REPORT

CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LEAFY VEGETABLES

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ABSTRACT:

The loss of methyl bromide as a soil fumigant, the limited efficacy of alternative fumigants and the emergence of new diseases, has necessitated fumigant-based crop production systems to radically restructure for continued productivity. In the coastal valleys of central California, the principal objective of soil fumigation has been to confine *Verticillium dahliae* population densities to levels below the threshold at which yield losses occur. The ability to harness native soil microbiomes to manage plant pathogens remains a promising but relatively unexplored area. Our studies focused on substrate-mediated changes in soil prokaryote communities with the goal to convert Verticillium wilt-conducive soils to Verticillium wilt-suppressive soils. The effect of broccoli residue and crustacean meal on soil microbiomes and suppression of Verticillium wilt were studied in soils with distinct land-use histories: intense agriculture, transition from pasture to agriculture, and native, undisturbed soil. Regardless of the soil type, the organic inputs significantly reduced *V. dahliae* microsclerotia and wilt incidence and severity in the greenhouse, and these reductions were correlated with an increase in the proportion of known fungal antagonists. Prokaryote communities identified were distinct in the wilt-suppressive soils as compared to the wilt-conducive soils. Three cycles of inputs nearly eliminated the pathogen in all three soil types. This approach was validated in a 12.2 ha commercial lettuce field in Salinas Valley with a known history of severe Verticillium wilt. After an 18-month broccoli-Romaine lettuce-broccoli rotation including amending the soil with crustacean meal before each crop, significant reductions in *V. dahliae* microsclerotia density were achieved. These studies demonstrated the potential for harnessing native soil microbiomes to manage plant pathogens using specific carbon

sources that favor fungal antagonists. We also studied the potential existence of race 3 among *V. dahliae* isolates from lettuce, and further confirmation of this are currently underway.

OBJECTIVES:

A. Determine the efficacy of non-chemical alternatives to methyl bromide on Verticillium wilt in greenhouse experiments.
B. Determine the effects of chitin amendment, and broccoli rotation on Verticillium wilt in lettuce in the field.
C. Greenhouse experiments with lettuce differentials for investigating a possible *Verticillium dahliae* race 3 in lettuce.
D. Greenhouse experiments to map gene(s) governing resistance to the possible new race of ***Verticillium dahliae***.

PROCEDURES:

Objective A. Determine the efficacy of non-chemical alternatives to methyl bromide on Verticillium wilt in greenhouse experiments.

Greenhouse experiments were conducted to determine the effects of a crustacean meal (Crab Life-Flake, Agricultural Solutions LLC, Safety Harbor, Florida, USA) and broccoli residue on soil microbial community dynamics and Verticillium wilt suppression. Amendments were evaluated in soils obtained from Salinas Valley, California with distinct land-use histories: intense production agriculture (Soil-A), native, undisturbed soil (Soil-B), and transition from pasture to agriculture (Soil C). Broccoli residue was incorporated at 10% w/w of dry soil, crustacean meal at 0.2% w/w of dry soil, and a combination of broccoli and crustacean meal with unamended soil as a control (Table 1). In addition to unique land-use patterns, these soils had a varied number of *Verticillium dahliae* soil microsclerotia (MS) as determined using the Anderson sampler technique to deposit soil on the NP-10 semi-selective medium. Soils were processed for DNA extraction and microbiome sequencing to determine baseline microbial populations prior to the introduction of soil amendments. The non-agricultural soil had no microsclerotia; thus, artificially grown MS in the laboratory were introduced for a final concentration of approximately 100 MS/g soil.

The greenhouse experiment included 12 treatments (three soil types x four amendment combinations: broccoli, crustacean meal, broccoli combined with crustacean meal, and an unamended control (Table 1). Two independent experiments (Exp. 1 Cycle 1 and Exp. 2 Cycle 1) and two additional repeats using leftover soil from each cycle - Exp. 1 Cycle 2, Exp. 1 Cycle 3; and Exp. 2 Cycle 1, Exp. 2 Cycle 2) were performed (Fig. 1). 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 the re-introduction of soil amendments.

All greenhouse experiments were set up in one-gallon plastic pots. Soil, amendments, and perlite were mixed and placed into pots arranged in a randomized complete block design (RCBD) with six, five, and three replications in cycles 1, 2 and 3, respectively. In each pot, three 21-day-old eggplant seedlings were transplanted after one month. Pots were irrigated every day until

transplanting to facilitate amendment decomposition. Each pot contained approximately 5.8 kg of soil and 225 g of perlite. Three 21-day-old seedlings of the susceptible eggplant variety 'Long Purple' were transplanted five weeks after soil treatments in each pot. Since eggplant is the most susceptible host for *V. dahliae*, treatments that work on this host are likely to work even better on other hosts including lettuce. Two pots in each treatment were included as unplanted controls.

