CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

April 1, 2012 - March 31, 2013

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) to assess the impact of immigrant populations of *V. dahliae* introduced from spinach seed into soil on crops that follow spinach; 2) to 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) to continue studies on the genetic relationships among populations of *V. dahliae* from spinach seed and other vegetables grown in coastal California; and 4) to conduct economic analyses of immigrant populations of *Verticillium dahliae* on coastal California agriculture. Lettuce became a host of *V. dahliae* in the mid-1990s and we have determined by genetic analyses and pathogenicity tests 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 two years, three spinach crops each in treatments with different proportions of *V. dahliae*-infested spinach seed were grown in replicated microplots during 2011 followed by two lettuce crops in all microplots during 2012. Verticillium wilt developed in both lettuce crops and the incidence during the spring season was proportional to the spinach seed infestation treatment established during the previous year. During the fall, however, incidence of Verticillium wilt on lettuce varied between 25-35% in all treatments except control. *Verticillium dahliae* was isolated from each infected plant and identified to species using the species-specific primers. The second objective was initiated to examine why Verticillium wilt of lettuce is confined to the two valleys in CA even though cropping patterns are similar in other areas was continued. Twenty plants from 100 commercial spinach fields in Salinas, Pajaro, Santa Maria and San Juan valleys were sampled between 2011 and 2012, and the roots and petioles from each were plated on the NP-10 medium. More than 885 isolates recovered were characterized to species. The majority of these isolates were *V. isaacii* followed by *V. dahliae*. The frequency of recovery of *Verticillium* species did not explain the current distribution of the disease in lettuce but was correlated with the magnitude of spinach production in the four valleys. This study also established *V. isaacii* as another species in the Verticillium wilt complex whose precise role remains to be determined. A global collection of isolates was analyzed to determine the migratory patterns of *V. dahliae*. In addition to confirming the migration of isolates from spinach seed production regions into California, the study also charted the global migratory patterns of this pathogen. Finally, the economic analysis of the impact of immigrant populations of *V. dahliae* on coastal California agriculture is currently underway in collaboration with Dr. Colin Carter and Ms. Christine Carroll from the Agricultural and Resource Economics Department at UC Davis.

CALIFORNIA ICEBERG LETTUCE RESEARCH PROGRAM

April 1, 2012 - March 31, 2013

PROJECT TITLE: **VERTICILLIUM WILT OF SPINACH: DETECTION, BIOLOGY AND CONTROL**

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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. Economic analyses of immigrant populations of *Verticillium dahliae* on coastal California agriculture.

PROCEDURES AND RESULTS:

Objective A. 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 not available for *V. dahliae* introduced via spinach seed nor can answers 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 conduct studies in state-of-the-art microplots, where we can control all factors. The previous report to the Leafy Greens Board described the progress of this experiment until May 15, 2012. At that point, we had documented the baseline levels of *V. dahliae* in the microplot soil and had observed an increase in soil levels of *V. dahliae* following three crops of spinach using five seed infestation level treatments.

Methods.

Six different spinach seed *V. dahliae* infestation level treatments were established (64%, 33%, 15%, 10%, 0% infested spinach seed) with four replications each in 2011. 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. The fungus isolates from plants were identified to species using species-specific markers. After three successive crops of spinach in 2011, lettuce cultivar Salinas was planted in all microplots in 2012 during spring and fall, and evaluated for Verticillium wilt at crop maturity. We sampled 20 lettuce plants from each microplot to evaluate for Verticillium wilt symptoms. Symptomatic roots were cleaned, dissected and plated on NP-10 medium (Fig. 1). If *Verticillium*-like fungi developed from the plated tissue, they were purified for molecular identification. The DNA was extracted and a *V. dahliae*-specific multiplex PCR was used to positively identify plants from specific microplots from which symptomatic plants were collected.

Results.

During both spring and fall seasons, lettuce plants in two plots of treatment 1 (67% infested seed), one plot of treatment 2 (33% infested seed) and one plot of treatment 3 (15% infested seed) exhibited Verticillium wilt symptoms (Fig. 2). The incidence of Verticillium wilt during the fall was higher than in the spring (Fig. 2). In both crops of lettuce in 2012, *Verticillium* – like fungus was isolated from the taproots of symptomatic plants. The isolated fungus was identified as *V. dahliae* using the species-specific primers (Fig. 3). The results of the second season were similar. The average number of *V. dahliae* isolates recovered from sampled spinach plants in each spinach seed infestation treatment during the three spinach crops in 2011 is shown in Figure 4. The soil assays are in progress and we will report on the inoculum augmentation in next year's report.

