CALIFORNIA LETTUCE RESEARCH PROGRAM

April 1, 2016 - March 31, 2017

PROJECT TITLE: BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LETTUCE AND SPINACH

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ABSTRACT:

The past year's project included two objectives: 1) to determine the efficacy of non-chemical alternatives to methyl bromide for Verticillium wilt management in lettuce, and 2) to determine the potential of host-directed evolution of *Verticillium dahliae* race 1. For objective 1, the effects of soil amendments on Verticillium wilt and soil microbiome dynamics were studied in three soils collected from fields with distinct land use histories. Treatments included: amendment with 10% Broccoli (w/w), 0.2% chitin (w/w), a combination of both broccoli+chitin, and an unamended control. Disease incidence and severity on eggplant (a highly susceptible host), microsclerotia (MS) density, chitinase enzyme activity, and soil microbiome community structure were analyzed over time. Broccoli and broccoli+chitin treatments resulted in significant reductions in disease severity and MS densities relative to the unamended control. Broccoli residue alone or in combination with chitin reduced Verticillium wilt in all soil types, and thus represents a cultural management approach for soilborne pathogens in the absence of fumigation. In objective 2, the host-directed evolution of a *V. dahliae* race 1 isolate was studied. A total of 427 isolates were recovered over six years from a lettuce wilt resistance screening nursery infested with only the *V. dahliae* race 1 isolate. Isolates recovered over six years from the disease nursery revealed no changes in race, mating type or virulence pattern. In separate pathogen collections from commercial fields, no significant genotypic variation in *V. dahliae* was observed between or within fields of either lettuce or tomato, but the pathogen populations were significantly differentiated between two hosts. These results indicated that the deployed race 1 resistance locus

in lettuce is likely to remain effective against the widespread race 1 pathogen populations at least for six years, unless novel pathogen races are introduced into the system.

OBJECTIVES:

A. Determine the efficacy of non-chemical alternatives to methyl bromide on Verticillium wilt incidence in lettuce.
B. Determine the potential of host-directed evolution of *V. dahliae* from a single race 1 genotype.

PROCEDURES:

Objective A. **Determine the efficacy of non-chemical alternatives to methyl bromide on Verticillium wilt incidence in lettuce.**

Introduction

Verticillium wilt caused by *Verticillium dahliae*, is a disease to which lettuce was once considered immune, has now significantly compromised lettuce production. The pathogen infested lettuce fields have suffered near total yield losses. Commercially acceptable lettuce cultivars with resistance to Verticillium wilt are being developed but none are available yet, limiting the use of host resistance as an effective disease management tool. *Verticillium dahliae* survives in soil in the absence of a host for over 14 years through microsclerotia (MS).

Lettuce growers with Verticillium wilt-affected fields prefer to rotate with strawberry, where pre-plant fumigation with methyl bromide and chloropicrin was a routine practice until 2015. This allowed growers to recover the cost of fumigation through a strawberry crop, and piggy-back on the residual fumigation effects when rotating back to lettuce production. The benefits of the residual fumigation effects, however, are short-lived, as Verticillium wilt typically returns with greater severity following the second consecutive lettuce crop. Methyl bromide is also banned from agricultural use now, and other fumigants that are still available are not as efficacious.

Verticillium inoculum dispersal is facilitated by tillage practices, equipment, and personnel. Seed transmission of *V. dahliae* occurs in lettuce and spinach. Crop rotations with several *Brassica* species including broccoli and Brussels sprouts have been shown to reduce *V. dahliae* MS number in soil and the subsequent disease incidence.

In this project, we are attempting to develop a systems-based approach to managing Verticillium wilt through enriching the soil environment to make it more supportive of biological regulation of microbial populations through a natural negative feedback mechanism. To accomplish this, pathogen populations must be present in levels that do not undermine the biological capacity of soils to regulate the disease. There are five primary components to this approach: 1) prevent the introduction of *Verticillium* spp. into fields. The use of clean seed and transplants. Introduction of *Verticillium* species through infected spinach seed lots continues to be problematic because of the long-term survival of *V. dahliae* microsclerotia in soil; 2) when pathogens are present in numbers high enough to cause significant losses, soil disinfestation using chemical fumigants or other means; 3) integrate beneficial crop rotations into long-term land

management plans; 4) make the most of the combined use of organic amendments and crop rotations to create beneficial shifts in soil microbial community composition associated with suppression of Verticillium wilt; and 5) mitigate inoculum build-up and dissemination, including microsclerotia, resulting from tillage of infected plant debris after harvest. This may be accomplished by crop termination treatments immediately after harvest using a material that prevents both inoculum formation on infected plants and reduces viable pathogen propagules in the root zone.

To implement and achieve our broader goal of integrated approaches to replace methyl bromide in crop production, we focused on two broader objectives: i) perform greenhouse experiments to determine the effects of a chitin-based blend of crustacean shell (formally known as RootGuard, Agricultural Solutions, Plant City, FL)) and broccoli crop residue on soil microbial community dynamics and Verticillium wilt suppression, and ii) to evaluate crop termination treatment using allyl isothiocyanate (AITC, a synthetic oil-based biopesticide) on severely infected lettuce in the field.

Methods

The effects of soil amendments on Verticillium wilt and associated microbiome dynamics were studied using multiple greenhouse experiments. The experiments were conducted using soil with three distinct land use histories- intense production agriculture (conventional soil-CS), transition from pasture to agriculture (organic transition soil-OTS), and native, undisturbed soil (nonagricultural soil-NAS), all collected from Salinas, California. The amendments tested were: broccoli residue applied at 10% w/w, a chitin-based blend of crustacean shell applied at 0.2% w/w, a combination of broccoli and crustacean shell, and an unamended control.

Our previous greenhouse studies on two soil types- Soil A (MS count 27.5/g) and Soil B (MS count 1.2/g) collected from grower fields under strawberry-lettuce-lettuce-lettuce rotations in Salinas, and amended with crabmeal, broccoli, and broccoli plus crabmeal, indicated uniquely enriched microbial community differences (Fig. 1A). In addition, differentially abundant OTUs were also detected between Soil A versus Soil B samples in the different treatment combinations (Fig. 1B). In Soil A, all treatments significantly reduced disease incidence relative to the control treatment but in Soil B, only broccoli and broccoli plus crabmeal treatments resulted in significant reductions in disease severity (Fig. 3C). The soil amendments significantly reduced soil microsclerotia count in soil A, but the number increased significantly in soil B in crabmeal application (Fig 1D).

These promising responses of soil microbiomes augmented with soil amendments for disease suppression prompted the current studies. The three soil types collected are more broadly representative of soil variation in agriculture systems practiced in coastal California that also included an uncultivated control. In addition to unique land use patterns, soils had varied concentrations of *Verticillium dahliae* soil microsclerotia as well. Complete treatment details are given in Table 1. The bulk soil required for all repeat experiments were collected once and stored until used. Native soilborne *V. dahliae* microsclerotia (MS) levels were determined using an Anderson sampler technique and a semi-selective growth medium. Soil was processed for DNA extraction and microbiome sequencing to study the baseline microbial populations prior to the introduction of soil amendments.

The non-agricultural soil had no microsclerotia, thus artificially grown MS were introduced for a final concentration of 100 MS/g soil.

