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Colonization of Resistant and Susceptible Lettuce Cultivars by a Green Fluorescent Protein-Tagged Isolate of *Verticillium dahliae*

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ABSTRACT

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Interactions between lettuce and a green fluorescent protein (GFP)-expressing, race 1 isolate of *Verticillium dahliae*, were studied to determine infection and colonization of lettuce cultivars resistant and susceptible to Verticillium wilt. The roots of lettuce seedlings were inoculated with a conidial suspension of the GFP-expressing isolate. Colonization was studied with the aid of laser scanning confocal and epi-fluorescence microscopes. Few differences in the initial infection and colonization of lateral roots were observed between resistant and susceptible cultivars. Hyphal colonies formed on root tips and within the root elongation zones by 5 days, leading to the colonization of cortical tissues and penetration of vascular elements regardless of the lettuce cultivar by 2 weeks. By 8 to 10 weeks after inoculation, vascular discoloration developed within the taproot and crown regions of susceptible cultivars well in advance of *V. dahliae* colonization. Actual foliar wilt coincided with the colonization of the taproot and crown areas and the eruption of mycelia into surrounding cortical tissues. Advance colonization of stems, pedicels, and inflorescence, including developing capitula and mature achenes was observed. Seedborne infection was limited to the maternal tissues of the achene, including the pappus, pericarp, integument, and endosperm; but the embryo was never compromised. Resistant lettuce cultivars remained free of disease symptoms. Furthermore, *V. dahliae* colonization never progressed beyond infected lateral roots of resistant cultivars. Results indicated that resistance in lettuce may lie with the plant's ability to shed infected lateral roots or to inhibit the systemic progress of the fungus through vascular tissues into the taproot.

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae* Kleb., poses a major threat to lettuce in California. While historically a problem on numerous crops grown in rotation with lettuce, such as artichoke, pepper, strawberry, and tomato, *V. dahliae* was not considered a pathogen of lettuce until an outbreak in 1995. The disease has since spread to over 400 ha, including production areas in the Salinas Valley of California, considered "America's Salad Bowl." Lettuce production in the Salinas Valley accounted for nearly half of the total in the United States with an estimated value of over a billion United State's dollars in 2006 (3).

Lettuce is unique among the hosts of *V. dahliae* in the severity and suddenness by which symptoms develop. Lettuce grown in fields infested with *V. dahliae* remains free of symptoms until the crop nears harvest maturity, when the symptoms of wilt develop rapidly. Foliar symptoms consist of angular chlorotic areas along the margins of the basal leaves that eventually coalesce and can become necrotic prior to wilting. Often, microsclerotia can be observed lining the veins of senescent basal leaves, a character unique to lettuce (54). Internally, vascular tissues become discolored yellow to dark brown with a distinctive olive-colored streaking. These symptoms progress acropetally and, depending on the cultivar and environment, lead to the rapid collapse and death of the plant (54,58). Before the onset of foliar symptoms, the presence of vascular discoloration in the taproot 1 to 2 weeks prior is the only diagnostic symptom (57). Other symptoms such as stunting and developmental abnormalities, such as premature bolting, occur occasionally. While all market types of lettuce are affected, Verticillium wilt is most severe in crisphead cultivars (58).

In many cases, entire crops of lettuce are lost to Verticillium wilt, forcing growers to disk the infected crop residue into the soil. The residues become covered with microsclerotia, the highly melanized survival structures produced by *V. dahliae*, which serve as the primary inoculum and hence, the propagule of choice for measuring populations in the soil (12). Typical lettuce fields afflicted with the disease have *V. dahliae* populations that exceed 50 microsclerotia per g of soil, and in severe cases as high as 2,400 microsclerotia per g of soil (54). The rate and severity of disease development in other susceptible crops is directly related to the number of microsclerotia in the soil (4,5,12,26,42,61). Seedborne transmission is an additional concern, especially if these lettuce-adapted isolates become established in the seed production areas of the San Joaquin Valley of California (56).

Isolates of *V. dahliae* recovered from lettuce are cross-pathogenic to several other crops grown in the Salinas Valley, CA (1,6, 46); not only limiting the effective use of crop rotations, but also threatening the agronomic sustainability of the region. Soil fumigants, such as methyl bromide and chloropicrin, reduce *V. dahliae* population in the soil, but the population rebounds quickly upon returning to lettuce production (58). The high costs associated with lettuce production make long-term soil fumigation economically infeasible, instead growers choose to rotate problematic fields to strawberry, also susceptible to Verticillium wilt, where soil fumigation is a standard practice. Therefore, host resistance is the most practical long-term solution to sustain lettuce production in the region.

Resistance to *V. dahliae* is best exemplified by the tightly linked resistance genes *Ve1* and *Ve2* in tomato (*Lycopersicon*

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains a figure showing an overview of the Verticillium wilt disease cycle.

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esculentum) that encode cell-surface glycoproteins (13,33). Subsequent studies have determined the allelic diversity of *Ve* homologs in Solanaceous crops (11,20). Furthermore, Hu et al. (28) recently characterized the *EDS1* gene, which is required for *Ve*-mediated resistance in tomato. Resistance conferred by the *Ve* genes in tomato, restricted the colonization of incompatible, race 1 isolates of *V. dahliae* and *V. albo-atrum* to the roots, allowing only limited spread to stem and foliar tissues (25,55). Several potential sources of resistance to *V. dahliae* were recently identified in a screen of heirloom and cultivated lettuce germplasm (27). Resistance appeared complete and specific against most isolates of *V. dahliae* pathogenic on lettuce (57). Some isolates of *V. dahliae* compromised this resistance and were found to be genetically distinct, forming a second race of lettuce isolates (46,57). Segregation of progeny from controlled crosses suggests that resistance to race 1 isolates is conferred by a single, dominant gene (R. Hayes, G. E. Vallad, and K. V. Subbarao, *unpublished data*). Little is known about the pathology of *V. dahliae* on lettuce, or the mechanism of resistance discovered to date.

While numerous microscopic studies have examined the interaction of various hosts with *V. dahliae*, most focused on anatomic aspects of colonization (7,18,21,22,43,44,62). Few studies followed the colonization process through the entire disease cycle, and even fewer examined the role of race-specific resistance. Relative to other hosts of *V. dahliae* studied previously, lettuce is unique, because of the growth habit, the growth stage, and acuteness of symptom development, the seedborne nature of the pathogen, and the existence of race-specific resistance. The objective of this study was to examine the infection and colonization of lettuce by *V. dahliae*, with special emphasis on symptom development, seed infection, and the differences between susceptible and resistant lettuce cultivars. With the use of a green fluorescent protein (GFP)-labeled, race 1 isolate of *V. dahliae*, not only were pertinent differences in colonization patterns between susceptible and resistant cultivars observed, but also biological clues regarding the pathology of this disease. This also offered the opportunity to investigate the spatial and temporal dynamics of colonization in a compatible and an incompatible interaction in lettuce.

MATERIALS AND METHODS

Isolate transformation and maintenance. The *V. dahliae* isolate VdLs14, a race 1 isolate recovered from a symptomatic lettuce plant in a field in Watsonville, CA, in 1996, was chosen for this study (57). Spheroplasts of VdLs14 were prepared and transformed with the pCT74 transformation vector, which expresses the sGFP and Hyg^R genes under the control of the ToxA promoter, as described in Visser et al. (59). Transformants were examined for morphology, GFP expression, and pathogenicity on lettuce relative to the wild-type isolate. The transformant VdLsGH1 was chosen for use in these studies. Isolate maintenance and storage, and inoculum preparation was as described previously (57), except that isolate VdLsGH1 was cultured on potato dextrose agar (PDA) amended with hygromycin B (75 μg/liter).

Lettuce inoculation and the assessment of disease severity. Seed of lettuce cultivars Salinas, Sniper, La Brillante, and Little Gem, and plant introduction line (PI) 251246 were sown in a pasteurized sand/potting soil mixture (2:1) in 200-well seedling plug trays (Hummert Int., St. Louis, MO) and initially maintained in a growth chamber (12 h photoperiod; 20°C constant temperature) for 4 to 5 weeks. Lettuce seedlings were inoculated using either a soil drench or a root dip method. For the soil drench method, the soil in each plug tray well was drenched with 3 ml of a spore suspension (2.0×10^6 conidia/ml of sterile distilled water) when the seedlings were 2-weeks-old and repeated after 1 week. Seedlings were then transplanted 1 or 2 weeks later to 0.5-liter foam-insulated cups filled with a pasteurized sand/-potting soil mixture (3:1) and maintained in a greenhouse. The root dip method was as previously described (57), except that seedlings were grown in growth chambers for 4 or 5 weeks prior to washing roots free of soil and dipping roots in a spore suspension (2.0×10^6 conidia/ml of sterile distilled water). Inoculated seedlings were transplanted and maintained in a greenhouse. Replicate experiments using each inoculation method was performed and always included control plants that were mock-inoculated appropriately with sterile distilled water. Plants were rated for disease severity 8 to 10 weeks after transplanting using a 0 to 5 scale based on the acropetal progress of root and foliar symptoms, as previously described (57).

Microscopy. Three to five plants per cultivar were periodically sampled after 24 h, 48 h, 5 to 7 days, 12 to 14 days, 4 weeks, 8 weeks, and 10 weeks postinoculation in the first set of two experiments using the root-dip inoculation method. The second set of two experiments used the soil-drench inoculation method with a similar sampling scheme, but with additional sampling of all cultivars during 3 to 8 weeks of growth; Salinas, Sniper, and PI251246 after 12 weeks of growth; and PI251246 during flowering and seed production after 14 to 16 weeks of growth. Additional seed was also collected from PI251246 for further microscopy after dehiscence of the capitulum. Plant samples were gently washed free of soil and either sectioned (both cross and longitudinal sections) by hand with a double-edged platinum razor blade into 1- to 2-mm slices for microscopy, or, in the case of fine roots, mounted whole.

