Colonization of Spinach by *Verticillium dahliae* and Effects of Pathogen Localization on the Efficacy of Seed Treatments

Karunakaran Maruthachalam, Steven J. Klosterman, Amy Anchietta, Beiquan Mou, and Krishna V. Subbarao

First and fifth authors: Department of Plant Pathology, University of California-Davis, c/o U.S. Agricultural Research Station, 1636 E. Alisal St., Salinas, CA 93905; and second, third, and fourth authors: U.S. Department of Agriculture–Agricultural Research Service, Salinas, CA.

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ABSTRACT

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Verticillium wilt on spinach (*Spinacia oleracea*) is caused by the soilborne fungus *Verticillium dahliae*. The pathogen is seedborne and transmission through seed is a major concern because of the dispersal of the pathogen to areas where fresh and processing spinach crops are grown in rotation with susceptible crops. Reduction in seedborne inoculum minimizes pathogen spread; therefore, knowledge of pathogen localization in seed is critical to develop methods to reduce seedborne inoculum. Spinach seedlings were inoculated with conidial suspensions of a green fluorescent protein-tagged strain of *V. dahliae* and colonization events were followed through seed production by confocal laser-scanning microscopy. Between 24 to 96 h postinoculation (PI), conidia germinated and

formed hyphal colonies on root tips and in root elongation zones. Hyphae colonized root cortical tissues both intra and intercellularly by 2 weeks, and colonized the taproot xylem with abundant mycelia and conidia that led to vascular discoloration coincident with foliar symptom expression by 8 weeks PI. At 10 weeks PI, the xylem of the upper stem, inflorescence, and spinach seed parts, including the pericarp, seed coat, cotyledons, and radicle, had been colonized by the pathogen but not the perisperm (the diploid maternal tissue). Maximum concentration of the fungus was in the seed coat, the outermost layer of the vasculature. Infection of *V. dahliae* in spinach seed was systemic and transmissible to developing seedlings. Additional analyses indicated that fungicide and steam seed treatments reduced detectable levels of the pathogen but did not eliminate the pathogen from the seed. This information will assist in the development of seed treatments that will reduce the seedborne inoculum transmission to crop production fields.

Verticillium dahliae Kleb. is a soilborne fungal pathogen that causes vascular wilt on a number of economically important crops (23,28). More than 200 plant species are hosts to *V. dahliae* and the list of susceptible hosts is expanding (15). *V. dahliae* can be transmitted (spores or microsclerotia) by vegetative material, farm equipment, water, soil, and air. The pathogen is seedborne in spinach (*Spinacia oleracea* L.), lettuce (*Lactuca sativa* L.), and other crops (7,32,37,39), representing an additional route of long-distance transmission.

The increasing production of spinach and the concomitant increased planting of *V. dahliae*-infected spinach seed has led to the introduction of *V. dahliae* strains into crop production fields (4). Spinach seed crops are produced only in areas of the world that receive ≈18-h photoperiods, which favors flowering (bolting) and elongation of the seed stem, such as in the Pacific Northwest, Chile, Denmark, the Netherlands, and New Zealand. Consequently, all of the spinach seed planted in California is imported, and imported seed has contained exotic strains of *V. dahliae* (4). These strains can be the major drivers of outbreaks of *Verticillium* wilt on lettuce and other crops grown in rotation with spinach in California (5), where ≈78% of the total fresh market spinach in

the United States is produced (16,40). The steep increase in spinach production in central coastal California in the 1990s has coincided with the appearance and spread of *Verticillium* wilt on lettuce in the region (3,34). The potential for transmission of *V. dahliae* from infected spinach seed to subsequent crops is supported by several observations. First, spinach seed produced in Europe and the Pacific Northwest have been infected with *V. dahliae* (8,9). The seed health testing of 62 commercial spinach seed lots for *Verticillium* spp. revealed 0 to 84.8% infected seed per lot (9). Second, *V. dahliae* strains from lettuce and other hosts grown in California are genetically similar to strains from spinach seed produced in Europe (The Netherlands and Denmark) and Washington State, as indicated by analyses of simple sequence repeat (SSR) markers (4). Furthermore, there are two races of *V. dahliae* affecting lettuce, tomato, pepper, and cotton (19,36). Both race 1 and race 2 isolates have been recovered from infected spinach seed and these are equally virulent on lettuce and other hosts (5). In addition, the vegetative compatibility groups (VCGs) of *V. dahliae* isolates infecting spinach seed are VCG4B and VCG2B (9), and these VCG strains cause disease on lettuce cultivars (*unpublished data*). Seedborne transmission of *V. dahliae* in spinach is a serious concern, especially if the transmitted isolates become established in coastal California, where lettuce and other hosts are rotated with spinach (4,35).

Resistance to *Verticillium* wilt in either the historical or contemporary spinach cultivars is unavailable, although efforts to identify wilt-resistant germplasm are currently underway (B. Mou, K. Maruthachalam, S. J. Klosterman, and K. V. Subbarao, *unpublished data*). In the interim, treatments to eliminate or reduce seedborne inoculum are being developed. One factor in determining the efficacy of seed treatment is the knowledge of the

Corresponding author: K. V. Subbarao; E-mail address: kvsbarao@ucdavis.edu

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location of the pathogen within the seed. Isolation of the pathogen from various seed parts has been successful but information on both the precise location and the amount of the pathogen within the seed are still lacking. Over the past decade, the expression of green fluorescent protein (GFP) has been reported in many fungal species, including *Verticillium* (10,18,21,27,33,37). Several *V. dahliae*-host interaction studies focusing on anatomical aspects have been conducted (6,11,21,24,25,42) but fewer studies such as interactions of *V. dahliae* and *V. longisporum* with oilseed rape and cauliflower have followed the complete colonization process, from plant infection through seed production (21,42). Using a GFP-tagged *V. dahliae* strain, the colonization events and symptom development were documented in lettuce (37). Similarly, colonization of broccoli and cauliflower in relation to phytochemical changes was studied using a GFP-tagged *V. longisporum* isolate (21). However, in spinach, little is known about the relationship between pathogen colonization and *Verticillium* wilt symptom development. Furthermore, it is unclear how *V. dahliae* subsequently reaches seed following infection, or the precise localization within spinach seed. This information is critical to develop effective seed treatments to eliminate or reduce the pathogen in the seed.