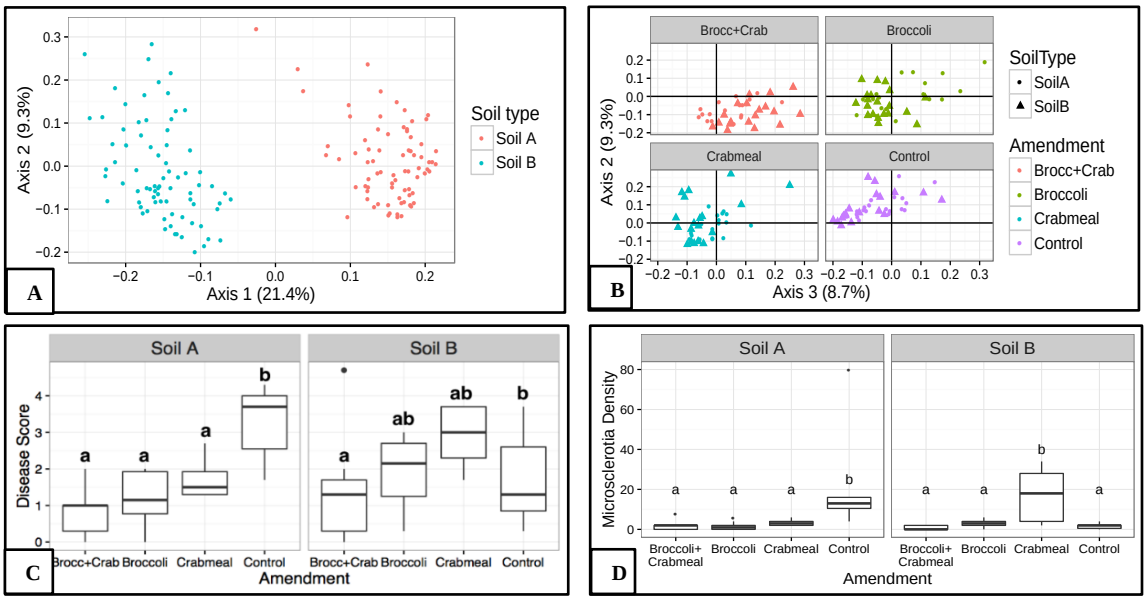

Fig 1. Effect of soil amendments in reducing soil microsclerotia dynamics, disease suppressiveness and microbial community richness after integrated approaches of Verticillium wilt disease management in the greenhouse. A) principal coordinate analysis (PCoA) of the microbial diversity of two major soil types delineated microbial community differences B) PCoA illustrated a shift in population structure between non-amended and amended soils C) Boxplots showing Verticillium disease scores of plants grown in two soil types (A and B) D) Boxplots showing microsclerotia dynamics in response to soil amendments in both soil types. The same letters above the bar represent no significant difference on treatment effect ($p < 0.05$).

Thus the greenhouse experiments included 12 treatments in total (three soil types x four treatment combinations: broccoli, RootGuard, a combination of broccoli and RootGuard, and unamended controls) (Table 1). Two independent experiments (Exp. 1 and Exp. 2), and an additional two repeats of each (Exp1R1, Exp1R2 or Exp2R1, Exp2R2) were conducted (Fig 2). Approximately two months after transplanting, plants were uprooted, washed and evaluated for disease symptoms. The remaining soil was carefully removed and stored for the next experiment. Soils from all replications of each treatment were pooled and allowed to air-dry before re-introduction of soil amendments as in the first experiment (Table 1).

All greenhouse experiments were set up in one-gallon plastic pots. Soil amendments and perlite were mixed using a cement mixer before pot fill, and the mixer was cleaned between treatments. Pots were then arranged in a randomized complete block design (RCBD) with six replications for the first round, and five and three replications in repeat 1 and repeat 2, respectively. The pots were watered day until eggplant transplanting to facilitate proper decomposition of amendments. The chitin source (RootGuard) consisted of crustacean shell @ 0.2% w/w and was applied according to the manufacturer's instructions. Broccoli leaves were collected form mature plants in the commercial fields, cut into small pieces (~0.5-1 cm long) with a sterile knife and mixed proportionally (10% w/w) with soil. Each pot contained approximately 5.8 kg soil and 225 g perlite. Three twenty-one-day-old seedlings of susceptible eggplant variety

Long Purple were transplanted five weeks after soil treatments in each pot, except for pots left as no-plant controls.

Table 1. Information on soil types and amendment treatment combinations applied in the greenhouse experiments.

Soil type	Amendments	Treatments combination
Conventional Soil (CS) (intense production agriculture)	0.2 % RootGuard (RG)	CS + RootGuard
	10% Broccoli (Br)	CS + Broccoli
	Combination (0.2% RootGuard+ 10% Broccoli)	CS + Combination
	Un-amended soils	CS + no amendment
Organic transitional Soil (OTS) (transition from pasture to agriculture)	0.2 % RootGuard (RG)	OS + RootGuard
	10% Broccoli (Br)	OS + Broccoli
	Combination (0.2% RootGuard+ 10% Broccoli)	OS + Combination
	Un-amended soils	OS + no amendment
Nonagricultural Soil (NAS) (native, undisturbed soil)	0.2 % RootGuard (RG)	NAS + RootGuard
	10% Broccoli (Br)	NAS + Broccoli
	Combination (0.2% RootGuard+ 10% Broccoli)	NAS + Combination
	Un-amended soils	NAS + no amendment

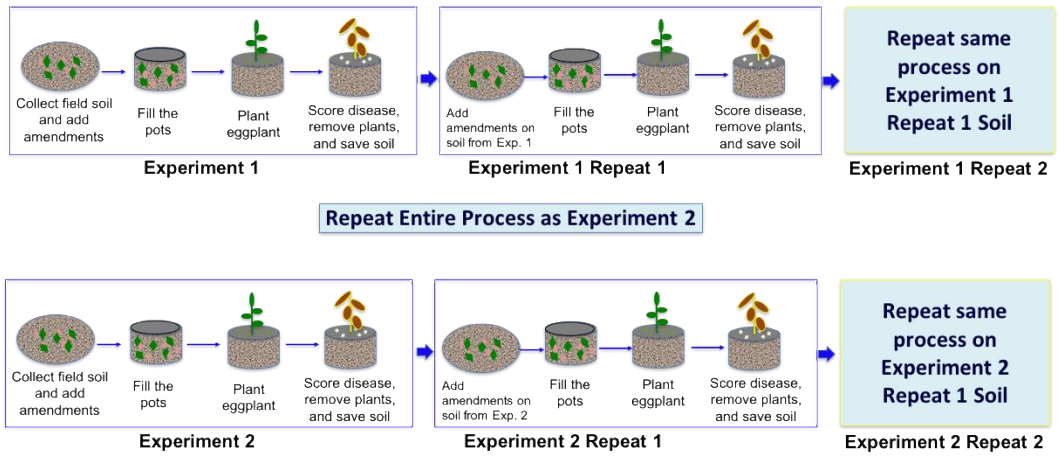

Fig 2. A generalized scheme for greenhouse experiments.

Soil samples were collected four times during each experiment: the day after soil amendment, at the time of transplanting, at one month after transplanting, and at the time of disease scoring. Soil collected at each time point were processed for microsclerotia count, chitinase enzyme assay and DNA extraction for both fungal and bacterial microbiome sequencing. Samples taken at the

beginning and end of the experiment (pot fill and harvest time) were further used to analyze soil physical and chemical properties (Table 2). For disease severity scoring, plants were carefully uprooted and washed. The tap root was sectioned vertically and scored based on degree of foliar symptoms and vascular tissue discoloration on a 0 to 5 scale (0 = no vascular discoloration; 1=1 to 25, 2 = 26 to 50; 3 = 51 to 75, and 4 = 76 to 100% of vascular tissues in the tap root exhibited discoloration in the absence of foliar symptoms; and 5 = 100% of vascular tissues in the tap root exhibited discoloration extending into the crown and the presence of foliar symptoms typical of Verticillium wilt). Taproot and crown tissues of inoculated and non-inoculated plants were sampled randomly and placed on NP10 medium to confirm the presence or absence of the pathogen. The mass of each washed, uprooted plant was recorded (including any fruit) along with the height of each plant, measured from the crown to the growing tip.

