root, two wpi (C) to (F), cross-sections four wpi (G) to (I); (D) (E) and (G) only fluorescent images. (G) Image stacking (ten images), depth= 60µm . a= Appressorium-like, h= hyphae.

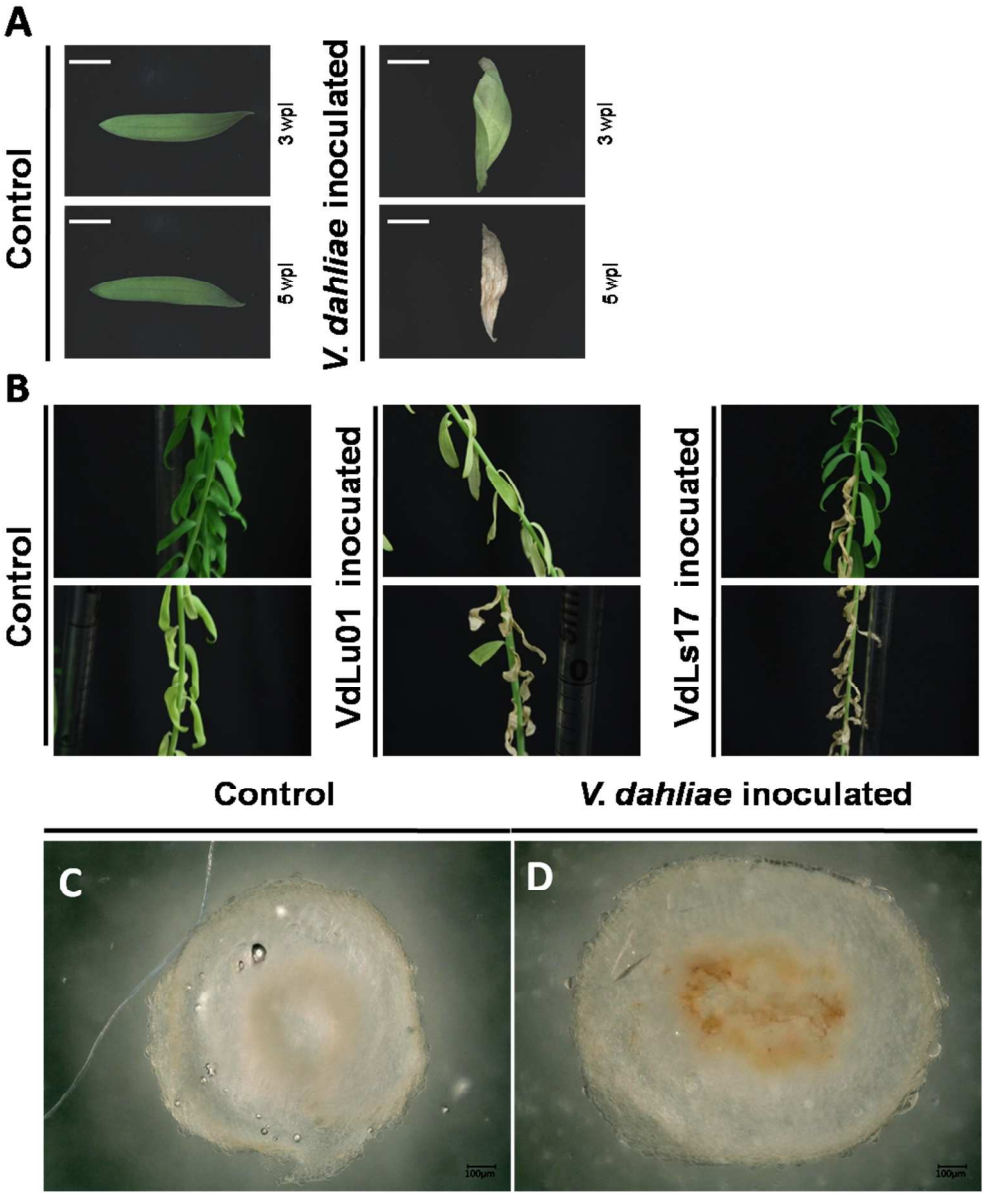

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Fig. 5. Symptoms of Verticillium wilt on stem and leave of flax. (A) Wilting symptom on a basal leaf at three and five wpi and control leaf. Bars= 5 mm. (B) Wilting symptoms at the plant level infected by VdLu01 or VdLs17, apical and basal level, five wpi and control plant for comparison. (C) Cross-sections of control (C) and inoculated (D) roots four wpi. Bars= 100 µm.