The first objective of this study was to examine the colonization of spinach by GFP-tagged *V. dahliae*, with an emphasis on observations of the fungus during systemic colonization, symptom development, and seed infection. The second objective was to evaluate the presence and location of *V. dahliae* in treated spinach seed. With the use of a GFP-labeled, race 2 isolate of *V. dahliae*, it was possible to track the fungus in planta in a susceptible cultivar that yielded valuable insights into the pathology of *V. dahliae* on spinach and differential colonization of male and female flowers and seed.

MATERIALS AND METHODS

Isolate transformation and maintenance. The *V. dahliae* isolate VdSo925 was recovered from spinach seed produced in The Netherlands. This isolate was determined to be race 2 based on molecular and pathogenicity assays (K. Maruthachalam and K. V. Subbarao, unpublished data). The VdSo925 isolate was grown on potato dextrose agar (PDA) (Difco Laboratories Inc., Detroit) for 7 days, and conidia were harvested and used for *Agrobacterium tumefaciens*-mediated transformation (ATMT). ATMT was carried out as previously described (20) using a T-DNA binary vector (pSK2241) encoding a hygromycin B resistance gene (*hph*) under the control of the *Aspergillus nidulans trpC* promoter and the ZsGFP gene under the control of the *Fusarium verticillioides* translation elongation factor 1 α gene promoter (20). The vector was introduced to *Agrobacterium tumefaciens* strain EHA105 by electroporation, then used for transformation of the conidia of *V. dahliae* by random insertional mutagenesis. Putative transformants were selected on PDA supplemented with hygromycin (50 μ g/ml) and examined under the compound microscope (Olympus BX60), which is equipped with a GFP filter (450- to 490-nm excitation, 500-nm longpass emission) for GFP expression. The insertion copy number was confirmed by Southern blot analysis as described by Selden and Schatz (30) using a DNA fragment of the hygromycin phosphotransferase gene and the probe was amplified using the *hph*-specific primers Hy_F (5'-TCA GCT TCG ATG TAG GAG GG-3') and Hy_R (5'-TTC TAC ACA GCC ATC GGT CC-3'), as previously described (20). A single transformant (VdSo925-316) with a single copy of T-DNA insertion, as determined by Southern blot analysis (20), and high GFP fluorescence intensity was selected and used throughout this study. The VdSo925-316 transformant was stored in 30% glycerol at -80°C as a conidial suspension. The transformant VdSo925-316 was examined for morphology (colony growth and microsclerotia production) and pathogenicity (by either soil drench or

root dip method) on both lettuce plant introduction 251246 and spinach cultivars 'Hector' and 'Avenger'.

Spinach inoculation. Seed of Hector spinach were planted in a pasteurized sand/potting soil mixture (2:1) in 200-well seedling plug trays (Hummert International, St. Louis), initially maintained in the dark for 3 days at 20°C, transferred to the greenhouse, and then germinated at 25 $\pm$ 2°C with a 12-h photoperiod. Plants were fertilized at least once a week with a solution of 20-20-20 fertilizer (Jack's Classic All Purpose; J. R. Peters, Allentown, PA). One particular seed lot of Hector was determined to have the lowest incidence of *V. dahliae* in seed health tests at 0.3% (7) and, thus, was used throughout these experiments. Meanwhile, isolate VdSo925-316 was grown on PDA for 7 days and conidia were harvested using sterile distilled water (SDW) and filtered through layers of sterile cheese cloth. Conidia were counted with a hemocytometer and the final inoculum density was adjusted to 1 $\times$ 10⁷ conidia/ml. Three-week-old seedlings were inoculated with conidia of VdSo925-316 at 1 $\times$ 10⁷ conidia/ml using a soil drench method (35). After 1 week postinoculation (PI), seedlings were transplanted into 0.5-liter foam-insulated cups filled with pasteurized sand/potting soil in a 3:1 mixture. The control plants were mock inoculated with SDW. Plants were maintained on benches in a greenhouse (24 $\pm$ 2°C) for $\approx$ 14 weeks PI or until seed maturation.

Microscopy. Three to five plants were sampled 24, 48, and 72 h and 1, 2, 4, 6, 8, and 10 weeks PI for microscopic observation. The inoculated plants were gently washed free of soil and sectioned with a double-edged platinum razor blade in both transverse and longitudinal directions. Whole-root mounts were examined with an epifluorescence Olympus BX60 compound microscope or a Nikon compound microscope with filter blocks for GFP (450- to 490-nm excitation, 590-nm longpass emission), coupled to an MRC 1024 Bio-Rad confocal laser-scanning microscope (CLSM) (Bio-Rad, Hercules, CA) or Leica CLSM (Leica Microsystems Inc., Buffalo Grove, IL). Confocal images were captured and processed as outlined by Vallad and Subbarao (37). In addition, seed were collected from inoculated plants at 12 to 14 weeks PI, hand sectioned (both cross and longitudinal sections) with a double-edged platinum razor blade into thin slices, and examined for the presence of *V. dahliae* by confocal laser-scanning microscopy.