Microscopic examination of samples utilized several microscopes equipped for viewing GFP. A Nikon compound microscope with filter blocks for GFP (450 to 490 nm excitation, 590 nm longpass emission), coupled to a MRC1024 Bio-Rad Confocal System (Bio-Rad, Hercules, CA) was used for all confocal scanning laser microscopy (CM). Confocal settings were optimized for GFP with excitation via a 488 Argon laser, detection of emitted light at 522 DF32 and autofluorescence detection at 680 DF32 through a 598/40 center bandpass filter. Digital images acquired from individual channels were merged using the Image J image processing program, an open source Java application available to the public through the Research Services Branch of the National Institutes of Health (47). Many of the images presented were prepared from a simple Z-series of images (two to four images) captured along the Z-axis of the specimen to a depth of 10 μm that were then merged into a 2-D image using the RGB_Zprojector Java plug-in (maximum intensity option selected for all channels) for Image J. For more complex Z-series images, the individual images were depth-coded, using varying colors to represent depths along the Z-axis. In these cases, the GFP signal was separated into a separate stack from the other channels for autofluorescence signal, depth-coded using the Z code stack plug-in and then recombined with the autofluorescence signal stack. The recombined stacks were then merged into a 2-D image using the Zprojector plug-in (maximum intensity option) in Image J.

All images from epi-fluorescence microscopy (EM) and light microscopy (LM) were captured using either an Olympus BX60 compound microscope or an Olympus SZX12 stereo microscope, both equipped with several filters for observing GFP (Longpass Endow GFP [450 to 490 nm excitation, 500 nm longpass emission], Bandpass Endow GFP [450 to 490 nm excitation, 500 to 550 nm bandpass emission], and a Blue longpass [450 to 490 nm excitation, 515 nm longpass emission]) and linked to a CCD device. Images captured with EM and LM were also prepared using Image J.

Fungal titer. The in planta titer of VdLsGH1 was examined in three separate experiments. In the first experiment, 50 seedlings of the cultivar Sniper were root-dip inoculated in a 2.0×10^6 conidia/ml solution, while 10 additional seedlings were root-dip inoculated in sterile distilled water for noninoculated controls. In the last two experiments, 12 seedlings of cultivars Salinas, Sniper, La Brillante, and Little Gem were root-dip inoculated, as de-

scribed previously, including a second set of plants as noninoculated controls. For all three experiments, plants were transplanted and maintained in a greenhouse. Ten weeks following inoculation, plants were carefully uprooted and washed free of soil. The taproot of each plant was collected using a sterile scalpel to remove the foliar tissues at the crown (1 cm above the beginning of the pith tissue) and all lateral roots. Individual taproots were then cut longitudinally, scored for disease severity as described previously, weighed, and homogenized with a mortar and pestle in 10 ml of sterile distilled water. A serial-dilution series was prepared for each homogenate with aliquots of each dilution plated on a modified Sorenson's NP10 medium (a semi-selective medium for *V. dahliae*; 31) and plated in replicate on a PDA medium amended with rifampicin (50 mg/liter), chloramphenicol (100 mg/liter), and hygromycin (100 mg/liter). The number of colony forming units (CFU) was recorded for each of the amended PDA plates and used to calculate the CFU of *V. dahliae* per gram fresh weight of taproot tissue. Colonies of *V. dahliae* on amended PDA plates were checked for fluorescence, while the replicate NP10 plates were checked to verify the absence of other *V. dahliae* isolates.

Statistical analysis. For comparisons of disease severity caused by VdLsGH1 and VdLs14, experimental units comprised of three to four plants for each isolate × cultivar combination were replicated three times in a randomized block design. Data from three separate experiments were pooled and the combined dataset analyzed using a nonparametric procedure for the analysis of ordinal data in factorial experiments (1,52). The overall effect of isolate on the severity of disease on each of the three lettuce cultivars was analyzed by the analysis of variance type statistic of ranked data using the Proc Mixed procedure in SAS (2004, version 9.1) to generate mean treatment rankings, from which relative marginal effects (RME) were calculated by the equation $RME = (R - 0.5)/N$, where R is the mean treatment ranking and N is the total number of experimental units in the analysis (52). Replications of experiment and blocks within experiment were considered random effects in the analysis. The LD_CI macro was used to generate 95% confidence intervals for RMEs (8).

In the first fungal titer experiment, experimental units consisted of individual plants that were randomly arranged on a greenhouse bench. For the following two experiments, examining the effect of cultivar on the in planta titer of VdLsGH1, experimental units comprised of groups of three to four inoculated and noninoculated plants for each cultivar that were replicated three times and arranged in a randomized block design. Recorded values (fungal titer, root weight, and disease severity) were averaged across plants within each experimental unit for further data analysis. In all experiments, fungal titer values (CFU/gram of taproot) were Log transformed and the noninoculated controls were removed prior to statistical analyses. The overall effect of cultivar on the in planta titer of VdLsGH1 was analyzed using the PROC Mixed procedure in SAS (2004, version 9.1) with blocks within experiment considered random effects. The PROC CORR procedure in SAS was used to examine the relationship between disease severity, root weight, and titer.

RESULTS

Virulence of GFP transformant relative to its wild type isolate. Of the stable transformants generated, VdLsGH1 was selected for the uniformity and strength of the resulting fluorescent signal in all fungal structures, including developing microsclerotia (data not shown). Pathogenicity tests revealed that the mean disease severity caused by isolate VdLsGH1 did not differ significantly ($P = 0.6521$) from the wild-type isolate, VdLs14, on the susceptible lettuce cultivars, Salinas and Sniper, or in avirulence towards the resistant cultivar, La Brillante (Table 1). Additionally, re-isolation of VdLsGH1 from inoculated plants with no observable loss of GFP fluorescence or hygromycin B resistance, confirmed the stability and robustness of the transformant. While the soils used in these experiments were initially pasteurized, the long duration of the experiments (>3 months) and incubation of plants in the greenhouse, made the exclusion of other microorganisms infeasible. Other fungal species exhibiting autofluorescence within the same range as the GFP protein were occasionally noticed in soil, and only in rare cases on plant roots, but the intensity of the signal paled greatly relative to that of the transformed isolate. In addition, the presence or absence of VdLsGH1 in specimen placed on PDA (with or without hygromycin B) or on a semiselective NP10 medium confirmed microscopic observations.

Time course of infection of lettuce by *V. dahliae*. Twenty-four hours after inoculation via the root-dip or soil-drench methods, roots were covered with conidia, of which only a small fraction (<5% estimate) exhibited signs of germination. By 48 h, at least half of the spores on root surfaces had germinated, with a germ tube emerging from one or both ends of the conidium (Fig. 1A and B). Germ tubes appeared to emerge from a germpore, because of the restricted diameter of the germ tube at the site of emergence relative to the larger diameter hypha (Fig. 1C).

Germinating hyphae continued elongating parallel with the longitudinal axis of the root along the junctions of epidermal cells before developing appressoria, which were evident by 48 h postinoculation (Fig. 1C). Appressoria developed along the junctions of root epidermal cells, but penetrated an adjacent epidermal cell directly (Fig. 1D). In other cases, hyphae growing along cell junctions would cross over an epidermal cell and begin growing along an adjacent cell junction prior to developing an appressorium (Fig. 1E). Some hyphae had a bulbous appearance, presumably due to several unsuccessful penetration attempts (Fig. 1F).

While many conidia developed appressoria shortly following germination, others developed more complex hyphal networks along the root epidermis growing within the grooves between epidermal cells before developing appressoria and colonizing cortical tissues. Whether appressoria were necessary for direct colonization of the root cap could not be resolved because of the rapid colony and root cap development. Hyphae exhibited thigmotropic

TABLE 1. The median, rank mean (R) and relative marginal effect (RME) based on the severity of Verticillium wilt caused by a transformed (VdLsGH1) and wild-type (VdLs14) isolate of *Verticillium dahliae* on three lettuce cultivars

		Verticillium wilt severity				
Cultivar	Isolate	Median	Range	R	RME	(95% CI)
La Brillante	Control	0.0	(0.0 – 0.0)	22	0.26	(0.22 – 0.31)
	VdLs14	0.0	(0.0 – 0.5)	26	0.31	(0.23 – 0.41)
	VdLsGH1	0.0	(0.0 – 1.0)	32	0.39	(0.28 – 0.51)
Salinas	Control	0.0	(0.0 – 0.5)	31	0.38	(0.27 – 0.49)
	VdLs14	1.0	(0.0 – 2.5)	50	0.61	(0.44 – 0.76)
	VdLsGH1	0.7	(0.0 – 2.7)	51	0.62	(0.44 – 0.76)
Sniper	Control	0.0	(0.0 – 0.3)	31	0.38	(0.28 – 0.50)
	VdLs14	1.3	(0.0 – 2.8)	63	0.77	(0.61 – 0.87)
	VdLsGH1	1.3	(0.0 – 2.8)	63	0.77	(0.61 – 0.87)

