CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

April 1, 2020, to March 31, 2021

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LEAFY VEGETABLES

Krishna V. Subbarao

Department of Plant Pathology

University of California, Davis

SUMMARY

Since the initial discovery of Verticillium wilt caused by *Verticillium dahliae* on lettuce in Watsonville in 1995, the disease has spread to the prime lettuce production regions of the Salinas and surrounding valleys. Isolates infecting lettuce belonged to either race 1 or race 2. A new *V. dahliae* race 3 was described on tomato in Japan in 2017. Whether this race is also present among the isolates affecting lettuce could not be ascertained due to the unavailability of lettuce differentials. Thus, we sequenced genomes of the race 3 isolates from tomato, developed race 3-specific primers, and validated it both in tomato and lettuce isolates. We will now test all isolates in our collection to determine possible race 3 in lettuce and other crops in coastal California as also the new isolates from lettuce. For genetic mapping of resistant loci in lettuce against new race, we completed phenotyping and genotyping of 200 $F_{2:3}$ mapping population developed from a cross between Sentry and LaBrillante. The preliminary results suggest that the resistant trait is quantitatively inherited in Sentry.

REPORT

CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

BIOLOGY AND EPIDEMIOLOGY OF VERTICILLIUM WILT OF LEAFY VEGETABLES

PRINCIPAL INVESTIGATOR: **Krishna V. Subbarao**

Department of Plant Pathology

University of California, Davis

COOPERATING PERSONNEL:

Krishna D. Puri

Department of Plant Pathology

University of California, Davis

and

Ivan Simko

USDA-ARS, Salinas

ABSTRACT:

Since the initial discovery of Verticillium wilt caused by *Verticillium dahliae* on lettuce in Watsonville in 1995, the disease has spread to the prime lettuce production regions of the Salinas and surrounding valleys. Isolates infecting lettuce belonged to either race 1 or race 2. A new *V. dahliae* race 3 was described on tomato in Japan in 2017. Whether this race is also present among the isolates affecting lettuce could not be ascertained due to the unavailability of lettuce differentials. Thus, we sequenced genomes of the race 3 isolates from tomato, developed race 3-specific primers, and validated it both in tomato and lettuce isolates. We will now test all isolates in our collection to determine possible race 3 in lettuce and other crops in coastal California as also the new isolates from lettuce. For genetic mapping of resistant loci in lettuce against new race, we completed phenotyping and genotyping of 200 $F_{2:3}$ mapping population developed from a cross between Sentry and LaBrillante. The preliminary results suggest that the resistant trait is quantitatively inherited in Sentry.

OBJECTIVES:

A. Investigate a possible *Verticillium dahliae* race 3 in lettuce.
B. Develop a PCR assay to rapidly identify race 3 strains.
C. Greenhouse experiments to map gene(s) governing resistance to the possible new race of pathogen.

PROCEDURES:

a. Investigate a possible *V. dahliae* race 3 in lettuce

Since race 3 has only been confirmed in tomato, much of the initial work comparing races 1, 2, and 3 was done with the tomato isolates. These isolates were collected from infected tomato plants in Japan and typed to races based on the response of differential tomato cultivars. We obtained these isolates from Dr. Usami (Japan) and single-spored them on potato dextrose agar. Cultures from each isolate were grown in 250 mL of the complete medium at 25°C, and high-quality genomic DNA was extracted from mycelia using the Quick-DNA^{TM} Fungal/Bacterial Midiprep Kit following the manufacturer's protocol.

High-quality genome sequences of all three races were obtained by both Illumina and PacBio sequencing methods at the Beijing Genome Institute, and assembled *de novo*. The genomes of the three tomato strains representing races 1 (JR2), 2 (TO22), and 3 (HoMCLT) were selected for structural comparisons and to identify race-specific sequences and identify and test candidate avirulence factors associated with races, and to develop and validate race-specific primers for detection by PCR.

