Frequency of *Verticillium* Species in Commercial Spinach Fields and Transmission of *V. dahliae* from Spinach to Subsequent Lettuce Crops

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ABSTRACT

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Verticillium wilt caused by *V. dahliae* is a devastating disease of lettuce in California (CA). The disease is currently restricted to a small geographic area in central coastal CA, even though cropping patterns in other coastal lettuce production regions in the state are similar. Infested spinach seed has been implicated in the introduction of *V. dahliae* into lettuce fields but direct evidence linking this inoculum to wilt epidemics in lettuce is lacking. In this study, 100 commercial spinach fields in four coastal CA counties were surveyed to evaluate the frequency of *Verticillium* species recovered from spinach seedlings and the area under spinach production in each county was assessed. Regardless of the county,

V. isaacii was the most frequently isolated species from spinach followed by *V. dahliae* and, less frequently, *V. klebahnii*. The frequency of recovery of *Verticillium* species was unrelated to the occurrence of *Verticillium* wilt on lettuce in the four counties but was related to the area under spinach production in individual counties. The transmission of *V. dahliae* from infested spinach seeds to lettuce was investigated in microplots. *Verticillium* wilt developed on lettuce following two or three plantings of *Verticillium*-infested spinach, in independent experiments. The pathogen recovered from the infected lettuce from microplots was confirmed as *V. dahliae* by polymerase chain reaction assays. In a greenhouse study, transmission of a green fluorescence protein-tagged mutant strain of *V. dahliae* from spinach to lettuce roots was demonstrated, after two cycles of incorporation of infected spinach residue into the soil. This study presents conclusive evidence that *V. dahliae* introduced via spinach seed can cause *Verticillium* wilt in lettuce.

Many plant pathogens have inadvertently been disseminated via infected plant material, including seed, on a global scale. In recent years, this dissemination has occurred at unprecedented rates (8,34). Often their spread may be overlooked until the pathogens become widely distributed and problematic (14,33). Identifying the actual vectors of pathogen dissemination therefore plays a key role in understanding the spread of diseases across geographic borders (25). One of the best documented plant pathogen invasions is the introduction of the oomycete *Phytophthora infestans*, via infected potato (*Solanum tuberosum*) tubers (into Europe in the mid-1840s), leading to the Irish potato famine (30). Other examples of emerging infectious diseases transmitted through infested plant material include rice blast (1) and karnal bunt of wheat (7). While some invasions can be eradicated if detected early, the prevention of biological invasions is the most cost-effective strategy of invasive species control (25).

Verticillium dahliae is a soilborne pathogen that causes economically significant wilt diseases of more than 400 plant species worldwide including lettuce (*Lactuca sativa*) (6,27,35). The disease has also been reported on seed crops of spinach (*Spinacia oleracea*) (10) but its economic significance in seed production is unknown. The pathogen is endemic in the spinach seed production

areas of northern Europe and Washington State in the United States and correspondingly, a high percentage of spinach seed lots produced in these areas can be infested with *V. dahliae* (10). Even though California (CA) produces more than 78% of the total spinach crop in the United States, and almost half of this crop is produced in Monterey County alone (2,3), no spinach seed is produced in CA because of the inadequate day length needed to induce flowering (=bolting). Seed produced in northern Europe, Chile, New Zealand, or Washington is the sole source of seed for crop production in CA. Because leafy spinach crops are harvested before the onset of bolting and, therefore the onset of *Verticillium* wilt symptoms, the disease is not an economic threat to spinach production in CA. However, the pathogen introduced by infested spinach seed has the potential to become a major problem on crops susceptible to *Verticillium* wilt that follow spinach.

In coastal CA, spinach and lettuce are often rotated with each other, and agricultural practices and cropping patterns are similar in both the northern and southern parts of this coastal region. *Verticillium* wilt has affected several fruit and vegetable crops for decades (16,38) in this area, but lettuce was historically considered immune to this pathogen. This changed, however, when *Verticillium* wilt in lettuce was first observed in a small number of fields in Watsonville (Santa Cruz County) during 1995 (35), and since then this pathogen has become a serious problem in lettuce in both the Salinas (Monterey County) and Pajaro (Santa Cruz County) Valleys. The disease has so far not been observed in other major lettuce producing valleys of CA, including the Santa Maria (Santa Barbara County) and San Juan (San Benito County) Valleys. The reasons the disease is currently confined to the Pajaro and Salinas Valleys of CA are unknown.

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Verticillium dahliae can be disseminated easily. The pathogen can be introduced into new fields or areas via infested seeds, planting material, and soil (11,27). Previously, it was shown that 92% of the commercial seed lots assayed from Denmark, New Zealand, The Netherlands, and the United States were infested with *Verticillium* species to varying degrees, while transmission of *V. dahliae* across spinach generations ranged from 0 to 100% (9). In infested seed, *V. dahliae* colonizes the pericarp, seed coat, cotyledons, and radicle, and can form approximately 250 microsclerotia per seed (24). Seedborne *V. dahliae* is transmitted systemically into the tissue of spinach seedlings and subsequently to the flowers and seeds in spinach plants grown for seed (24). In spinach production fields, the crop is harvested prior to symptom development, though the pathogen can become a part of the resident soil microbial community when the crop residue is incorporated into the soil. Fungicide treatments of infested spinach seeds tested to date reduce but do not eliminate viable inoculum of *V. dahliae* from seeds (24).

