

## CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

April 1, 2011 - March 31, 2012

### VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL

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#### SUMMARY

There were four objectives during the current funding cycle and included: 1) assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach; 2) sample spinach seedlings in organic and conventional production systems in Monterey, San Benito, Santa Barbara and Santa Cruz counties and assay for *V. dahliae*; 3) continue studies on the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables grown in coastal California; and 4) determine the localization of *V. dahliae* in spinach seed. Lettuce became a host of *V. dahliae* in the mid-1990s and we have determined by genetic analyses that the pathogen that is carried on spinach seed has had a significant role in this host range expansion. We initiated a controlled field experiment to assess the role of *V. dahliae* introduced via spinach seed into soil in causing Verticillium wilt on lettuce. Over the past year, three spinach crops, each in treatments with different proportions of *V. dahliae*-infested seed, were grown in replicated microplots. Plant and soil samples were assayed for *V. dahliae*. Both plant and soil assays showed that the level of *V. dahliae* in both corresponded with the proportion of infected spinach seed planted. Concrete conclusions from this study will only be possible after additional years of experimentation. The second objective was also initiated this past year to examine why Verticillium wilt of lettuce is confined to the two valleys in CA even though cropping patterns are similar in other areas. Twenty plants from 61 commercial spinach fields in Salinas, Pajaro, Santa Maria and San Juan valleys were sampled and the roots and petioles from each were plated on the NP-10 medium. More than 500 isolates recovered were characterized as *V. dahliae* or *V. tricorpus*-like species based on colony morphology. Of these, 150 isolates were randomly selected and identified to species using the *Verticillium* species-specific primers, and were predominantly composed of *V. isaacii* and *V. dahliae*. The frequency of recovery of *Verticillium* species did not explain the current distribution of the disease in California but the disease on lettuce was correlated with the magnitude of spinach production in the four valleys. A global collection of isolates to determine *V. dahliae* migratory patterns has been assembled and the analyses of these isolates are currently in progress. To determine the location of the pathogen in spinach seed, seedlings were inoculated with a green fluorescent protein (GFP)-tagged strain of *V. dahliae* and colonization events were followed through seed production by confocal laser scanning microscopy. The xylem of the upper stem, inflorescence and various spinach seed parts, including the pericarp, seed coat, cotyledons and radicle were colonized but not the perisperm. Maximum concentration of the fungus was in the seed coat, the outermost layer that contains the vasculature. Seed treatments significantly reduced levels of the pathogen, but did not eliminate it from the seed. Infection of *V. dahliae* in spinach seed was systemic and transmissible to developing seedlings. This information will be particularly useful for administering effective seed treatments that in turn reduce the seedborne inoculum transmission to crop production fields.

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### OBJECTIVES:

- A. Assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach.
- B. Sample spinach seedlings in organic and conventional production systems in Monterey, San Benito, Santa Barbara and Santa Cruz counties and assay for *Verticillium dahliae*.
- C. Continue the research of the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables grown in coastal California.
- D. Determine the localization of *Verticillium dahliae* in spinach seed.

### PROCEDURES AND RESULTS:

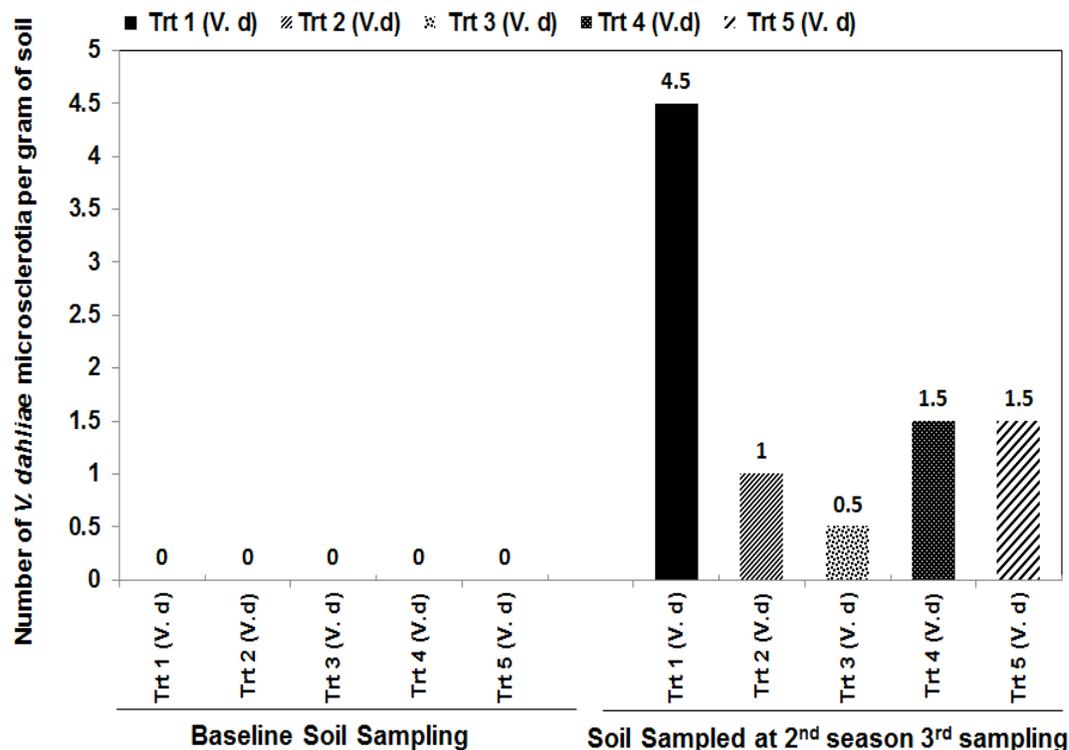
**Objective 1. Assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach.**

Many unanswered questions remain regarding the epidemiology of Verticillium wilt in lettuce. If we assume seed as the sole source of pathogen introduced into a field, how many microsclerotia are added to the soil after planting infested seed in a field, how *V. dahliae* populations change over time in response to the cropping patterns in the field following pathogen introduction, and how soon does the density of microsclerotia in soil reach a level at which Verticillium wilt develops in lettuce crops, etc. We have already found answers to these questions for *V. dahliae* introduced via infested lettuce seed using a simulation model that we developed a few years ago. Such information is unavailable for *V. dahliae* introduced via spinach seed nor can answers to the above questions be found using a simulation model, as the parameters for spinach Verticillium wilt are unavailable. Conducting these studies in growers' fields would require many years of commitment

and it is highly unlikely that growers are able to accommodate such long-term studies. We therefore decided to find answers to these questions by conducting studies in microplots where we can control all factors.