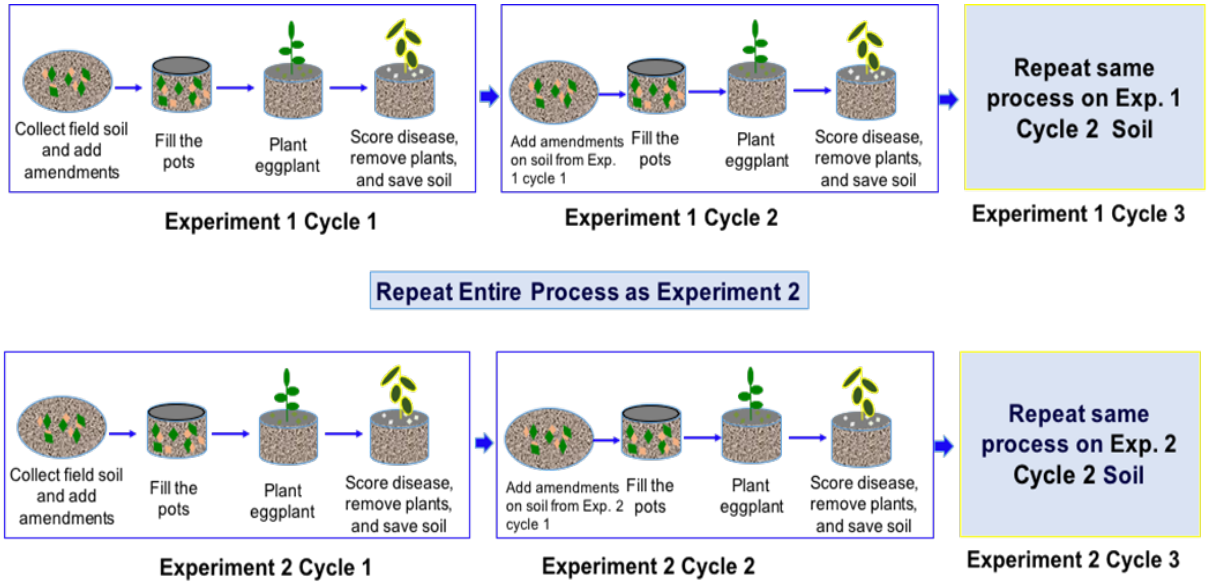

Fig. 1. Schematic of the greenhouse experiments.

Soil sampling was done four times during each experiment cycle: the day after the incorporation of the amendment, at the time of transplanting, one month after transplanting, and at the final disease rating. Soil collected at each time point was processed for microsclerotia count, chitinase activity, and DNA extraction for both fungal and bacterial microbiome sequencing. For disease severity scoring, plants were carefully uprooted and washed. Each root was sectioned vertically and scored based on the 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 discoloration in the absence of foliar symptoms; and 5 = 100% of vascular tissues in the taproot exhibited discoloration extending into the crown and the presence of foliar symptoms). The entire plant biomass (including fruit), plant height from the soil line to growing tip, and the number of fruits were counted.

Total genomic DNA was extracted from 0.25 g soil 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. The ITS1 and ITS2 primers were used for fungal community analysis. Library preparation and indexing were done using a Nextera XT IndexKkit following the standard protocol developed by Illumina (Illumina, Inc, CA, Part # 15044223 Rev. B). Paired-end (2x300bp) sequencing was done on Illumina MiSeq. The amplicon library and sequencing of the V3/V4 region of the 16S ribosomal RNA gene were performed using an Illumina workflow. The sequence analysis and ordination

were performed using various R packages (DADA2, phyloseq) and Quantitative Insights into Microbial Ecology (Qiime).

A chitinase enzyme activity assay was also performed on selected 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 of 5.5.

Objective B. Determine the effects of chitin amendment and broccoli rotation on Verticillium wilt in lettuce in the field.

A field trial was established in a commercial lettuce field with a known history of severe Verticillium wilt located in Salinas Valley. A timeline of activities performed in the fields is presented in Table 3. Prior to initiating the treatments, the incidence of Verticillium wilt in a mature crisphead lettuce crop was recorded. A section of the infected lettuce crop was terminated (sooner than normal drying of plants to prevent microsclerotial formation on infected lettuce) with allyl-isothiocyanate (AITC, Dominus, ISAGRO USA, Morrisville, NC) at 155 kg ha^{-1} applied through the drip irrigation system. Allyl-isothiocyanate (AITC) occurs naturally in broccoli and brussels sprouts, two crops common to coastal California. However, the amounts returned to the soil are less than 10 kg ha^{-1}, whereas the amount registered for application is well above 224.2 kg ha^{-1}. Since endemic soil microbial communities have been exposed to AITC for decades from decomposing crucifer crops, they have had ample time to adapt to the chemical as a carbon source.

A total of 72 soil samples (18 per reps) were collected following standard sampling method prior to AITC application and approximately one month after AITC application for *V. dahliae* microsclerotia counts and DNA extraction for microbial community analysis. After the harvest of the first iceberg lettuce crop and AITC application, the field was left fallow for approximately eight months due to heavy rain and flooding. The field was then prepared for transplanting of broccoli as the second crop in the sequence. At the time of land preparation, the entire field was amended with crustacean meal at 336 kg ha^{-1} incorporated to a depth of 12 cm. Soil sampling was done two days after chitin amendment as described earlier and used to monitor chitinase enzyme activity, Verticillium microsclerotia count, and DNA extraction.

The cropping sequence consisted of iceberg lettuce- broccoli- Romaine lettuce- broccoli. Following broccoli harvest, the remaining broccoli biomass was incorporated into the soil through disking to facilitate tissue decomposition. One month after the incorporation of the residue, the soil was re-amended with chitin material and prepared for a Romaine lettuce crop. Soil samples were taken before lettuce transplanting for chitinase enzyme activity analysis, soil microsclerotia count, and DNA extraction as before.