During 2013, we are repeating the cycle of two spinach crops followed by one lettuce crop in the same soil. We have thus far grown one crop of spinach and have taken soil samples before and

after the spinach crop incorporation. We have also sampled 20 spinach seedlings per microplot during the season, which were plated on NP-10 medium and scored for the presence of *V. dahliae*.

This is the first demonstration of *V. dahliae* introduced by infested spinach seed as the cause of Verticillium wilt in lettuce. The repeat experiments during 2013 will provide confirmation of these results.

Fig 1. Lettuce cv. Salinas tap root longitudinally sectioned, showing severe vascular discoloration. A petri dish containing NP-10 medium with sections of discolored taproot plated is also shown.

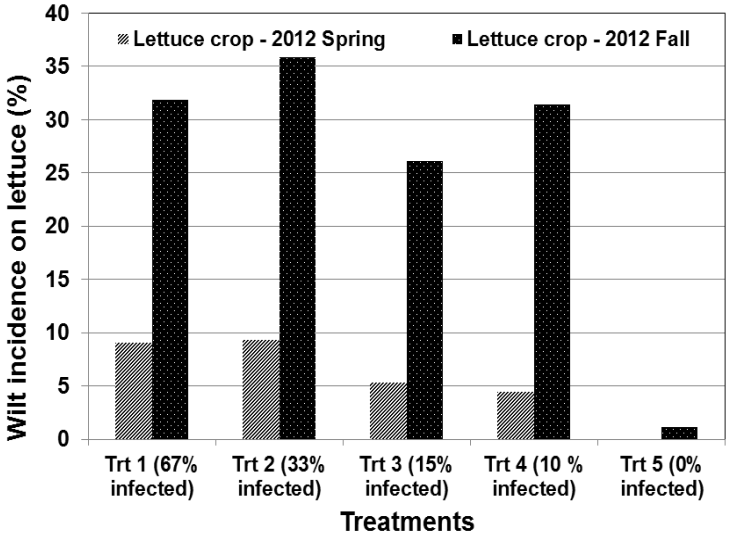

Fig. 2. Incidence of Verticillium wilt on lettuce cv. Salinas in microplots in treatments previously established with different levels of spinach seed infestation by *Verticillium dahliae*.

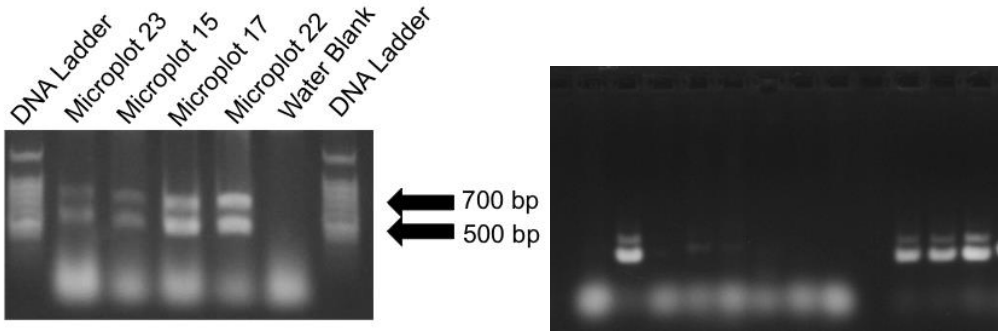

Fig 3. Results of *V. dahliae*-specific PCR from the first crop (left) and second crop (right) of lettuce grown in the microplots. Left: double bands indicate that *V. dahliae* was detected in four microplots. Right: the three double bands on the right were used as positive controls. The double bands in the third well from the left indicate a sample tested positive for *V. dahliae.*