## Methods.

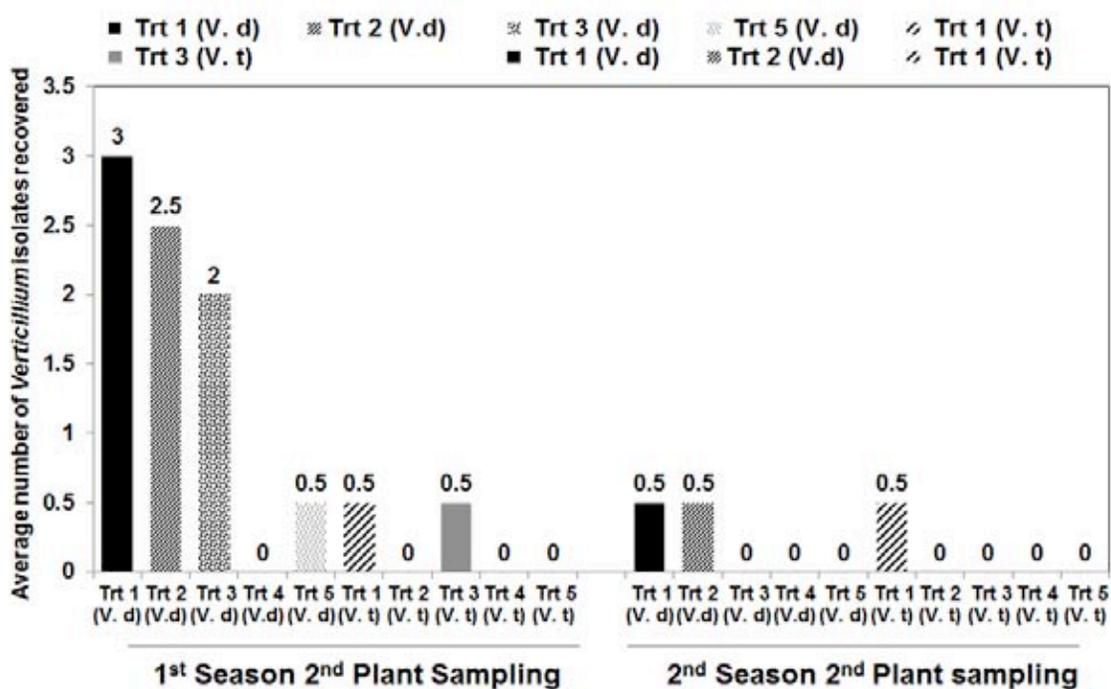
These studies were delayed by a year because of lack of highly infested spinach seed. Over the past year, we obtained large quantities of highly infested seed from two sources as well as uninfested seed, so that we could begin these studies in microplots. Six different spinach seed *V. dahliae* infestation level treatments were established (64%, 33%, 15%, 10%, 0% *Verticillium* in spinach seed and no spinach) with 4 replications each. Prior to planting spinach, soil samples were collected from each microplot and assayed for *V. dahliae* microsclerotia and determined that soil in these microplots did not contain *V. dahliae*. Thus, any *V. dahliae* microsclerotia recovered from soil in subsequent samplings is likely to have come from spinach seed planted in the microplots. Three spinach crops representing each treatment were grown in the microplots in 2011. Both soil and spinach root samples were collected twice each season and assayed for *Verticillium* spp. and were identified to species using species-specific markers.



**Fig 1.** Recovery of *Verticillium dahliae* microsclerotia from soil collected from microplots at two sampling times. Treatment 1= 67% spinach seed infected with *V. dahliae*; Treatment 2= 33%; Treatment 3= 15%, Treatment 4= 10%, and Treatment 5= 0%.

## Results.

None of the soils sampled prior to the first planting of spinach crop in 2011 contained *V. dahliae* microsclerotia (Fig. 1). However, after the very first spinach crop, soils from all infestation treatments except control in all replications yielded *V. dahliae* and plant samples yielded both *V. dahliae* and *V. isaacii*. Similar trends persisted in the two subsequent spinach crops (Fig. 2). Lettuce has been planted in all treatments in 2012 and the plan is to plant two crops of lettuce and evaluate potential disease development on these crops.



**Fig. 2.** Recovery of *Verticillium* species from spinach plants at two different times of plant sampling.

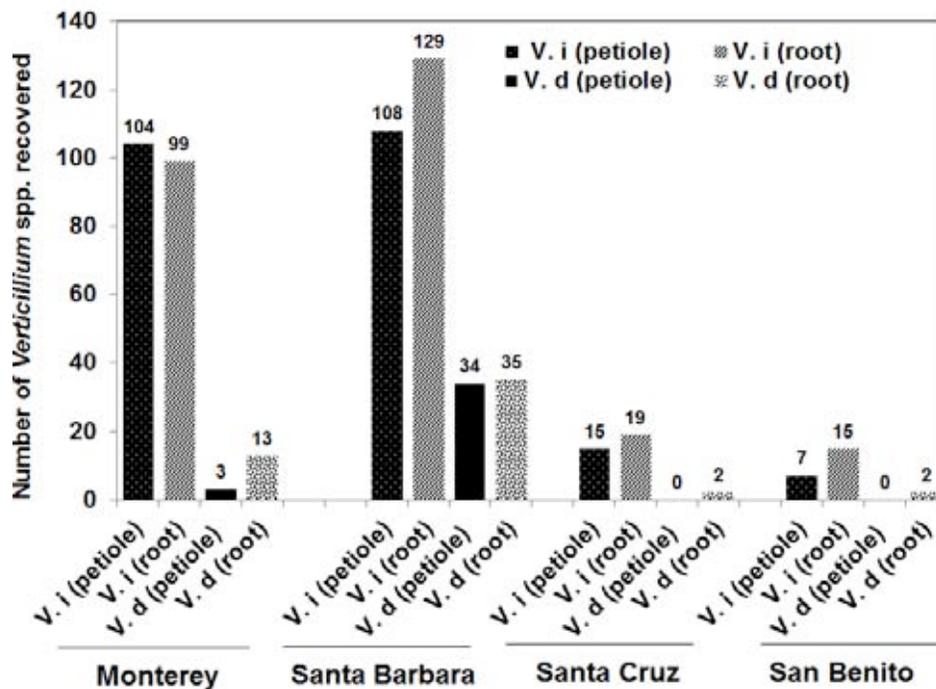
### Objective 2. Sample spinach seedlings in organic and conventional production systems in Monterey, San Benito, and Santa Barbara counties and assay for *Verticillium dahliae*.