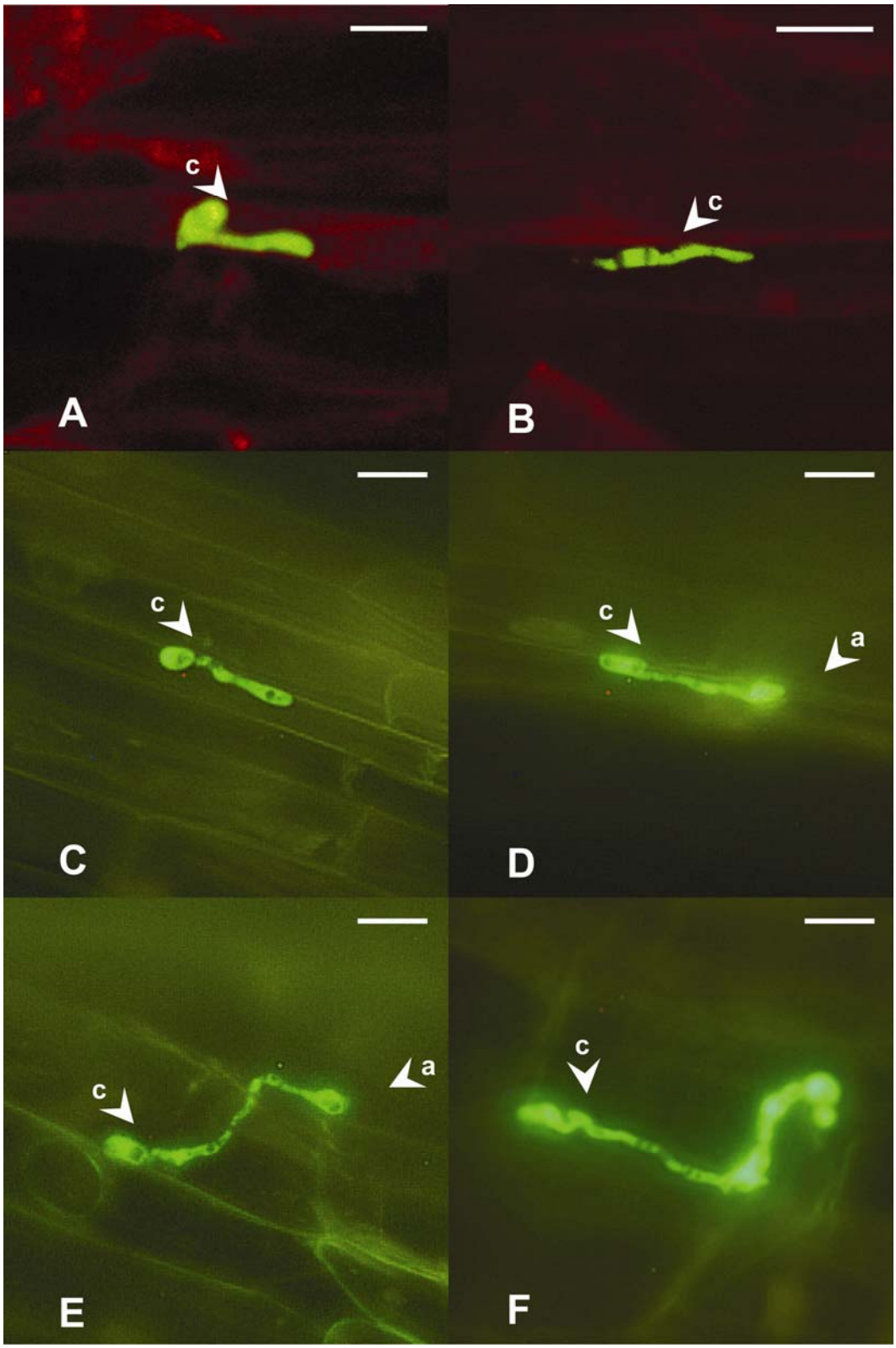

Fig. 1. Germination of conidia from a green fluorescent protein (GFP) expressing isolate of *Verticillium dahliae* and the formation of appressoria on the surface of lettuce roots 12 to 48 h following the inoculation of of 4- to 5-week-old seedlings. **A,** A conidium lying on the root surface (cv. Salinas) with a single germ tube emerging from its distal end, 12 h after inoculation. **B,** Two germ tubes emerging from the distal ends of a conidium on the root surface (cv. La Brillante), 24 h after inoculation. **C,** Conidium with a single germ tube extending parallel along the longitudinal junction between root epidermal cells (cv. Little Gem), 24 h after inoculation. **D,** Conidium with a single germ tube and appressorium (a) developing at the junction of root epidermal cells (cv. Salinas), 48 h after inoculation. **E,** Conidium with a germ tube extending across a root epidermal cell before growing parallel along the longitudinal junction between two root epidermal cells (cv. La Brillante) and developing an appressorium, 48 h after inoculation. **F,** Conidium with a germ tube extending along longitudinal junction between root epidermal cells (cv. Sniper) and developing bulbous growths at apparent penetration sites, 48 h after inoculation. Arrows denote conidia (c) and appressoria (a). Analyses of images captured using confocal laser scanning microscopy (**A to B**) and epifluorescence microscopy (**C to F**). **A to F,** Scale bars = 10 µm.

growth, always elongating parallel to the longitudinal axis of the root within the junctions of epidermal cells and developing appressoria along the junctions. It is unclear if any structural features triggered appressorium development.

Three to five days following inoculation, substantial colonization of epidermal and cortical tissues was observed at the tips of lateral roots and within the root elongation zone (Fig. 2A and F). Some advance colonies even produced conidia from simple conidiophores at the root surface (Fig. 2F). Colonies established at root tips appeared most successful, being the first to invade vascular tissues (Fig. 2B), and led to the formation of necrotic lesions, collapsed tissue, and the loss of meristematic activity (Fig. 2E). Colonies established within root elongation zones seemed to produce more elaborate networks of hyphae along the root surface, exhibiting growth within the grooves between epidermal cells, and intracellular growth before developing appressoria (Fig. 2C). While conidia germination was also observed at root eruption sites, along root hairs and at areas with noticeable root damage, no substantial colony formation, in regards to successful colonization of cortical tissues, was ever observed at these sites (data not shown). As colonization progressed from 6 to 14 days postinoculation, necrotic lesions and tissue collapse became more apparent. Within initial infection areas, colonies became more defined with hyphae stratifying all internal tissues, including vascular tissues, and further encompassing the root surface (Fig. 3A and B). Hyphae from the leading edge of the colony progressed acropetally up the xylem vessels of infected lateral roots (Fig. 3C and D). Hyphae appeared to penetrate neighboring xylem vessels directly, via pit pairs and perforation plates, exhibiting only slight swelling at the site of penetration (data not shown).

Occasionally, a change in the size of hypha following penetration to neighboring cells or xylem vessels was observed in confocal imagery (Fig. 3A and B). This reduction in hypha size was also noted as hyphae progressed from the root surface into internal tissues (Figs. 2D, 3A, and 3B). However, this observed change was found to be an artifact of the confocal imagery caused by a reduction in fluorescent signal in relation to the increased amounts of refraction that occurs as the mycelia progresses deeper into the tissue in some cases. In other cases, the increased fluorescent signal was due to several hyphal strands oriented in parallel to each other, but couldn't be effectively resolved at low magnification. Few apparent changes in the size of hyphae were observed during the course of epi-fluorescence microscopy, and hyphae oriented in parallel were often observed within infected xylem vessels (Fig. 4C). The slow methodical progression of hyphae up tertiary and secondary lateral roots continued from 2 to 8 weeks postinoculation. During this time, no conidia were observed in xylem vessels or at xylem perforation plates within root tissues.

From 6 to 8 weeks following inoculation, depending on the inoculation method, plants began developing symptoms of vascular discoloration characteristic of Verticillium wilt in the absence of foliar symptoms. The vascular discoloration consisted of a yellowing of xylem vessels, especially protoxylem vessels, and the appearance of a dark gum-like substance within as the vessel matured. The vascular discoloration progressed acropetally from the infected lateral roots, up through the taproot and into the crown. Interestingly, even though the taproot and crown region exhibited severe vascular discoloration, the fungus was absent upon closer examination (Fig. 3E and F). Careful serial sectioning revealed the leading edge of hyphae within infected xylem vessels of lateral roots at the junction of the taproot or just encroaching into the apex of the taproot (Fig. 4A). It is also important to note that hyphae did not progress through vessels exhibiting discoloration. The apical ends of these infected lateral roots were often degenerated and exhibited massive colonization with the production of conidiophores and microsclerotia (Fig. 4B). This level of vascular discoloration (75% or greater) in the taproot in the absence of foliar symptoms corresponds with a disease severity rating of four (57).

Over the next 2 weeks (8 to 10 weeks postinoculation), hyphae progressed rapidly through the xylem vessels of the taproot, crown, and into basal foliar tissues (Fig. 4C and D). This acropetal progression was immediately followed by an eruption of hyphae from xylem vessels into the surrounding cortical tissues (Fig. 4E and F), coinciding with the development of foliar symptoms and corresponding with a disease severity rating of five (Fig. 5A and B). The enumeration of CFUs of *V. dahliae* in homogenized taproot tissues of the susceptible cultivars Salinas and Sniper corroborated microscopic observations, and correlated directly (Spearman's rank correlation; $\rho = 0.731$, $P < 0.0001$) with disease severity (DS) ratings (Table 2). While a subtle 6.3-fold (1.8 to 2.6 $\log_{10}$ CFU/g root tissue), but significant (*t* test DS 0 versus DS 4; $t = -3.14$, $P = 0.0028$), increase in fungal titer was detected in lettuce taproots as vascular discoloration progressed from a DS score of 0 to 4. A drastic 55-fold increase (2.6 to 4.3 $\log_{10}$ CFU/g root tissue; *t* test DS 4 versus DS 5; $t = -6.87$, $P < 0.0001$) was detected as the DS score progressed from 4 to 5 (Table 2).