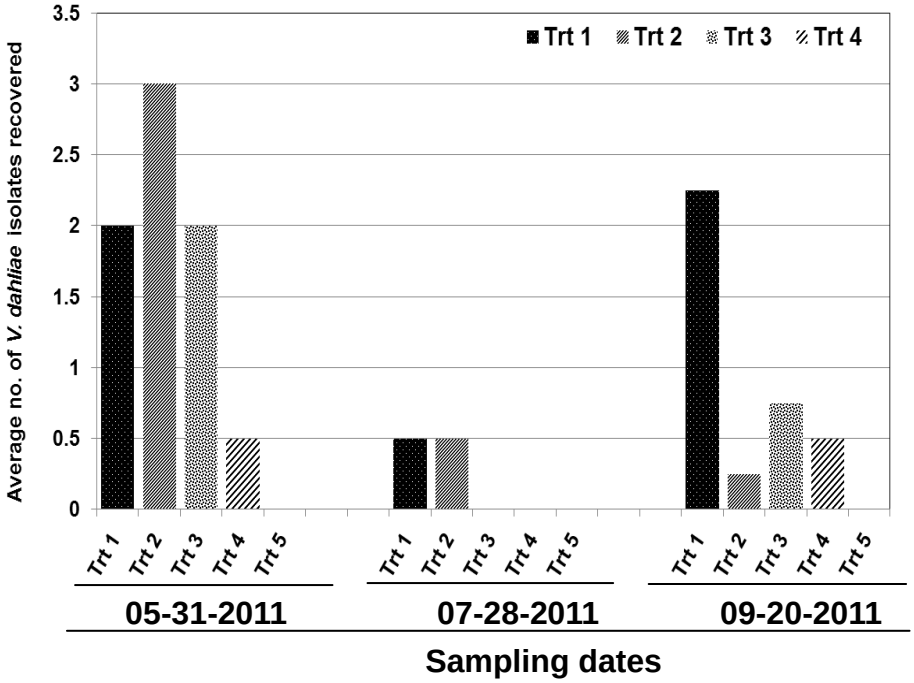

Fig. 4. Number of *Verticillium dahliae* isolates recovered from sampled spinach seedlings in the five seed infestation level treatments during each spinach crop in 2011.

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 206,200 acres of lettuce in 2011. 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 on lettuce in Watsonville, Santa Cruz County. 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 the disease 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 in 2011 and continued in 2012.

Methods.

A total of 69 and 31 major commercial spinach fields from Monterey, San Benito, Santa Barbara and Santa Cruz counties in California were extensively surveyed for Verticillium wilt diseases during 2011 and 2012, respectively. 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 all spinach plants were washed free of soil, surface sterilized with 10% bleach, rinsed 2-3 times with sterile water, dissected and plated on NP-10 media. The numbers of plants yielding *Verticillium* colonies were examined 9 to 14 days after plating. *Verticillium* species were identified based on the colony morphology and later confirmed using the species-specific PCR tests.

Results.

Prior to this study, the incidence of species like *V. isaacii* and *V. klebahnii* were never associated with Verticillium wilt either in spinach or other crops susceptible to the disease. However, *V. isaacii* and *V. dahliae* were predominant species isolated from the sampled spinach roots and petioles in all counties (Figs. 4 and 5). Of the total 1380 and 620 spinach plants plated on NP-10 media, 585 and 300 *Verticillum* species were recovered during 2011 and 2012, respectively. In total, 496 *V. isaacii* and 89 *V. dahliae* during 2011, and 218 *V. isaacii* and 82 *V. dahliae* during 2012 were identified based on colony morphology and pigment production. The data analyzed on a per field basis also indicated *V. isaacii* as the most predominant species isolated in all counties (Figs. 4 and 5).

In total, 160 and 198 isolates, identified to species based on colony morphology, were randomly selected for identification to species level using the species-specific PCR assays described in the lettuce report. Eight of the Monterey County samples from 2012 could not be amplified from the PCR assays. The PCR assays again confirmed that *V. isaacii* as the most predominant species followed by *V. dahliae* recovered from all four counties with the exception of San Benito County, where *V. dahliae* was the most predominant species in 2011. In addition, four samples from Monterey and two samples from San Benito County from 2012 samples were identified as *V. klebahnii*. As outlined in this year's report on lettuce, the majority of *V. isaacii* and *V. kelbahnii* isolates tested were non-pathogenic on lettuce and other crops but some were as pathogenic as *V. dahliae*. Thus, in addition to *V. dahliae*, growers may also need to manage these two species of *Verticillium* in the future.