Total genomic DNA was extracted from soil samples using PowerSoil® DNA Isolation Kits (MO BIO Laboratories, Inc, CA). DNA was purified, quantified and diluted to 5 ng/µl for library preparation. The V3 and V4 region of the 16s gene was sequenced to assess bacterial communities, and ITS1 and ITS2 primers were used for fungal community analysis. Library preparation and indexing was done using a Nextera XT Index kit following the standard protocol developed by Illumina (Illumina, Inc, CA, Part # 15044223 Rev. B). Paired-end (2x300bp) sequencing of samples was done on an Illumina MiSeq. Quality of filtered and de-multiplexed reads were analyzed using the bioinformatics pipeline Qiime and R packages (DADA2). The sequence data was further analyzed using principal coordinate analyses to visualize broad differences in microbial communities.

A chitinase enzyme activity assay was also performed for select soil samples. The b-glucosaminidase enzyme, which hydrolyses the N-deacetylated chitin derivative found in cell walls of phytopathogenic fungi was measured using acetate buffer at the optimal pH 5.5.

The field study was conducted in grower fields with a history of strawberry/lettuce rotations with recurrent epidemics of Verticillium wilt in Salinas. Prior to initiating a systems-based approach, the incidence of Verticillium wilt ranged from 70 to 100% in the standing lettuce crop. The termination of the infected lettuce crop was accomplished by application of allyl-isothiocyanate (AITC, Dominus, ISAGRO USA, Morrisville, NC) applied at 155 kg ha^{-1} through the drip irrigation system. AITC occurs naturally in broccoli and brussels sprouts, two crops common to coastal California and associated with suppression of Verticillium wilt. Since endemic soil microbial communities have been exposed to AITC for decades from decomposing crucifer crops they have had ample time to adapt it as a carbon source. The experiment used a complete randomized block design with four replications within each AITC treated and untreated plots (Fig. 3). Prior to application, the field was left fallow for approximately eight months due to heavy rain. Before transplanting of successive broccoli crops, the entire field was augmented with chitin-rich crustacean shell @ 1.1 t ha^{-1} incorporated to a 12 cm depth. One additional broccoli crop will be planted in the field followed by crisphead lettuce for the next rotation. Soil samples were taken before and after each treatment application, and the process will be continued until the end of the experiment. Soil samples are being/will be processed to monitor density of *V. dahliae* microsclerotia (MS) in soil, and used for microbiome sequencing to study microbial community dynamics, similar to the greenhouse experiment.

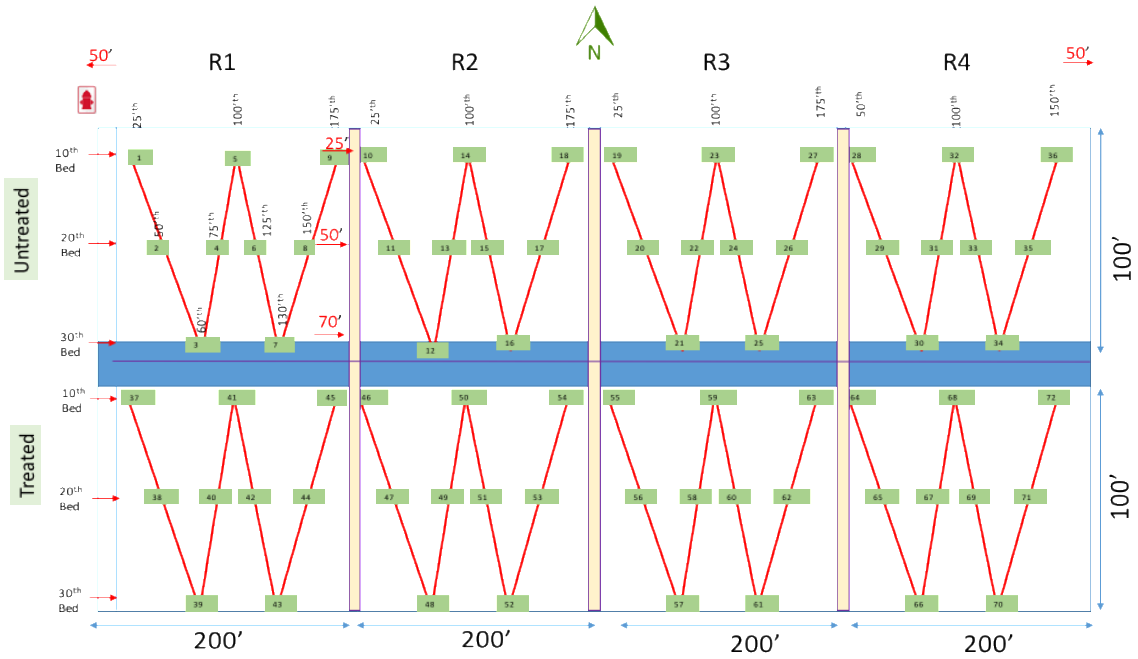

Fig 3. Schematic layout of the field experiment. The field was marked into two 800 x 100 sq ft plots and each plot divided into four replications (200 x 100 sq ft). One plot received AITC as a crop termination treatment while the other remain untreated. A chitin-source was applied on both plots before transplanting the broccoli crop after lettuce crop termination. Soil sampling was/will be done at each green block and processed for microsclerotia count and DNA extraction at the beginning and end of each crop rotation, and before and after treatment applications.

Results and Discussion

Soil chemical and physical property analyses indicated highly variable soil attributes among three soil types. The Organic Transition Soil (OTS) and Non-agricultural (NAS) soil were sandy soil with at least 82% sand, whereas the conventional soil was clay loam with approximately 40% clay, 23% sand and 37% silt (Table 2). The NAS and OTS were acidic (pH 5.7 and 6.7, respectively), whereas the conventional soil was slightly basic (pH 7.6). The NAS was low in all macro- and micronutrients. The OTS had high nitrate N content but lower organic matter content than the CS soil. Incorporation of amendments did not change soil structure but adjusted soil pH towards neutral, except in the NAS soil. In general, chitin amendments increased both ammonium and nitrate N significantly in soil compared to the broccoli amendments. The broccoli amendment significantly contributed to Olsen P, organic matter, total carbon, micronutrients, and improved cation exchange capacity (CEC) compared to the chitin amendment (Table 2).