Closer examination of the taproots of inoculated plants exhibiting a DS score of 0, revealed the presence of single, scattered microsclerotia embedded in the root epidermal tissues (Fig. 5C and D). These scattered microsclerotia were devoid of any vegetative hyphae, and were not derived from a systemic infection, since no internal colonization was observed in serial sections of the surrounding tissues. These scattered microsclerotia were also observed in the taproots of plants exhibiting progressive systemic colonization and disease symptoms, but in stark contrast to the dense patches of microsclerotia that developed in the internal tissues of severely diseased plants (Fig. 5E and F).

Colonization of resistant and susceptible lettuce cultivars by *V. dahliae*. No difference in the initial colonization of lateral roots of susceptible and resistant cultivars was observed. Limited colonization of vascular tissues observed among the resistant lettuce cultivars Little Gem and La Brillante up to 2 weeks following inoculation, including the development of necrotic lesions and the collapse of infected lateral roots. In later observations, it became apparent that the fungus failed to progress into the taproots of resistant cultivars, since the taproots remained free of any internal colonization through vegetative and reproductive growth. The enumeration of *V. dahliae* in taproot homogenates detected an average 159-fold difference in CFU/g root tissue between resistant (0.88 log CFU/g root tissue) and susceptible (3.36 log CFU/g root tissue) cultivars across two independent experiments, corroborating microscopic observations (Table 3). Closer examination of resistant plants found scattered microsclerotia embedded within the epidermis of the taproot and crown, similar to what was observed in nonsymptomatic susceptible plants (Fig. 5C and D). Nonetheless, a significant (*t*-test; $t = -5.52$, $P < 0.0001$) difference in CFU/g of root tissue was still detected among nonsymptomatic plants (DS = 0) of susceptible (2.34 log) and resistant (0.91 log) cultivars 10 weeks after inoculation.

Vascular discoloration progressed well in advance of the hyphae into the stems of susceptible plants. Microcolonies and isolated conidia were observed within the xylem vessels, the first evidence of the production and systemic transport of conidia within the xylem vessels (Fig. 6A). In addition, hyphae were also found progressing into the pith tissues of plants exhibiting severe symptoms (data not shown). Foliar symptoms quickly developed, beginning as regions of angular chlorosis that became flaccid, enlarged, often asymmetrically, and developed into angular areas of necrosis. The discoloration of vascular tissues within leaves was also followed by hyphae moving through the vascular tissues of the leaf mid-rib and veins within the chlorotic regions (Fig. 6B). Hyphae eventually erupted from vascular tissues into surrounding mesophyll (data not shown).

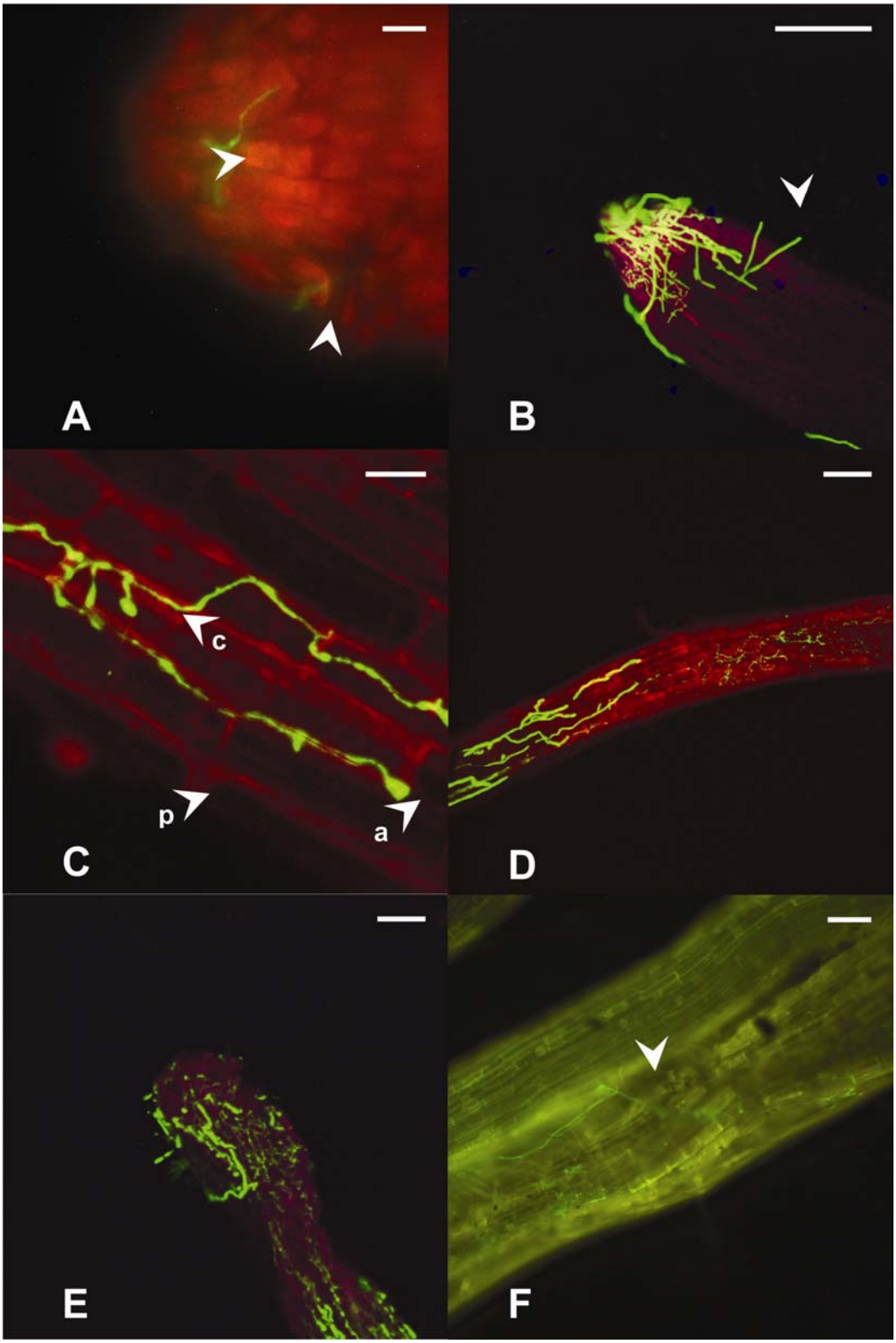

Fig. 2. Early stages of lettuce root colonization by a green fluorescent protein (GFP) expressing isolate of *Verticillium dahliae*. Images captured 48 h to 2 weeks following the inoculation of seedlings with conidia of *V. dahliae*. **A,** Small colonies (arrows) growing on the tip of a lateral root (cv. Salinas), 48 h after inoculation; signal from autofluorescence was partially subtracted to enhance visualization of GFP. **B,** Tip of a lateral root (cv. La Brillante) colonized by *V. dahliae* with simple conidiophores (arrow) protruding from root surface, 12 days after inoculation. **C,** Early colonization of a lateral root (cv. Sniper) that originated at the zone of root elongation, 5 days after inoculation. **D,** Lateral root (cv. Little Gem) showing advance colonization with mycelium extending from the epidermis into cortical tissues, 5 days after inoculation. **E,** Structural breakdown of lateral root tip (cv. Salinas) following colonization, 12 days after inoculation. **F,** Verticilliate conidiophore (arrow) extending from surface of lateral root colonized by *V. dahliae*, 5 days after inoculation. Arrows denote root hair primordia (p), conidia (c), and appressoria (a). Analyses of images captured using confocal laser scanning microscopy (**A and F**) and epi-fluorescence microscopy (**B to E**). **A and C,** Scale bars = 25 µm; **B, D, E, and F,** 100 µm.