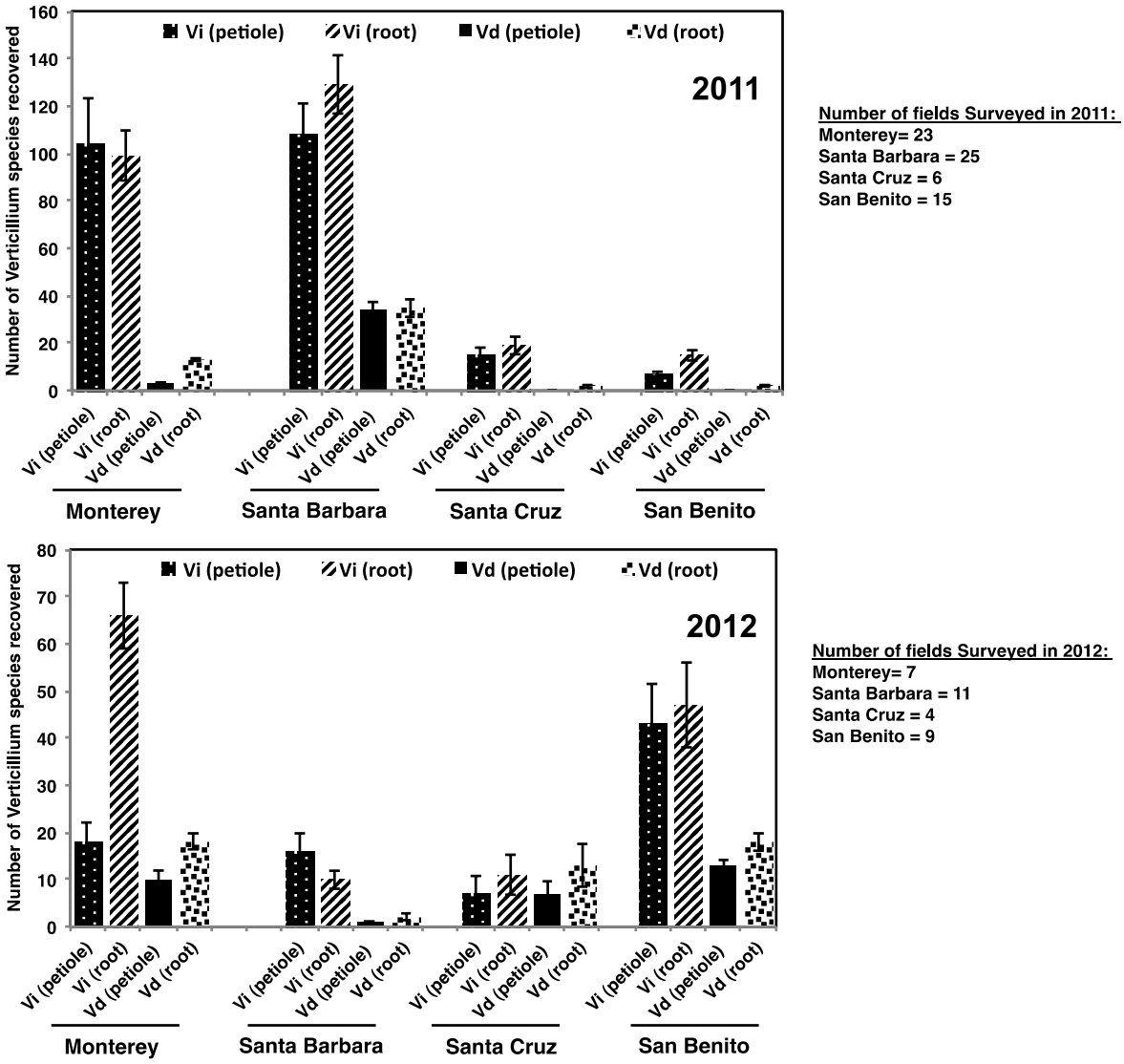

Fig. 4. Distribution of *Verticillium* species in commercial spinach fields in four counties in 2011 (top) and 2012 (bottom). For 2011, the number of fields surveyed in Monterey, Santa Barbara, Santa Cruz, and San Benito were 23, 25, 6, and 15, respectively. For 2012, the number of fields sampled were 7, 11, 4 and 9, respectively.