Romaine lettuce was transplanted during the fall season after the broccoli crop was disked in and additional crustacean meal was applied. Soil samples were again collected at the beginning and end of the cropping season, and have been processed for microsclerotia density, microbial community structure, and chitinase enzyme assay. Data analysis is currently in progress. The disease incidence and severity on lettuce was also recorded just prior to harvest by uprooting 25 plants in each replication.

Results

Objective 1: Greenhouse study

Soil chemical and physical properties analyses indicated highly variable soil attributes among the three soil types. Soil in transition from pasture to the agricultural soil (Soil C) and native undisturbed soil (Soil B) were sandy soils with at least 82% sand, whereas the soil under intense

agriculture (Soil A) was clay loam with approximately 40% clay, 23% sand, and 37% silt (Table 2). Soils B and C were acidic (pH 5.7 and 6.7, respectively), whereas Soil A was slightly basic (pH 7.6). Soil B was low in all macro- and micronutrients. Soil C had high nitrate N content but lower organic matter and micronutrients than Soil A. Incorporation of amendments did not change soil structure but adjusted soil pH towards neutral, except in Soil B. In general, chitin amendments increased both ammonium and nitrate N significantly in soil compared to the broccoli amendments. The broccoli amendment significantly contributed to higher Olsen P, organic matter, total carbon, micronutrients, and improved cation exchange capacity (CEC) compared to the chitin (crustacean meal) amendment (Table 2).

Combined analyses of variance (ANOVA) of Verticillium wilt disease severity on eggplant from the first cycle of Exp. 1 and Exp. 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. 2). The native undisturbed soil- (Soil B) had the highest disease severity compared to the soil under intense production agriculture- (Soil A) and soil in the transition from pasture to agriculture (Soil C) (Fig. 2A). Such variation in disease severity likely occurred due to the initial density of *V. dahliae* microsclerotia in different soil types (Soil A = 20, Soil B=100, and Soil C=15). We hypothesized a gradual reduction of disease severity and microsclerotia count during repeat experiments. Soil amendments resulted in a significant reduction in Verticillium wilt disease severity on eggplant (P < 0001). The broccoli amendment alone or in combination with crab meal reduced disease severity significantly compared to crab meal alone or unamended control (Fig. 2B).

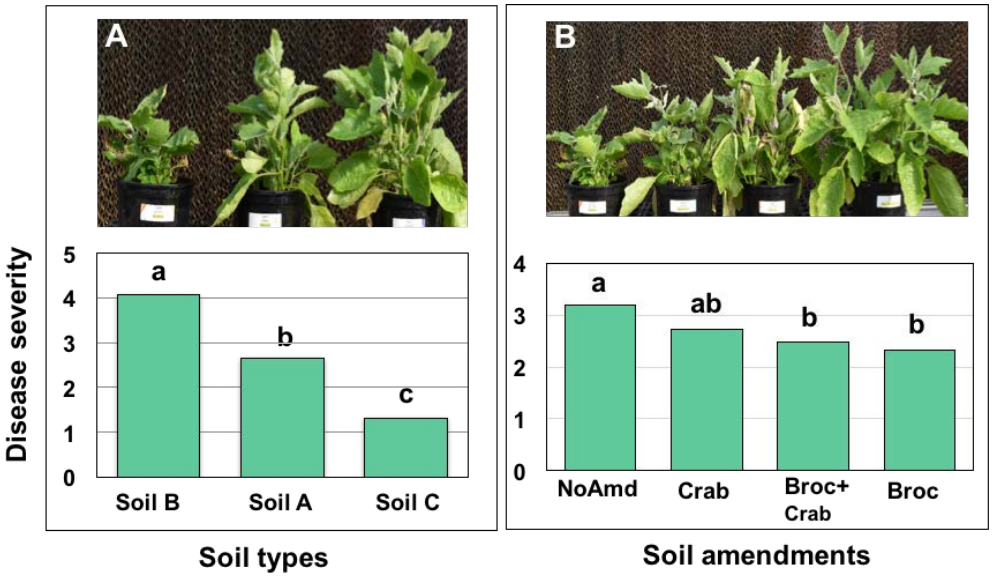

Fig. 2. Combined analysis of the effect of soil types and amendments in Verticillium wilt reduction under greenhouse conditions. Vertical bars with the same letter above are not significantly different from each other based on the least significant difference test (LSD) at a probability of P < 0.05. Bars with different letters are statistically different according to the LSD test at a probability of P < 0.05. Values indicate an average disease severity scores combined between cycle 1 of Experiment 1 and 2. Soil A has a long history of crisphead lettuce and strawberry cropping sequences and associated epidemics of Verticillium wilt. Soil B is native undisturbed

soil amended with microsclerotia of *Verticillium dahliae*, Soil C was recently transitioned from perennial pasture grass to organic strawberry production.

Number of microsclerotia count over time indicated a gradual increase in the number of MS from transplant to disease evaluation. Broccoli amendment, in general, had lower numbers of microsclerotia than other treatments irrespective of the soil type (Fig. 3).