Population genetic analyses using microsatellite markers revealed that spinach populations of *V. dahliae* from Denmark, The Netherlands, and the United States (Washington) were similar to populations collected from infested lettuce in coastal CA (5). This suggested an association between *V. dahliae* in spinach seed and lettuce. Subsequent work documented significant gene flow within the spinach populations and among populations from infested spinach seed and lettuce (6) and concluded that anthropogenic movement of infested spinach seed had contributed to the epidemics of Verticillium wilt in lettuce in the Salinas and Pajaro valleys (6).

Immigration of novel genotypes or races is a particularly serious concern as it interferes with the ability of breeders to select for durable resistance (26). Previously, more than 90% of the *V. dahliae* isolates collected from spinach seed were identified as race 2 based on polymerase chain reaction (PCR) assays (31). Although *V. dahliae* populations sampled from lettuce fields in the Salinas and Pajaro valleys between 1995 and 2013 were highly clonal and dominated by race 1 (15), the unrestricted influx of novel *V. dahliae* genotypes or races via infested spinach seed (6) may lead to changes in the population and race structure of *V. dahliae* in these regions. Thus, the successful efforts over the past 15 years in developing *V. dahliae* race 1 resistance in lettuce cultivars (17) may be compromised. Additionally, *V. dahliae* isolates from spinach seed have been shown to cause severe Verticillium wilt in several crops, including cotton, lettuce, and tomato under greenhouse conditions (18,23). Likewise, 53 arbitrarily selected *V. dahliae* isolates from spinach seeds caused Verticillium wilt in the race 1-resistant lettuce cultivar La Brillante (which contains the *Vr1* gene that confers resistance against race 1) (31). Despite these greenhouse and laboratory experiments, direct evidence linking *V. dahliae* introduced into a field from spinach seeds and Verticillium wilt in subsequent lettuce crops is lacking. Furthermore, species of *Verticillium* other than *V. dahliae* have also been isolated from spinach seed (18,37). The frequency of these species introductions into spinach production fields and their eventual impact on other crops are currently unknown.

The current study investigated the potential role of *V. dahliae* inoculum introduced via infested spinach seed on Verticillium wilt in subsequent lettuce crops and assessed the reasons why the disease is currently restricted to the Salinas and Pajaro valleys in CA. Specifically, the objectives of this study were (i) to investigate why Verticillium wilt of lettuce is confined to two valleys in CA by determining the diversity and frequency of isolation of *Verticillium* in commercial spinach fields and the area under spinach production; and (ii) to determine the ability of *V. dahliae* inoculum from infested spinach seeds to spread to the roots of the subsequent lettuce crop using an alternating spinach-lettuce cropping pattern, and by using spinach seedborne green fluorescence

protein (GFP)-tagged *V. dahliae* isolate to track its invasion of soil and lettuce roots.

MATERIALS AND METHODS

Spinach field survey and isolation of *Verticillium* species.

The incidence and identity of *Verticillium* species in major commercial spinach fields from Monterey, San Benito, Santa Barbara, and Santa Cruz counties in CA were investigated. There were 69 and 31 fields surveyed during 2011 and 2012, respectively (Fig. 1). Twenty spinach plants were arbitrarily collected from each commercial spinach field when the crops were at market maturity. The plants were washed free of soil and debris, surface-sterilized in 1% NaOCl (Clorox, The Clorox Company, Oakland, CA), and rinsed for 20 to 30 s with sterile distilled water (SDW). After surface disinfestation, the plant samples were left in a laminar flow hood on paper towels for 1 to 2 h to dry. The roots and petioles were then dissected and plated on sodium polypectate agar (NP-10 agar) (22). *Verticillium* colonies were putatively identified to species after 9 to 14 days on NP-10 agar based on the colony morphology, conidiophore branching, microsclerotia production, and yellow pigmentation (19). Since *V. isaacii* and *V. klebahnii* are morphologically cryptic (19), no attempt was

Fig. 1. Map showing the four counties (Monterey, San Benito, Santa Barbara, and Santa Cruz) of California where 69 and 31 major commercial spinach fields were surveyed during 2011 and 2012, respectively, for determining the incidence and species identity of *Verticillium* species. Whole spinach plants were sampled randomly from each field, and fungal isolations from the roots and petioles using semiselective media were performed to determine the incidence of plants infected.

made to distinguish the two through microscopy. Cultures were then transferred to potato dextrose agar (PDA) and single-spored. Colonies derived from single spores were transferred to potato dextrose broth (PDB) and grown for 7 to 10 days. The mycelium was harvested, washed with SDW, lyophilized, and ground to a fine powder with a high-speed mixer mill (Model MM301, Retsch Inc., Newtown, PA). Genomic DNA of each isolate was extracted using a FastDNA Kit (MP Biomedicals, LLC, Solon, OH). A NanoDrop (Model ND-1000, Thermo Scientific Inc., Waltham, MA) instrument was used to quantify the DNA in each sample and 1 µl of DNA (10 ng/µl) was used for PCR assays.