b. Develop a PCR assay to rapidly identify race 3 strains

From the above comparative genomic analyses, unique sequences specific to race 3 were identified and labeled as the '*VdAve3'* locus, race 3 (*Ave3*)-specific primers were designed using the Primer3 (v0.4.0) software. The primers, namely Avr3_F (ATGAAGCTATCCGTCCTCGT) and Avr3_R (CTACCAACCCCACATGTTGG), were first validated on tomato isolates, which amplified approximately 350 bp fragment specific to race 3. The PCR conditions included initial denaturation at 95°C for 5 min followed by 25 cycles at 95°C for15 s, 60°C for15 s, and 72°C for 30 s, with the final extension of 5 min at 72°C. The PCR conditions and primer specificity are currently being validated on *V. dahliae* isolates from other hosts including lettuce. Upon confirmation, these primers will be used to identify the presence of race 3 isolates among our collection from lettuce. We are currently collecting new isolates from lettuce that will be evaluated in the next funding cycle. We encourage anyone with a diseased field to send Verticillium wilt-infected plant samples to my lab at the USDA Station in Salinas. These isolates will be a valuable resource to validate primers and understand pathogen race dynamics in coastal California.

For both objectives A and B, the race differentiation was done based on the presence or absence of amplification for molecular loci specific to race 3. The data will be summarized in Excel or R to perform a Chi-square test.

c. Greenhouse experiments to map gene(s) governing resistance to the possible new race

Plant material

Previous confirmation tests indicated an apparent differential disease reaction in Sentry and LaBrillante by *V. dahliae* race 3 isolate 303 isolated from the spinach. Thus, a biparental mapping population was developed from a cross between *V. dahliae* partially resistant genotype Sentry and the susceptible genotype LaBrillante, and used for genetic mapping study to reveal resistance mechanism in Sentry against *V. dahliae* isolate 303. The F_1 progeny was selfed to get F_2 families, subsequently grown to get $F_{2:3}$. A total of 450 F_2 seeds were randomly selected and used for seed increase. Subsequently, 200 of the $F_{2:3}$ family lines were screened against *V. dahliae* isolate 303 in a greenhouse using a replicated trial (Fig. 1).

Phenotyping

For phenotyping, approximately thirty seeds of each F_3 family were sown in 128-well plug trays filled with sterilized Sunshine Mix number 4. Fourteen days after seedling emergence, the

seedlings were inoculated with a conidial suspension of 1×10^7 conidia/ml of *V. dahliae* isolate 303 by nearly saturating soil in each well (3 ml/well). Two additional inoculations were performed at one and two weeks of the first inoculation. Thirty-day-old seedlings were then transplanted into 500 ml Styrofoam cups filled with pasteurized potting mix and sand mixture in 2:1 vol/vol. One week after transplanting, a fourth inoculation was performed by pouring 10 ml of the *V. dahliae* isolate 303 conidial suspensions per cup. The seedlings from each family were arranged in a randomized complete block design, replicated three times with 7 - 8 plants per replication ($202 \times 3 \times 7 = 4{,}242$ total cups). The control plants for each parent were mock-inoculated with sterile distilled water and placed on a separate bench. Plants were then grown for approximately ten weeks before disease evaluation. For evaluation, each plant was uprooted, washed, and sectioned longitudinally to assess disease severity based on the extent of taproot vascular discoloration along with the above-ground symptoms using the previously developed 0 (symptomless) to 5 (most severe symptoms) scale.