Similarly, soil chitinase activity (p-Nitrophenol concentration) was significantly higher in Soil B compared to soils C and A (Fig. 4A). The soil amended with broccoli alone or in combination with chitin had significantly higher chitinase activity (Fig. 4B) ($P < 0.05$).

Initial microbial community analysis of soil collected one month after soil amendments indicated a highly significant community enrichment among the three soil types. The principal coordinate analysis (PCoA) grouped communities based on soil type (Fig. 5A). Soil type itself and soil amendments had a unique influence on microbial communities. Soils amended with broccoli alone, or both broccoli and chitin were associated with unique microbial community enrichment, compared to chitin- alone and unamended controls (Fig 5). In addition, microbial communities gradually shifted from the baseline samples taken at amendment mix to plant maturity (Fig. 5B). The relative proportions of DNA sequence reads of the top five phyla at the beginning and end of experiments indicated a proportional decrease in Proteobacteria but an increase in minor bacterial populations in all soil types and amendments. Notably, the broccoli + crustacean meal amended soil had increased Bacteroidetes in soils A and B, but a reduction in soil C. An opposite interaction was observed for Firmicute populations, which increased in soil C but decreased in the other two soil types (Fig 6).

The differential abundance analysis of prokaryote communities in amended vs. unamended soil at one month after transplanting identified microbes (cl.=class, or. =order, fa. =family, ge. =genus, sp.=species) with biocontrol potential in all soil types as shown in Fig. 7. Broccoli plus crustacean meal amended soil favored highest numbers of a known fungal antagonist such as *Sporocytophaga* sp. (cellulose degrading), *Streptomyces*, *Pseudomonas* sp., *Bacillus* sp. (cell wall degrading), *Nannocystis* sp. (biocompetitive), etc.

Objective 2: Field trial

The disease incidence (DS) ranged from 70 to 100% on the iceberg lettuce crop at the time of harvest prior to crop termination treatment (data not shown). The number of soil microsclerotia increased significantly when the infected crisphead lettuce was incorporated into the soil (Fig. 8). However, the numbers decreased significantly when broccoli was incorporated into the soil along with chitin. Spatial analyses of the density of *V. dahliae* microsclerotia (MS) in the field soil indicated that MS densities varied within the field and were not uniformly distributed (Fig. 9). Considerably higher numbers of MS were found in the plot that was selected for the AITC application relative to the untreated control plots. The tillage operation and the inoculum derived from infected lettuce plants contributed an increase of microsclerotia in the field. There was a 1,250% increase in the MS population in AITC-untreated plots. It does not appear that the crop termination treatment was successful in reducing MS populations. In fact, MS continued to increase after the crop termination treatment in the field. The rate of increase (338%) was much less in treated plots compared to untreated control plots (1250%), but the mean numbers were significantly higher.

Table 1. Treatment combinations used in the greenhouse study

Intense Production Agriculture- (Soil A)	0.2 % crustacean meal
	10% broccoli
	Combination (0.2% crustacean meal+ 10% broccoli)
	Unamended soils
Native undisturbed soil- (Soil B)	0.2 % crustacean meal
	10% broccoli
	Combination (0.2% crustacean meal+ 10% broccoli)
	Unamended soils
Transition from pasture to agriculture Soil- (Soil C)	0.2 % crustacean meal
	10% broccoli
	Combination (0.2% crustacean meal+ 10% broccoli)
	Unamended soils

Table 2. Analysis of physical and chemical properties of soils used in greenhouse experiment at the time of field collection and one month after amendment incorporation

Soil Type	NH_4-N)ppm(	NO_3-N)ppm(	Olsen-P (ppm)	C)%(	Total N)%(	Clay)%(	Sand (%(	Silt)%(	OM)%(	pH	Zn (ppm)	CEC (meq /100g)
Soil A	9.7	51.7	55.0	1.5	0.2	40	23	37	3.1	7.4	111	38.3
Soil B	5.9	3.5	7.5	1.1	0.1	4.5	92	3.5	2.0	5.6	32.0	7.6
Soil C	2.4	121.3	15.3	1.0	0.1	7.0	86	7.0	2.0	6.7	44.0	11.3

Table 3. Time lime of activities in the field demonstration trial

Date	**Activity**
9/25/16	Harvest Iceberg lettuce crop, assess disease incidence (Vert wilt) sample soil for Vert MS
9/28/16	Apply AITC (Dominus) thru drip for crop termination
10/24/16	Sample soil for Vert MS, chitinase enzyme, and DNA
10/26/16	Crustacean meal applied to the soil
4/3/17	Sample soil for Vert MS, chitinase enzyme and DNA
4/5/17	Planting beds prepared, Crustacean meal applied to the soil Broccoli planted
7/10/17	Broccoli harvested and disked in
8/14/17	Planting beds prepared, crustacean meal applied to the soil
8/16/17	Sample soil for Vert MS, chitinase enzyme and DNA
8/17/17	Romaine lettuce planted (transplants)
10/12/17	Romaine harvested, Disease incidence recorded (Vert wilt) Sample soil for Vert MS, chitinase enzyme and DNA
11/7/2017	Planting beds prepared, Crustacean meal applied to the soil
4/26/2018	Crustacean meal applied to the soil
4/27/2018	Sample soil for Vert MS, chitinase enzyme and DNA Broccoli planted