Verticillium species identification using PCR assays. Three hundred fifty-eight *Verticillium* isolates from spinach field surveys that were putatively identified to species using morphological and cultural characteristics were characterized to phylogenetic species using PCR assay as previously described (20). PCR assays were performed in 25-µl reactions using GoTaq Green Mastermix (Promega, Madison, WI). All of the PCR assays used in this study were carried out in a PTC-100 Peltier Thermal cycler (MJ Research, Inc., Waterman, MA). The following thermal profile was used for PCR: 2 min of initial denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s min at 68°C, and 1 min at 72°C; and 10 min final extension at 72°C. Six microliters of SyberGold (Invitrogen, Carlsbad, CA) was added to PCR amplicons, which were then separated in 2% (wt/vol) agarose gels in 0.5× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Gels were each run for 120 min at 75 V and visualized with a transilluminator (UVP, Upland, CA). A 100-bp DNA ladder (Invitrogen Corporation, Carlsbad, CA) was used as a size marker in each gel.

Microplot soil preparation. Microplots (each 1.2 × 1.2 m²) established at the U.S. Department of Agriculture Research Station in Salinas, CA, were used for this study. To prevent potential interference from background of *Verticillium* spp. that may have been present, soil in each microplot was excavated to a depth of 90 cm, and the excavated microplots were refilled with soil obtained from a local nursery (McShane Nursery, Salinas, CA). The soil used in the experiment was an Antioch sandy loam (45.8% sand, 43.4% silt, 8.8% clay, and 1.8% organic matter) with a pH of 7.1. Soil from each microplot was collected and assayed for *V. dahliae* by impaction plating onto NP-10 agar using the Anderson sampler technique (9,22). The assay revealed that *V. dahliae* colony forming units, such as microsclerotia, in microplot soils were undetectable.

Spinach seed infestation treatments. A sample of spinach seed obtained from a commercial seed company was surface-disinfested in 1% NaOCl for 1 min, rinsed in SDW, air dried, and plated on NP-10 agar to assess the percentage of seed infested with *V. dahliae*. In each of two separate experiments, 600 seeds were plated and the number of seeds harboring *V. dahliae* was recorded, for a total of 1,200 seeds. Based on these data, the infestation level of the seed lot was determined to be approximately 67% infested seeds. Seeds from this lot were then mixed with treated seeds of the same cultivar from a different lot (treated with proprietary material to eliminate seedborne *Verticillium* spp. by the seed company that provided the seed) to produce five infestation levels (=treatments). Five treatments established in the microplots consisted of planting spinach seed with infestation levels of approximately 0% (all seed treated), 10%, 15%, 33% (these latter three treatments contained a mixture of treated and nontreated seed), and 67% (all nontreated seed). Four replications of each treatment were planted (≈7 million per hectare, equivalent to ≈1,600 seeds per plot) into microplots that were arranged in a complete random block design. Four microplots were not sown with any spinach seed and were used as negative controls. The microplots for each treatment were fixed for the duration of the study. Cultural practices were followed in accordance with the standard procedures for spinach production. The microplots were

regularly irrigated with drip tape throughout the experiment. A urea-nitrate fertilizer (Wilbur-Ellis County, Salinas, CA) was applied to microplots at the rate of 124 kg/ha at 3-week intervals. To simulate harvesting practices typical of commercial production, twice during each crop, spinach leaves were harvested with hand shears that were cleaned between microplots. Three successive crops of spinach were grown in each microplot during the first year (2011) followed by one lettuce crop. The next year (2012), two successive spinach crops were followed by one crop of lettuce.

Soil and spinach plant sampling. To obtain the baseline inoculum density of *V. dahliae* for each microplot, soil samples were collected from each microplot before planting any crops. To estimate inoculum added to the soil from infested spinach seed, soil was assessed 1 to 2 weeks after the first spinach leaf harvest and 1 to 2 weeks after the incorporation of spinach crop residue. Five soil samples were collected from the top 10 cm of each microplot using a trowel, which was cleaned with 70% ethanol in between collecting soil from different microplots. Soil was pooled from each microplot, air dried, pulverized, and plated onto NP-10 agar (22) using an Anderson sampler (9,22) and incubated in the dark at ambient temperature. After 2 weeks, plates were washed and examined under a dissecting microscope for the presence of microsclerotial colonies characteristic of *V. dahliae* (19).

To assess the incidence of plants colonized by *V. dahliae* within spinach seedlings, plants were collected from each microplot at least twice during each spinach crop in the first year (2011), and sampled once during each spinach crop in the second year (2012). During each sampling, 20 spinach seedlings were arbitrarily collected from each microplot, cleaned with SDW, soaked in 1% NaOCl for 1 min, rinsed in SDW, and air-dried in a laminar flow hood for 1 h. The roots were dissected into 0.5 to 1 cm pieces and plated on NP-10 agar, incubated for 2 to 3 weeks in the dark at ambient temperature, and examined under a dissection microscope. To mimic residue incorporation in commercial fields, after each spinach crop, residual plant tissue was incorporated into the microplot soil with a gardening hoe, which was cleaned with water in between treatments.