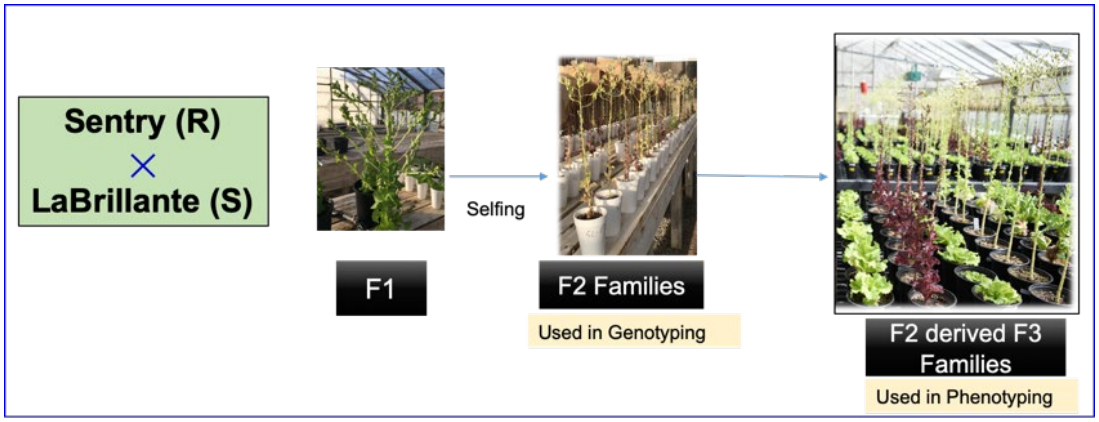

Fig. 1. Outline of biparental population development for QTL mapping to identify genetic locus linked to *V. dahliae* isolates 303 (race 3) resistance in lettuce cultivar Sentry.

Genotyping

Initially, we used a set of ~ 200 single nucleotides polymorphic (SNP) markers randomly distributed in the lettuce genome to test polymorphisms between two parents. Later, a complete SNP genotyping of a total of 288 (F_2 families + control) was done using tGBS® Genotyping by Sequencing technology at Data2Bio® using the restriction enzyme *Bsp1286I*. Samples were sequenced in an Illumina HiSeq X platform, and reads were aligned to *Lactuca sativa* cv Salinas V8 reference genome following debarcoding and trimming of reads. The SNP calling was conducted using only those reads aligned to a single location in the reference genome.

Data Analysis

The statistical analysis of phenotypic variation in disease severity was done by the analysis of variance (ANOVA) fitting in the linear model (*lm*) in SAS or using the 'Stats' Package in R. The broad-sense heritability (H^2) of phenotypic data calculated using mixed model equations (*mmer*) function of 'sommer' package in R using formula $H^2 = \sigma^2 g / (\sigma^2 g + \sigma^2 e / r)$ ($\sigma^2 g$, genetic variance; $\sigma^2 e$, residual variance; and r, number of replications). The obtained SNP markers from the sequencing facility will be filtered using the TASSEL SNP discovery pipeline. Linkage map based on informative markers will be generated using MSTmap software. Fully linked markers with no recombination between them will be collapsed into a single combined marker. Collinearity between physical distance of markers determined by alignment with the reference genome and

their position on the molecular linkage map will be assessed. Only markers with the matching positions on both physical and molecular linkage maps will be used in QTL analysis. The QTL analysis will be performed in QGene 4.0 software and declare significance at a logarithm of the odds (LOD) value of 3.0 and above.

Results and Discussion

a. Investigate a possible Verticillium dahliae race 3 in lettuce

Race 1 isolates in the *V. dahliae*-tomato and lettuce interactions are identified by the *Ave1* effector. In this study, we identified avirulence factors in the genomes of *V. dahliae* races 2 and 3 that are not shared by other races. The results showed that only 5, 87, and 68 genes from strain-specific regions among three races displayed sequence divergence (Fig. 2A). Of the five genes in the HoMCLT genome, only one (HoMCLT_V04212) was predicted as a secreted protein with characteristics of an effector (Fig. 2C). Interestingly, PCR assays showed that HoMCLT_V04212 was present in nearly all race 3 strains except for three and was absent in the strains from races 1 and 2 from various hosts and countries (Fig. 2B), strongly suggesting that HoMCLT_V04212 (named *VdAve3*) was a specific secretory protein in the race 3 population. PCR tests were performed to identify the three races using new primers developed from these specific regions of DNA sequence for each of the three races.

