CALIFORNIA LEAFY GREENS RESEARCH PROGRAM

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SCREENING OF COMMERCIAL LETTUCE CULTIVARS FOR RESISTANCE AGAINST RACES 1, 2, AND 3

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SUMMARY

Planting resistant cultivars is a key strategy to manage Verticillium wilt in lettuce. We therefore evaluated 48 lettuce germplasm lines and one endive line, comprising commercial cultivars (cv.) and breeding lines, including the race 1-resistant heirloom cv. La Brillante and the susceptible cv. Salinas as controls. Resistance against races 1, 2, and 3 along with VdLs17, a virulent isolate of *V. dahliae* from lettuce that is currently not assigned to a race was evaluated in replicated greenhouse experiments. Two crisphead lettuce lines, HL28 and HL29, exhibited resistance against race 1 and partial resistance against race 2 while all other lines were highly susceptible to races 1 and 2 and VdLs17. The majority of lines exhibited higher resistance to race 3 relative to the other two races. Cultivars developed using the sources of resistance identified in this study should be effective or partially effective against these races for targeted deployment as soon as they are available. The study also demonstrated that breeding efforts against VdLs17 should continue as it would facilitate identifying resistance against the other three races.

REPORT

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ABSTRACT:

Planting resistant cultivars is a key strategy to manage Verticillium wilt in lettuce. We therefore evaluated 48 lettuce germplasm lines and one endive line, comprising commercial cultivars (cv.) and breeding lines, including the race 1-resistant heirloom cv. La Brillante and the susceptible cv. Salinas as controls. Resistance against races 1, 2, and 3 along with VdLs17, a virulent isolate of *V. dahliae* from lettuce that is currently not assigned to a race was evaluated in replicated greenhouse experiments. Two crisphead lettuce lines, HL28 and HL29, exhibited resistance against race 1 and partial resistance against race 2 while all other lines were highly susceptible to races 1 and 2 and VdLs17. The majority of lines exhibited higher resistance to race 3 relative to the other two races. Cultivars developed using the sources of resistance identified in this study should be effective or partially effective against these races for targeted deployment as soon as they are available. The study also demonstrated that breeding efforts against VdLs17 should continue as it would facilitate identifying resistance against the other three races.

OBJECTIVE:

Screen commercial lettuce cultivars and the newly available race-1 resistant cultivars for their responses to *Verticillium dahliae* races 1, 2, and 3.

PROCEDURES:

Experimental plan to evaluate lettuce lines against *V. dahliae* races

Four *V. dahliae* isolates representing the three races, (Table 1), namely VdLs16, the type isolate for race 1 in lettuce; VdLs4325, the newly identified effector-based race 2 from lettuce and VdCv4323, the race 3 isolate from watermelon; and VdLs17, a virulent isolate from lettuce that is currently not assigned to a race, were tested for their virulence against 48 lettuce lines (breeding lines/commercial cultivars) and an endive line. Included among these lines were La Brillante that exhibits resistance against race 1, and the universally susceptible cultivar Salinas (Hayes et al. 2011) to serve as controls. The lettuce breeding lines were contributed for the study by six seed companies and since these lines are proprietary, they were coded to ensure anonymity. The 48 lettuce lines and an endive line screened for Verticillium wilt resistance were grouped into six phenotypic types: crisphead type (36 – named sequentially HL1-HL36), followed by green leaf lettuce (6 -GLL1-GLL6), romaine (4 -RL1-RL4), red leaf lettuce (1 - RLL1), Endive (1 - E1), and Batavia type (1 - La Brillante, BT1).

Table 1. *Verticillium dahliae* isolates used in this study along with their hosts, geographic origin, mating type and race

Isolate	Original host	Geographical origin	Mating	Race
VdLs16	Lettuce (*Lactuca sativa* L.)	CA (Santa Cruz Co.)	MAT-2	Race 1
VdLs17*	Lettuce (*Lactuca sativa* L.)	CA (Santa Cruz Co.)	MAT-2	-
VdLs4325	Lettuce (*Lactuca sativa* L.)	CA (Monterey Co.)	MAT-2	Race 2
VdCv4323	Watermelon (*Citrullus lanatus* L.)	CA (San Luis Obispo Co.)	MAT-2	Race 3

* Remains as an undefined race according to the effector-based markers.