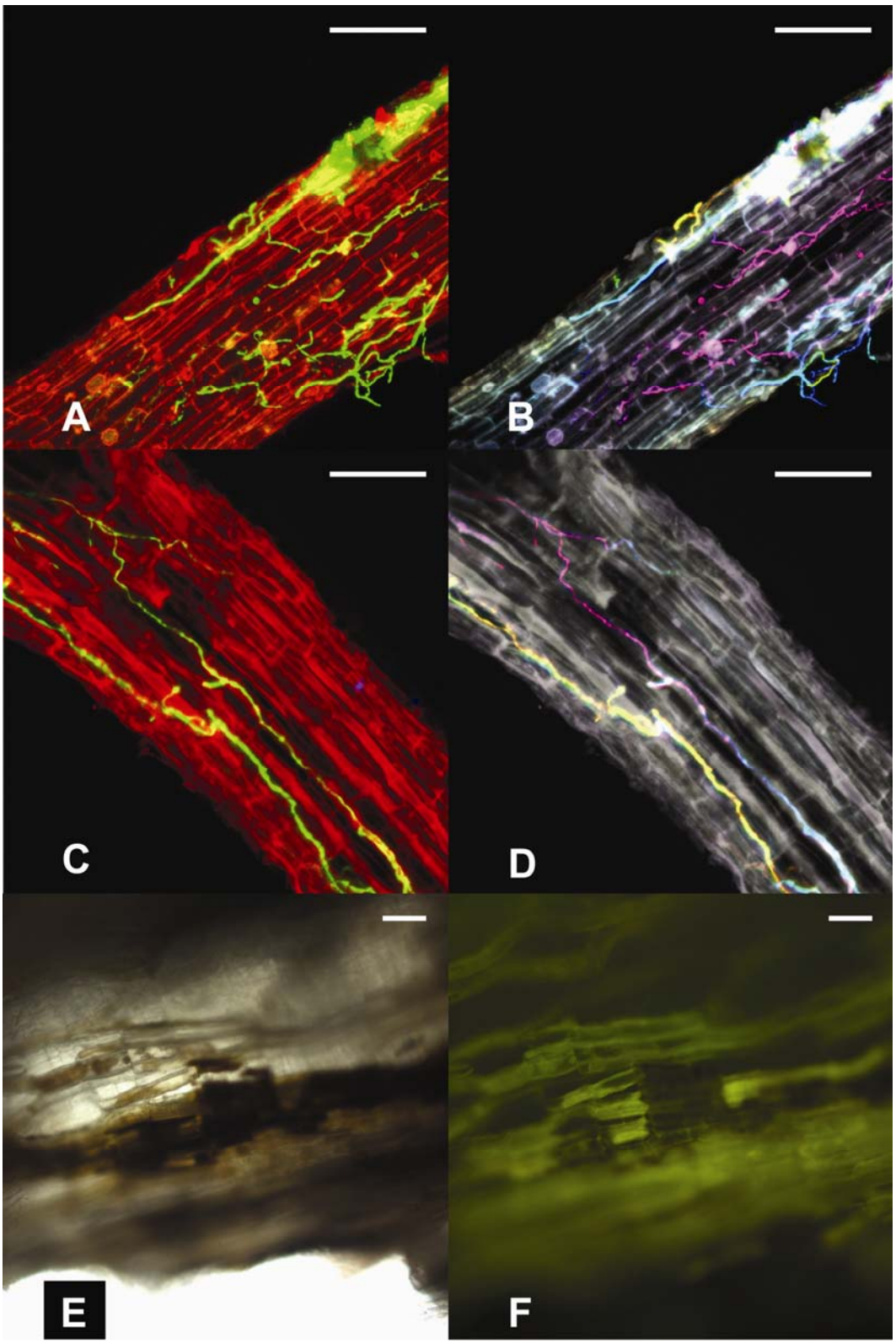

Fig. 3. Systemic infection of lettuce roots by a green fluorescent protein (GFP) expressing isolate of *Verticillium dahliae* and the development of vascular discoloration in the taproot of lettuce plants. Images captured 2 to 8 weeks following inoculation of seedlings with conidia of *V. dahliae*. **A and B,** Composite of images from a confocal laser scanning microscope of a lettuce root (cv. Salinas) exhibiting advance colonization of epidermal and cortical tissues, 2 weeks after inoculation. **B,** Signal from GFP was color-coded, violet, blue, green, yellow, and orange, corresponding with the root surface (violet) to a depth of 90 µm into the sample (orange); the signal from root autofluorescence was averaged across the 18 image stack. **C and D,** Composite of an image stack from a confocal laser scanning microscope of a longitudinally dissected lettuce root (cv. La Brillante) exhibiting advance colonization of cortical and vascular tissues, 2 weeks after inoculation. **D,** Signal from GFP was color-coded, violet, blue, green, yellow, and orange, corresponding with the longitudinally dissected surface (violet) to a depth of 55 µm into the sample (orange); the signal from root autofluorescence was averaged across the 22 image stack. **E and F,** Respective light and epi-fluorescence microscopy image showing the vascular tissues in the taproot of a lettuce plant (cv. Salinas) 8 weeks after inoculation, exhibiting vascular discoloration prior to the development of foliar symptoms; note the absence of GFP signal associated with the discolored vascular tissues in **F**. **A to F,** Scale bars = 100 µm.

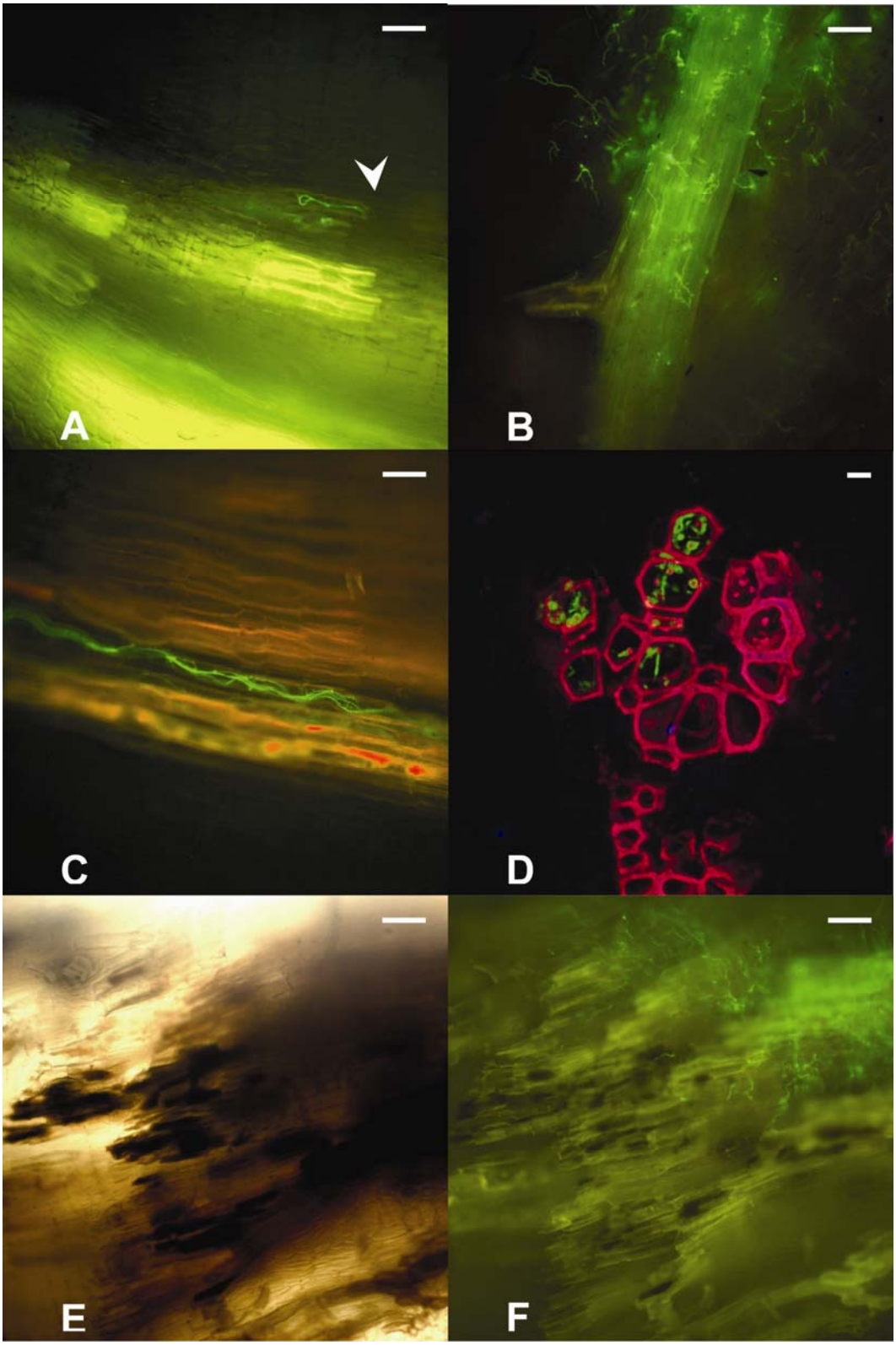

Fig. 4. Later stages of lettuce root colonization by a green fluorescent protein (GFP) expressing isolate of *Verticillium dahliae*. Images captured 6 to 10 weeks following the inoculation of seedlings with conidia of *V. dahliae*. **A,** Hyphae (arrow) within xylem vessels leading from a systemically infected lateral root into the apex of a lettuce (cv. Salinas) taproot. **B,** A degenerating lateral root of lettuce (cv. Salinas) exhibiting massive colonization and sporulation, 8 weeks after inoculation. **C and D,** Respective longitudinal section (cv. Salinas) and cross section (cv. Sniper) of a lettuce taproot showing the acropetal, but restricted, advancement of mycelia through a xylem vessel (tracheary element), 8 weeks after inoculation. **E and F,** Respective light and epi-fluorescence microscopy image of a longitudinal section of a lettuce taproot (cv. Salinas) showing the discolored vascular tissues (**E**) and the massive eruption of mycelia from infected xylem vessels into the surrounding cortical tissues (**F**), 10 weeks after inoculation. Analyses of images captured using epi-fluorescence microscopy (**A to C**) and confocal laser scanning microscopy (**D**). **A, B, E, and F,** Scale bars = 100 µm; **D,** 10 µm; **C,** 25 µm.