Production of infected staminate and pistillate spinach plants to study pathogen transmission. Seed of Hector spinach were planted and grown as described above. Three-week-old seedlings were inoculated with conidia (1 $\times$ 10⁷ conidia/ml) of GFP-tagged *V. dahliae* strain VdSo925-316 by the soil drench method as above (35). Plants were maintained on the greenhouse benches at 24 $\pm$ 2°C under a 16-h photoperiod to initiate bolting (flowering). Spinach is a predominantly dioecious species with an even ratio of female (pistillate) and male (staminate) plants (22,29). The staminate and pistillate spinach plants were separated to determine pathogen transmission either from the staminate flowers into pistillate plants or vice versa. In the first experiment, inoculated male plants were surrounded by healthy female plants to mimic natural hybridization. Similarly, in the reverse experiment, inoculated female plants were surrounded by healthy male plants. At 7 weeks PI, male and female flowers from inoculated plants were collected and observed under a confocal microscope. Mature seed were collected from both experiments and plated on NP-10 medium to determine whether male or female plants are able to transmit *V. dahliae* into each other through developing seed. In a separate experiment, pollen from inoculated male plants was collected and dusted manually onto healthy female flowers to determine pollen-mediated pathogen transmission into developing seed. Pollen was collected from inoculated and noninoculated plants and plated on NP-10 medium to screen for the presence of *V. dahliae*. The experiment was repeated once. To test the attachment of *V. dahliae* conidia on the

spinach pollen, both conidia and pollen were mixed and co-incubated on water-agar medium. Plates were observed after 12, 24, and 48 h and images were captured using epifluorescence microscopy.

Infected seed production and seed treatment. To produce a large quantity of infected and healthy seed for seed treatment, ≈500 spinach seedlings of Hector were inoculated with VdSo925-316 as described above. After seed set, ≈200 g of seed were collected from VdSo925-316-inoculated spinach plants, and delivered to Germains Seed Technology (Gilroy, CA) for a fungicidal seed treatment with Pro-Gro (Germains Seed Technology), a mixture of thiram and carboxin. The healthy seed, ≈200 g from Hector spinach, were also treated to compare the seed treatment effect on infected and healthy spinach seed. Pro-Gro-treated commercial seed lots 1 to 5 (Table 1) were obtained from Germains Seed Technology, and seed lot 6 (Table 1), treated with the steam treatment Seedgard (IncoTec. Inc., The Netherlands), was obtained from Dr. L. du Toit (Washington State University, Mt Vernon). The protocols used for the application of these seed treatments are proprietary. After seed treatment, 100 seeds (10 to 15 seeds/plate) were plated on NP-10 medium and microscopically examined for the presence of *V. dahliae* following incubation at room temperature for 10 days. The experiment was repeated once.

Post-seed-treatment assessment of *V. dahliae*. The presence of *V. dahliae* in the spinach seed was confirmed based on production of the microsclerotia and verticillate conidiophores. In addition, a portion of the treated and untreated seed was stored at room temperature for 2 to 3 months to assess the storage effect on *V. dahliae* growth.

To assess the effects of treatment on percent seed germination, both treated and untreated seed ($n = 100$) were planted in 100-well seedling plug trays and maintained as described previously. Three weeks after planting, the number of seedlings emerged and their growth and development were visually assessed. In addition, developing seedlings from both treated and untreated groups were sampled and examined microscopically for growth of *V. dahliae*.

Evaluation of seed treatment by quantifying the DNA of *V. dahliae* in treated and untreated commercial seed lots by quantitative polymerase chain reaction. A quantitative polymerase chain reaction (qPCR) assay (8) was conducted on five infected commercial seed lots (lots 1 to 5) that had been treated with Pro-Gro and seed from the same seed lots that were untreated. The qPCR assay was conducted on a sixth sample of seed from a commercial seed lot that had been treated with the steam

treatment from Seedgard (seed lot 6). Three replicate samples of 1,000 seeds each were randomly selected from each of the five different seed lots from seed lots 1 to 5 to evaluate the effect of seed treatment on the DNA quantity from *V. dahliae*. Two replicate samples of 750 seeds were tested for seed lot 6, the seed of which were treated with Seedgard or untreated. The seed were ground for 45 s to a fine powder using an IKA A10 analytical grinding mill (IKA Laboratory, Germany). DNA extractions were conducted from 200-mg fractions of ground seed using a FastDNA kit following the manufacturer's protocol (MP Bio-medicals, Solon, OH), except that the IKA A10 mill was used for sample homogenization in place of the FastPrep Instrument. DNA extracts were further purified with AMPure magnetic beads and a SPRI 96-Ring Magnet Plate (Agencourt, Beverly, MA) to cleanse DNA from potential PCR inhibitors. DNA concentration was measured using a Qubit fluorometer and Quant-iT[™] dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). All DNA samples were diluted to 1 ng/μl in nuclease-free water and 5 μl of the diluted DNA was used for qPCR. The qPCR was carried out using SYBR Green methodology in a LightCycler 480 model II (Roche Diagnostics, Indianapolis, IN) using β-tubulin-specific primers, as described by Duressa et al. (8). The quantity of DNA from *V. dahliae* in the seed samples was estimated using copy-number-based, five-point (500,000, 50,000, 5,000, 500, and 50 copies) standard curves constructed by serial dilution of plasmid vector DNA carrying the β-tubulin gene fragment from *V. dahliae*, as described by Duressa et al. (8). The absolute quantification module of the LightCycler 480 software (version LCS480 1.5.0.38) was used to determine quantification cycle and β-tubulin copy number, in addition to mean and standard deviation. The spinach actin (GenBank accession JN987183) was used as an external control for DNA amplification in each sample using an actin-specific primer pair, as previously described (8).