California growers planted 209,500 acres of lettuce in 2010. A large portion of this acreage is located in the Monterey and Santa Cruz counties. In 1995, *Verticillium* wilt, caused by *V. dahliae*, appeared suddenly and unexpectedly in lettuce in Watsonville, Santa Cruz County. Since the initial appearance of the disease, the number of lettuce fields affected has increased dramatically, reaching more than 175 by 2010. More than 50% of the newly affected fields occurred in 2009 and 2010, indicating that the problem has spread more rapidly in recent years. The disease is appearing increasingly in the southern end of the Salinas Valley. Despite the nearly 18-year

history of *Verticillium* wilt on lettuce, the disease is largely confined to the Monterey and Santa Cruz counties. Since our discovery of *V. dahliae*-infected spinach seed as a pathway for the introduction of the pathogen into lettuce production fields, a frequent but very legitimate question that is asked is why *Verticillium* wilt is currently confined to lettuce fields in Monterey and Santa Cruz counties despite the similarity in cropping patterns in San Benito and Santa Barbara counties. In an attempt to answer this question, we initiated this objective this year.

### Methods.

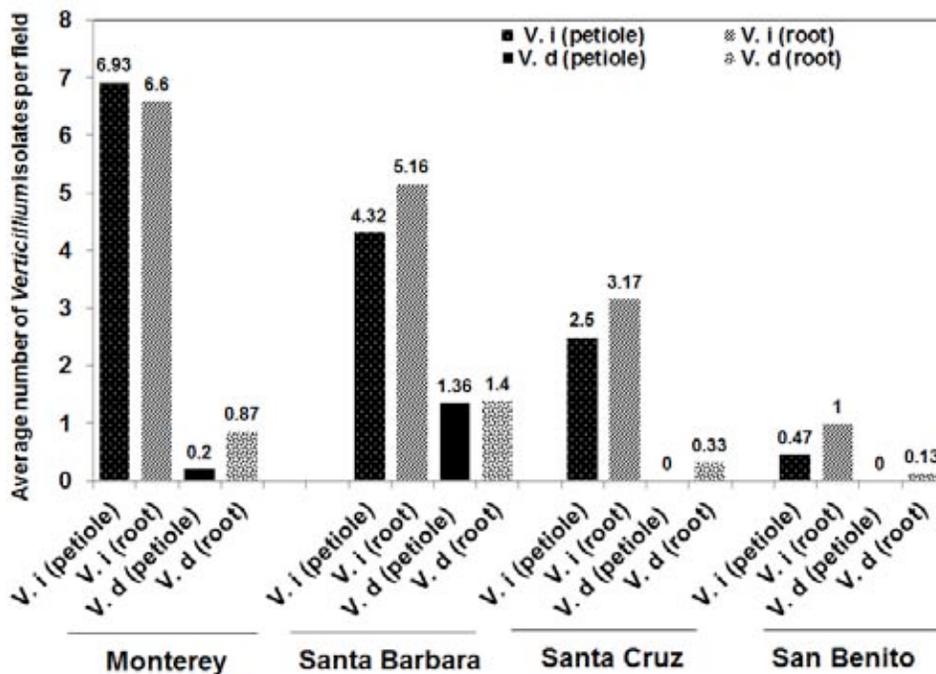
We conducted extensive surveys of spinach fields between April and September 2011 in Monterey, Santa Barbara, San Benito and Santa Cruz counties. Each field, regardless of the cultivar or crop age, was walked in an X pattern and 20 plants were randomly sampled and returned to the laboratory. The roots and petioles of each plant were plated on NP-10 medium and the number of plants yielding *Verticillium* colonies was counted. In total, 20 plants each from 61 spinach fields in all four counties were evaluated. We also retrieved the spinach acreage data from Monterey and Santa Barbara counties from the respective Agricultural Commissioner's offices for the 1990-2010 periods and the acreage versus year was plotted.



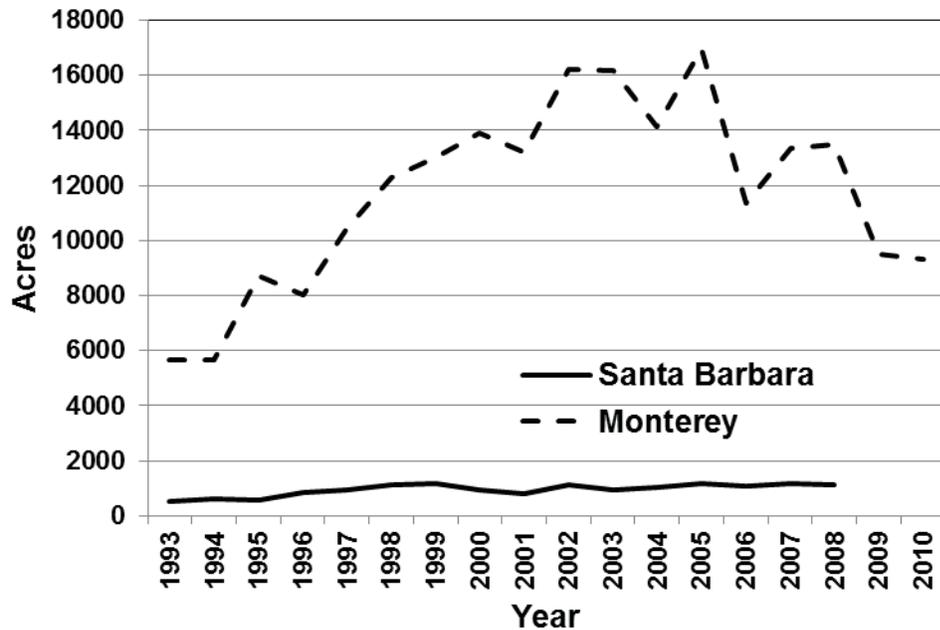
**Fig. 3.** Distribution of *Verticillium dahliae* (V.d) and *Verticillium isaacii* (V.i) in four counties of California. In total 69 fields were surveyed from Monterey ( $n = 23$ ); Santa Barbara ( $n = 25$ ); Santa Cruz ( $n = 6$ ); and San Benito ( $n = 15$ ) counties.