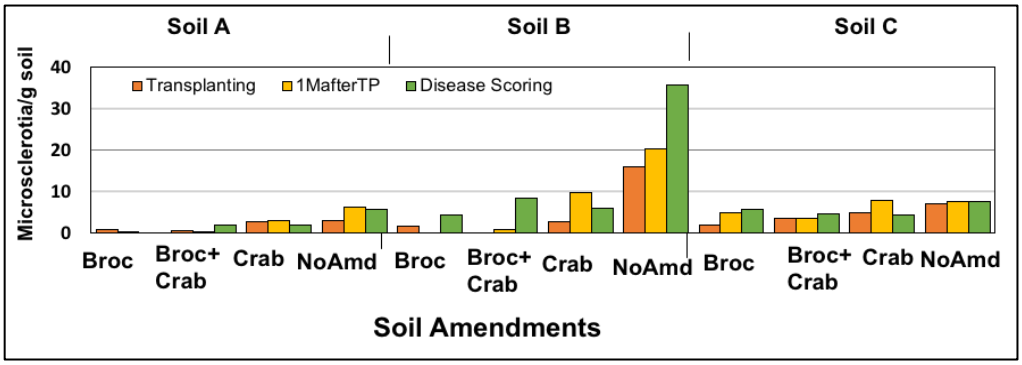

Fig. 3. The average number of *V. dahliae* microsclerotia in three soil types under various treatment combinations. (Data from experiment 1 cycle 1 only are shown). Microsclerotia densities were determined at transplanting, 1 month after transplanting and when disease ratings for Verticillium wilt were conducted. Soil A has a long history of crisphead lettuce and strawberry cropping sequences and associated epidemics of Verticillium wilt. Soil B is native undisturbed soil amended with microsclerotia of *Verticillium dahliae*, Soil C was recently transitioned from perennial pasture grass to organic strawberry production.

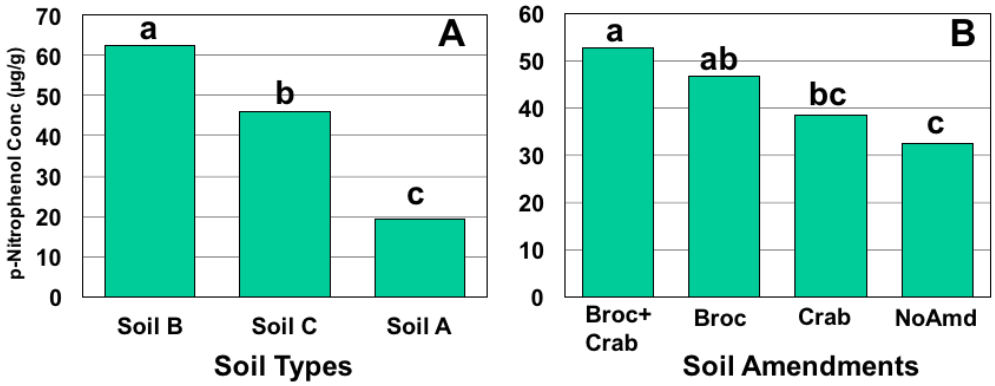

Fig. 4. Soil chitinase enzyme activity (p-Nitrophenol concentration) in greenhouse experiment 2 cycle 1. Vertical bars with the same letter above are not significantly different from each other based on the least significant difference test (LSD) at a probability of $P < 0.05$. Bars with different letters are statistically different according to the LSD test at a probability of $P < 0.05$.

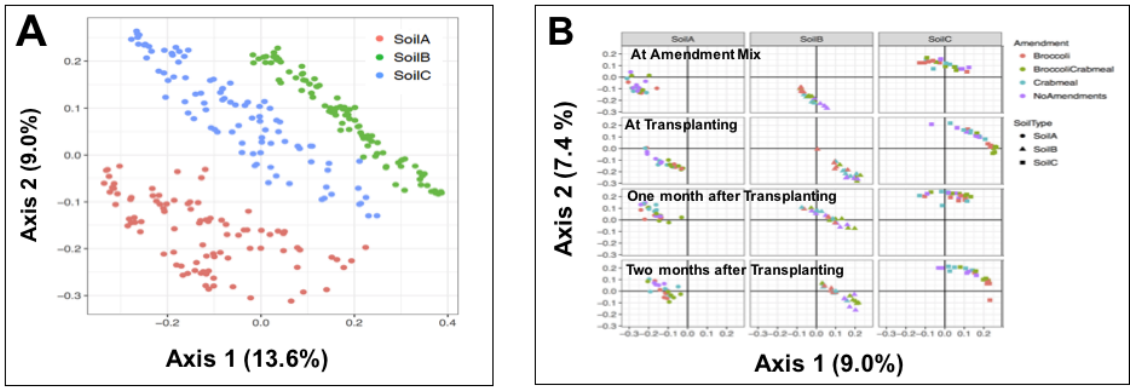

Fig. 5. PCoA ordination plot using unweighted UniFrac distance measures of prokaryote communities based on soil types (A) and over time (data from experiment 1 only are shown).