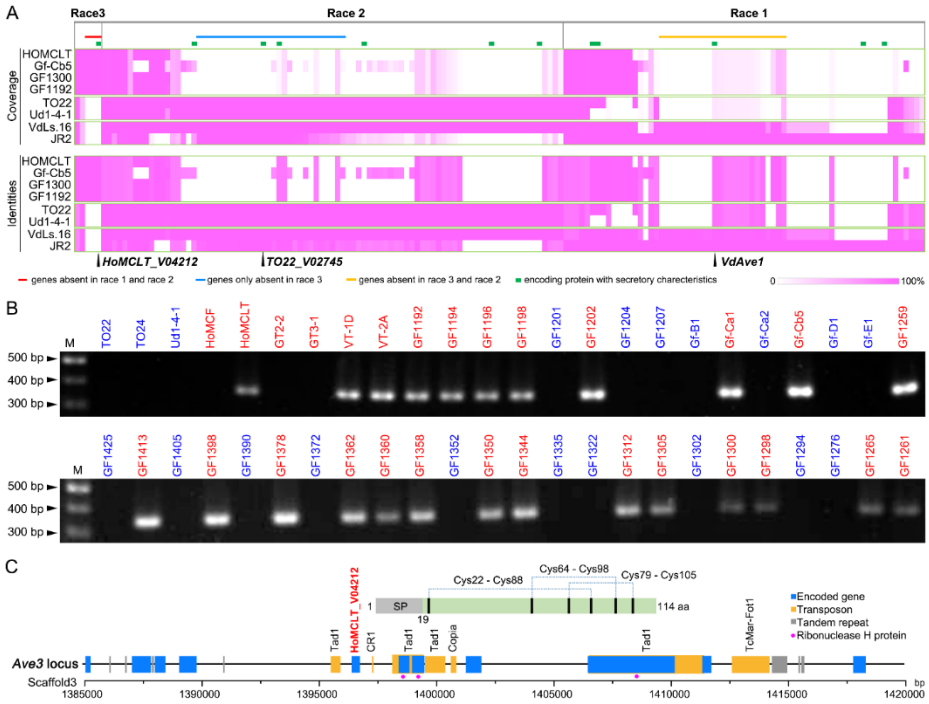

Fig. 2. Analysis of the genomes of the three races in *Verticillium dahliae*. (**A**) Comparison of total genes encoding pathogenicity-related proteins in the three races. (**B**) PCR validation of the marker specific to race 3 among the tomato isolates from various regions of Japan. (**C**) Location of the *Ave3* marker on the genome.

b. Develop a PCR assay to identify race 3 strains.

The race 3 primers PCR conditions and primer specificity were validated with additional isolates from tomato and other hosts. These primers will be used to screen all isolates in our collection that were not confirmed as either race 1 or 2 and all new isolates collected from lettuce.

Instead of waiting for new isolates for evaluation, we mined over 3,500 resequenced genomes for HoMCLT_V04212 (*VdAve3*)-specific secretory protein sequences. These sequences came from isolates from 15 hosts, including cotton, eggplant, lettuce, sunflower, tomato, spinach, potato, strawberry, artichoke, olive, pepper, watermelon, horseradish, and cantaloupe. Most of these isolates originated in the USA and China and included a few from other countries as well. The results showed nearly 8.5% (n= 294) of the genomes contained race 3-specific sequences. The percentages of race 3 isolates detected in different hosts are presented in Fig. 3 below. About 69.9% (total=166) of isolates from spinach, 62.4% (total=186) from tomato, and 28.1% (total=146) from strawberry were confirmed as race 3. However, only a single isolate out of 275 from lettuce and none from artichoke (total =96) had *Ave3* sequences. Almost all isolates from these five hosts originated in the USA, while most isolates in cotton or other hosts were either from China or other countries.