Seeding and *V. dahliae* inoculation

Approximately four hundred seeds (2-3 seeds per cell) of each line were sown in 128-well plug trays filled with sterilized sand and Propagation Mix (Sun Gro Horticulture, Brantford, AB, Canada) mixture (1:2) and kept saturated with water. The seeded trays were covered with dark plastic sheets for 48 hr to ensure uniform seedling emergence, and later transferred to a growth chamber for further development at 20-25°C with an 8-hr dark and 16-hr light cycle. Seedlings were thinned to maintain a single seedling per cell 10 days after emergence and returned to the greenhouse for acclimatization to ambient conditions.

The three isolates of different races and VdLs17 were cultured, inoculum prepared, and inoculum density adjusted to 1×10^7 conidia/ml as described in Simko et al. (2022). Briefly, glycerol stock isolate suspensions were initially transferred into PDA plates and allowed to grow for 7 days. Aliquots of conidial suspensions prepared from fully grown plates from each isolate was then transferred into new PDA plates and incubated at room temperature (~24 ± 2°C) for 7 days to increase inoculum production. Inoculum was prepared by flooding the cultures with autoclaved ddH_2O, and the contents were filtered through a double-layered cheesecloth and used for seedling inoculation. Inoculations with each race were performed on three different occasions to prevent escapes. The first inoculation was performed by pipetting 3 ml of conidial suspension (~1×10^7 conidia/ml) and drenching the soil surrounding the 14-day-old seedlings in each cell. The seedlings were inoculated a second time 7 days after the first using the method described

above. Thirty-day-old inoculated and uninoculated seedlings were then transplanted into 500 ml Styrofoam cups filled with a 2:1 vol/vol mixture of pasteurized potting mix and sand. About 3-4 days after transplanting, the third and final inoculation was performed by pouring 5 ml conidial suspension per cup. For each race, 10 seedlings were inoculated and an equal number of uninoculated seedlings were maintained as controls. The entire experiment was repeated once. The pots were arranged in a split plot design [49 lines × 5 treatments (4 isolates + 1 water control) × 10 plants = 2,450 total cups per experiment] in which isolates served as the main plot and cultivars served as the split plot and incubated on greenhouse benches. The uninoculated control plants for each cultivar were treated with sterile distilled water (CW treatment) and placed on separate benches to prevent contamination from the inoculated plants during watering. Plants were grown for approximately 10 weeks before being evaluated for Verticillium wilt severity.

Verticillium wilt severity

For disease evaluation, plants were uprooted, washed free of soil, and the taproot cut longitudinally to assess Verticillium disease severity (VDS) based on the extent of vascular discoloration along with the above-ground symptoms using the previously developed 0-to-5 scale, where 0 = no vascular discoloration; 1 = 1 to 25% of the vascular tissue exhibiting discoloration; 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% discoloration in the absence of foliar symptoms; and 5 = 100% discoloration and the presence of foliar symptoms typical of Verticillium wilt (Hayes et al., 2011b).

Statistical analyses

The disease severity was analyzed as a split-plot design with isolates as the main plot and breeding lines/cultivars as the split plot using a statistical software SAS following Shah and Madden (2004). The analysis was conducted with the non-parametric method of the ranked means (Shah and Madden 2004) using PROC MIXED in SAS. In order to obtain the ranked means and the relative marginal effects (RME) with their respective confidence intervals, the macros F1_LD_F1 and LD_CI were employed. RMEs were calculated for each breeding line/cultivar × isolate interaction. RMEs were also obtained for the 49 breeding lines/cultivars, five pathogen isolates plus control (water, VdCv4323, VdLs4325, VdLs16, and VdLs17), and for six lettuce types (Batavia, romaine, green leaf, crisphead, endive, and red leaf). In all instances, orthogonal contrasts were calculated to compare cultivars within an isolate, breeding lines/cultivars, and across isolates and lettuce types.