Lettuce cultivation in microplots. Lettuce cultivar Salinas was planted in each microplot after the completion of three crops of spinach in the first year and two crops of spinach in the second year. The lettuce seed lot was obtained from the laboratory of R. Hayes, USDA-ARS, and screened for the presence of *V. dahliae* prior to planting by plating 50 replicates of 20 seeds per plate on NP-10 agar. The lettuce seed lot was determined to be uninfested. Two raised beds were prepared within each microplot and each was direct seeded with two rows of lettuce seed 25 cm apart. In each row, lettuce was planted at 20 cm plant spacing for a total of 24 plants per microplot. Plants were irrigated with drip tape running parallel to the rows and the lettuce was grown according to standard commercial practice. At market maturity, plants were uprooted and the taproots were cut longitudinally up to the crown and the presence or absence of vascular discoloration typical of *Verticillium* wilt in lettuce was determined. Plants with symptoms typical of *Verticillium* wilt were collected and *Verticillium* spp. isolates were obtained in the laboratory as described above for spinach.

Molecular identification of fungi isolated from lettuce. *Verticillium* isolates from lettuce grown in microplots were transferred from NP-10 agar with a pipette tip, mixed with 80 µl of SDW, and spread onto PDA with a cell spreader. Single-spore colonies were obtained, transferred to PDB, and grown for 7 to 10 days. DNA extraction and PCR assays were performed as described above. The presence of *V. dahliae* on lettuce within microplots was confirmed using a *V. dahliae*-specific PCR assay (20).

Transmission of a GFP-tagged strain of *V. dahliae* from spinach to lettuce. Seeds of spinach cultivar Hector, infested with the GFP-expressing spinach strain VdSo925-316 (24), were

planted in 10 cups (10 seeds/0.94 liter Styrofoam cup) in a pasteurized 3:1 sand/all-purpose potting soil mix (Sun Land Products, Inc., Watsonville, CA). The experiments were conducted in a greenhouse located at the USDA-ARS Station, Salinas, CA under natural light conditions between 30 July and 14 December 2013. For temperature control, fan-forced radiant heat was on below 18°C and an automatic exhaust fan with evaporative cooling was on above 24°C. Seventy-five percent of the spinach seeds were infested with *V. dahliae* (234/312) as determined by NP-10 agar plate analyses described previously (12). Approximately 6 weeks after planting, whole spinach plants were uprooted, chopped into 1 cm pieces, and allowed to dry on the surface of the sand soil mixture for 5 days. After the drying period, more of the SoVd316-GFP-infested spinach seeds were planted into the same 10 cups and soil mix (10 seeds per cup) and grown for approximately 6 weeks. The plants were uprooted, chopped into pieces as before, and allowed to dry on the surface of the sand/potting soil mix for approximately 1 week. Following two sets of spinach, lettuce cultivar Salinas was planted, also at 10 seeds per cup, in five of the same cups containing the dried spinach debris. Seeds of Hector not infested with SoVd316-GFP were planted into the other five cups at a rate of 10 seeds per cup for the final spinach planting. After 5 weeks, both lettuce and spinach plants were uprooted, the roots were rinsed vigorously with distilled water, and examined for the presence of the GFP-expressing *V. dahliae* strain.

Whole root mounts were examined with an epifluorescence Olympus BX60 compound microscope or a Nikon compound microscope with filter blocks for GFP (450-490 nm excitation, 590 nm longpass emission), coupled to a Leica confocal laser scanning microscope (CLSM; Leica Microsystems Inc., Buffalo Grove, IL). Confocal images were captured as outlined by Maruthachalam et al. (24). Out of five lettuce root systems from

five plants examined, a portion of the root system of a single plant was determined as positive for GFP expression by epifluorescence microscopy. Similarly, of the roots of five spinach plants examined, one was positive for GFP expression. The positive samples were examined further by CLSM analyses (24,36).

Spinach and lettuce production in coastal CA. The areas under spinach (1993 to 2012) and lettuce (1994 to 2012) production in Santa Barbara, San Benito, Santa Cruz, and Monterey counties were compared and plotted against the year of production. Data were obtained from the respective Agricultural Commissioners Offices: Santa Barbara (<http://www.countyofsb.org/agcomm/agcomm.aspx?id=11562>), San Benito <http://www.cosb.usd.com/county-departments/agriculture/crop-report>), Santa Cruz (<http://www.scparks.com/crop02.html>), and Monterey (<http://ag.co.monterey.ca.us/resources/category/crop-reports>).

RESULTS

Distribution of *Verticillium* species in Monterey, Santa Barbara, San Benito, and Santa Cruz counties of CA. Commercial spinach fields were surveyed in four counties in the current study (Fig. 1). Of the 1,380 total spinach plants analyzed in 2011, 585 plants were infected with a *Verticillium* species; of the 620 plants analyzed in 2012, *Verticillium* was recovered from 300 plants. Based on an initial assessment of colony morphology and pigment production, 496 *V. isaacii*/*V. klebahnii* and 89 putative *V. dahliae* isolates were collected in 2011, while 218 *V. isaacii*/*V. klebahnii* and 82 *V. dahliae* isolates were collected in 2012. The recovery of *V. dahliae* and *V. isaacii*/*V. klebahnii* from spinach roots and petioles were similar in four counties during 2011 and 2012, except in Monterey County during 2012, where the recovery of *V. isaacii*/*V. klebahnii* from spinach roots were almost threefold higher than those from petioles (Fig. 2).