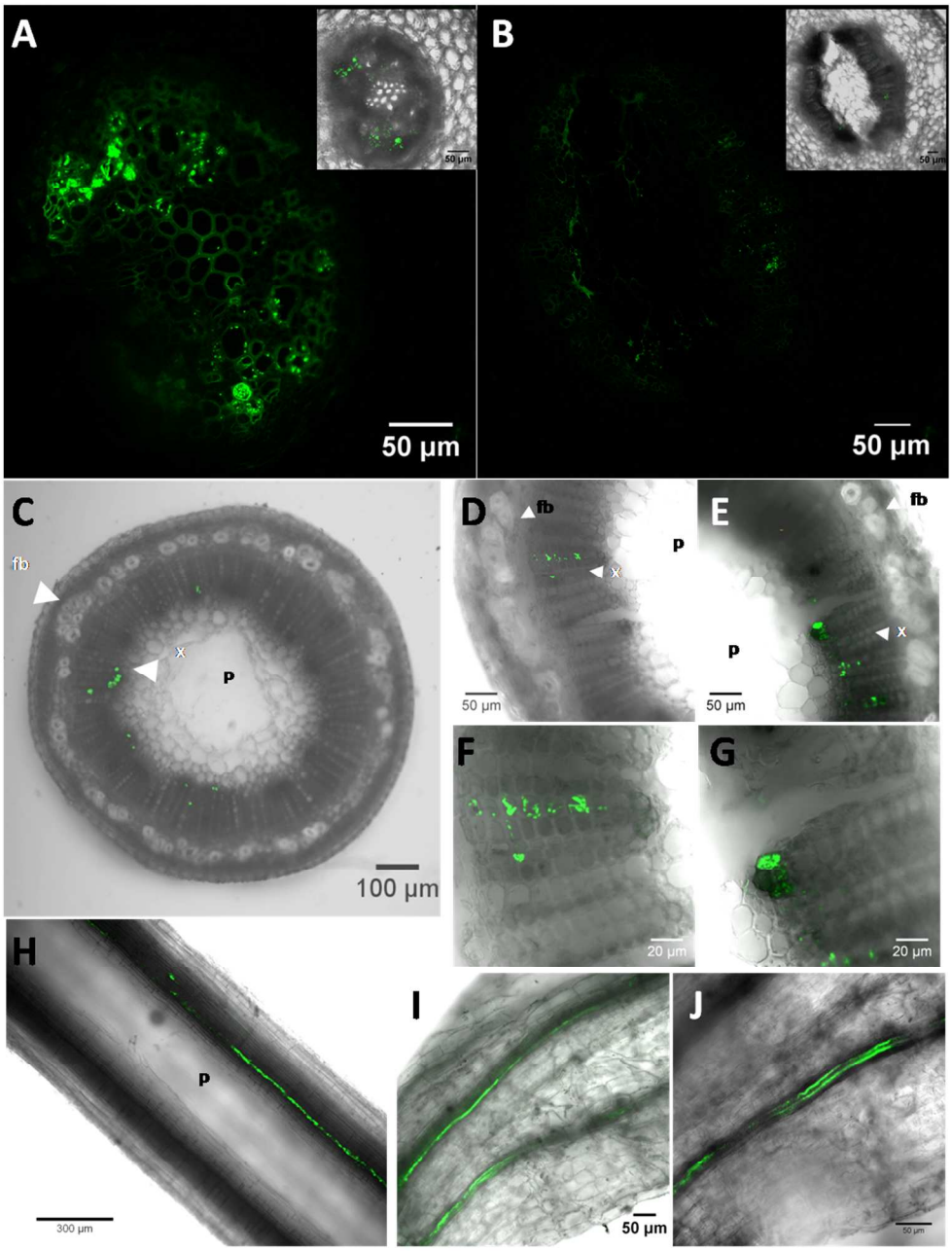

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Fig. 6. Vascular colonization of VdLs17 strain within the above-ground parts. Overlay of a bright field image and a fluorescent image. (A) and (B) Comparison of pathogen fluorescence within xylem cross-sections of root and stem (hypocotyl) on the same plant, only fluorescent images, four wpi. (C) to (J) Between six and eight 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. Fb= Fiber bundle, P= Pith, X= xylems.