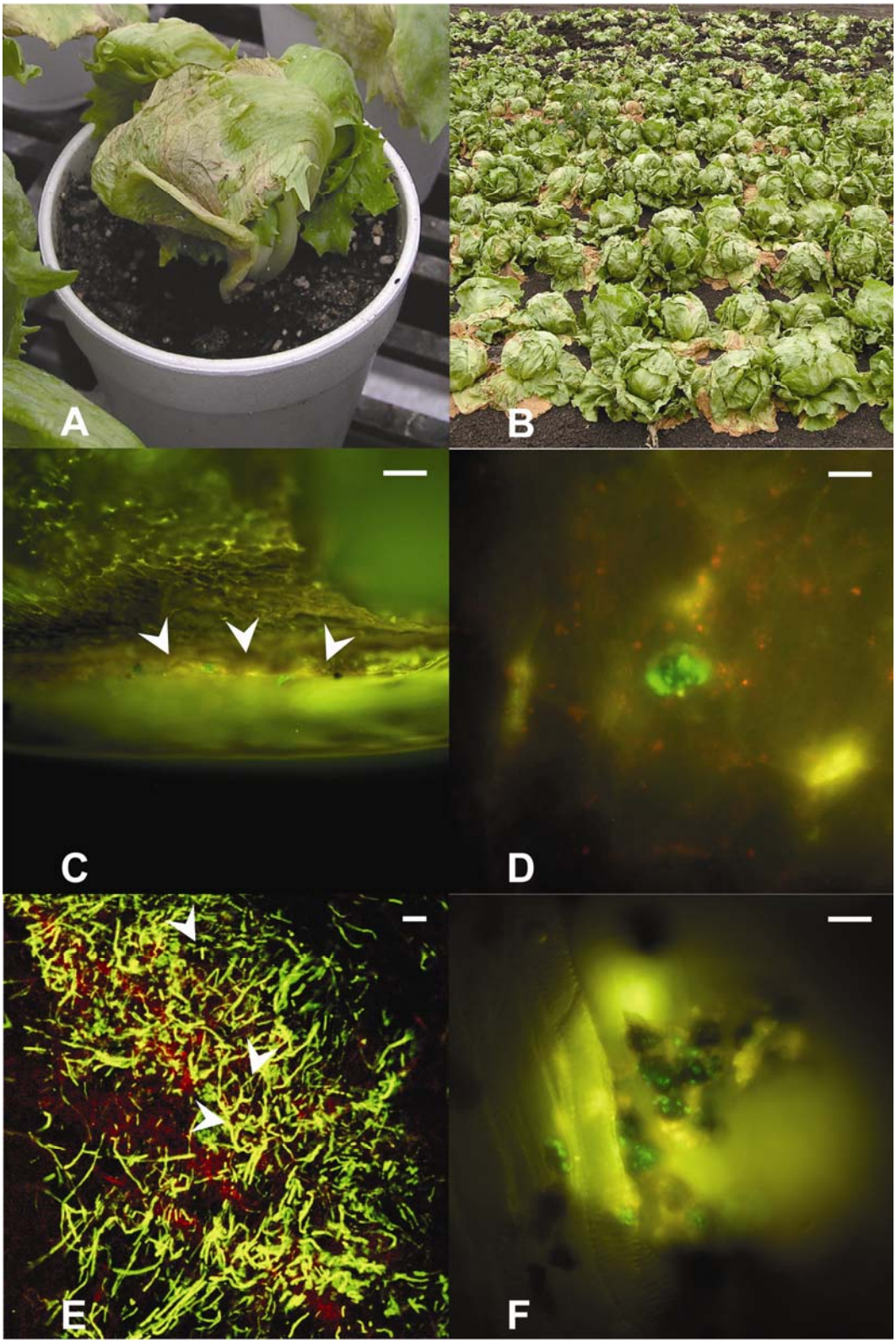

Fig. 5. The development of foliar wilt symptoms in greenhouse and field grown lettuce plants and the presence of microsclerotia of *Verticillium dahliae* embedded on the surface and within lettuce taproots, 10 to 14 weeks following the inoculation of seedlings with conidia of *V. dahliae*. **A,** Greenhouse grown lettuce (cv. Sniper) plant exhibiting typical foliar symptoms of Verticillium wilt, 10 weeks after being inoculated with a conidial suspension of *V. dahliae*. **B,** A crop of lettuce in Santa Cruz County, CA, afflicted with Verticillium wilt being plowed into the soil (note plant debris in the background). **C,** Taproot surface of a nonsymptomatic plant (cv. Salinas) with lone microsclerotia (arrows) embedded in the epidermal tissues, 10 weeks following inoculation. **D,** Close-up of a microsclerotium orphaned, lacking any supporting vegetative tissues, within the epidermal tissues of a nonsymptomatic taproot (cv. Salinas), 10 weeks following inoculation. **E,** Massive colonization of vascular and cortical tissues within the taproot of a diseased plant (cv. Sniper) with microsclerotia developing, 12 weeks following inoculation. **F,** Mature microsclerotia lining the vascular tissues within senesced tissues of a diseased plant (cv. Sniper), 14 weeks following inoculation. Analyses of images captured using confocal laser scanning microscopy (**E**) and epi-fluorescence microscopy (**C, D, and F**). **C,** Scale bars = 100 µm: **D, E and F,** 25 µm.

Pathogen growth in lettuce inflorescence. Symptoms progressed up the bolting stem into the developing tissues of the determinate inflorescence. Lettuce produces a corymbose panicle, with each branch containing clusters of capitula or flower heads. Each capitulum is composed of 10 to 25 ray florets that develop and reach anthesis simultaneously. Because symptomatic plants of the susceptible cultivars, Salinas and Sniper, rarely survived through reproductive growth, the susceptible plant introduction line (PI) 251246 was used to further study the colonization of floral tissues. PI 251246, is an early maturing line with an erect plant architecture and is susceptible to *V. dahliae*. As symptoms progressed, leaves on individual stems of the panicle and individual bracts of the involucre became chlorotic and flaccid. Symptoms progressed generally along the most apical branch of the panicle, before developing on lateral branches. However, plants that developed severe symptoms throughout the panicle led to capitula that aborted prior to anthesis. Upon microscopic examination of pedicels and adjoining capitula, it was apparent that small micro-colonies had developed from conidia produced within xylem vessels and then progressed into the capitula (data not shown). Capitula compromised prior to anthesis would usually abort development, wilt, and become necrotic. Capitula compromised during or following anthesis, developed normally, but yielded a mixture of fully developed achenes, stunted achenes, and achenes that aborted as seed developed. Regardless of when the capitula were compromised, copious amounts of conidia were produced within the infected tissues of the involucre (Fig. 6C and D). No verticilliate hyphae were observed on the exterior tissues of the capitula. However, as the achenes ripened, hyphae and verticilliate conidiophores developed on the exterior surface of the pericarp and the pappus (Fig. 6E and F). Further microscopic examination of whole and dissected achenes also revealed considerable internal colonization of all maternal tissues, including the pappus, pericarp, integument, and endosperm (Fig. 7A and F). Conidia were also produced internally in infected tissues within the capitulum, including the achenes, through hyphal budding; especially in the pappus of achenes where conidia exuded out when detached (Fig. 7A and B). During the examination of over 100 mature achenes from several plants, neither microsclerotia nor colonization of the embryo was ever observed (Fig. 7D and F). All plants from the resistant cultivars and inoculated, non-symptomatic plants of the susceptible cultivars produced seed free of *V. dahliae*.

DISCUSSION

Colonization of lettuce by *V. dahliae* in relation to the development of Verticillium wilt in compatible and incompatible interactions was determined employing a GFP-transformed, race 1 isolate. Relative to other hosts of *V. dahliae* studied to date, lettuce exhibits many features that are unique. Principal among them are its well-defined taproot system, rosette pattern of growth, the acuteness of symptom development that coincide with vegetative maturity, the development of microsclerotia in basal leaves prior to plant death, and the seedborne nature of the pathogen (56,58). Of even greater importance to the disease management is the presence of two genetically distinct races and several lettuce cultivars that can differentiate the two races (27,46,57). This being the only characterized race structure for *V. dahliae* other than that described for tomato (1,48).

The use of a transformed isolate of *V. dahliae*, VdLsGH1, expressing a GFP construct in conjunction with the appropriate illumination and optical filters allowed for easy discrimination of the fungus in planta, while requiring only minimal preparation of plant tissues. Additional staining was unnecessary for this pathosystem. However, a similar study of *V. dahliae* in oilseed rape, *Brassica napus*, found that conventional dyes were still necessary for microscopic observations (18). Eynck et al. (18) also examined the colonization of *V. dahliae* and *V. longisporum* on oilseed rape and found differences in colonization between the two *Verticillium* species; presenting evidence that the *V. dahliae* isolate from flax, *Linum usitatissimum*, was nonpathogenic on oilseed rape (18). *V. longisporum* colonization began with root hairs in oilseed rape, a pattern similar to that described for *Fusarium oxysporum* on tomato (37). Our observations of *V. dahliae* on lettuce differed considerably. This is the first report, to our knowledge, to use GFP to study *V. dahliae*, in planta, from the perspective of a compatible and incompatible interaction. While confirming many of the findings from earlier histochemical and immunoenzymatic studies in potato and cotton (6,21,22,43,44), this study also offered novel insights into *V. dahliae* pathogenesis from the early stages of root

TABLE 2. The titer of *Verticillium dahliae* isolate VdLsGH1, expressed as Log_{10} colony forming units (CFU) per gram of homogenized tissue in the taproots of susceptible lettuce cultivars 10 weeks after inoculation, pooled by disease severity rating[a]

		V. dahliae titer (Log_{10} CFU·g^{-1})				
Disease severity	*N*	Range	Median	Mean	SD	95% CI
0	36	0 – 2.98	2.01	1.81	0.84	(1.52 – 2.09)
1	1	–	–	0	–	–
2	0	–		–	–	–
3	6	0 – 3.61	2.38	2.10	1.21	(0.83 – 3.37)
4	18	0 – 4.83	2.56	2.61	0.98	(2.11 – 3.10)
5	20	3.41 – 5.03	4.47	4.34	0.45	(4.13 – 4.55)

[a] Spearman's correlation of Log_{10} CFU·g^{-1} versus disease severity ($N = 81$), $r = 0.73$, $P < 0.0001$.

TABLE 3. The mean titer of *Verticillium dahliae* isolate VdLsGH1, expressed as Log_{10} colony forming units (CFU) per gram of homogenized tissue, in the taproots of several lettuce cultivars 10 weeks after inoculation and corresponding median disease severity rating

			V. dahliae titer (Log_{10} CFU·g^{-1})	
Experiment	Cultivar	Disease severity: median (range)	Range	Mean (95% CI)
1	La Brillante	0.0 (0)	0.00 –1.63	0.65 (–0.29 – 1.60)
	LittleGem	0.0 (0)	0.00 – 2.05	1.12 (0.16 – 2.08)
	Salinas	4.0 (0 – 5)	0.00 – 4.47	2.71 (1.77 – 3.65)
	Sniper	4.0 (0 – 5)	1.90 – 4.81	2.91 (1.95 – 3.87)
2	La Brillante	0.0 (0 – 1)	0.00 – 1.81	0.88 (0.24 – 1.52)
	Salinas	3.5 (0 – 5)	0.00 – 4.82	3.01 (2.37 – 3.65)
	Sniper	4.5 (0 – 5)	1.90 – 5.83	3.70 (3.06 – 4.34)

colonization through the advance stages of disease, including seed infection.