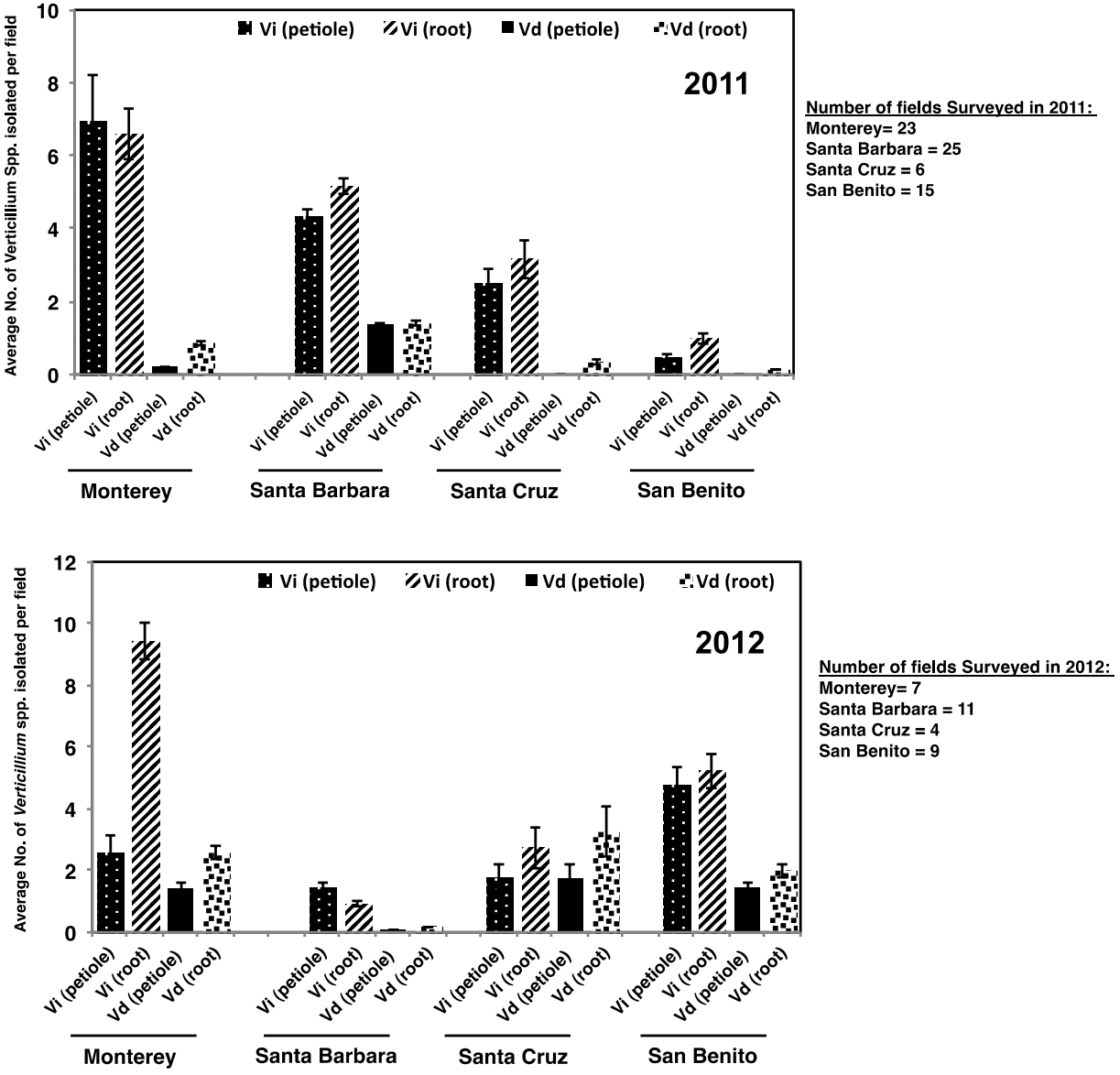

Fig 5. Recovery of *Verticillium* spp. per spinach field. For 2011, the number of fields surveyed in Monterey, Santa Barbara, Santa Cruz, and San Benito were 23, 25, 6, and 15, respectively. For 2012, the number of fields sampled were 7, 11, 4 and 9, respectively.

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

Since 1995, lettuce in coastal California, where more than half of the crop in North America is grown, has consistently suffered from severe outbreaks of Verticillium wilt. The disease is confined to this region, although the pathogen (*Verticillium dahliae*) and the host are present in

other crop production regions in California. Migration of the pathogen with infested spinach seed was previously documented, but the geographic sources of the pathogen, as well as the impact of lettuce seed sparsely infested with *V. dahliae* produced outside coastal California on the pathogen population in coastal California had remained unclear. Population analyses of *V. dahliae* were completed using 16 microsatellite markers on isolates from lettuce plants in coastal California, infested 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). California produces 80% of spinach in the US and all seed planted (the majority infested by *V. dahliae)* comes from the above four sources. Three globally distributed genetic populations were identified, indicating sustained migration among these distinct geographic regions with multiple spinach crops produced each year and repeated every year in coastal California. The population structure of *V. dahliae* from coastal California lettuce plants was heavily influenced by migration from spinach seed imported from Denmark and Washington. Conversely, the sparsely infested lettuce seed had limited or no contribution to the Verticillium wilt epidemic in coastal 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 likely affecting disease epidemiology. We have expanded this study to determine the global movement of *V. dahliae* and its contribution to Verticillium wilt on a variety of hosts.