RESULTS

Characterization of the *V. dahliae* transformant strain VdSo925-316. *A. tumefaciens*-mediated transformation of *V. dahliae* with the binary vector, pSK2241, led to the appearance of hygromycin-resistant colonies 7 days after transfer to PDA medium containing hygromycin. In all, ≈125 to 150 transformants per plate were obtained at 48 h of co-cultivation. Among these transformants, 69% harbored a single copy of T-DNA insertion (20). A single transformant, VdSo925-316, was selected because this strain contained a single copy of the T-DNA insert, as

TABLE 1. Quantification of DNA from *Verticillium dahliae* by quantitative polymerase chain reaction in six different commercial spinach seed lots treated with fungicide and steam

Seed lot, treatment ^a	β-Tubulin Cq ^b	β-Tubulin SD Cq ^c	Copy number ^d	SD copy number	Actin Cq ^e	SD Actin Cq ^f
1, Pro-Gro	30.79	0.11	30.63	2.38	22.16	0.17
Untreated	27.56	0.75	265.34	135.17	22.52	0.63
2, Pro-Gro	34.08	1.23	3.40	3.16	22.68	0.66
Untreated	26.63	0.26	494.04	88.69	22.62	0.30
3, Pro-Gro	31.47	0.76	21.39	10.84	22.49	0.33
Untreated	28.77	0.11	118.18	8.33	22.38	0.12
4, Pro-Gro	28.90	0.38	108.35	24.63	22.68	0.63
Untreated	26.32	0.53	607.78	186.33	23.17	0.30
5, Pro-Gro	30.82	1.12	36.25	24.06	23.06	0.77
Untreated	27.78	0.68	245.94	103.44	22.66	0.74
6, Seedgard	27.72	0.35	243.26	51.84	21.64	0.18
Untreated	25.98	0.46	793.85	233.20	21.48	0.32

^a Seed were either treated with a fungicide or steam (see Materials and Methods) or untreated. Seed samples 1 to 5 were treated with Pro-Gro while seed lot 6 was treated with steam treatment Seedgard. For seed lots 1 to 5, the results from the untreated and treated samples were derived from three replicates of 1,000 seed.

^b Mean value of the quantification cycle (Cq). Cq values obtained from the untreated seed from seed lots 1 to 5 were previously reported (8) and used in the table for comparison.

^c SD = standard deviation.

^d β-tubulin copy number.

^e Actin sequence from spinach (DNA amplification inhibition check and control).

^f Based upon two replicates of 750 seeds for each of the steam-treated and untreated seed batches from seed lot 6.

determined by Southern blot analysis (data not shown). In addition, the VdSo925-316 transformant showed a strong and uniform green fluorescence signal in all parts of the thallus, including hyphae, conidiophores, and conidia (Fig. 1B, C, and D). Transformant VdSo925-316 did not show differences in colony morphology, growth rate, and microsclerotia production or pathogenicity (foliar and vascular symptoms by 3 weeks PI) relative to the wild-type strain VdSo925 from spinach (data not shown).

Infection and colonization of spinach plants by *V. dahliae*.

Of the conidia observed on the root surface using epifluorescence, $\approx 67\%$ germinated at 24 h PI, and half of these conidia formed appressorium-like structures (ALS) by 48 h PI (Fig. 2A and B). The hyphae from germinated conidia elongated in parallel with the longitudinal axis of the root at the junctions of epidermal cells at 48 h PI (Fig. 2C). Conidia of *V. dahliae* near the wound site of roots germinated and penetrated the tissues to establish hyphal colonization (Fig. 2F). At 1 week PI, hyphae colonized the root tips and invaded the vascular tissues of several lateral roots (Fig. 2D and E). In the root elongation zone, colonization by hyphae was observed in both inter- and intracellular spaces of the cortical tissues and vascular elements (Fig. 2G, H, and I). Colonization at 2 weeks PI within root elongation zones consisted of more complex networks of hyphae along the root surface, in addition to growth within the grooves between epidermal cells (Fig. 2G and H), and some hyphae exhibited swelling at the tip before penetrating adjacent cells (Fig. 2H). Hyphal colonies progressed acropetally to the xylem vessels of infected lateral roots.

At 6 weeks PI, colonization progressed into the root xylem vessels in inoculated plants (Fig. 3B) but GFP fluorescence was not observed in the root xylem vessels of mock-inoculated seedlings (Fig. 3A). Subsequently, brownish vascular discoloration progressed acropetally from the infected lateral roots to the crown

region of the plant (Supplemental Figure 1). At 10 weeks PI, many numbers of hyphal colonies were observed in the xylem vessels of the stem (Fig. 3C to F), coinciding with necrotic lesions of root tissues and leaves and foliar symptom development. All of the xylem vessels of the stem were heavily colonized by *V. dahliae* (Fig. 3D) at 10 weeks. Closer examination of xylem vessels in the stem region revealed hyphal growth toward the pith of the spinach stem (Fig. 3E).