Fig. 2. Number of *Verticillium dahliae* and *V. isaacii* isolates recovered from spinach petiole and root samples from commercial spinach fields in four counties of California, after plating plant tissue on NP-10 agar. Each data point represents the mean and standard error of the incidence of plants that tested positive for that species in the respective county, out of 20 plants sampled per field per year. Number of fields sampled from each county in A, 2011 and B, 2012, respectively, were as follows: Monterey Co., 15 and 7; Santa Barbara Co., 25 and 11; San Benito Co., 15 and 9; Santa Cruz Co., 6 and 4.

V. isaacii/*V. klebahnii* were the predominant *Verticillium* species isolated per field in all counties, with the exception of Santa Cruz County in 2012 (Fig. 3). In 2011, the highest number of *V. isaacii*/*V. klebahnii* isolates were recovered from Monterey County, followed by Santa Barbara County. In 2012, the highest number of *V. isaacii*/*V. klebahnii* isolates were recovered in Monterey County, followed by San Benito County. *V. dahliae* was recovered with the highest frequency from Santa Barbara County followed by Monterey County during 2011, and Santa Cruz County followed by Monterey County during 2012 (Fig. 3).

Verticillium species identification using PCR assays. Three hundred fifty-eight *Verticillium* isolates were arbitrarily selected for molecular identification to species using a *Verticillium* species-specific PCR assay (20). This total included 160 and 198 isolates from 2011 and 2012, respectively, to obtain a representative sample of isolates from both years. Using PCR assays, three *Verticillium* species (*V. dahliae*, *V. isaacii*, and *V. klebahnii*) were identified from spinach plants (Table 1). *Verticillium isaacii*

was the most frequently isolated, followed by *V. dahliae*, and then *V. klebahnii*, in each of the four counties (Table 1). With the exception of San Benito County, where *V. dahliae* was the most predominant species in the 2011 sample, PCR assays confirmed that *V. isaacii* was the predominant species, followed by *V. dahliae* in all four counties (Fig. 2, Table 1). Notably, 10 isolates which were putatively identified as *V. isaacii* based on morphological traits were identified as *V. dahliae* based on PCR assays (Fig. 2, Table 1), indicating that estimates of *V. dahliae* in spinach production fields presented here should be considered conservative and highlighting the importance of molecular identification of *Verticillium* species (21). In addition, *Verticillium* isolates from four 2012 samples obtained from Monterey County and two samples from San Benito County were identified as *V. klebahnii*, based on PCR assays (Table 1).

Isolation of *V. dahliae* from microplot soil. Soil collected before the first spinach crop from all microplots was free of *V. dahliae* (Fig. 4). Microsclerotial colonies of *V. dahliae* from

Fig. 3. *Verticillium* species incidence per field in four counties of California during **A**, 2011 and **B**, 2012. *Verticillium* isolates were recovered from spinach petiole and root samples from commercial spinach fields in four counties of California, after plating plant tissue on NP-10 agar. Each data point represents the mean and standard error of the incidence of plants that tested positive for that species, per field, in the respective county.

TABLE 1. Species identity and number of *Verticillium* isolates collected from spinach seedlings in commercial spinach crops grown in different counties of California, as determined with *Verticillium* species-specific polymerase chain reaction (PCR) assays (19)

Location	Sample size ^a		<i>V. isaacii</i>		<i>V. dahliae</i>		<i>V. klebahnii</i>	
	2011	2012	2011	2012	2011	2012	2011	2012
Monterey	52	62	50	46 ^b	2	4	0	4
Santa Barbara	80	4	70	4	10	0	0	0
San Benito	13	102	1	85	12	15	0	2
Santa Cruz	16	30	14	17	2	13	0	0
Total	160	198	135	152	26	32	0	6

^a A total of 160 and 198 isolates from 2011 and 2012 that were putatively identified to *Verticillium* species based on morphological and cultural characteristics were arbitrarily selected for molecular identification using a *Verticillium* species-specific PCR assay (20).
^b Eight isolates from Monterey could not be amplified using *Verticillium* species-specific primers (20).

soil plated on NP-10 agar were first observed in soil samples collected approximately a month after sowing spinach seeds (time point designated Y1.S1.SMP1 in Fig. 4) in microplots that had levels of seed infestation corresponding to approximately 67, 33, or 15% infestation treatments (treatments 1, 2, and 3). At this time point, the amount of *V. dahliae* inoculum recovered was the highest in microplots sown with $\approx 67\%$ infested spinach seed, followed by ≈ 33 and $\approx 15\%$ (Fig. 4).