Microscopic observations demonstrated that resistance in La Brillante and Little Gem is complete, corroborating earlier findings of the race-specific nature of this resistance (57). The described colonization pattern on incompatible lettuce cultivars also differed from incompatible tomato–*Verticillium* interactions conferred by the *Ve* genes (14,25,53), where colonization by incom-

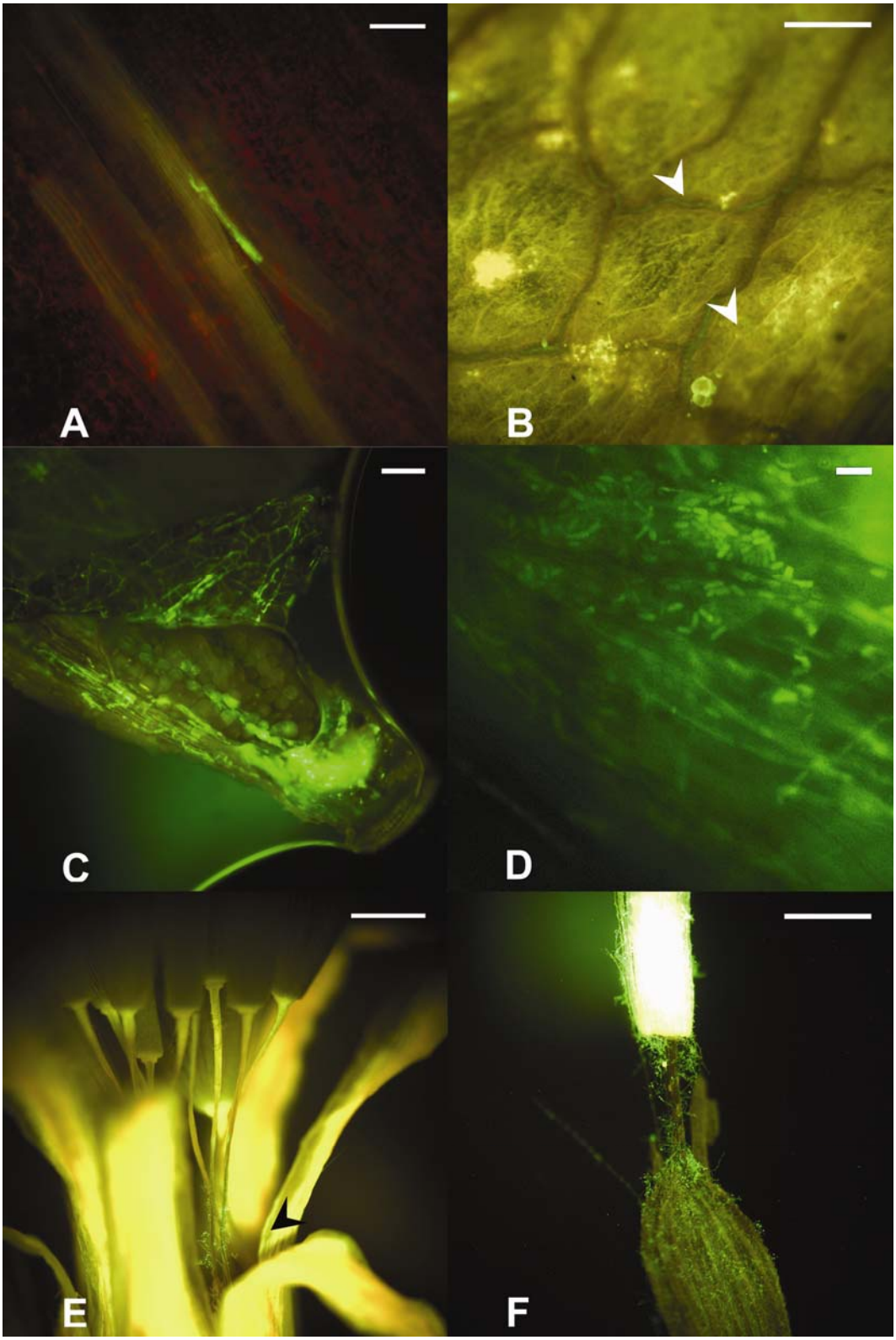

Fig. 6. The colonization of the stem and inflorescence of lettuce by *Verticillium dahliae*, 10 to 16 weeks following inoculation of 4- to 5-week-old seedlings with conidia. **A,** Small colony isolated within the xylem vessels of a pedicel from an infected plant (cv. Salinas), 12 weeks following inoculation. **B,** Necrotic region of a basal leaf from a symptomatic plant (cv. Salinas) with colonization (arrows) of minor veins by *V. dahliae*, 12 weeks following inoculation. **C and D,** Respective images showing the developing capitulum with massive colonization by *V. dahliae* prior to anthesis and the accumulation of conidia within the interior tissues of the developing capitulum. **E and F,** A dehiscent capitulum collected after flowering (PI 251256), approximately 16 weeks after inoculation, containing several ripened achenes with pappi covered with sporulating conidiophores (arrow) of *V. dahliae*. Analyses of images captured using epi-fluorescence microscopy (**A to F**). **A,** Scale bar = 100 µm; **C,** 500 µm; **D,** 10 µm; **B, E, and F,** 1 mm.

patible isolates of *V. dahliae* and *V. albo-atrum* was restricted to the roots, with limited spread to stem and foliar tissues (25,55). In cotton, the difference in susceptible and resistant (or tolerant) cultivars lies in the number of xylem vessels colonized and the resulting production of conidia within the transpiration stream (21). Resistance in lettuce cultivars La Brillante and Little Gem limited the fungus to lateral roots and prevented systemic spread to the taproot. In many cases, resistance appeared to eliminate the

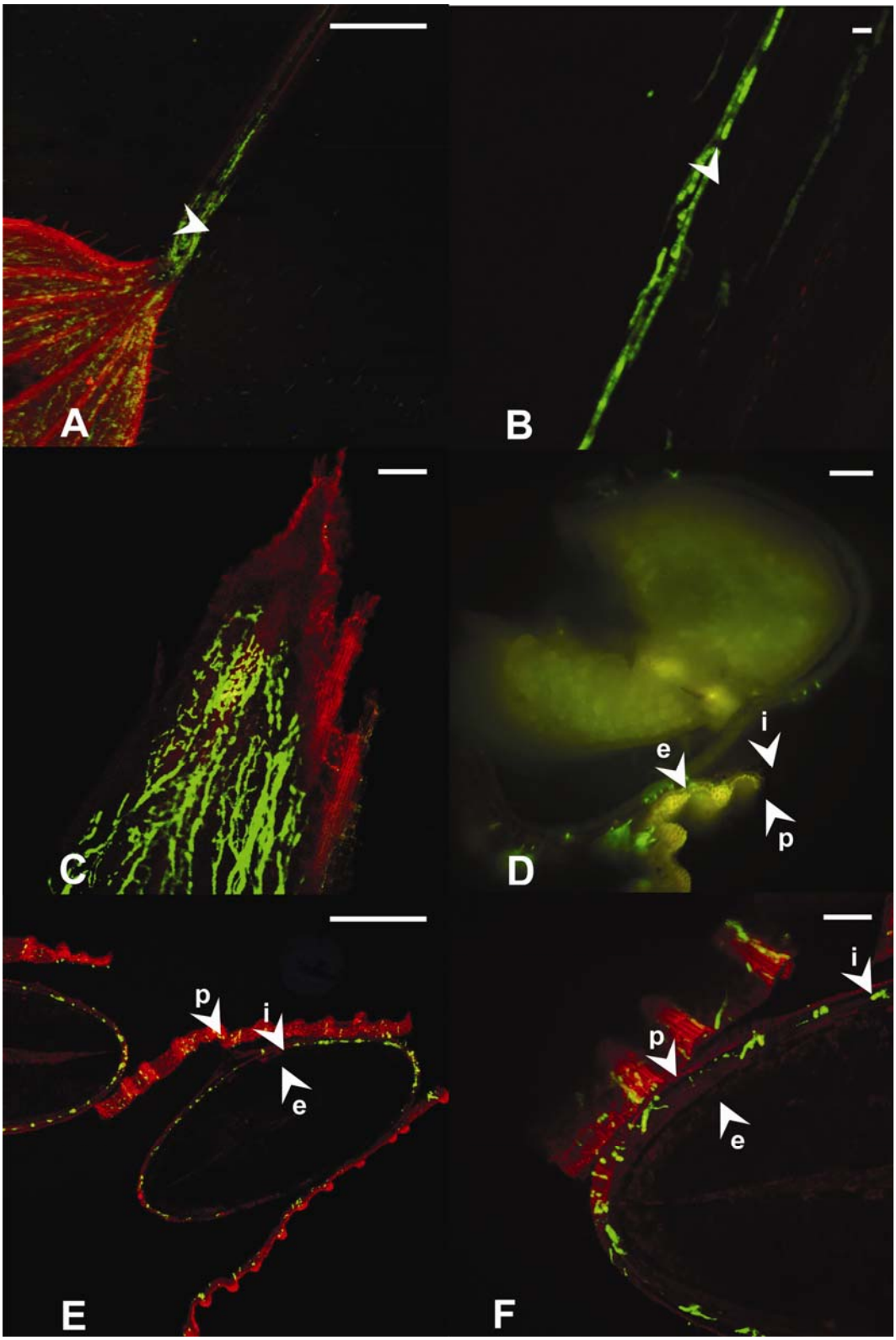

Fig. 7. Colonization of external and internal tissues of ripened achenes collected from lettuce (PI 251246) infected with *Verticillium dahliae* as seedlings. **A,** Ripening achene showing colonization of pericarp surface and internal colonization of pappus (arrow). **B,** Conidia production via hyphal budding within the pappus (arrow). **C,** Inner pericarp surface from a ripened achene ramified with hyphae. **D, E, and F,** Cross-section of a mature achene with hyphae ramified through all maternal tissues, but not into the embryo. Arrows denote tissues of the achene, pericarp **(p)**; integument **(i)**; endosperm **(e)**. Analyses of images captured using confocal laser scanning microscopy (**A, B, C, E, and F**) and epi-fluorescence microscopy (**D**). **A and E,** Scale bars = 500 µm; **B,** 10 µm; **C, D, and F,** 100 µm.