Table 2. Physical and chemical properties analysis of soils used in greenhouse experiment at the time of field collection and one months after amendment incorporation

Treatments	NH4-N (ppm)	NO3-N (ppm)	Olsen-P (ppm)	Total C (%)	Total N (%)	Total OC (%)	Clay (%)	Sand (%)	Silt (%)	OM (%)	pH	Total Cu (ppm)	Total Fe (ppm)	Total Mn (ppm)	Total Zn (ppm)	CEC (meq /100g)
NAS Original	5.9	3.5	7.5	1.1	0.1	1.1	4.5	92.0	3.5	2.0	5.6	54.0	6435	136.0	32.0	7.6
NAS NoAmd	6.8	5.7	7.3	1.0	0.1	1.0	4.5	90.0	5.5	1.8	6.0	6.0	4765	86.0	20.5	7.7
NAS Broccoli	21.7	0.7	26.1	1.2	0.1	1.2	4.0	89.0	7.0	2.4	5.7	5.5	4345	77.0	18.5	8.3

NAS RootGuard	35.1	5.0	8.6	1.0	0.1	1.0	4.0	90.5	5.5	1.8	6.2	7.0	5025	85.0	22.0	7.3
NAS Broccoli + RootGuard	27.0	0.8	28.4	1.4	0.1	1.3	4.5	89.5	6.0	2.6	6.1	5.5	4470	81.0	20.0	7.5
CS Original	9.7	51.7	55.0	1.5	0.2	1.5	40.0	23.0	37.0	3.1	7.4	52.5	31730	530.0	111.0	38.3
CS NoAmd	5.7	70.9	53.7	1.4	0.1	1.4	38.0	24.0	38.0	3.1	7.5	28.0	26900	467.5	86.0	36.9
CS Broccoli	6.2	48.1	98.0	2.3	0.2	2.2	37.5	28.5	34.0	4.7	7.0	30.5	27820	472.0	89.5	36.5
CS RootGuard	13.9	64.4	57.3	1.4	0.2	1.5	36.0	28.0	36.0	3.0	7.6	31.5	28140	477.5	91.5	36.5
CS Broccoli + RootGuard	11.7	56.4	72.9	1.9	0.2	1.8	39.0	25.5	35.5	3.8	7.3	31.5	27410	482.0	91.5	36.7
OTS Original	2.4	121.3	15.3	1.0	0.1	1.0	7.0	86.0	7.0	2.0	6.7	24.5	7160.0	264.0	44.0	11.3
OTS NoAmd	2.6	85.7	14.9	0.9	0.1	0.9	7.0	84.5	8.5	1.6	7.0	14.5	6405	252.0	42.0	10.6
OTS Broccoli	28.7	37.7	31.0	1.1	0.1	1.1	6.5	85.0	8.5	2.1	7.0	31.0	7500	273.5	44.0	10.5
OTS RootGuard	20.1	80.9	16.3	0.9	0.1	0.8	6.0	87.0	7.0	1.5	7.3	44.0	6160	244.0	56.5	9.6
OTS Broccoli + RootGuard	23.4	34.1	59.9	1.4	0.1	1.4	7.5	82.5	10.0	2.7	6.9	13.5	6605	254.5	43.0	11.3

Combined analyses of variance (ANOVA) of Verticillium wilt disease severity on eggplant in greenhouse experimental data (Experiment 1 and Experiment 2) showed no significant interaction between soil types and amendments (p = 0.176). The disease severity varied significantly among the soil types, and between soil amendments (Fig. 4). The nonagricultural soil (NAS) had the highest disease severity compared to organic transition soil (OTS) or conventional soil (CS) (Fig. 4A). Such variation in disease severity is likely due to the initial density of *Verticillium* microsclerotia in different the three soil types (NAS=100, CS= 20, and OTS=15). We hypothesized a gradual reduction of disease severity and microsclerotia count during repeat experiments. Some amendment treatments resulted in a significant reduction in Verticillium wilt disease severity on eggplant (p <.0001). The broccoli amendment alone or in combination with RootGuard significantly reduced disease severity compared to RootGuard alone or the unamended control (Fig. 4B). Preliminary data analysis has indicated that treatment affects may be influenced by soil properties (data not shown). This work is still in progress and the results obtained will be included in future reports.

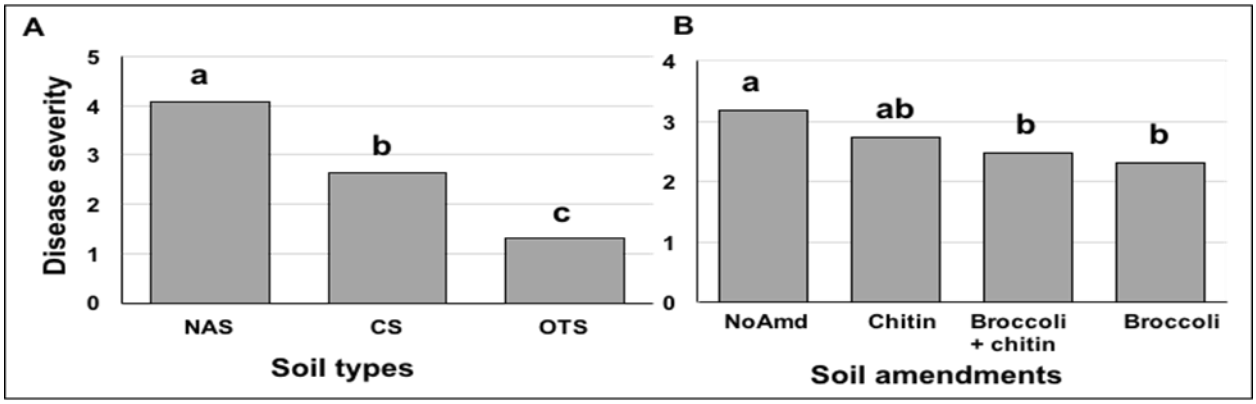

Fig. 4. Effect of soil types (NAS= nonagricultural soil, CS= conventional soil, and OTS= organic transition soils) and soil amendments (NoAmd= no amendments applied, and amendments) on suppression of Verticillium wilt of eggplant in the greenhouse. A) Bar plots showing average disease severity among three soil types; B) bar graphs showing average disease severity among amendments. Identical letters above the vertical bars indicate no significant difference

between treatments based on the least significant difference (LSD) test. Values indicate average disease severity scores combined over Experiments 1 and 2.

Initial microbial community analysis of soil collected one month after soil amendment incorporation indicated a highly significant community enrichment among the three soil types. The principal coordinate analysis (PCoA) grouped communities based on soil type and amendments applied (Fig. 5). Soil type itself, and soil amendments had a unique influence on microbial communities. Soils amended with broccoli alone or broccoli and chitin were associated with unique microbial community enrichment, compared to chitin-amended and unamended control (Fig 5). The soil microbiome DNA sequencing and community analyses of each experiment in various time points are in progress, and the results obtained will be included in future reports. Our studies demonstrate that providing appropriate organic inputs into the soil will enhance a certain microbial community that may contain beneficial agents that promote soil health and suppress soilborne diseases.

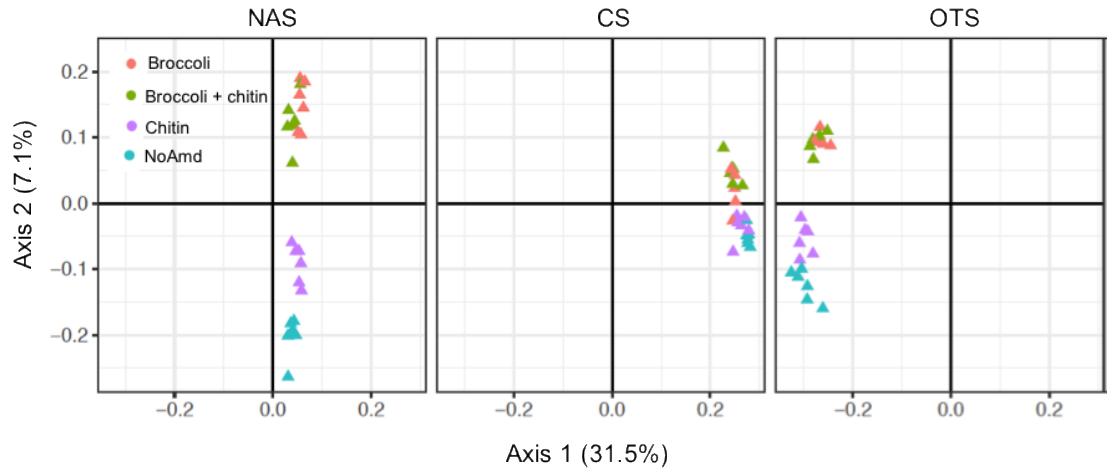

Fig 5. A representative soil microbial community richness analysis in three soil types (NAS= nonagricultural soil, CS= conventional soil, and OTS= organic transition soils) and in response to incorporation of soil amendments (NoAmd= no amendments applied, and amendments) under greenhouse conditions in soil samples collected one month after amendment incorporation. The Principal coordinate analysis delineates microbial diversity by soil type on axis 1 and by amendments in axis 2.