### Results.

The number of fields surveyed in the four counties included, 6, 15, 15, and 25 from Santa Cruz, Monterey, San Benito, and Santa Barbara, respectively. Two species of *Verticillium* were predominantly isolated from the sampled plants in all counties. *Verticillium isaacii* was isolated more frequently than *V. dahliae*, even though *V. isaacii* is rarely recovered from infected spinach seed lots that are planted in California (Fig. 3). Whether *V. isaacii* is the first colonizer of spinach plants, and if so, what is the source of this fungus and its role is currently unknown. This is a newly described species of *Verticillium* and its role in the Verticillium wilt complex is currently being elucidated. The frequency of isolation of the different *Verticillium* species differed among counties with the highest frequency of *V. isaacii* being from Monterey County followed by Santa Barbara, Santa Cruz and San Benito counties (Fig. 4). It appears that similar levels of *V. dahliae* are being introduced at least in Monterey and Santa Barbara counties with the least amount of *V. dahliae* being introduced into Santa Cruz and San Benito counties (Fig. 4). Since spinach crops in both Monterey and Santa Barbara counties are introducing similar levels of *V. dahliae*, why has the disease not been observed on lettuce in the Santa Barbara production fields? Figure 5 shows the increase in spinach production in Monterey County, where Verticillium wilt is a problem, compared with the relatively stable levels of spinach production in Santa Barbara County, where the disease has not appeared. This may explain why the disease has not yet appeared on lettuce outside of Monterey and Santa Cruz counties. Although historically the increase in spinach is linked to the increase in Verticillium wilt on lettuce, spinach production has declined in Monterey County since the *E. coli* outbreak in 2006 (Fig. 5).



**Fig. 4.** Number of *Verticillium dahliae* and *V. isaacii* colonies recovered from spinach petiole and root samples from individual fields in four California counties.



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**Fig. 5.** Acres of Spinach Grown in Monterey and Santa Barbara Counties from 1993.

We also reassessed the taxonomy of the genus *Verticillium* using phylogenetic analyses, morphological investigations and comparisons to herbarium material and the literature, and identified five new species which are new to science. Combined analyses of the data for 74 isolates revealed two major groups within *Verticillium*, Clade Flavexudans and Clade Flavnonexudans, reflecting the respective production and absence of yellow hyphal pigments. Clade Flavexudans comprised *V. albo-atrum* and *V. tricorpus* as well as the new species *V. zaregamsianum*, *V. isaacii* and *V. klebahnii*, of which the latter two were morphologically indistinguishable from *V. tricorpus* but may differ in pathogenicity. Clade Flavnonexudans comprised *V. nubilum*, *V. dahliae* and *V. longisporum*, as well as the two new species *V. alfalfae* and *V. nonalfalfae*, which resembled the distantly related *V. albo-atrum* in morphology. This information is important as a few research groups have made claims that the majority of fungi isolated from spinach seed are not the pathogenic *V. dahliae*, but the mildly pathogenic *V. tricorpus*. With the discovery of new species within the *V. tricorpus* group, we are now able to assess the role of these new species in the disease complex.

**Objective 3. Continue the research of the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables grown in coastal California.**

Lettuce became a host of *V. dahliae* in the mid-1990s. The mechanisms involved in the expansion of the host range to include lettuce have remained elusive. Three possible hypotheses examined were: a shift or adaptation in the local *V. dahliae* populations toward lettuce; or a sudden increase in population numbers in the region; or recurrent introductions of the pathogen into the area. These scenarios were primarily derived from the knowledge of the significant increase in the area

planted with salad spinach in coastal California, and the published information on the high incidence of *V. dahliae* in spinach seed lots produced in multiple regions of the world. The disease is confined to this region, although *V. dahliae* and lettuce are present in other agricultural environs of California. Gene flow with heavily infested spinach seed was previously described, but the geographic sources of this immigration, as well as the impact of the imported sparsely-infested lettuce seed on the population causing disease on lettuce in coastal California were examined this year. Population analyses of *V. dahliae* were completed using 16 microsatellite markers on strains from diseased lettuce plants in coastal California, infected lettuce seed produced in the neighboring Santa Clara Valley of California, and spinach seed produced in four major spinach seed production regions: Chile, Denmark, the Netherlands, and the United States (Washington State). Three globally distributed genetic populations were identified, indicating that sustained gene flow occurs among these distinct geographic regions. The population structure of *V. dahliae* from coastal California lettuce plants was heavily influenced by gene flow from spinach seed imported from Denmark and Washington. Conversely, the sparsely-infested lettuce seed showed no evidence of contribution to the Verticillium wilt epidemic. These conclusions were drawn from our analyses of about 250 isolates of *V. dahliae*. We are currently studying the global migratory patterns among populations of *V. dahliae* collected from many parts of the world and the potential avenues by which they make their way into California. The global trade in plant and seed material is likely contributing to sustained shifts in the population structure of *V. dahliae*, affecting the equilibrium of native populations, and possibly affecting disease epidemiology.

#### **Methods.**

One thousand four hundred twenty four isolates of *V. dahliae* from ten countries have been assembled to determine the global migratory patterns (Table 1). The isolates come from 11 hosts, many of which are vegetables (Table 2). We have obtained the DNA from all of these isolates and are currently analyzing them using simple sequence repeats (SSRs), race 1 and race 2-specific PCR markers and mating type primers. We are also testing virulence phenotype for randomly selected *V. dahliae* isolates from worldwide our collection. Since these studies are in progress, only partial results are available. Detailed results would be made available in next year's report.

#### **Objective 4. Determine the localization of *Verticillium dahliae* in spinach seed.**

The seedborne nature of *V. dahliae* in spinach has long been established. All spinach seed in California is imported, and the seed imported from Washington State or abroad are heavily infested with *V. dahliae*. The seed health testing of 68 commercial spinach seed lots for *Verticillium* spp. revealed between 0.3 to 84.8% of infested seed per lot. Wilt symptoms on spinach, however, are only apparent in seed production fields, in plants that have reached reproductive maturity. Spinach crops are harvested well before symptom expression, and thus, Verticillium wilt is not a significant threat in fresh and processed spinach production. The pathogen is seedborne and transmission of *V. dahliae* through seed is a major concern because of the dispersal potential 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,

and therefore knowledge of pathogen localization in seed is critical to develop methods to reduce seedborne inoculum. The goal of this objective is to assess where *V. dahliae* is localized in spinach seed.