fungus from the root system. It is possible that the resistant cultivars are able to impede the progress of the fungus within lateral roots that are then shed through root turnover, or perhaps the fungus is trapped within infected xylem vessels where it may be eradicated through active defenses or by a combination of the two processes. Few studies have reported the presence of appressoria or any specialized structures during the colonization of roots by *V. dahliae*. During the infection of potato by *V. dahliae* in a hydroponic system, "small protuberances" were reported to develop on hyphae along the longitudinal grooves of epidermal cells that resulted in intracellular infection (43). Garber and Houston (21) reported the presence of appressoria during the infection of cotton roots by *V. albo-atrum*, but only when hyphae developed intracellularly in the epidermis or at the pit border between xylem vessel members. However, within the first 48 h following inoculation of lettuce roots, germinating conidia with distinct appressoria were observed. The germ tubes extended along root epidermal cells longitudinally and appressoria usually formed within the cell junctions and directly penetrated an adjoining cell. These observations conflict with a recent study of *V. dahliae* and *V. longisporum* on oilseed rape, where only slight hyphal swelling was observed before penetration (18).

While conidial suspensions were administered either to the bare roots of lettuce seedlings or to the soil surrounding seedlings to initiate infection in this study, microsclerotia are recognized as the long-term survival structures and the primary source of inoculum in infested soils. There are benefits to using conidia rather than microsclerotia as inoculum. Conidia are quite uniform, making them easier to prepare and quantify relative to microsclerotia. Conidia are also short-lived in soil (49), do not exhibit repeat germination that is observed with microsclerotia (17,19, 38), and still require root exudates to overcome soil fungistasis and germinate (50). Therefore, any apparent colonies along the surface of lettuce roots would be the result of a single inoculation event rather than from the repeat germination of microsclerotia. The single, root-dip inoculation with conidia helped synchronize infection and resulted in the colonization of one to two whorls of lateral roots. While the soil drench method, inoculating plants three times over a 3-week period, resulted in a more random pattern of root colonization over a larger root area and resulted in a higher level of disease incidence (G. E. Vallad and K. V. Subbarao, *unpublished data*). Regardless of the inoculation method employed, our observations were consistent with those obtained with microsclerotia (6).

The relative contribution of conidia to the Verticillium wilt disease cycle is still open to debate. Numerous researchers reported sudden increases in *V. dahliae* soil populations during the production of a crop (24,30,36,38), and either attributed this increase to sporulation (24,36,38) following the germination of microsclerotia, or to the release of microsclerotia from the breakdown of residues from a previous crop (30). Early sporulation on infected rootlets is also a possibility. Sporulation was also described for *V. albo-atrum* on the surface of infected tomato roots following their death (51). While sporulation on lettuce also occurred following root death, it was first observed during early colonization when apical growth had ceased, suggesting that the roots were physically compromised and also later at sites of successful systemic infection. The relative contribution of conidia and mycelia from infected crop residue on subsequent crops grown within the same season requires further consideration; especially in the coastal production areas of California. In the cooler environs of the Salinas Valley of California, two to three crops of lettuce per year may be planted within a single field, with as little as 2 weeks of fallow between crops. The severity of Verticillium wilt on lettuce is usually most severe on fall harvested lettuce crops (54), even though there is not necessarily a corresponding increase in the number of microsclerotia in the soil. While there is some evidence to suggest that the increased severity is related to plant development (G. E. Vallad, R. Grube, R. Hayes, and K. V. Subbarao, *unpublished data*), increases in soil inoculum due to infected residue from the previous lettuce crop including mycelia and conidia could also explain these observations.

In soil naturally infested with microsclerotia, direct colonization of root caps by *V. dahliae* leading to early colonization of vascular tissues in potatoes was observed (6), analogous to the colonization of cotton roots by *V. albo-atrum* (21). However, others reported that root cap colonization never led to successful systemic infection (44), or that colonization of the root cap and root tip was never observed (22). Such differences may be due to the limitations in the labeling technique, such as the inability of reagents to penetrate host tissues (22) or variations in experimental conditions (43,44). In our study, it was clear that *V. dahliae* preferentially colonized the tips of lettuce roots and within the zone of root elongation.

Colonization of lettuce by *V. dahliae* was spatially and temporally dynamic. Colonies of VdLsGH1 in the cortex of lettuce were sparse, with hyphae growing towards vascular tissues with little hindrance. These successful vascular infections differed from the colonies that developed along other regions of the root, root hairs, lateral root eruption zones, and in areas with apparent root damage. These superficial colonies failed to persist with time. As colonization progressed in the lateral roots, two colonization fronts became apparent; one front enveloped the epidermis and cortical tissues and the other within the vascular tissues. The former led to the eventual collapse of the infected root tip whereas, the latter continued its acropetal progression through infected xylem vessels of the infected lateral root, well in advance of the mycelia enveloping the infection site. Besides the noticeable collapse of the root tip, no symptoms were evident beyond the infection site as the mycelia advanced systemically through xylem vessels towards the taproot. Huisman (30) reported that the number of *V. dahliae* colonies per root length remained constant and randomly distributed in direct relation to soil inoculum levels initially, but subsequently an increase in the number of colonies per root length and a deviation from a random distribution occurred. In lettuce, initial colonies also appeared randomly distributed as conidia germinated following the root-dip or soil drench methods. With time, colonies became noticeably aggregated near the apical ends of lateral roots. Over time the systemic colonies progressed acropetally through infected xylem vessels, which would appear as an increase in colony density. While Huisman (30) estimated an average colony size of only 2.3 mm by analyzing the recovery of *V. dahliae* from cotton root segments, later studies using immunoenzymatic staining of *V. dahliae* increased this estimate to 7.3 mm and found an increase in colony length correlated with increased distance from the root apex (22). Despite the distinctly different methods employed between this study and Huisman (30) and Gerik and Huisman (22), the observations are nearly similar. In cotton, cortical root infections occur 1,000-fold in excess of systemic infections (29). Our observations suggest that despite the high correlation between inoculum density and high infection, the number of colonies per root is probably unrelated to the frequency of infections at the root tip and within the zone of root elongation (30).

Vascular discoloration developed well in advance of the encroaching hyphae of *V. dahliae.* For growers, vascular discoloration is a key diagnostic symptom that can be used prior to harvest to assess disease prior to crop failure. Growers can utilize such symptoms as a warning to harvest the field early before the development of acute wilting symptoms (G. E. Vallad, Q-.M. Qin, R. Hayes, and K. V. Subbarao, *unpublished data*). The spatial and temporal development of vascular discoloration in advance of the colonizing mycelia could be due to a phytotoxin(s) produced by *V. dahliae*. Various lipopolysaccharide and proteinaceous fractions from the culture filtrates of *V. albo-atrum* and *V. dahliae*

have been isolated and characterized, demonstrating their ability to cause vascular discoloration when infiltrated into susceptible plants (9,10,15,23,34,39–41,45). The strongest evidence came from a heat-stable, protein-lipopolysaccharide complex recovered from the culture filtrates of various *V. dahliae* isolates that caused vascular discoloration and wilting in susceptible hosts (34,40), and when isolated from race 1 and race 2 isolates caused a differential host reaction when infiltrated into tomato near-isogenic lines with or without the *Ve* resistance genes (41). Further purification of this protein-lipopolysaccharide complex identified a 3-kDa peptide with high activity (9,39). Recently, Wang and colleagues (60) identified a *V. dahliae* necrosis and ethylene-eliciting protein (VdNEP) while screening an expressed sequence tag library for cDNAs that encoded putative secreted proteins. An isolated His-tagged VdNEP fusion protein expressed in *Escherichia coli*, caused wilt symptoms when infiltrated into cotton and the accumulation of gossypol and other phytoalexins in cotton cell cultures (60). However, the relative importance, if any, of these proteinaceous factors in the pathogenicity of *V. dahliae* remains unresolved.

Seedborne infection may play an important role in the epidemiology of Verticillium wilt on lettuce (56) and other crops (2,16,32,35) by not only transmitting *V. dahliae* to the seedling, but also facilitating pathogen spread to new production areas, both domestic and international. Of more concern, is the potential of spreading *V. dahliae* through airborne inoculum during the production of lettuce seed. While the airborne dispersal of conidia from the achene may be limited by size, the pappus is easily dislodged from the rest of the achene on nonshattering commercial lettuce plants resulting in the long-distance dispersal of conidia. The introduction of pathogenic lettuce isolates of *V. dahliae* to the lettuce seed production fields could further threaten the lettuce industry, by accelerating the spread of these lettuce-adapted isolates. Such a scenario is already present in spinach production, but unlike lettuce, spinach does not exhibit symptoms until flowering (16 and references therein). How the warmer climate in the San Joaquin Valley may impact infection of lettuce during seed production and the subsequent expression of Verticillium wilt symptoms is unclear.

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