Verticillium wilt symptoms first appeared on the bolting stem and then the tissues of the inflorescence. As symptoms progressed, leaves on individual stems as well as the inflorescence and bracts of the involucre became chlorotic and flaccid. A longitudinal section of the inoculated staminate and pistillate flowers revealed *V. dahliae* colonization (Fig. 4). Furthermore, the anther walls from inoculated staminate flowers were colonized by the pathogen (Fig. 4C) but the pollen grains were devoid of the GFP signal and no *V. dahliae* was recovered on the NP-10 medium (Fig. 4D). Anthers and pollen of staminate flowers from mock-inoculated plants were not colonized by *V. dahliae* (Fig. 4A and B). In the inoculated pistillate flowers, *V. dahliae* was detected in the perianth, style, and stigma (Fig. 4I to L) but no pathogen was detected in the noninoculated pistillate flowers (Fig. 4G and H). An in vitro pollen germination assay failed to reveal *V. dahliae* in the pollen tube of the inoculated staminate flowers (Fig. 4E and F). *V. dahliae* conidia firmly attached to spinach pollen following 24 h of co-incubation in the water-ager medium and penetration and hyphal colonization were observed after 48 h of incubation (Fig. 4M to P). In addition, *V. dahliae* or GFP fluorescence was not detected in seed collected from healthy female plants that surrounded the infected male plant.

Attempts to demonstrate whether male seed stock or female seed stock is the source of *V. dahliae* transmission to the devel-

Fig. 1. *Verticillium dahliae* isolate from spinach seed (VdSo925) expressing green fluorescent protein (GFP). Additional details on the transformation are provided in the Materials and Methods. **A**, Culture morphology of the transformant, VdSo925-316, on potato dextrose agar medium. **B**, Verticillate conidiophores of VdSo925-316. **C**, GFP-expressing VdSo925-316, released conidia (c) on the tip of a verticillate conidiophore. **D**, Conidia of GFP-tagged VdSo925-316. **B to D**, Scale bars = 100 μm .

oping spinach seed also provided useful information. Results from the crossing experiment suggested that the male seed stock was not the source of the pathogen because the seed plated from the crosses between *V. dahliae*-inoculated male plants with healthy female plants did not result in *V. dahliae* infection. Seed plated from inoculated female plants (experimental set-up 2) were infected with *V. dahliae* and this was confirmed by both NP-10 plating and microscopic observations (data not shown).

Further microscopic examination of whole seed 10 weeks PI revealed low internal colonization by *V. dahliae* of the thick and corky region of the pericarp (Fig. 5A to C). At 12 weeks PI, the

entire pericarp of the spinach seed was heavily colonized (Fig. 5D and F) and was limited only to the pericarp. Over the next 2 weeks (12 to 14 weeks PI), hyphae rapidly progressed into the seed coat, cotyledons, and radicle from the pericarp of the spinach seed (Fig. 6A to H). Conidiophores and massive amounts of secondary conidia were also observed internally in the infected pericarp tissues (Fig. 6A and C). A transverse section of the spinach seed showed that *V. dahliae* had colonized the cotyledons, plumule, hypocotyl, and radicle (Fig. 6D to H). The analyses of 50 mature seeds from several infected plants revealed that *V. dahliae* was not present in the perisperm of the seed. At this stage, microsclerotia

Fig. 2. Early and advanced stages of root colonization by the green fluorescent protein-tagged spinach isolate of *Verticillium dahliae*, VdSo925-316 at 48 h, 1 week, and 2 weeks postinoculation. **A**, Conidia (c) on the root surface with one or two germ tubes. **B**, Conidium (c) with a germ tube and an appressorium-like structure (ALS). **C**, Germinated conidium on the root surface with the primary mycelia. **D**, Lateral root tip colonized by *V. dahliae*. **E**, Lateral roots showing advanced hyphal colonization (HC), with mycelium extending from the epidermis into cortical tissues. **F**, Wound site of the lateral root colonized by *V. dahliae*. **G**, Composite of images from a confocal laser-scanning microscope (CLSM) showing advanced colonization of epidermal and cortical root tissues of spinach by *V. dahliae*. **H and I**, Composite image stack from CLSM exhibiting inter- (yellow arrows) and intra- (blue arrows) cellular colonization of cortical and vascular tissues of spinach by *V. dahliae*. Swelling of the hyphae before penetration of the next cell is denoted by HS.

Fig. 3. Later stages of spinach root stem colonization by the green fluorescent protein (GFP)-tagged spinach isolate of *Verticillium dahliae*, VdSo925-316. Images were captured 6 and 10 weeks postinoculation (PI) of seedlings with conidia of *V. dahliae*. **A**, Cross section of a noninoculated plant main root without GFP fluorescence. **B**, Cross section of inoculated plant exhibiting restricted advancement of fungal mycelia through the xylem vessels at 6 weeks PI. **C and D**, Cross section of a spinach stem showing colonization of *V. dahliae* in the vascular bundles (in a ring like arrangement) at 10 weeks PI. **E and F**, Magnified images of the spinach stem cross section, representative of the vascular bundles in D, exhibiting heavy colonization in every vascular bundle by *V. dahliae*. Scale bars = 100 μ m. White arrowheads indicate fungal hyphae with GFP fluorescence. Yellow arrowhead indicates hyphal growth toward pith.

were not observed in the pericarp or seed coat. However, when the infected seed collected from inoculated plants were stored at room temperature for nearly 3 months and then observed after taking cross sections, massive numbers of microsclerotia in the pericarp and seed coat were present (Fig. 7A and B). Observations of 20 seeds under a light microscope revealed that each infected spinach seed carried ≈ 250 microsclerotia. In addition, fungal hyphae with GFP fluorescence were not observed because the fungus may have switched almost entirely to the dormant

microsclerotial stage. Microsclerotia on either pericarp or seed coat regions were not present on healthy seed that were stored and examined similarly (Fig. 7C and D).