Isolation of *V. dahliae* from spinach. Fungal colonies and microsclerotia characteristic of *V. dahliae* were recovered from spinach roots and petioles collected during the first spinach crop after plating on NP-10 agar. *Verticillium dahliae* was recovered from spinach grown in microplots with the seed infestation treatments of approximately 0, 10, 15, 33, and 67% (Fig. 5). *Verticillium dahliae* recovery from spinach in samples drawn from the $\approx 67\%$ infestation treatment in the second sample of the third season of year one (Y1.S3.SMP2), and both of the seasons during the second year of the experiment (Y2.S1.SMP1 and Y2.S2.SMP1) was significantly higher than all other treatments (Fig. 5).

Isolation of *V. dahliae* from lettuce in microplots. Verticillium wilt symptoms were visible in a few plants during the first lettuce crop that followed three spinach crops. Planted tissue from symptomatic plants yielded fungal colonies with cultural and morphological traits characteristic of *V. dahliae*, i.e., zonate growth, microsclerotia, and verticillate conidiophores (19). The *Verticillium* species-specific PCR assay on these isolates confirmed that symptoms of four lettuce plants grown the first year and three lettuce plants grown the second year were indeed caused by *V. dahliae* (gels not shown). Following the first lettuce planting, the incidence of vascular discoloration in lettuce taproots in treatments with approximately 10, 15, 33, and 67%

infested spinach seed was 4.44, 5.26, 9.02, and 9.29, respectively. Similarly, in the second lettuce crop, the incidence of vascular discoloration in lettuce taproots in treatments with approximately 10, 15, 33, and 67% infested spinach seed was 21.43, 26.11, 35.83, and 31.82, respectively.

Transmission of a GFP-tagged strain of *V. dahliae* from spinach to lettuce. The GFP-tagged spinach strain VdSo925-316 colonized the lettuce root surface 5 weeks after planting lettuce in soil following two prior plantings of the infested spinach seeds and incorporation of the residue (Fig. 6A). In addition to the result in Figure 6A, another portion of the same lettuce root system was clearly positive for the GFP-expressing fungus (data not shown).

The spinach-to-spinach transmission of strain VdSo925-316 was carried out similarly, and the strain also colonized the spinach root surface (Fig. 6C). The GFP-tagged spinach strain penetrated the root surface as observed by CLSM (arrow in Fig. 6C).

Spinach and lettuce production in coastal CA. Monterey County planted the largest area of spinach and lettuce during 1993 to 2012, and was at least 10- to 12-fold higher than other coastal counties (Fig. 7). Production of spinach in Monterey County increased significantly over the past two decades. From 1994 to 1999, the year when Verticillium wilt of lettuce was first reported in Monterey County, spinach production more than doubled. The spinach and lettuce production areas in the other three counties (Santa Barbara, San Benito, and Santa Cruz) were relatively constant throughout years 1993 to 2012 (Fig. 7).

DISCUSSION

This work provides an assessment of *Verticillium* species that occur in spinach seedlings in commercial production fields in CA.

Fig. 4. Recovery of *Verticillium dahliae* from microplots at different times of soil sampling. Each data point represents the mean and standard error of the number of microsclerotia per gram soil in microplots of the same treatment. Tr1-Tr6 = treatments 1 to 6 (varying levels of spinach seed infestation ranging from ≈ 67 to 0%, and no spinach planted), Y1 = first year (2011), S1 = first spinach crop, SMP1 = first sampling, SMP2 = second sampling, S2 = second spinach crop, S3 = third crop, L = lettuce crop, Y2 = second year (2012). Vertical bars represent standard errors.

Verticillium dahliae, along with *V. isaacii* and *V. klebahnii* (19), were recovered from spinach sampled in production fields from four coastal CA counties with significant spinach production. The relative frequency of recovery of individual *Verticillium* species was unrelated to the occurrence or incidence of Verticillium wilt on lettuce in the four counties. However, in the case of Monterey County, the area of spinach production was highly correlated with the incidence of Verticillium wilt on lettuce (4). The relative importance of the three *Verticillium* species in the disease complex on lettuce and other coastal CA crops is currently being assessed. These data also clearly establish the missing epidemiological link between *V. dahliae* introduced into the soil by infested spinach seed and the occurrence of Verticillium wilt on lettuce. Based on field and greenhouse studies, a direct connection between *V. dahliae* in infested spinach seed and Verticillium wilt on subsequent lettuce crops was documented.

Based on assessments of cultural characteristics, *V. dahliae* and isolates consistent with the morphological descriptions of *V. isaacii* and *V. klebahnii* were recovered from all spinach production counties sampled during 2011 and 2012. With the molecular characterizations of a subset of the isolates collected, *V. isaacii* was recovered in greater frequencies from spinach fields than *V. dahliae* and *V. klebahnii*. The recovery of *V. isaacii* from petioles of young spinach plants in this study corroborates previous work that shows that at least one species consistent with the morphological descriptions of *V. isaacii* and *V. klebahnii* (collectively referred to as the outdated morphospecies '*V. tricornis*') occur inside spinach seeds (18,37). We hypothesize that it is virtually impossible to recover *Verticillium* species from petioles at the seedling stage of spinach growth if the pathogen did not originate from within the spinach seed, especially since the fungus is known to colonize seedlings from infected seeds (24). Therefore, based on the average number of *V. isaacii* isolates recovered from each commercial field, it appears that spinach

crops introduce *V. isaacii* into commercial fields at comparable frequency, regardless of where the crop is grown.