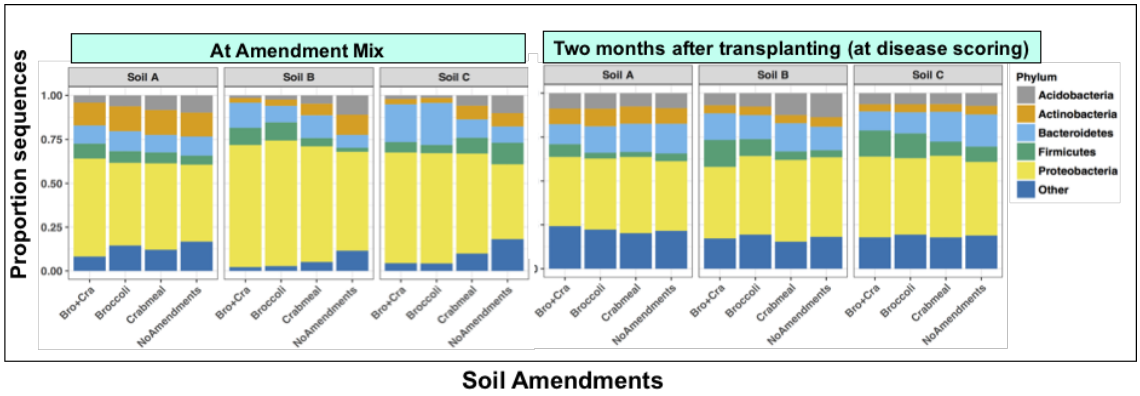

Fig. 6. Relative proportions of the DNA sequence read of the top five phyla at the beginning and end of experiments 1.

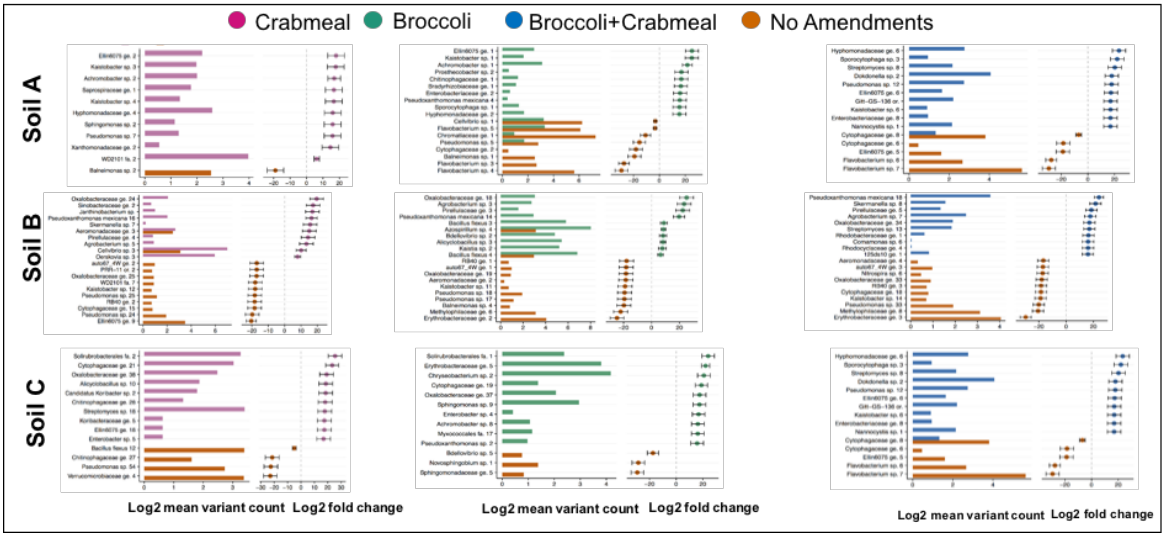

Fig. 7. Differential abundant analysis of prokaryote communities in amended-vs-unamended soil at one month after transplanting of experiment 1 cycle 1.

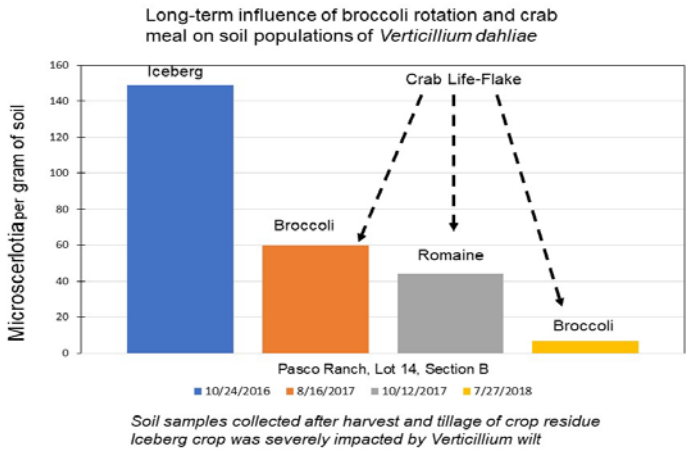

Fig. 8. Temporal changes in soil populations of *Verticillium dahliae* microsclerotia in a field that received chitin and broccoli residue treatments over a period of two years.