The schematic diagram presented in Fig. 6 represents a template for implementing a systems-based approach in field experiments. Over the 3-year cropping cycle depicted, one strawberry crop and two lettuce crops will be cultivated. However, adjustments to the system may be made based on the level of soil infestation by *V. dahliae* microsclerotia. For example, cropping cycles can be extended if pathogen populations do not decline sufficiently. Alternatively, crop termination applications and/or organic amendments can be omitted if pathogen populations decline below detectable thresholds. A version of this systems-based approach is being evaluated on commercial farms in the Salinas Valley.

The soil microsclerotia density analysis of *Verticillium dahliae* collected from the field site indicated that MS densities varied within the field and were not uniformly distributed. Considerably higher numbers of MS were found in the plot selected for the AITC application relative to the plot selected for the control (Fig 7A). The tillage operation, and the inoculum derived from infected lettuce plants contributed to a significant increase in microsclerotia in the

field (Fig. 7B). While control plots without the AITC treatments had a 1250% increase in the number of microsclerotia per gram soil post-lettuce crop, a 338% increase was observed in the AITC-treated plots (Fig. 7C). Regardless, the crop termination treatment with AITC did not reduce microsclerotia populations (Fig. 7C), the reasons for which are unclear at this time. It is possible that lettuce is a poor host for the uptake of AITC and thus, the chemical may have had little effect on inoculum production on infected lettuce plants. Due to the ineffectiveness of AITC as a crop termination treatment in reducing the inoculum returned to the soil, we have adjusted the system plans and will include two successive broccoli planting following amendments with chitin, as stated above.

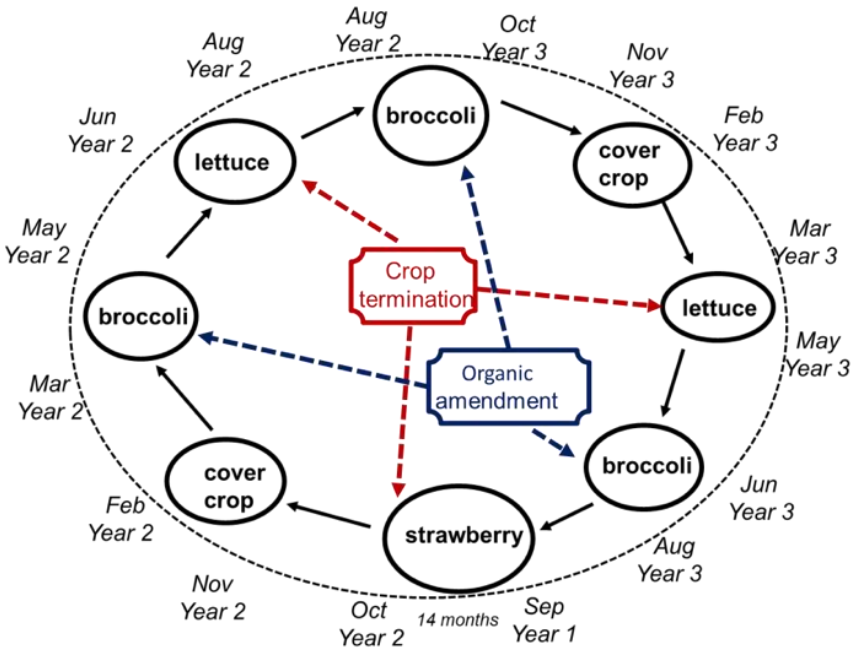

Fig. 6. Framework for systems-based management of Verticillium wilt in the coastal valleys of central California.

Objective B. Determine the potential of host-directed evolution of *Verticillium dahliae* from a single race 1 genotype.

Verticillium dahliae is a destructive soilborne pathogen that causes Verticillium wilt on many economically important crops including artichoke, cotton, lettuce, peppers, strawberry and tomato. The resting structures produced by the fungus (microsclerotia) can remain viable in soil for more than 14 years in the absence of susceptible host tissue. Thus, in the absence of soil fumigants like methyl bromide and chloropicrin, yield losses can reach up to 100% for lettuce crops. Regardless of the Verticillium wilt management technique employed, the inoculum can quickly rebound with repeated planting of the susceptible host. The deployment of effective host resistance will be the most effective and economically and environmentally sustainable option for controlling Verticillium wilt in lettuce. Thus, understanding pathogen evolution over time is vital for plant breeding and deployment of host resistance. Deployment of major resistance genes in commercial cultivars and sustaining their efficacy over time under agricultural conditions is a significant challenge. Generally, plant disease resistance governed by a single major gene has a

higher chance of being compromised than resistance controlled by multiple genes. Widespread deployment of a resistance gene in a field crop could select for pathogens that compromise deployed resistance leading to evolution of virulent forms of the pathogen. Plant pathogens with a higher mutation/substitution rate, a higher rate of migration/gene flow, larger effective population sizes, and mixed reproductive systems are predicted to have a relatively high evolutionary potential and therefore are able to overcome major resistance genes more quickly than those with lower evolutionary potential. It is therefore useful to understand how pathogen populations evolve, so as to predict the durability of resistance genes over time.