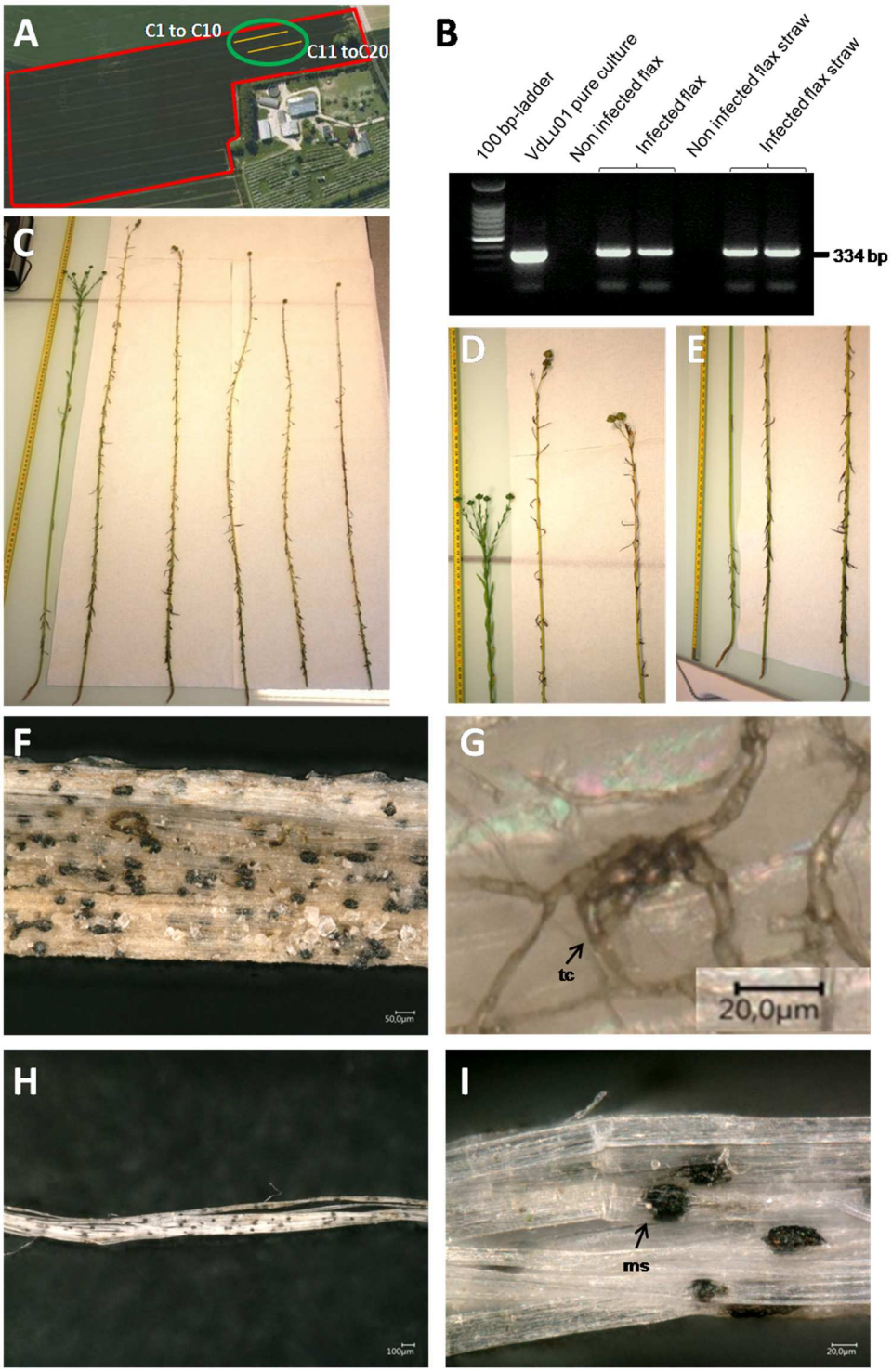

Fig. 7. Verticillium wilt caused by *V. dahliae* on flax at the capsule stage and after retting. (A) Delimitation of the fiber flax plot investigated (red lines), area where the infectious site was detected (green circle), C1 to C20 (yellow lines), points where the infected plants were sampled. (B) PCR amplification using primers targeting ITS specific regions of *V. dahliae* on *V. dahliae* strain VdLu01, non infected flax, infected flax, non infected flax straw and infected flax straw. Expected amplicon size 334 bp. (C) to (E) Wilting symptoms on above-ground parts (non affected 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. ms = microsclerotium, tc = torulose hyphal cells. Bars= 50 µm (F), 20 µm (G) and (I), 100 µm (H).