**TABLE 1.** Isolates of *Verticillium dahliae* collected from various countries to determine global migratory patterns of the pathogen

<b>Country</b>	<b>No. Isolates</b>
<b>United States</b>	<b>951</b>
<b>Italy</b>	<b>127</b>
<b>Denmark</b>	<b>119</b>
<b>Turkey</b>	<b>58</b>
<b>China</b>	<b>48</b>
<b>Netherlands</b>	<b>34</b>
<b>Israel</b>	<b>22</b>
<b>Japan</b>	<b>18</b>
<b>Australia</b>	<b>17</b>
<b>Chile</b>	<b>30</b>
<b>Total</b>	<b>1424</b>

## **Methods.**

The *V. dahliae* isolate VdSo925 recovered from spinach seed produced in The Netherlands was determined to be race 2 based on molecular and pathogenicity assays. This isolate was transformed 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 ZsGreen fluorescent protein gene under the control of the *Fusarium verticillioides* translation elongation factor 1 $\alpha$  gene promoter. The vector was introduced to *A. tumefaciens* strain EHA105 for transformation of the conidia of *V. dahliae* by random insertional mutagenesis. Putative transformants were selected and

a single transformant (VdSo925-316) with a single copy of T-DNA insertion, as determined by Southern blot analysis and high GFP fluorescence intensity was selected and used in this study.

**TABLE 2.** Collection of isolates of *Verticillium dahliae* from various hosts to determine potential gene flow among populations from different hosts

<b>Plant Host</b>	<b>No. Isolates</b>
Spinach Seed	678
Tomato	149
Olive	124
Cotton	93
Lettuce	89
Artichoke	68
Potato	63
Strawberry	55
Mint	49
Pepper	30
Eggplant	14
<b>Total</b>	<b>734</b>

Seeds of spinach cultivar Hector were planted in a pasteurized sand/potting soil mixture (2:1) to produce seedlings. The isolate VdSo925-316 was grown on PDA for 7 days and conidia were harvested and the final inoculum density was adjusted to  $1 \times 10^7$  conidia/ml. Three-week-old seedlings were inoculated with conidia of VdSo925-316 using a soil drench method. At 1-wk post-inoculation, seedlings were transplanted into 0.5 liter foam-insulated cups filled with pasteurized sand/potting soil in a 3:1 mixture. Plants were maintained on benches in a greenhouse for approximately 14 wks after inoculation or until seed maturation.

Three to five inoculated plants were sampled at 24, 48, 72 hr, and 1, 2, 4, 6, 8, and 10 wk post-inoculation (PI) for microscopic observation. The inoculated plants were 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 a MRC 1024 Bio-Rad confocal laser-scanning microscope (CLSM) (Bio-Rad, Hercules, CA) or Leica confocal laser-scanning microscope (Leica Microsystems Inc. Buffalo Grove, IL). Confocal images were captured and processed. In addition, seeds were collected from inoculated plants at 12-14 weeks PI and observed for the presence of *V. dahliae*.

To produce a large quantity of infested and healthy seeds for seed treatment, approximately 500 spinach seedlings were inoculated with VdSo925-316 as described above. After seed set, ~200 g of seeds were collected from VdSo925-316-inoculated spinach plants, and delivered to Germains Seed Technology (Gilroy, CA, USA) for a proprietary fungicidal seed treatment. The healthy seeds, ~200g, from spinach cultivar Hector, were also treated to compare the seed treatment effect on infested and healthy spinach seeds. After seed treatment, 100 seeds (10-15 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.

Treated and untreated spinach seeds were plated on NP-10 medium upon receipt from the Germains Seed Technology to assess the levels of *V. dahliae* in the seeds. Both treated and untreated seeds were plated in whole or as pieces (single seed approximately broken into 10 pieces) on NP-10 medium. Plates were incubated at 25°C in the dark for 10 days and screened for *V. dahliae*. The presence of *V. dahliae* in the spinach seeds was confirmed based on production of the microsclerotia and verticillate conidiophores. In addition, some of the seeds from treated and untreated seeds were stored at room temperature for several months to assess the storage effect on *V. dahliae* growth on these seeds.

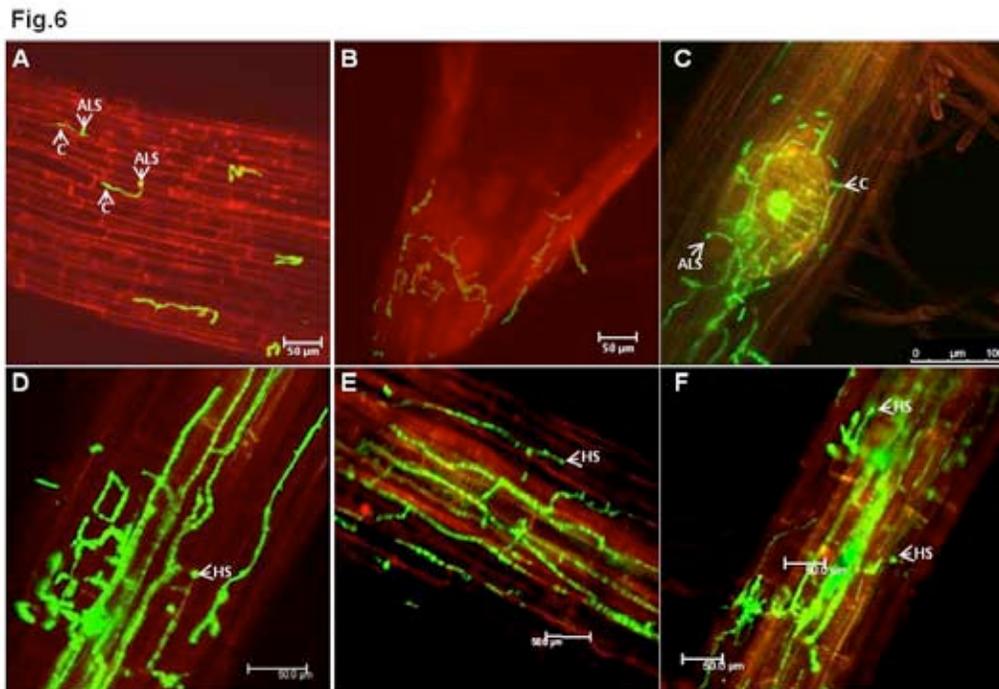
To assess the effects of treatment on percent seed germination, both treated and untreated seeds were planted in 100-well seedling plug trays and maintained as described previously. Three weeks after planting, the number of seedlings emerged and their growth and development were visually assessed. In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

## **Results.**

*Agrobacterium tumefaciens*-mediated transformation of *V. dahliae* yielded hygromycin-resistant colonies. A single transformant, VdSo925-316 was selected as it contained a single copy of the T-DNA insert and this transformant also showed a strong and uniform green fluorescence signal in the hyphae, conidiophores and conidia. VdSo925-316 did not show differences in colony morphology, growth rate, or pathogenicity relative to the wild type strain VdSo925 from spinach.