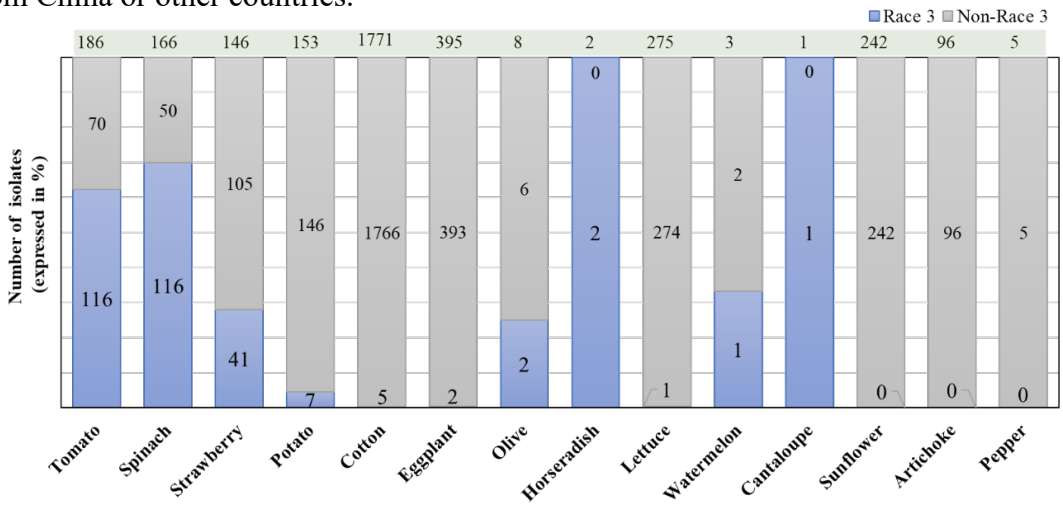

Fig. 3. Distribution of *V. dahliae* race 3 isolates from 15 different hosts based on genetic sequences similarity of *Ave3* loci in HoMCLT isolate genome from tomato. The number inside the bar represents isolates belonging to either race 3 or non-race 3. The number on the top of the bar represents the number of isolates evaluated host-wise.

Interestingly, however, VdLs.17 that we have considered as a type isolate for race 2 since 2006 in lettuce, has none of the molecular signatures that define races 1, 2, and 3. It suggests to the complexity of the race structure in *V. dahliae*. If we define the races at the individual host level as it is traditionally done, VdLs.17 still qualifies as a race 2 strain. If we begin to define races at the pathogen level owing to the very broad host range and virulence of isolates across hosts, then VdLs.17 does not qualify as a race 2 strain and the race structure itself becomes more complex in *V. dahliae*. We are working towards resolving this complication.

c. **Mapping gene(s) governing resistance to the possible new race in lettuce**

Phenotypic disease evaluation in parents

Both parents, i. e. Sentry and LaBrillante, showed a significant differential disease reaction to *V. dahliae* isolate 303 (Fig. 4, $P<0.05$). The Sentry and LaBrillante had median disease ratings of 1 and 3.5, with Relative Marginal Effect (RME) of 0.42 and 0.69, respectively. Both parents were resistant to *V. dahliae* race 1 but susceptible to race 2 isolates from the lettuce. Isolate 303 previously had an inclusive classification as race 2 had a differential infection to Sentry and LaBrillante, now confirmed as race 3 based on primers developed above.

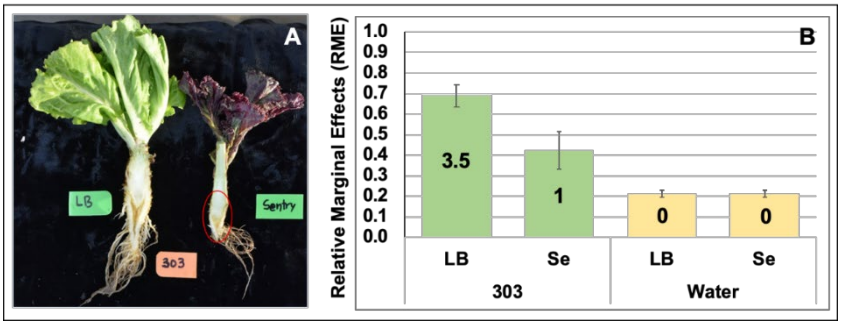

Fig. 4. The differential vascular discoloration caused by *V. dahliae* isolates 303 in LaBrillante (LB) and Sentry (A); and the Relative Marginal Effects (RME), median disease score (the numbers within the bars scored on a 0-5 scale), and confidence intervals (shown as error bars) of disease severity (B).

Phenotypic evaluation of the mapping population

The frequency of disease severity rating among the $F_{2:3}$ families from the mapping population approximated a normal distribution as expected for a polygenic inheritance of disease resistance (Fig. 5). The phenotypic variation in disease resistance ranged from 0.14 - 4.71 (across replications). Transgressive segregation was observed in the offspring with underperforming and outperforming genotypes in both directions compared to parents (Fig. 5). The analysis of variance (ANOVA) of phenotypic data indicated a significant variance among the $F_{2:3}$ family lines ($P < 0.001$). The broad-sense heritability (H^2) was 0.618, with a standard error of 0.047 demonstrating a strong genetic effect on disease resistance trait.