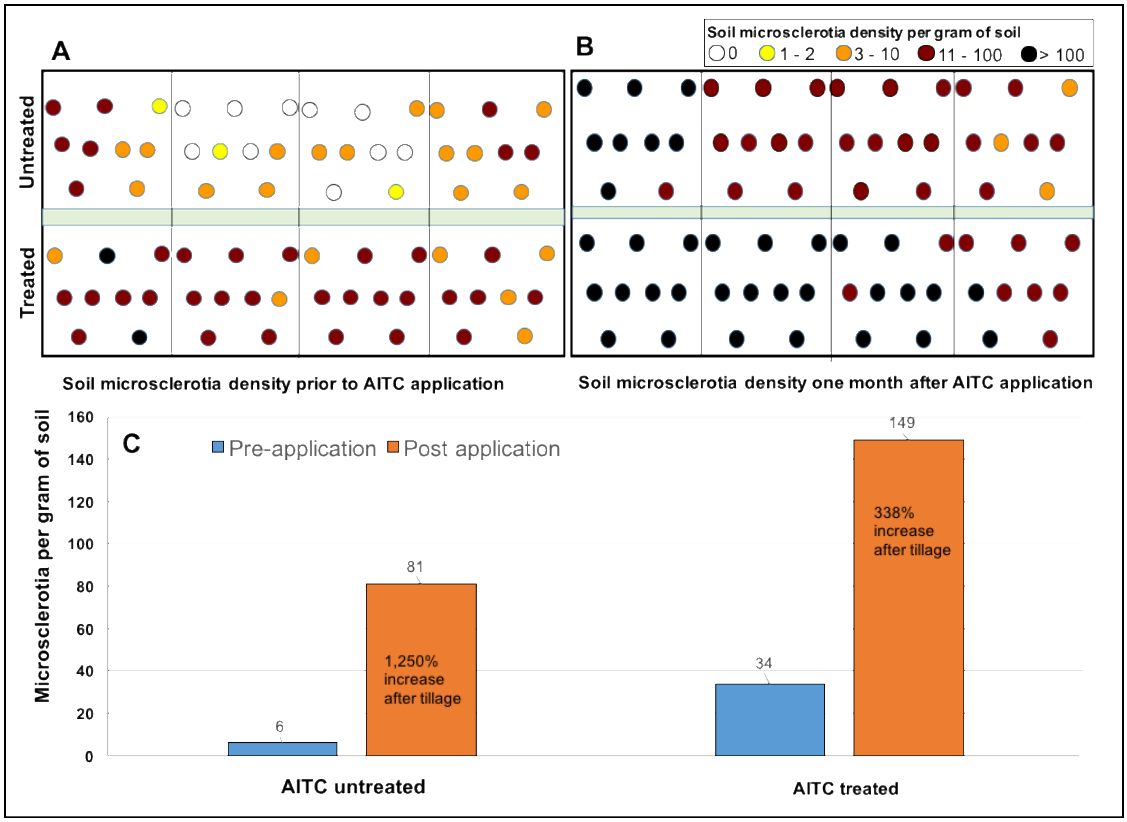

Fig. 7. Dynamics of *Verticillium dahliae* microsclerotia (MS) in soil in response to AITC application after lettuce harvest in a grower filed: A) Field distribution of soil MS before AITC application in the lettuce crop; B) soil MS distribution one month after AITC application following soil tillage operations; C) bar diagram showing average MS difference before and after AITC application and in the untreated plots.

Two pathogenic races, race 1 and 2 have been characterized for *V. dahliae* using differential lettuce and tomato cultivars. The *Verticillium Resistance 1* (*Vr1*) gene locus in lettuce cv. La Brillante confers resistance to *V. dahliae* isolates that secrete the effector *Ave1*. Recently, a new race 3 has been documented in tomato.

In the Salinas valley, *V. dahliae* race 1 is more prevalent and widespread than race 2 in lettuce. Thus, the deployment of lettuce cultivars with *Vr1* will be useful for Verticillium wilt management in lettuce. There is circumstantial evidence that the evolution of race 2 through loss of *Ave1* has occurred multiple times in a multitude of *V. dahliae* lineages, but estimating the evolutionary potential of individual *V. dahliae* genotypes over the time has not been attempted,

and no information is available on the impact of host resistance gene-selective pressure on the evolution of *V. dahliae* races. The resistant host genotypes could eventually select for mutants lacking the avirulence gene, though the time frame in which this would happen in lettuce is unclear.

In this study, we evaluated the effects of a lettuce crop consisting of multiple cultivars and breeding lines varying in race 1 resistance on a monoclonal pathogen population. We investigated whether host-R-gene directed pathogen evolution is a possibility in *V. dahliae* by testing if mutation/loss of the avirulence gene *Ave1* could be detected over the course of a multiyear study. We also compared genotypic variation within and between populations of *V. dahliae* collected from commercial lettuce and tomato fields within California. In addition, genotypic variation in populations of *V. dahliae* isolates collected form lettuce and tomato form Salinas Valley and Yolo county, California were compared.

Methods

A *Verticillium dahliae* race 1 disease nursery (45 x 40m^2) was established in a research field at the USDA Research Station, Salinas, California. The nursery had no previous history of *V. dahliae* infestation and was fumigated with a methyl bromide (67%) and chloropicrin (33%) mixture at 361 kg/ha in 2009. The uniformity of inoculum distribution in the disease nursery soil was established by transplanting *V. dahliae* race 1 inoculated susceptible lettuce cv. Salinas. Briefly, the disease nursery was transplanted with approximately 10,000 lettuce seedlings of cv. Salinas that had previously been inoculated with a *V. dahliae* race 1 isolate- Ls16 in a greenhouse. Inoculated transplants were grown in two rows per 1 m wide beds, which is standard for lettuce production in coastal California. Plant spacing was approximately 28 cm between rows and 28 cm between plants within a row. Plants were grown until maturity and incorporated entirely into the soil by disking and redistributing the soil. The process was repeated in the spring and fall seasons of 2009 and 2010. From 2010 to 2015 the nursery was used for screening lettuce germplasm and breeding lines. Soil inoculum density was maintained at a high level (>100 microsclerotia g^{-1} soil) through inoculum generated in Verticillium wilt-susceptible lines. Each spring and fall, varying numbers of F_1 and F_2 families from crosses between cv. La Brillante (*Vr1*) and crisphead lettuce cultivars or the recombinant inbred line populations derived from cv. Salinas (*vr1*) and La Brillante were evaluated in the disease nursery for resistance to Verticillium wilt. After disease evaluations, roots from arbitrarily selected diseased plants were collected in the spring of 2010, 2011, 2012, 2013, 2014, 2015, and fall of 2013 (a total seven 'populations'). Each year, approximately 100 to 120 symptomatic root samples representing the entire disease nursery were collected and processed for *V. dahliae* isolation. The roots were transported to the laboratory, washed thoroughly to remove any soil, surface disinfested with 0.5% sodium hypochlorite for 3-5 minutes, followed by a 2-3 min rinse with sterilized distilled water. Roots were dried in a laminar flow hood, sliced into ~1 mm cross-sections and plated on the semi-selective NP-10 medium enriched with two antibiotics (Streptomycin sulfate and Chlortetracycline HCl @ 100 ppm each). A small section of the agar with mycelium was transferred to Potato Dextrose Agar (PDA) plates and incubated for an additional week. Conidia were then streaked onto a new PDA plate and a single conidial colony was further transferred onto a new plate. Cultures were then prepared for long term storage in 25% glycerol or for DNA extraction.

Mycelium growing in potato dextrose broth (PDB) was harvested, washed, and lyophilized for 24 hours. The genomic DNA was extracted from lyophilized tissue using a FastDNA Kit and used to characterize species, mating type, and race using previously established molecular protocols. Further, thirteen simple sequence repeat (SSR) markers designed from the genome of *V. dahliae* isolate Ls17 were used for genotypic characterization. Isolates identified as variants of a particular SSR locus were classified as mutants and SSR loci were sequenced to verify mutant alleles.

SSR mutants were tested for functional changes to *Ave1* phenotypically by pathogenicity tests using the lettuce differential cultivars La Brillante (*Vr1*) and Salinas (*vr1*) in the greenhouse. Further, susceptible eggplant cultivar Long Purple was inoculated with mutant isolates in greenhouse assays and disease severity was compared to severity inflicted by the wild type Ls16 isolate. Greenhouse plants were grown in a Styrofoam cups (350 ml) filled with sterilized sand:potting soil mix (2:1 v/v) and inoculated three time using 3 ml of 1×10^7 /ml conidial suspension. The standard *V. dahliae* race 1 isolate Ls16 and water-inoculated plants were used as controls. Disease severity was assessed at 10 weeks after the final inoculation using a standard subjective ordinal rating scale (0-5) based on root discoloration and foliar symptoms.

Population genetic analysis of SSR genotypic data was performed using statistical software POPGENE v1.32, R package for population genetics analysis (*Popper*), NTSYSpc, and GenAlex v6.5.

For field studies, three commercial lettuce fields and one commercial tomato field, all with a history of Verticillium wilt, were sampled. Lettuce roots were collected in 2009 and 2010 from diseased fields in Monterey County, while tomato roots were collected in 2012 from a field in Yolo County, California. A systematic hierarchical sampling method was adopted in all years using three 25×25 m^2 grids approximately 75 m apart in each field. Within a grid, an infected plant was uprooted from every 5-10 m distance and returned to the laboratory for fungus isolation. The sample processing, single spore isolation, DNA extraction, PCR reactions for species composition, race structure, mating type and SSR genotyping were done as described above.