Conidia of *V. dahliae* on the root surface germinated at 24 hr PI (Fig. 6A). Following germination, hyphae colonized the root tips and invaded the vascular tissues (Fig. 6B). In the root elongation zone, colonization by hyphae was observed in both inter- and intracellular spaces of the cortex and vasculature (Fig. 6C, D, E, and F). Colonization at 2 wks 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. 6D and E).

At 6 wk PI, colonization progressed into the root xylem vessels in inoculated plants (Fig. 7B), but GFP fluorescence was not observed in the root xylem vessels of water-treated seedlings (Fig. 7A). Ten wk PI, massive numbers of hyphal colonies were observed in the xylem of the stem (Fig. 7C, D), coinciding with necrotic lesions of root tissues, leaves and foliar symptom development. All the xylem vessels of the stem were heavily colonized by *V. dahliae* (Fig. 7D).

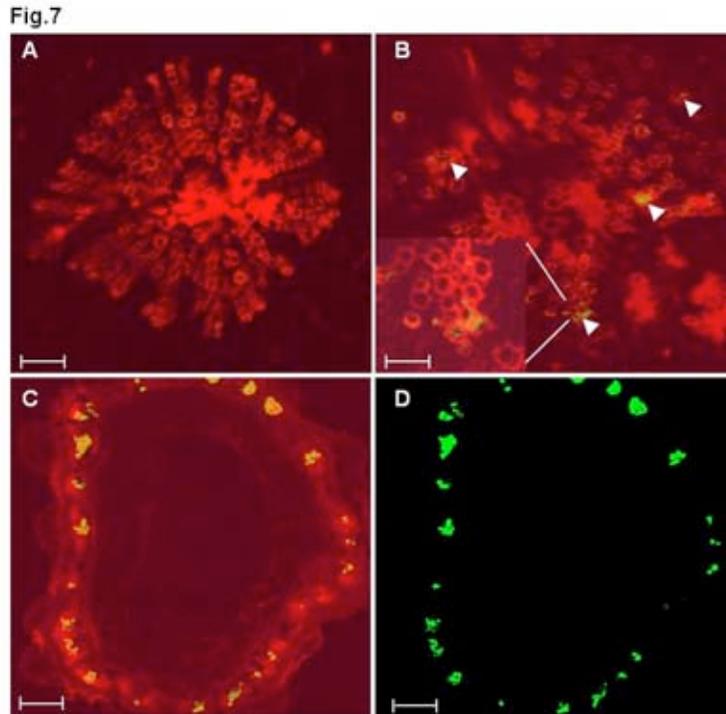


**Fig.6.** Early and advanced stages of root colonization by the green fluorescent protein-tagged spinach isolate of *V. dahliae*, VdSo925-316 at 48 hr, 1 wk and 2 wks PI. **A.** Conidia (C) on the root surface with a solitary or two germ tubes. **B.** Lateral root tip colonized by *V. dahliae*. **C.** Wound site of the lateral root colonized by *V. dahliae*. **D.** Composite of images from a confocal laser scanning microscope showing advanced colonization of epidermal and cortical root tissues of spinach by *V. dahliae*. **E** and **F.** Composite image stack from CLSM exhibiting inter and intra-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.

As symptoms progressed, leaves on individual stems, as well as the inflorescence and bracts of the involucre became chlorotic and wilted. Inoculated male and female flowers revealed *V. dahliae* colonization (Fig. 8) and the anther walls from inoculated male flowers were colonized by the pathogen (Fig. 8C), but not the pollen grains (Fig. 8D). Anthers and pollen from uninoculated male flowers did not contain *V. dahliae* (Fig. 8A and B). In the inoculated female flowers, *V. dahliae* was detected in the perianth, style and stigma (Fig. 8G and H), but pathogen was not detected in the uninoculated female flowers (Fig. 8E and F).

The entire pericarp of the spinach seed was heavily colonized by 12 wk PI (Fig. 9A, C, D, and E) that progressed into the seed coat and inner parts from the pericarp of the spinach seed (Fig. 9F, G

and H) by 14 wk PI. Conidiophores and massive amounts of secondary conidia were also observed in the infected pericarp (Fig. 9D, E, and F). A section of the spinach seed showed that *V. dahliae* had colonized the cotyledons, hypocotyl and radicle (Fig. 9H).

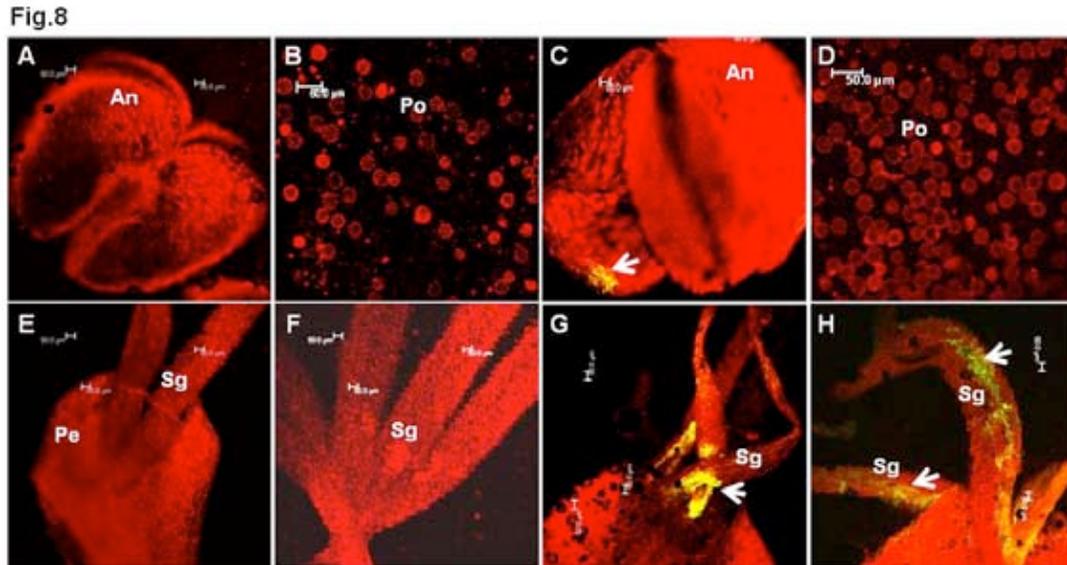


**Fig. 7.** Later stages of spinach root stem colonization by the GFP-tagged spinach isolate of *V. dahliae*, VdSo925-316. Images were captured 6 and 10 wks post inoculation (PI) of seedlings with conidia of *V. dahliae*. **A.** Cross section of an uninoculated plant main root without GFP fluorescence. **B.** Cross section of inoculated plant exhibiting restricted advancement of fungal mycelia through the xylem vessels, at 6 wks post inoculation. **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 wks post inoculation. **A to D,** Scale bars = 100  $\mu$ m. Arrow heads indicate fungal hyphae with GFP fluorescence.