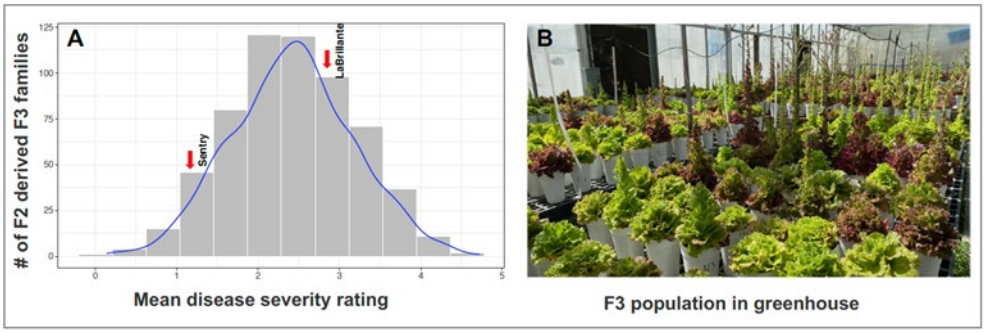

Fig. 5. Distribution of the mean disease severity across replications in Sentry × LaBrillante $F_{2:3}$ mapping population (A) and the $F_{2:3}$ families at the time of disease rating (B) in the greenhouse.

Genotypic evaluation of the mapping population

The initial set of approximately 200 single nucleotides polymorphic (SNP) markers distributed randomly in the lettuce genome identified approximately 40% polymorphic markers between two parents. Later, a complete genotyping of 288 families, including parents, identified 265,056 SNP sites without any filtering criteria. While using robust criteria for filtering high-quality reading named MCR50 (Minimum Call Rate SNP that genotyped in at least 50% of the samples) identified a set of 132,508 SNP sites.

The quantity of missing data, minor allele frequency, heterozygosity, and genotype number are summarized in Fig 6A, while the numbers SNPs in each genotype that were homozygous with

reference (REF) allele, homozygous for the alternative (ALT) allele, heterozygous, and missing are presented in Fig. 6B for MCR50 SNP dataset.

The SNP data from 200 genotypes used in phenotyping will be extracted, imputed, and processed to obtain high-quality SNP markers. The genotypic and phenotypic data will then be analyzed to identify QTLs confirming resistance for race 3 of *V. dahliae* and will be reported once available.

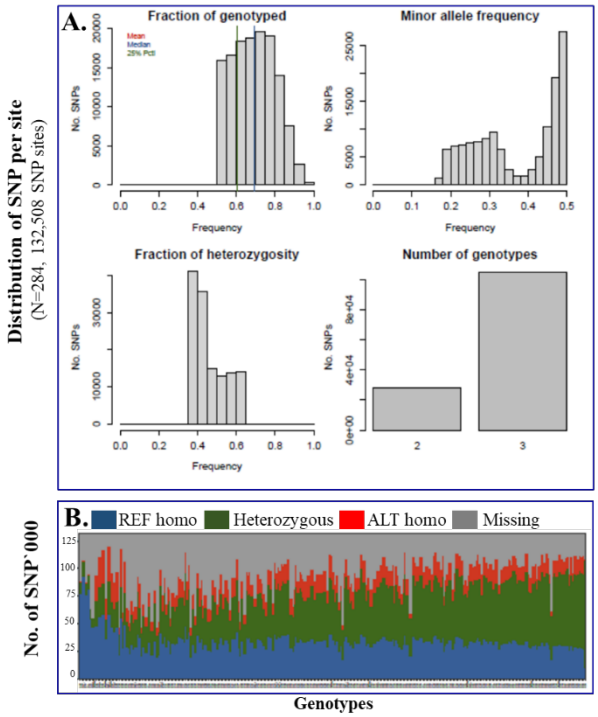

Fig. 6. Various characteristics of the MCR50 SNP dataset (A) and portions of various SNPs sites in each sequenced genotype (B).