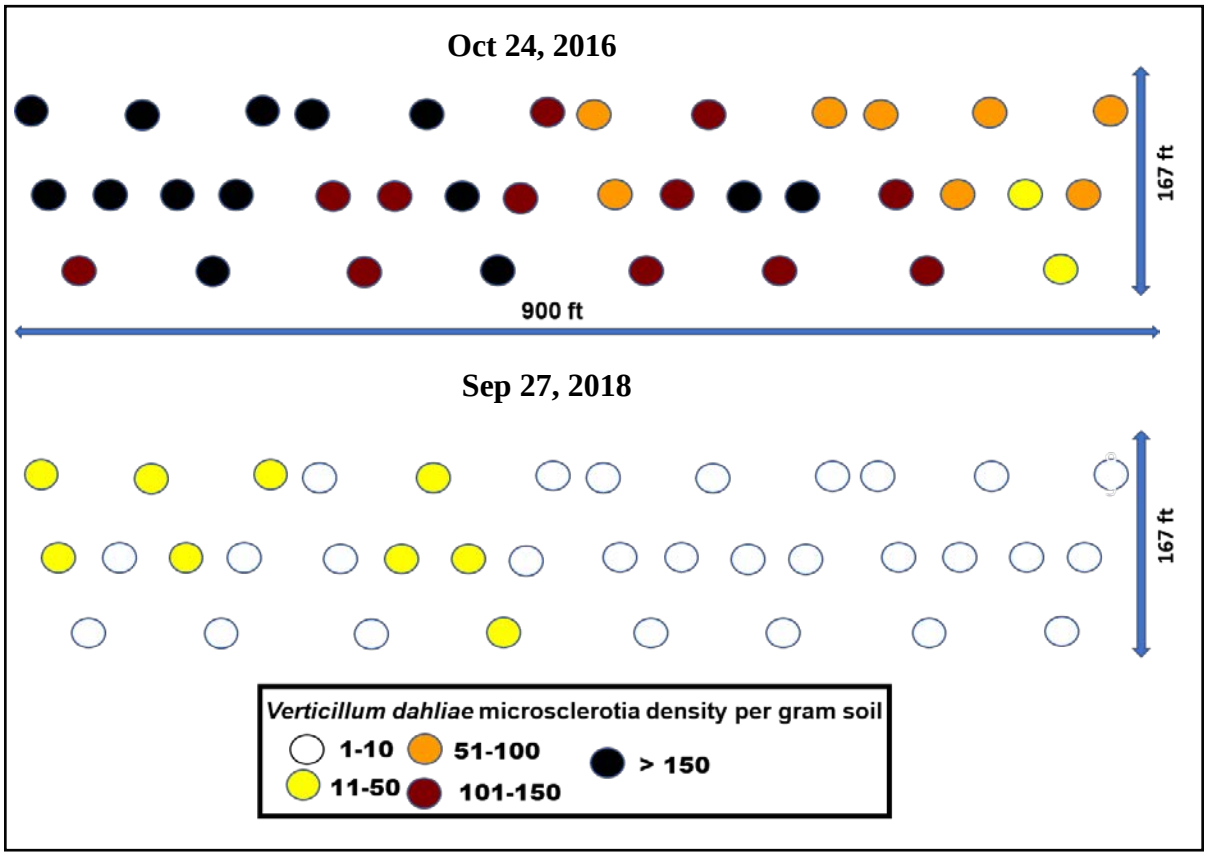

Fig. 9. Spatial changes in soil populations of *Verticillium dahliae* microsclerotia in a field that received chitin and broccoli residue treatments over a period of two years. Notice the significant reductions in the microsclerotia numbers in the bottom panel.

Objective C. Greenhouse experiments with lettuce differentials for investigating a possible *Verticillium* race 3 in lettuce.

Host resistance is one of the best alternatives to the long-term, sustainable Verticillium wilt management in lettuce. *Verticillium dahliae* on lettuce exists as two pathogenic races (race 1 and race 2). However, more recently, a new race (race 3) was identified within the previously classified race 2 groups in tomato from Japan, though the existence of race 3 is unknown in California. A good source of resistance to race 1 has been identified in a Batavian cultivar La Brillante and several other heirloom cultivars, whereas no source of complete resistance to race 2 is known. In our previous study, we had noticed that a few isolates of *V. dahliae* from spinach and potato belonging to race 2 had different reactions on LaBrillante and Sentry cultivars, while both cultivars were highly susceptible to the race 2 isolate Ls17 from lettuce.

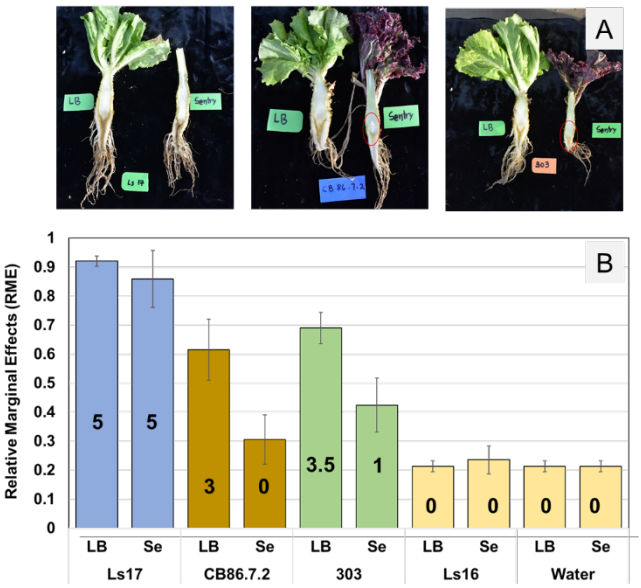

Fig. 10. Vascular tissue discoloration (A); and Relative Marginal Effects (RME), median disease score (inside bars in 0-5 scale), and confidence intervals (as error bars) of disease severity in La Brillante (LB) and Sentry (Se) (B) after inoculation with *V. dahliae* race 2 isolates Ls17, CB86.7.2 and 303 (A), and disease scores including race1 isolate Ls16 (B).