Results

Virulence differences among races

Both the isolates and the lettuce lines showed a significant ($P < 0.0001$) impact on VDS (Table 2). All four isolates had a significant ($P < 0.0001$) effect on wilt severity relative to the uninoculated control (Figure 1, Table 3). The overall disease severity caused by the race 2 isolate VdLs4325 (RME 0.682) across lines was similar to that caused by the race 1 isolate VdLs16 (RME 0.676) and the race-undefined VdLs17 (RME 0.652). Differences in the wilt severity caused by VdLs16 and VdLs17 were not significant ($P > 0.05$) from each other in this study. The race 3 isolate VdCv4323 (RME 0.378) had a relatively lower virulence than the other isolates (Figure 1).

Table 2. Summary analyses of variance based on the effects of individual isolates representing the three races of *Verticillium dahliae* and VdLs17 on the ranked means of Verticillium wilt severity on 48 lines of different lettuce types and endive across two independent experiments

Effect	ANOVA[a]			
	Num DF	Den DF	*F* Value	*P* > F
Isolate	3.14	67.3	366.38	<0.0001
Germplasm	45.10	3797.0	13.79	<0.0001
Isolate*Germplasm	150.00	3797.0	5.70	<0.0001

[a] ANOVA = analysis of variance, NumDF = numerator degrees of freedom, and DenDF = denominator degrees of freedom.

Figure 1. The relative marginal effects (RME) obtained from nonparametric analysis of the Verticillium wilt severity caused by different isolates of *Verticillium dahliae* on 49 lettuce cultivars in two independent experiments.

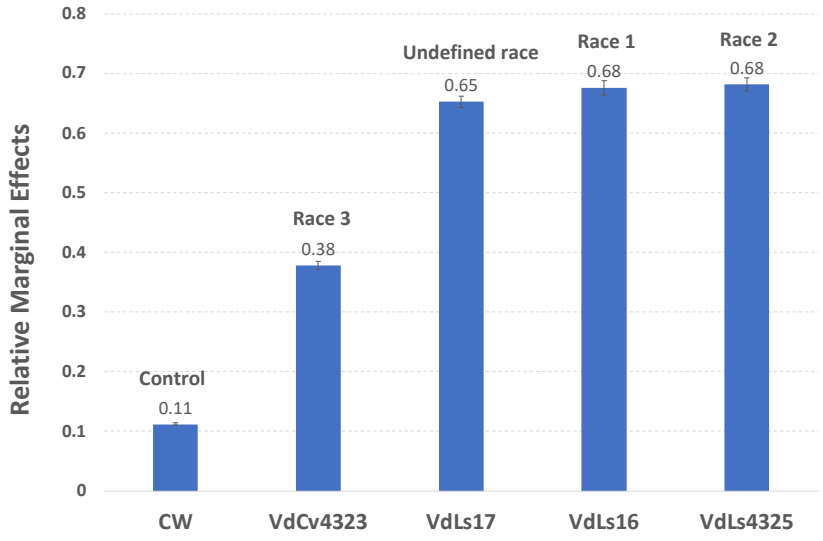


Table 3. Orthogonal contrasts of the Verticillium wilt severities caused by isolates representing different races of *Verticillium dahliae* as well as the untreated control (CW)