Results and Discussion:

A total of 427 isolates - 63, 33, 37, 96, 73, 31 and 94, were collected in 2010, 2011, 2012, Spring 2013, Fall 2013, 2014, and 2015 from lettuce roots from the disease nursery, respectively. *Verticillium* species-specific PCR identified all recovered isolates as *V. dahliae*. The DNA from all isolates amplified with effector *Ave1*-specific primers, confirming that they were race 1, identical to the original genotype introduced into the field, and had only MAT1-2-1-specific amplicons. This suggests that the resistant *Vr1* locus will likely remain effective against the race 1 populations that are currently commonplace in field soils for at least six years, unless novel pathogen genotypes or races are introduced into the production system.

Genotyping of all 427 isolates from the disease nursery with 13 SSR markers indicated that all SSR loci except four- VD1, VD8, VD10, and VD7, generated monomorphic alleles identical to the original isolate Ls16. Among these four polymorphic SSR loci, two, three, two and two alleles were detected, respectively. In total, 18 alleles were observed at 13 SSR loci, resulting in six distinct multilocus genotypes (MLGs) (Fig. 8). Of these, 420 isolates (98.36%) belonged to a single locus genotype (MLG.1). The MLG.3, 4, and 6 were singletons collected in 2012, 2015, and 2012, respectively. MLG.2 consisted of two isolates from fall 2013, while MLG.5 consisted of two isolates - one from spring 2013 and one from fall 2015 (Fig. 8). As the distinct alleles were

rare and recovered from the third year of sampling onwards, our observations provide direct evidence of active molecular evolution of this soilborne plant pathogen.

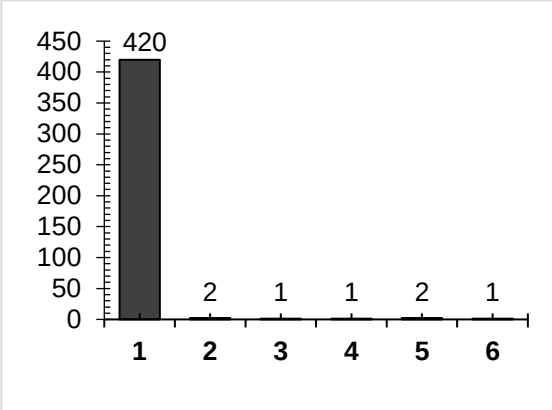

Fig. 8. Histograms of multilocus genotype frequency of *Verticillium dahliae* in race 1 disease nursery.

No significant population differentiation (Φpt= 0.002, P=0.245) was observed among isolate collections from the disease nursery in different years. The standardized index of association (*rBarD*) test further indicated a significant multilocus linkage disequilibrium in the collection of all populations from all years, indicating an extremely clonal population structure in the field nursery (Table 3).

TABLE 3. Analysis of molecular variance (AMOVA) of *Verticillium dahliae* populations sampled from the race 1 disease nursery (N = 427), and commercial lettuce (N = 163), and tomato (N = 43) fields.

Source of variation[a]	**Degrees of freedom**	**Estimated variance**	**% molecular variance**	***PhiPT/PhiPR/PhiRT*[b]**	**Number of migrants (*Nm*)[c]**
Among collection years	6	0.000	1.21	0.012[ns]	40.8
Within collection years	420	0.032	98.79		

[a] Variances were partitioned within and among population based on sampling years in the race 1 disease nursery.

[b] *PhiPT*, *PhiPR*, and *PhiRT* are the distributions of variation among populations, fields, and populations within fields, respectively.

[c] Haploid number of migrant genotypes (N_m) was calculated using $N_m = [(1/\phi_{PT})-1]/2$ using GeneAlex 5.2.

* Significant at P=0.001. [ns] Not significant at P<0.05.

Isolates with at least one allele distinct from the alleles in Ls16 genome were classified as mutant genotypes. Seven isolates, Ls2936, Ls2938, Ls3243, Ls3246, Ls3355, Ls3364, and Ls3662, were identified as mutant genotypes due to either a gain or loss of at least one SSR repeat motif in one of 13 the SSR loci used (Table 4).

TABLE 4. Single locus mutant genotypes of *Verticillium dahliae* isolates sampled from a race 1 disease nursery during 2012-2015

Isolate	Year	Cultivar	Disease score[a]	Race	Mating type	Vd1[b]	Vd73	Vd8	Vd10	Disease severity[c]	
										Salinas	La Brillante
Ls16	**-**	***Salinas***	***5***	***Race 1***	***MAT1-2-1***	***299***	***289***	***401***	***254***	5	0
Ls2936	2012	Honcho II	1.4	Race 1	MAT1-2-1	299	289	398	254	4	0
Ls2938	2012	Green Lightning	2.6	Race 1	MAT1-2-1	303	289	401	254	4	0
Ls3355	2013fall	RH12-1633	3.0	Race 1	MAT1-2-1	299	289	401	250	4	0
Ls3662	2013spring	Salad Crisp	3.0	Race 1	MAT1-2-1	299	295	401	254	4.5	0
Ls3364	2013fall	RH12-1586	3.0	Race 1	MAT1-2-1	299	289	401	250	5	0
Ls3243	2015	Quest MT	5.0	Race 1	MAT1-2-1	299	295	401	254	5	0
Ls3246	2015	Salinas	4.7	Race 1	MAT1-2-1	299	289	371	254	5	0

[a] Disease ratings were assigned at the time of sampling based on vascular discoloration using an ordinal scale (0-5). Scores were an average of 10 plants in a row per cultivar.

[b] SSR locus and alleles (PCR amplicon size).,

[c] Median disease severity from 30 plants across three replications was evaluated at 10 weeks after inoculation based on vascular discoloration using disease score (0-5).

Sequencing the mutant loci revealed a 30 bp deletion (10 copies of motif GAT) in locus VD8 for isolate Ls3246, and a six bp insertion (one copy of motif CGTTGC in VD73) in isolates Ls3243 and Ls3632 relative to the wild type Ls16 (Table 4, Fig. 9), respectively. The remaining four mutants either lost or gained a single motif (Table 4). In addition to SSR variation, three single nucleotide polymorphisms were detected in locus VD73 compared with the reference genome sequence of isolate Ls17 from which primers were designed (Fig. 9).

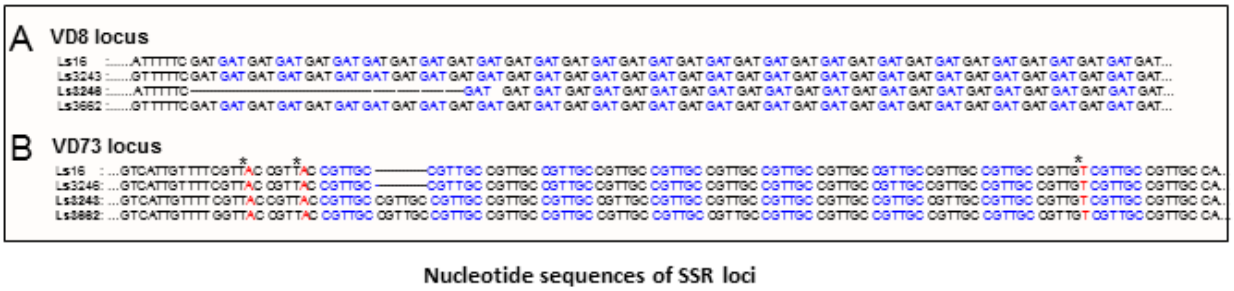

Fig. 9. Nucleotide sequences of SSR loci of a representative mutant isolate. Deletion of 10 copies of the trinucleotide microsatellite GAT in locus VD8 in Ls3246 (A); a gain of the six nucleotide motif CGTTGC in VD73 in isolates Ls3243 and Ls3632 (B) are aligned with sequences of Ls16. The single nucleotide polymorphisms are indicated by an asterisk.