Post-seed-treatment observation of infected seed. To examine the effect of a fungicide treatment on *V. dahliae* in spinach seed, mature seed were collected from VdSo925-316-inoculated (>90% infection rate) and noninoculated (0% infection rate) spinach plants, subjected to Pro-Gro fungicide treatment, and examined on NP-10 medium. When *V. dahliae*-infected seed were

Fig. 4. *Verticillium dahliae* colonization in staminate and pistillate flowers. Confocal images captured in confocal 12 weeks following the inoculation of seedlings with conidia of green fluorescent protein (GFP)-expressing *Verticillium dahliae*. **A**, Noninoculated staminate flower anther (An) without GFP fluorescence. **B**, Pollen grains (Po) from noninoculated staminate flower. **C**, Inoculated staminate flower anther (An) shows *V. dahliae* infection. **D**, Inoculated staminate flowers pollens (Po) did not show *V. dahliae* infection. **E** and **F**, In vitro pollen germination assay did not show *V. dahliae* growth in the pollen tubes (Pt) of pollen grains from inoculated plants. **G**, Noninoculated pistillate flower without GFP fluorescence in perianth (Pe) and stigma (Sg). **H**, Stigma (Sg) of the staminate flower shows no fungal infection. *V. dahliae*-inoculated plants shows GFP fluorescence in **I**, perianth (Pe) and **J**, style (St). **K** and **L**, *V. dahliae* colonization in stigma of the pistillate flower. **M** and **N**, Germination of *V. dahliae* conidia (Co) on spinach pollen surface, when co-incubated on water-agar medium. **O** and **P**, Spinach pollens colonized by *V. dahliae* hyphae (Hy).

treated with Pro-Gro and plated on NP-10 medium, growth of *V. dahliae* was not apparent at 10 days post-planting. However, growth of *V. dahliae* from the spinach seed was apparent when the whole fungicide-treated seed were broken into small pieces and then plated (Fig. 8C). In contrast, all of the untreated seed from the inoculated plants were infected with *V. dahliae* (Fig. 8A). The seed from noninoculated plants that were treated with fungicide or untreated were devoid of *V. dahliae* growth (Fig. 8B and D). The GFP-fluorescence signal in the untreated, infected seed was strong (Fig. 8E). Microscopic observation of fungicide-treated seed from the inoculated plants revealed growth of *V. dahliae* in the pericarp region but GFP-fluorescence of the fungal mycelia showed a bleached appearance in the observed seed samples (Fig. 8G). No growth of *V. dahliae* was observed on healthy seed from noninoculated plants (Fig. 8F and H). Furthermore, slight thickening of the cell walls in the pericarp region of the fungicide-treated seed from both inoculated and noninoculated plants occurred. In addition, seedlings that grew from either group of fungicide-treated seed exhibited slower growth and leaves were etiolated (Fig. 8J) relative to the seedlings that grew from the seed untreated with fungicide (Fig. 8I). Early stages of seed germination on PDA plates (7 days after plating) of 100 randomly selected fungicide-treated seeds from inoculated plants revealed hyphal colonization and secondary conidia in the pericarp, hypocotyl, plumule, and radicle regions (Supplemental Figure 2) and, subsequently, (after 2 weeks) massive colonization with the production of conidiophores and microsclerotia (Fig. 8K to M).

Quantification of *V. dahliae* DNA in fungicide-treated and untreated commercial seed lots by qPCR. The β -tubulin gene in *V. dahliae* was quantified by qPCR in six infected commercial seed lots that were either fungicide/steam treated or untreated

(Table 1). After seed treatment with the fungicide Pro-Gro (seed lots 1 to 5), the β -tubulin copy number of the pathogen was reduced in all of the fungicide-treated seed lots compared with untreated seed lots. Among the five seed lots assessed for β -tubulin copy number following treatment with Pro-Gro, the β -tubulin copy number was most markedly reduced in seed lot 2. The recorded β -tubulin copy number from seed lot 2 was 494.04 ± 88.69 for the untreated seed and 3.40 ± 3.16 following seed treatment (Table 1). The steam treatment Seedgard (seed lot 6) also caused a reduction in the detection of the β -tubulin gene by qPCR (Table 1). A reduction in the β -tubulin copy number was observed in response to both fungicide and steam treatments but the β -tubulin sequence was detectable in all samples, even after treatments (Table 1).

DISCUSSION

The major emphasis of this research was to observe the colonization of spinach plants by a GFP-tagged strain of *V. dahliae*, analyze seed infection, determine the relative efficiency of the male and female plants to transmit the pathogen into the seed, and, finally determine the impact of seed treatments currently practiced on the fate of the pathogen within seed. Determining where *V. dahliae* resides in spinach seed is critically important for the development of effective seed treatments. The analyses of the GFP-tagged spinach isolate of *V. dahliae* by confocal microscopy confirmed that *V. dahliae* is present in deeper seed tissues such as the plumule, hypocotyl, and radicle, suggesting that complete elimination of the pathogen using seed treatment may be difficult.

Transmission of *V. dahliae* in spinach seed and the potential for the immigration of exotic strains in spinach seed poses a serious

Fig. 5. Early and latter stages of spinach seed colonization by a green fluorescent protein (GFP)-tagged spinach isolate of *Verticillium dahliae*, strain VdSo925-316, at 10 and 12 weeks postinoculation (PI) of 3-week-old spinach seedlings. **A to C**, Cross section of young spinach seed exhibiting low colonization of *V. dahliae* on the pericarp fruit wall of the spinach seed at 10 weeks PI. **D**, Cross section of spinach seed showing extensive colonization of *V. dahliae* on the pericarp 12 weeks PI. **E**, Same spinach seed shown in D, without filter to detect fungal GFP fluorescence. **F**, Same spinach seed shown in D, without filter to detect plant auto fluorescence; thus, only GFP fluorescence from the fungus is detectable. Scale bars = 100 μ m.

threat to many crop production regions of the world. In California, spinach is rotated with other *V. dahliae*-susceptible crops. *V. dahliae* produces microsclerotia, which remain viable up to 14 years in the soil in the absence of a host. We observed that a spinach seed could carry up to 250 microsclerotia. Seed health tests have detected infection rates of up to 85% by *V. dahliae* in spinach seed lots (8,9). With an average seeding rate of 7.5 to 10 million spinach seeds/hectare for baby leaf spinach production, there is every likelihood of introducing a large amount of *V. dahliae* inoculum (and possibly exotic strains) with infected spinach seed into the field. Verticillium wilt symptoms are not

observed on spinach crops because of the harvest within 4 weeks after planting; therefore, the disease is rarely considered important on this crop. However, the inoculum introduced with each spinach crop can remain in the soil for a number of years to affect susceptible crops that follow spinach.