The analyses of over 50 mature seeds from several infected plants revealed that *V. dahliae* was not present in the perisperm nor were microsclerotia observed in the pericarp or seed coat. However, when the infected seeds were stored at room temperature for nearly three months and then observed after taking cross sections, massive numbers of microsclerotia in the pericarp and seed coat were present (Fig. 10A and B). Examination of more than 20 seeds under light microscope revealed ~250 microsclerotia/seed. Microsclerotia on either pericarp or seed coat regions were not present on uninfected healthy seeds that were stored and examined similarly (Fig. 10C and D).

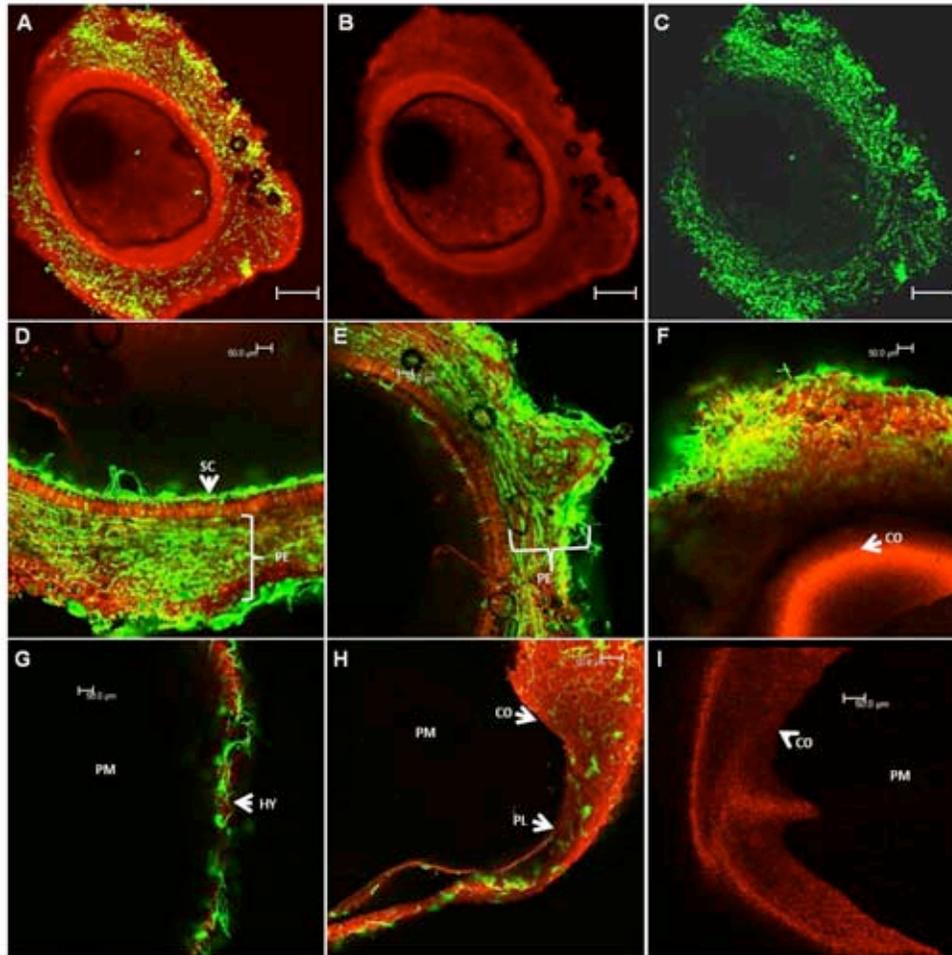
Growth of *V. dahliae* was not apparent at 10 days post-planting of the infected fungicide-treated seed. However, growth of *V. dahliae* from the spinach seeds was observed when the whole fungicide-treated seeds were broken into small pieces and then plated (Fig. 11C). In contrast, all of the untreated seeds from the inoculated plants were positive for *V. dahliae* (Fig. 11A). The non-inoculated seeds that were treated or untreated with fungicide did not yield *V. dahliae* growth (Fig.

11B and D). The GFP-fluorescence signal in the untreated-infected seeds was strong and widespread (Fig. 11E). Microscopic observation of fungicide-treated seeds from the inoculated plants revealed growth of *V. dahliae* in the pericarp region. Healthy seeds from uninoculated plants, whether fungicide treated or untreated, did not display growth of *V. dahliae* (Fig. 11F and H). In addition, seedlings that grew from either group of fungicide-treated seeds exhibited slower growth and leaves were etiolated (Fig. 11J) relative to the seedlings that grew from the seeds untreated with fungicide (Fig. 11I), perhaps related to the deleterious effect of the seed treatment. Early stages of seed germination on PDA plates (7 days after plating) of fungicide-treated seeds from inoculated plants revealed hyphal colonization and secondary conidia in the pericarp, hypocotyl, plumule and radicle regions, and subsequently (after two weeks) massive colonization with the production of conidiophore and microsclerotia (Fig. 11K, L, and M).



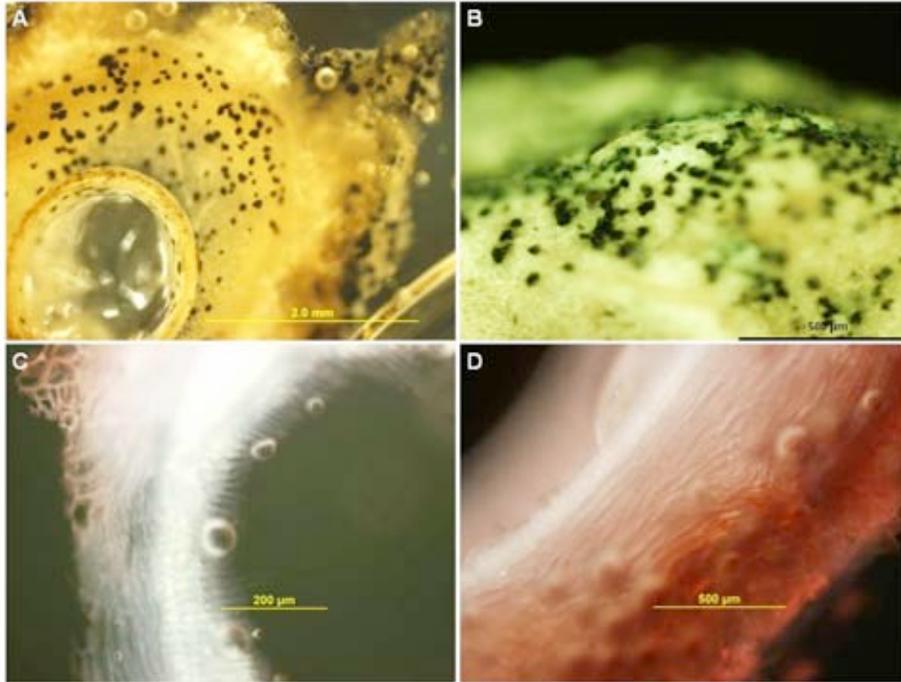
**Fig. 8.** *Verticillium dahliae* colonization in staminate and pistillate flowers. Confocal images captured in confocal 12 weeks following the inoculation of seedlings with conidia of GFP expressing *V. dahliae* **A.** Uninoculated staminate flower anther (An) without GFP fluorescence. **B.** Pollen grains (Po) from uninoculated 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.** Uninoculated pistillate flower without GFP fluorescence in perianth (Pe) and stigma (Sg). **F.** Stigma (Sg) of the staminate flower shows no fungal infection. **G. and H.** *V. dahliae* colonization in stigma of the pistillate flower.