Dissemination of *Verticillium* species, especially *V. dahliae*, via contaminated spinach seed represents a potential threat to agriculture in CA. *Verticillium dahliae* is known to be associated with Verticillium wilt in many of the agronomically important crops grown in CA (4,6,35). Spinach and lettuce production acreages in Monterey County are 10- to 12-fold higher than in Santa Barbara, San Benito, and Santa Cruz counties, and likely explains why soil inoculum densities of *V. dahliae* may not have increased to the threshold level required for Verticillium wilt development in lettuce crops in Santa Barbara and San Benito counties. While lettuce seeds can also harbor *V. dahliae*, Atallah et al. (6) provided genetic evidence that suggested that *V. dahliae*-infested spinach seeds are a more likely source of *V. dahliae* inoculum in CA lettuce production than lettuce seeds themselves. Future experimental work following the procedures outlined in the current study should be conducted to elucidate the potential contributions of lettuce seedborne *V. dahliae* in wilt epidemics. Verticillium wilt on lettuce has not been observed in Santa Barbara and San Benito counties. The disease is confined to the Pajaro and Salinas valleys of coastal CA, a region that also includes the other two counties (Monterey and Santa Cruz) compared in this study. Since *V. isaacii* was also commonly recovered from Santa Barbara County, where Verticillium wilt on lettuce has not been reported, we are unable to make any association between *V. isaacii* and Verticillium wilt in lettuce. Interestingly, cauliflower (*Brassica oleracea* var. *botrytis*) fields infested with both *V. longisporum* and *V. isaacii* showed fewer Verticillium wilt disease symptoms relative to cauliflower fields with *V. longisporum* alone (13), suggesting that specific *V. isaacii* isolates may suppress disease caused by *V. longisporum* and other *Verticillium* species in certain cases (13). Preliminary co-inoculation studies have also demonstrated that colonization of lettuce by certain

Fig. 5. Recovery of *Verticillium dahliae* from spinach plants at different times of spinach sampling. Tr1-Tr6 = treatments 1 to 6 (varying levels of spinach seed infestation), Y1 = first year (2011), S1 = first spinach crop, SMP1 = first sampling, SMP2 = second sampling, S2 = second spinach crop, SMP3 = third sampling, S3 = third spinach crop, Y2 = second year (2012). Vertical bars represent standard errors.

strains of *V. isaacii* may reduce wilt caused by *V. dahliae* (29). Although *V. isaacii* was recovered frequently along with *V. dahliae* in the same commercial spinach fields, further studies are necessary to elucidate the role of this species, if any, in the etiology of Verticillium wilt in lettuce and other crops grown in CA.

The occurrence of *V. isaacii* and *V. klebahnii* in spinach fields suggests that these species may be present in major spinach seed-supplying regions, such as northern European countries and WA, and that these *Verticillium* species might also be associated with other crops grown in those regions. This hypothesis is supported by recent reports of *V. isaacii* associated with Verticillium wilt in a lettuce crop on a research farm in WA (28). Although Powell et al. (28) described several pathogens (Vt.Ls.2010, Vt.Ls.2011-1, and Vt.Ls.2011-2) as “*V. tricorpus*,” sequences of the internal transcribed spacer (ITS) regions of rDNA reveal these isolates to be conspecific with *V. isaacii* (19). Therefore, it is reasonable to suspect that some isolates of *V. isaacii* could cause disease on multiple crops, including lettuce, following rotation with spinach. Given their distribution and potential seedborne nature, future analyses of the pathogenicity of *V. isaacii* and *V. klebahnii* are

essential for understanding the relative importance of these two species as pathogens.

Verticillium dahliae in infested spinach seeds or infected plant tissues can serve as primary sources of inoculum to cause Verticillium wilt in subsequent crops, as demonstrated for lettuce in this study. *Verticillium dahliae* can produce more than 200 microsclerotia per infested spinach seed (24), and these microsclerotia can remain viable for more than 14 years in the soil in the absence of a host (27). Previous analyses also revealed that it is common to find commercial spinach seed lots with up to 85% infested seed (10,12). Thus, infested spinach seeds planted at a rate of 7.5 to 10 million seed per hectare can introduce considerable amounts of *Verticillium* inoculum into the field (24). Since the leaves of spinach production crops are harvested before Verticillium wilt symptoms become evident, economic losses caused by *V. dahliae* on spinach are nonexistent. After harvest, spinach plant residue is incorporated into the soil, along with any *V. dahliae* inoculum that may be present in the plant tissue. This inoculum, which can remain viable for several years, may be amplified through infections of successive lettuce crops to eventually reach the threshold levels to threaten lettuce and other crops. In contrast, a maximum

Fig. 6. Analyses of the transmission of *Verticillium dahliae* strain VdSo925-316 from spinach debris in soil to lettuce or spinach roots by confocal laser scanning microscopy. Strain VdSo925-316 expresses green fluorescent protein (GFP) and was visualized using the appropriate UV excitation and emission filters (see materials and methods). **A**, Analysis of lettuce root sample (cultivar Salinas) 5 weeks after planting in soil infested with VdSo925-316-infected spinach debris. **B**, Same lettuce root sample shown in **A**, without the filter to detect fungal GFP fluorescence. **C**, Analysis of spinach root sample (cultivar Hector) 5 weeks after planting in soil infested with VdSo925-316-infected spinach debris. **D**, Same spinach root sample shown in **C**, without the filter to detect fungal GFP fluorescence. Scale bars: **A** and **B** = 50 μ m; **C** and **D** = 100 μ m.