In spinach, colonization was initiated at the root tips, through wounds on the roots, and at the junctions of the lateral and main roots. The initial colonization events of *V. dahliae* on spinach were similar to those described on lettuce, cauliflower, and broccoli, except that there was hyphal swelling during cell penetration (21,37). Hyphal swelling has been observed during the coloni-

Fig. 6. Advanced colonization of spinach seed by green fluorescent protein-tagged *Verticillium dahliae*, strain VdSo925-316, at 14 weeks postinoculation of 3-week-old spinach seedlings. **A to C**, Cross sections of the spinach seed showing massive colonization of *V. dahliae* on the pericarp (PE) and seed coat (SC). **D and E**, Cross section of spinach seed showing *V. dahliae* colonization on the plumule (PL) and radicle (RA). **F**, Cross section of seed showing *V. dahliae* infection on cotyledons (CO). **G**, Cross section of spinach seed without pericarp, exhibiting colonization on the hypocotyl (HY). **H**, Cross section of spinach seed without pericarp showing heavy colonization of *V. dahliae* on the cotyledons (CO) and plumule (PL). **I**, Cross section of a noninoculated germinating spinach seed indicating the cotyledons that are not colonized with *V. dahliae* (CO). PM indicates perisperm region of the seed and CP is conidiophore.

zation of oilseed rape by *V. longisporum* (10), at points prior to hyphal entry to the adjacent plant cell. However, in *V. dahliae* on lettuce, the hyphal swellings were not reported during penetration of adjacent root cells (37). It is possible that the structural variation in the root cells between plant species and the thigmotropic response that they elicit may determine whether or not *V. dahliae* produces hyphal swelling during colonization.

After successful penetration of the roots, *V. dahliae* strain VdSo925-316 quickly invaded the root cortex both inter- and intracellularly, producing an extensive hyphal network. On spinach roots, *V. dahliae* grew with no apparent direction, similar to *V. dahliae* and *V. longisporum* on oilseed rape (10). After dense colonization of the cortex, *V. dahliae* invaded xylem vessels, as observed following colonization of the cortex in olive, oilseed rape, and lettuce (10,27,37). However, the entire root vascular tissues were colonized by mycelia of *V. dahliae* strain VdSo925-316. This is in contrast to the incomplete colonization of the vascular tissue by *V. dahliae* in olive and *V. longisporum* on oilseed rape (10,27).

The pollen-mediated transmission of *V. dahliae* through spinach flowers in female plants into developing seed was investigated. Even though *V. dahliae* was observed in the anther wall, the pathogen was not transmitted into the developing pollen grains that reside inside the locule. The absence of a direct vascular link between the anther wall and pollen or absence of vascular elements in the pollen may prevent the colonization of

pollen grains by *V. dahliae*. Also, the timing of pollen maturation and *V. dahliae* colonization may preclude pollen colonization. The role of structural or physiological components that prevent infection of pollen by *V. dahliae* requires further study. In contrast, *V. dahliae* colonized the perianth, style, and stigma of the female flowers. The spinach stigma is composed of two vascular bundles in a single axis and *V. dahliae* moves through these vascular elements of the stigma into the flower and fruit stalk of the mother plant. Results from previous studies suggest that the internal ovary infection of mother plants by many fungi, including *Verticillium* spp., is through vascular elements in the stigma, integument, or other female flower parts (31,32,38). Infection through the female floral reproductive tissues results in the typical internal fruit colonization of *V. dahliae* and production of infected spinach seed. Most of the seed produced from infected female floral parts revealed verticillate conidiophores and fungal hyphae on the surface of the seed coat. It is likely, therefore, that transmission of *V. dahliae* to the developing seed is through systemic infection of maternal tissues and not the paternal tissues. This result correlates with the earlier observation of high incidence of *V. dahliae* in the female stock of a hybrid spinach seed crop when compared with male stock seed (9). Our study further confirms that the infected female spinach plant is the major source for inoculum transmission and systemic seed infection. These results indicate that it is critically important to use clean female spinach stocks for seed production to prevent or reduce

Fig. 7. Examination of spinach seed stored at room temperature nearly 3 months following collection from noninoculated plants, and from infected plants. **A and B,** Cross section of a spinach seed exhibiting microsclerotia (MS) on the pericarp and seed coat. **C and D,** Seed collected from noninoculated spinach plants did not show microsclerotia (MS) on the pericarp of the spinach seed.

the seedborne infection of *V. dahliae* and reduce the impact of this disease.

Verticillium dahliae was recovered from most of the seed collected from infected plants, as reported previously (9,38). However, the precise localization of the fungus in spinach seed had not been reported. Spinach seed contain a fleshy outer layer called the pericarp or fruit wall, seed coat, embryo, and perisperm (26). We observed heavy infections of *V. dahliae* in the pericarp region of the spinach seed. Subsequently, the fungus colonized the seed coat and inner parts of the seed. The way in which the fungus colonizes the pericarp and seed coat indicates that the infection develops directly from the xylem vessels. These observations are very similar to a previous study, where internal colonization of pathogen on the spinach seed was reported (38).