Methods

To further investigate the population structure and genetic diversity of a global population of *V. dahliae*, we collected 1186 isolates from a variety of hosts and countries. Isolates were verified as *V. dahliae* using a PCR assay with *Verticillium* species-specific primers. We employed 13 of the same microsatellite markers that we had previously used successfully to characterize just the California and spinach populations of *V. dahliae*. Structure analysis was used to determine the affiliation of individual isolates of *V. dahliae* to specific genetic clusters and the possibility of admixture.

The software Migrate-n was used to estimate the effective population size and inter-population migration. Mutation rates at all 13 SSR loci were assumed to be constant for estimating migration rates. Five independent runs were performed using the Brownian motion model, and the results of the run that generated the smallest Ln(Likelihood) value. Five short chains were performed with 1,000 samples and 20 sampling increments and 1,000 trees were recorded; whereas, five long chains were performed with 5,000 samples, a burn-in of 10,000, and 5,000 trees were recorded.

Nearly all of the California populations of *V. dahliae* regardless of the host from which they were originally isolated contained only the *MAT1-2* idiomorph. However, our analyses of the mating type genes carried by isolates from spinach seed suggested that about 5% of the isolates also carried *MAT1-1* idiomorph. The mere presence of the two mating types together does not assure sexual reproduction in *V. dahliae*. We therefore investigated the *V. dahline* genome for the presence of genes orthologous to those associated with mating in other fungal systems, including

the heterothallic, sexually reproducing fungus *N. crassa*. Sequences of a total of 87 fungal genes associated with various biological functions related to sex were acquired and we investigated whether the *V. dahliae* genome contained orthologs to those genes. In addition to these 87 genes, we investigated seven pheromone-related genes in *V. dahliae*, which are essential for mating in other fungal systems.

Results

Six hundred and fifty two unique multilocus SSR types were detected using 13 genome-wide loci harboring polymorphic microsatellite repeats. We discovered five major clones of *V. dahliae* with widespread geographic distributions (Fig. 6). Isolates from Denmark had a strikingly high allelic richness as calculated using rarefaction (Fig. 7).

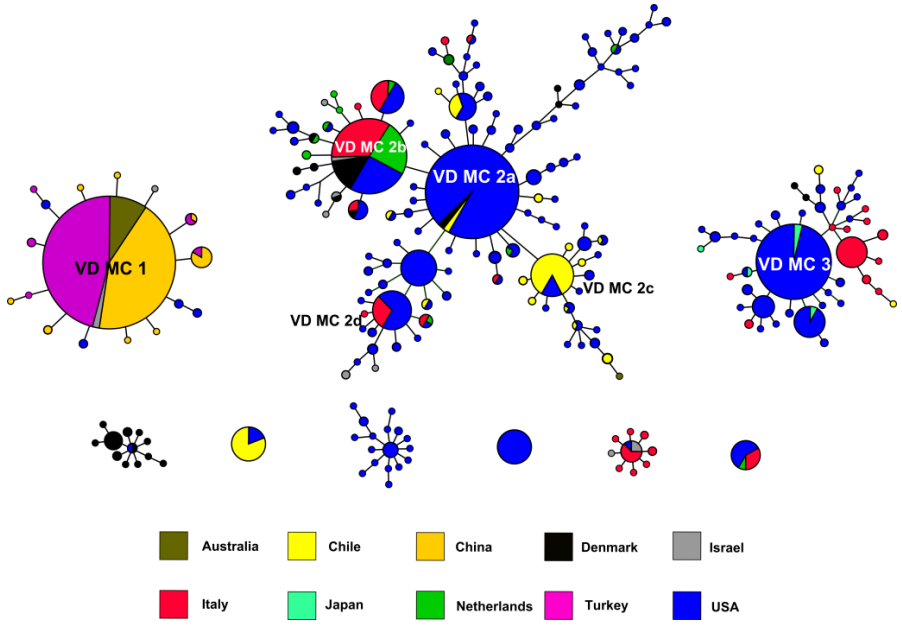

Fig. 6. Relationships and frequencies of *V. dahliae* microsatellite types from 10 countries. Each circle represents a unique microsatellite type.