Fig.9



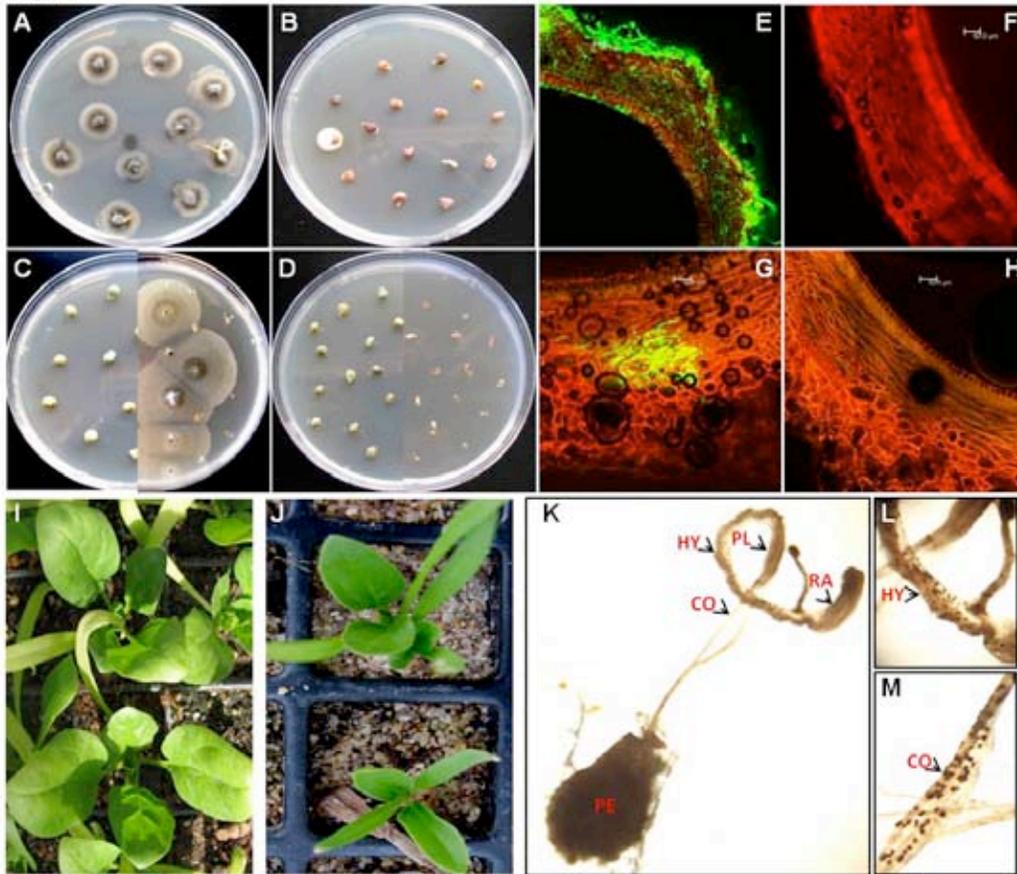
**Fig. 9.** Early and advanced stages of spinach seed colonization by a green fluorescent protein-tagged spinach isolate of *V. dahliae*, strain VdSo925-316, at 10 and 12 weeks post inoculation (PI) of 3 week-old spinach seedlings. **A.** Cross section of spinach seed showing massive colonization of *V. dahliae* on the fruit wall 12 weeks PI. **B.** The same spinach seed shown in A, without filter to detect fungal GFP fluorescence. **C.** The same spinach seed shown in A, without filter to detect plant auto fluorescence, and thus only GFP fluorescence from the fungus is detectable. **D, E** and **F.** Cross sections of the spinach seed showing massive colonization of *V. dahliae* on the pericarp (PE) and seed coat (SC). **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 an uninoculated germinating spinach seed indicating the cotyledons that are not colonized with *V. dahliae* (CO). PM indicates perisperm region of the seed. **A** to **C**, Scale bars = 100  $\mu$ m.

Fig.10



**Fig. 10.** Examination of spinach seeds stored at room temperature nearly three months following collection from uninoculated plants, and from infected plants. **A** and **B.** Cross section of a spinach seed exhibiting microsclerotia on the fruit wall and seed coat. **C** and **D.** Seeds collected from uninoculated spinach plants did not show microsclerotia on the fruit wall of the spinach seed.

Fig.11



**Fig. 11.** Evaluation of a spinach seed treatment by comparing fungicide-treated and untreated seeds that were inoculated or not inoculated with *V. dahliae*, strain VdSo925-316. **A.** Untreated spinach seeds from inoculated plants were plated on NP-10 semi-selective medium exhibiting growth of *V. dahliae* 10 days after plating. **B.** Untreated spinach seeds from non-inoculated plants did not exhibit growth of *V. dahliae* on NP-10 medium. **C.** Treated spinach seeds were plated as whole (no growth of *V. dahliae*) or broken (growth of *V. dahliae* is evident) on NP-10 medium. **D.** Treated seed from non-inoculated 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 non-inoculated 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. **H.** Cross section of a treated spinach seed from non-inoculated plants without fungal GFP fluorescence. **I and J.** Phenotypic differences of developing seedlings (3 week-old) from untreated (**I**) and treated seeds (**J**). **K, L, M.** A developing seedling from fungicide-treated seed shows microsclerotia on the pericarp (**PE**), hypocotyl (**HY**), cotyledons (**CO**) plumule (**PL**), and radicle (**RA**).