Because *V. dahliae* is systemic in spinach seed, when seed collected from infected plants germinate, the fungus multiplies simultaneously and a substantial amount of inoculum is transmitted to different parts of the seedlings, such as cotyledon, hypocotyl, and radicle. The thick, corky nature of the spinach pericarp (26) combined with the systemic infection of spinach plants by *V. dahliae* suggest that complete removal of *V. dahliae* from infected seed lots may be difficult. Furthermore, analyses of seed stored at room temperature revealed that the fungus switched into a dormant stage consisting of the long-lived microsclerotia. This may also explain why the GFP fluorescence observed on the stored seed was reduced. The presence of hundreds of microsclerotia may make it increasingly difficult to eliminate the pathogen from the infected seed because these tough survival structures

Fig. 8. Evaluation of a spinach seed treatment by comparing fungicide-treated and untreated seed from plants that were inoculated with *Verticillium dahliae* strain VdSo925-316 or noninoculated. **A**, Untreated spinach seed from inoculated plants were plated on NP-10 semiselective medium exhibiting growth of *V. dahliae* 10 days after plating. **B**, Untreated spinach seed from noninoculated plants did not exhibit growth of *V. dahliae* on NP-10 medium. **C**, Treated spinach seed was plated as whole (no growth of *V. dahliae*) or broken (growth of *V. dahliae* is evident) on NP-10 medium. **D**, Treated seed from noninoculated plants (both whole and broken) showed no growth of *V. dahliae*. **E**, Cross section of the untreated spinach seed from inoculated plants showing fluorescence of green fluorescent protein (GFP) under confocal microscopy. **F**, Cross section of the untreated spinach seed from noninoculated plants did not show any GFP fluorescence. **G**, Cross section of a treated spinach seed from an inoculated plant showing a reduction in the amount of *V. dahliae* and bleached GFP fluorescence due to fungicide effect. **H**, Cross section of a treated spinach seed from a noninoculated plant without fungal GFP fluorescence. **I and J**, Phenotypic differences of developing seedlings (3 weeks old) from **I**, untreated and **J**, treated seed. **K to M**, Developing seedling from fungicide-treated seed shows microsclerotia (MS) on the pericarp (PE), hypocotyl (HY), cotyledons (CO), plumule (PL), and radicle (RA). Conidiophores (CP) are on the radicle region of the seed (**K** insert).

are generally more resistant to environmental extremes than hyphae and conidia of the fungus. The number of microsclerotia required for causing *Verticillium* wilt symptom varies among different hosts (3). In lettuce, >100 to 150 microsclerotia/g of soil is required for *Verticillium* wilt symptom development but, in cauliflower, <50 microsclerotia/g of soil is sufficient to cause near-total loss (41).

Currently, effective seed treatments are unavailable to completely eradicate *V. dahliae* from the infected seed. Efforts are underway to develop physical, chemical, and biological seed treatments to reduce or eliminate seedborne *V. dahliae* on spinach (9). In this study, we assessed the impact of two currently used seed treatments with different modes of action to eliminate *V. dahliae* in spinach seed. The results of the qPCR clearly indicated a reduction in detectable levels of the pathogen DNA in the treated relative to untreated seed. Alternatively, this suggested that the fungal DNA was degraded following seed treatment. Plating whole seed in plates with the NP-10 medium also confirmed the absence of *V. dahliae* growth from treated seed. In contrast, when broken seed pieces from treated seed were plated on NP-10 medium, the pathogen was readily recovered, suggesting that the seed treatments eliminated the pathogen only from the pericarp region but not from layers below the pericarp, which remains viable. These results suggest that both of the seed treatments examined in this study are fungistatic as applied to spinach seed, and not fungicidal. Other seed treatments such as the fungicides, thermal, NaOCl, mercuric chloride, and so on reduce seedborne *V. dahliae* below the economic or phytosanitary thresholds (9,40) established by countries such as Mexico (2).

Improved methods for rapid diagnosis and quantification of *V. dahliae* in spinach seed may help to slow or reduce the spread of *V. dahliae* via seed. Testing spinach seed for *V. dahliae* infection has primarily relied upon the use of semiselective NP-10 medium. A major drawback of the NP-10 plating is the difficulty in adapting the technique for high-throughput usage to test a large number of seed lots produced by commercial growers in a short period of time. In recent years, qPCR assays are gaining popularity for pathogen quantification in seed, environmental samples, and symptomatic plants from fields because it is rapid, ultrasensitive, and reliable (12,17). Potentially, the qPCR assay previously developed to quantify *V. dahliae* in spinach seed (8) could be applied to assess the efficacy of seed treatments that aim to eliminate *V. dahliae*.

Seedborne transmission of *V. dahliae* in spinach is expected to play an important role in the epidemiology of *Verticillium* wilt, similar to other crops (1,13,14,35). In spinach, *V. dahliae* was recovered in >90% of the seed collected from infected plants. The results of this study clearly indicated that *V. dahliae* in spinach seed is systemic and not restricted to the external surface of pericarp. Furthermore, internal colonization of *V. dahliae* led to its transmission to developing seedlings. Thus, reducing the *V. dahliae* inoculum from infected spinach seed is a worthy goal but seed treatments that completely eliminate the pathogen from contaminated seed are currently not available. The development of seed treatments to eliminate *V. dahliae* is difficult; however, their development may be achieved with new fungicides or fungicide combinations of diverse chemistries.

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