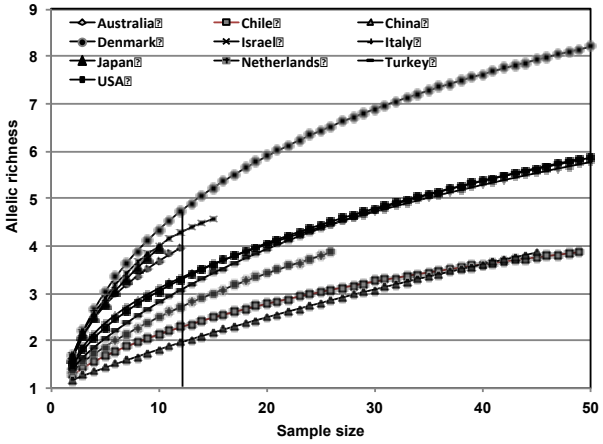

Fig. 7. Allelic richness in samples collected from 10 countries. The sample from Denmark was the most diverse, and contained the most unique genotypes.

The STRUCTURE analyses revealed five genetic clusters. Each genetic cluster had a defined distribution among the 10 countries (Fig. 8). Isolates from the United States were affiliated with all 5 genetic clusters in varying degrees. Migration analyses using Migrate-n and the software BayesAss detected gene flow between countries (Fig 9).

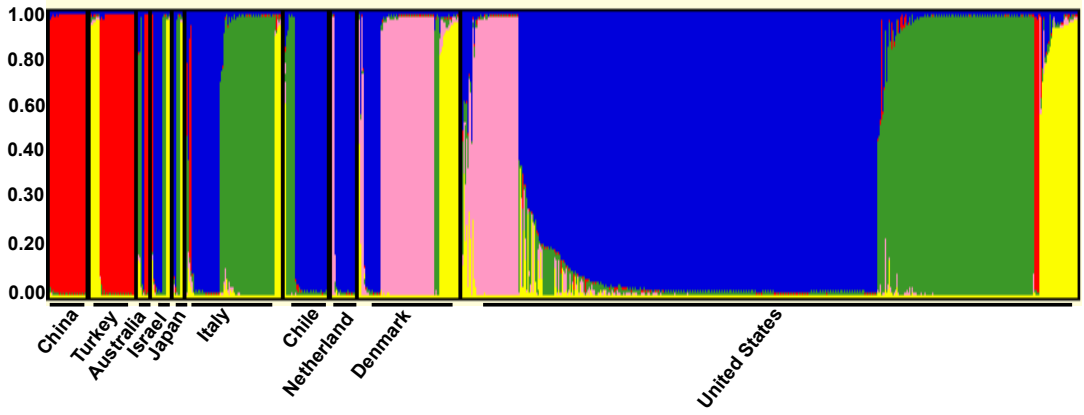

Fig. 8. Distribution of 5 unique genetic clusters detected in the global sample of *V. dahliae* from the STRUCTURE analyses. Colored bars represent isolates; bars within each color represent isolates that belong to the same genetic cluster.