V. dahliae infestation rate of 5% has been detected in lettuce seed (4). However, the proportion of infested seed actually contributing inoculum to lettuce production fields is diminished by processes involved in lettuce production. Lettuce is direct-seeded at a density three to five times higher than the plants retained for crop production post-thinning. Thus, nearly 75 to 83% of the seedlings regardless of whether they came from healthy or infested lettuce seed are removed during thinning. Depending on the production system, the number of lettuce plants retained per hectare vary from 55,357 to 116,250 (32). The probability that seedlings from infested lettuce seed are successfully retained post-thinning that result in mature, diseased lettuce plants is therefore $\leq 1.25\%$. Apart from the significant differences between the seeding rates in lettuce ($\approx 166,071$ to 581,250 per hectare) and spinach (≈ 8 to 9 million per hectare), spinach seedlings are not thinned, and thus the probability of introducing *Verticillium* species into the soil is proportional to the level of *Verticillium* infestation in the planted seed.

Prior to this study, direct evidence linking *V. dahliae* introduced into soil from infested spinach seed with wilt incidence in lettuce was unavailable. This was despite the analyses that had established the genetic similarity of populations of *V. dahliae* from

spinach and lettuce (6). By planting alternating crops of spinach and lettuce in microplots in which the soil was devoid of *V. dahliae* microsclerotia or other forms of resting structures, we demonstrated a detectable increase in the *V. dahliae* inoculum in soil after infested spinach seeds were used in crop production. The recovery of *V. dahliae* inoculum from soils in microplots is clear evidence that *V. dahliae*-infested spinach seed introduced microsclerotia into the soil. In addition, the percentage of infested spinach seed planted was correlated with the quantities of microsclerotia in the soil. By the end of the second year of the experiment, the amount of *V. dahliae* inoculum recovered was significantly higher in the microplot soils planted with $\approx 67\%$ infested spinach seed relative to other treatments where a lower percentage of infested seeds were planted. Similarly, at the end of the first year and during the second year of spinach plant sampling, the amount of *V. dahliae* recovered from spinach seedlings also increased relative to the increasing percentage of infested seed planted in the microplots. This demonstrated that the soil inoculum accumulated relative to the inoculum in seed. *Verticillium* wilt was observed on the very first lettuce crop following three spinach crops and the incidence levels correlated with the percentage of infested spinach seed planted in the corresponding

Fig. 7. Area planted with spinach and lettuce in Santa Barbara, San Benito, Santa Cruz, and Monterey counties in different years (Santa Barbara, San Benito, Santa Cruz, and Monterey counties Agricultural Commissioners Offices).

microplots. It is important to note that economically relevant build-up of microsclerotia in field soil, which could cause higher incidence than what was observed in the microplot experiment, would likely take additional cropping cycles of lettuce, as predicted by recent epidemiological modeling of *Verticillium* wilt of lettuce (39). The lettuce seed planted in the microplots were not infested with *V. dahliae*; therefore, the sole source of *V. dahliae* for infection of lettuce was the contaminated spinach seed planted in the microplots along with any increases that occurred on spinach crop residue following incorporation into the soil.

The greenhouse studies with the GFP-tagged spinach strain of *V. dahliae* further confirmed the role of *V. dahliae* in infested spinach seed in colonizing subsequent lettuce crops. Previous studies (24) revealed that a GFP-tagged spinach strain of *V. dahliae* colonized the seed of spinach, including deeper seed tissues such as the plumule, hypocotyl, and radical, and that the fungus was transmitted to developing seedlings (24). Seed-to-seed transmission of *V. dahliae* in spinach was also demonstrated by du Toit et al. (10). The studies described herein following the GFP-tagged spinach strain of *V. dahliae* provide definitive evidence that *V. dahliae* naturally occurring in infested spinach seed can and does in fact colonize the root surfaces of subsequent lettuce plants that are planted in the same soil. The lettuce root system colonized by the GFP-expressing strain was observed following two cycles of planting spinach seed with the GFP expressing strain and incorporating spinach residue in soil. Based on the body of evidence, the planting of *V. dahliae*-infested seed is an epidemiologically relevant contributor to the recent, widespread distribution of *Verticillium* species in the commercial spinach fields surveyed (6). At present, the actual role and impact of *V. isaacii* and *V. klebahnii* on crops grown in CA is unknown. Thus, it is imperative that pathogenicity tests are conducted for these *Verticillium* species in the near future. These findings have implications in other crop systems where seedborne pathogens pose problems.

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