The lettuce plants from which mutant isolates were recovered showed symptoms of outer wilted leaves and vascular taproot discoloration in the disease nursery. The genotype Honcho II had the lowest disease severity (<2) followed by the genotypes Green Lightning, RH12-1633, Salad Crisp, and RH12-1586 with disease rating scores of 2 or 3. The genotypes Quest MT and Salinas were the most susceptible and received the highest disease rating scores of 4 to 5 (Table 4). At least one mutant genotype was recovered from both of these genotypes over the years.

Inoculation of mutant isolates in two host differentials indicated that all mutants were pathogenic on susceptible lettuce cv. Salinas but nonpathogenic to resistant cv. La Brillante (Table 4, Fig. 10A) demonstrating that these mutants still carried a functional *Ave1* homolog. No *V. dahliae* was recovered from symptomless La Brillante taproots indicating a lack of latent infection. Inoculation of the highly susceptible eggplant cultivar 'Long Purple' with isolates Ls16, Ls3243 and Ls3246 all resulted in severe disease symptoms including severe stunting and vascular discoloration in the tap root (Fig. 10B). Thus the lack of significant variation in virulence of the seven mutant isolates relative to the parental isolate further demonstrates that such mutations were not associated with functional changes to *Ave1* or other as yet unknown pathogenicity-related genes. This provides evidence that a single race 1 clonal population would need longer than six years to overcome *Vr1*-mediated resistance through somatic mutations.

For the isolates collected from commercial fields, the analysis of molecular variance indicated no significant differentiation within field populations of either lettuce or tomato. In addition, the nine grid sub-populations and three lettuce field populations had very low genetic variation. Similarly, the tomato grid sub-populations had very low variation (0.12 %). However, a very high genotypic variation (78.62%) existed between lettuce and tomato populations, which were significantly differentiated (*PhiPT* = 0.786, *P* = 0.00. The principal component analysis (PCoA) further delineated tomato and lettuce genotypes into two distinct clusters correlating with the host plant of origin (Fig. 11).

The field population of *Verticillium* from lettuce comprised 163 isolates collected from three fields (Harkins = 32, sampled in 2009) and (Cooper1 = 84 and Cooper2 = 47, sampled in 2010) in Salinas. While *Verticillium* samples from tomato comprised 43 isolates. Isolates from both fields were confirmed to be *V. dahliae* using species-specific PCR. All lettuce isolates and all except one tomato isolate (Le1806) were MAT1-2-1. All field lettuce isolates also amplified with effector-specific *Ave1* PCR, indicating they were race 1, but no tomato isolates amplified with the *Ave1* PCR. Putative race 2 specific primers amplified DNA from the majority of tomato isolates but none from lettuce isolates.

Thus, a strictly monoclonal race 1 population of *V. dahliae* established in a disease nursery was exposed to selection pressure of *Vr1* in different genetic backgrounds and yet, it did not result in the loss of *Ave1*. Consistent with a model of clonal expansion, the field populations of *V. dahliae* in lettuce and tomato were dominated by a single multilocus genotype with a few other rare genotypes, whereas populations were distinct between these hosts. A strong quarantine measure that prevents the direct movement of inoculum or inoculum sources will help maintain the efficacy and durability of race 1 resistance in lettuce. Thus, race 1-resistance lettuce cultivars released for commercial production are likely to remain resistant for at least for 6 years.

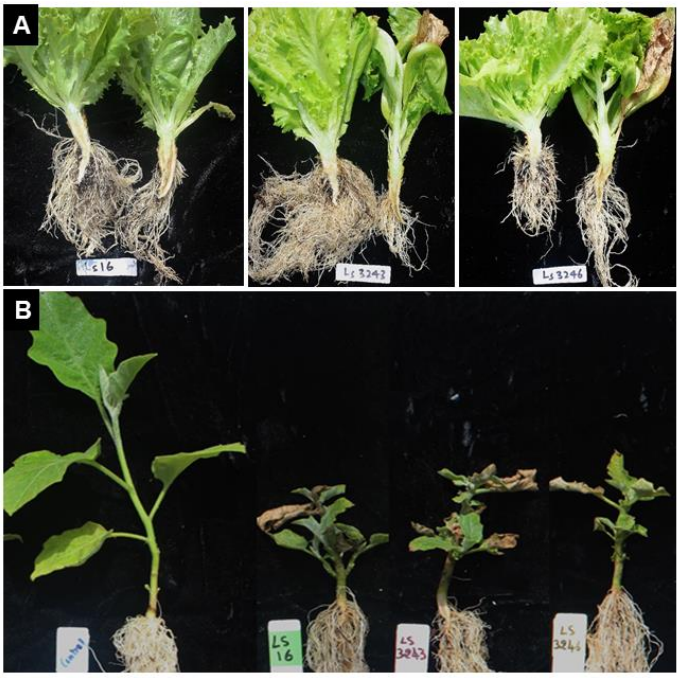

Fig. **10**. Inoculation test of simple sequence repeat (SSR) mutant genotypes in a lettuce differential and a susceptible eggplant cultivar. Two representative mutants Ls3243 and Ls3246 were used to inoculate *V. dahliae* race 1 resistant cv. La Brillante (Top: left photo of each isolate) and susceptible cv. Salinas (Top: right photo of each isolate) cultivar (**A**), and Eggplant cultivar 'Long Purple' (**B**) (Bottom: Left to right: mock, wild-type and mutant inoculated).

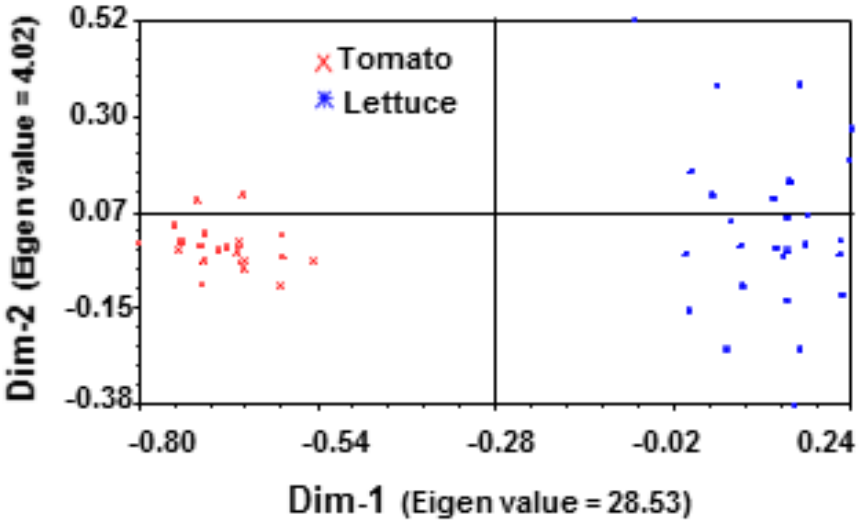

Fig. 11. Principal component analysis of *V. dahliae* isolates collected from lettuce and tomato. Fifty-four alleles generated by thirteen SSR markers were analyzed using NTSYSpc on a similarity matrix using DECENTER and EIGEN functions.