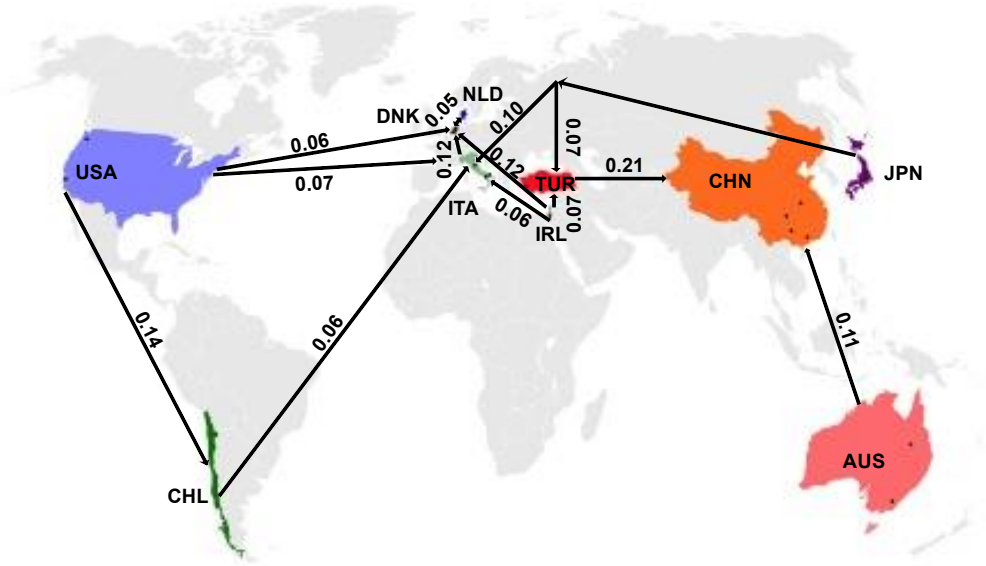

Fig. 9. Gene flow due to migration in *V. dahliae*. Arrows indicate the direction of movement and values represent the share of genotypes in a population composed of migrants over the last few generations on a scale of 0-1.

Orthologs to 85 out of 87 *Neurospora* genes involved in meiosis were found in the genome of *V. dahliae.* Orthologs to the 7 pheromone-related genes and an ortholog to the *rid* gene were also found in the *V. dahliae* Ls17 genome. We then investigated the hypothesis that mating-related genes in *V. dahliae* are not being maintained by purifying selection, a process that removes deleterious alleles from populations. This approach has been recently applied to other systems, including *Penicillium*. We used two evolutionary models to contrast two types of substitutions (synonymous and non-synonymous) within the mating type idiomorphs and 19 other mating-relating genes of *V. dahliae* strain Ls17, relative to orthologs from fungi with known sexual cycle. Both mating type idiomorphs and all 19 sex-related genes investigated displayed a high frequency of sites under purifying selection suggesting that genes involved in sexual reproduction are being maintained in functional form in *V. dahliae* between generations.

Objective 4. Economic analyses of immigrant populations of *Verticillium dahliae* on coastal California agriculture (Christine Carroll, Colin Carter, and Krishna Subbarao).

Invasive plant pathogens, including fungi, cause an estimated $21 billion in crop losses each year in the United States (Rossman, 2009). California, as a major global trader and agricultural producer, sustains much damage from such pathogens. Fungi damage a wide variety of crops, resulting in yield and quality related losses, reduced exportability, and increased fungicide expenditures (Palm, 2001).

California lettuce growers are extremely concerned about Verticillium wilt, given the persistence of the pathogen in the soil and the potential for large tracts of land to become unsuitable for

growing lettuce and other crops. The value of California's 2011 lettuce crop, which represents the majority of the United States' lettuce production, was more than $1.5 billion. Measured by value, lettuce ranks in the top ten agricultural commodities produced in California. In an effort to address these concerns, we study the economic causes of Verticillium wilt of lettuce, evaluate policy options to control the disease, and develop a simulation model for understanding growers' optimal decision-making.

Agricultural economics is an important tool for pest management research because human behavior and economic activities affect pests. Bioeconomic modeling incorporates aspects from the fields of economics and biology to accurately model natural systems. Whereas economics often relies on abstraction, we explicitly model the interaction among crops and the fungus. The main components of the model are a description of the biological relationship among the fungus, the crops, and the control methods and the profit maximization problem of the grower.

The seminal work of Rust (1987) uses a nested fixed-point algorithm to estimate a dynamic programming model of discrete choice. In this case, the discrete choices are crop and fumigation combinations, based on data from the California Department of Pesticide Regulation. Dynamic programming refers to the method of finding the optimal solution to a complex problem by breaking it down into overlapping sub-problems. We use a per-period (monthly) profit function for each grower as a sub-problem. The dynamic component arises from growers' expectations about the impact of today's actions on future profits, for example, the consequences of microsclerotia persisting in the soil for many years.

We adopt this approach to estimate a structural econometric model of growers' profit maximizing behavior, explaining crop choice decisions made by growers in Monterey County. A simulation model allows for the incorporation of biological parameters estimated from the work of plant pathologists. We adopt this model because it allows us to perform counterfactuals and simulations. This will be useful to simulate the effects of different control options, such as a resistant lettuce variety or rotation to broccoli that are not widely available or used.

In future work, we will consider models beyond the individual grower. Many potential solutions have a much wider impact than at the farm level. Fumigation imposes externalities on neighbors and the environment. The use of resistant varieties uses up the "stock of resistance" and imposes externalities on other users. Testing or quarantining imported seeds requires either the cooperation of seed companies or the enforcement of the federal government. Analyzing these externalities will allow us to determine the feasibility of solutions that may be outside growers' control, such as the development of resistant lettuce varieties.

References

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