We further confirmed that the race interactions of four *V. dahliae* isolates (Ls16, Ls17, 303, and CB86.7.2) using three lettuce cultivars (La Brillante, Salinas, Sentry). The results suggest that the race 1-resistant cultivars, La Brillante and Sentry, had differential reactions to the contemporary race 2 isolates (Ls17, CB86.7.2 and 303) (Fig. 10). Notably, the cultivar Sentry which is as equally susceptible as La Brillante to Ls17, consistently shows a high level of resistance to the other two race 2 isolates CB86.7.2 and 303 (the possible race 3). These results have now confirmed that the previously classified race 2 isolates CB86.7.2 and 303 had a differential reaction to Sentry and La Brillante. Sentry consistently shows a higher level of resistance, while La Brillante was susceptible to these two isolates (Fig. 10). To explore and understand the genetic makeup of these two isolates in comparison to previously known race 1 and race 2 isolates, we have processed all four isolates for whole genome sequencing using PacBio and Illumina MiSeq. The comparative genomics analysis of all races will facilitate developing race 3-specific primers that can accurately differentiate Ls17 vs. CB86.7.2 or 303. Once primers are available, we will screen all our race 2 isolate collection and check if any race 3 isolates are present in coastal California. This information will be crucial for breeding programs.

Objective D. Greenhouse experiments to map gene(s) governing resistance to the possible new race of *Verticillium dahliae*.

Because of the differential responses of Sentry and La Brillante to some isolates of *V. dahliae* (Fig. 10), we need to understand the resistance mechanisms in Sentry relative to the susceptibility in La Brillante. A biparental mapping population has been developed from a cross between Sentry x La Brillante. A set of 200 single nucleotide polymorphic (SNP) markers linked with Verticillium resistant loci were tested to detect polymorphisms between two parents and the F_1. Approximately 40% of the markers linked with Verticillium resistant loci were polymorphic between the two parents.

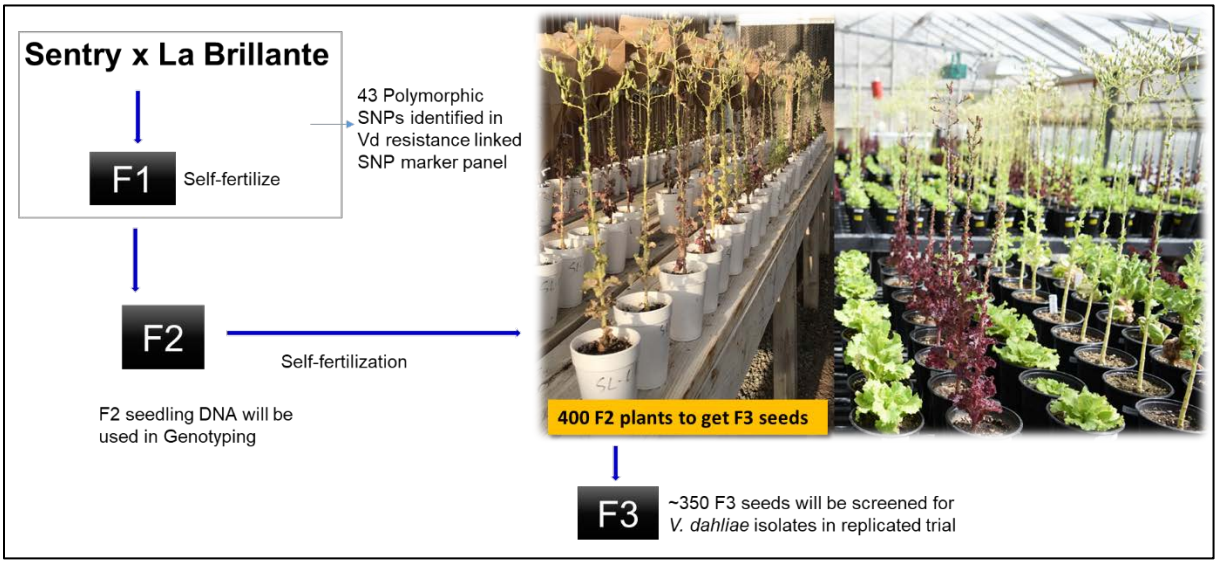

Fig. 11. Biparental population development and QTL mapping approach to identify genetic locus linked to CB86.7.2/303 resistance in Sentry.

A total of 400 F_2 derived families were self-pollinated to obtain F_3 seeds. The leaf tissue from the F_2 seedlings was harvested and used in DNA extraction for genotyping. For phenotyping, seeds are currently being threshed to get F_2-derived F_3 family seeds. Approximately 350 families will be screened with *V. dahliae* isolate CB86.7.2/303 in replicated greenhouse trial (Fig. 11). Greenhouse screening will begin in July/Aug 2019 and repeated over the coming years.