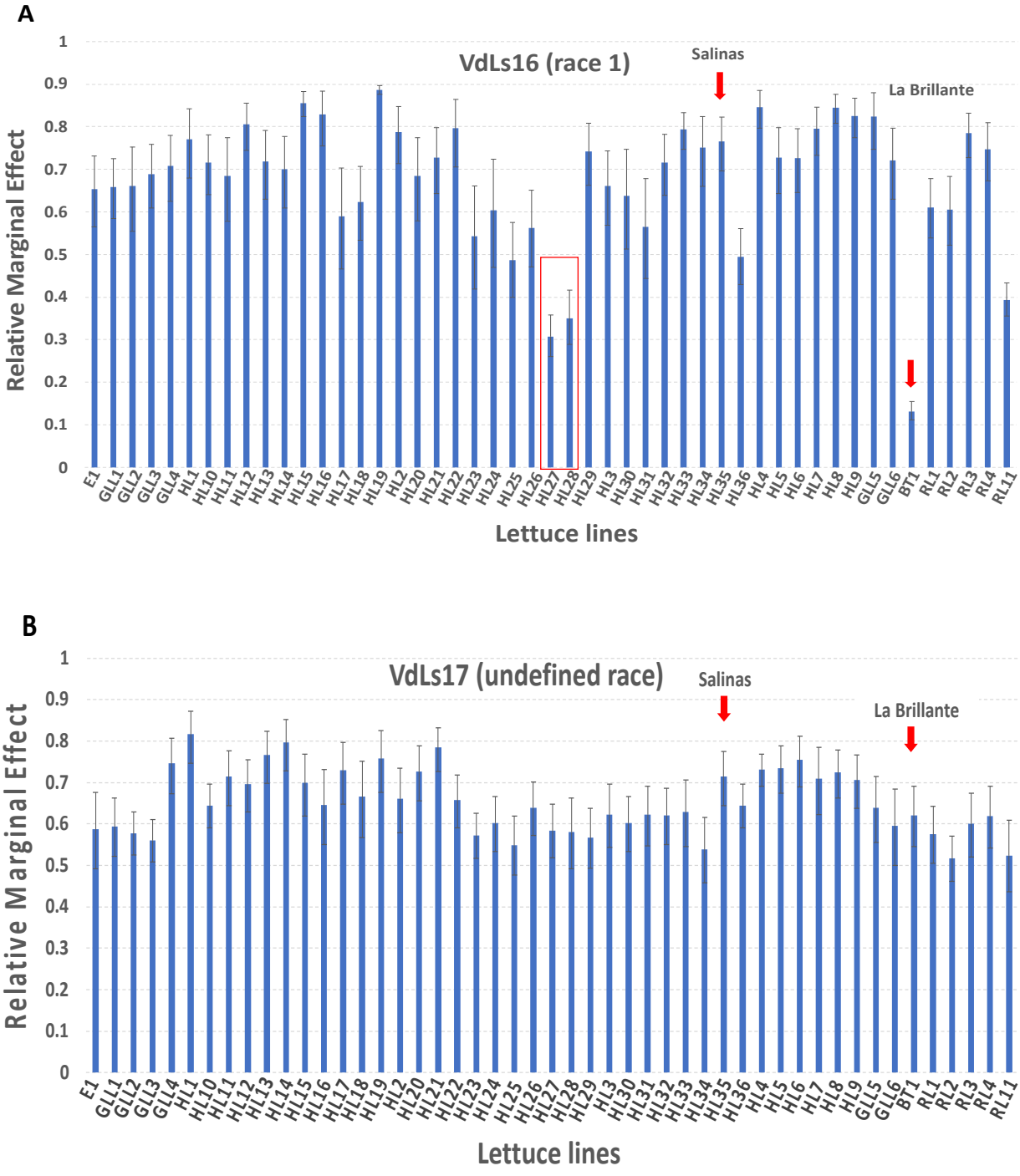
Contrasts	Num DF	Den DF	*F* Value	*P* > *F*
CW vs VdCv4323	1	118	140.63	<0.0001
CW vs VdLs4325	1	90.6	918.87	<0.0001
CW vs VdLs16	1	274	955.53	<0.0001
CW vs VdLs17	1	88.6	703.57	<0.0001
VdCv4323 vs VdLs4325	1	90.6	317.76	<0.0001
VdCv4323 vs VdLs16	1	123	263.24	<0.0001
VdCv4323 vs VdLs17	1	86.6	190.76	<0.0001
VdLs4325 vs VdLs16	1	102	12.87	0.0005
VdLs4325 vs VdLs17	1	86.8	18.86	<0.0001
VdLs16 vs VdLs17	1	100	1.26	0.2646

Performance of breeding lines/cultivar to specific *V. dahliae* races 1, 2 and 3

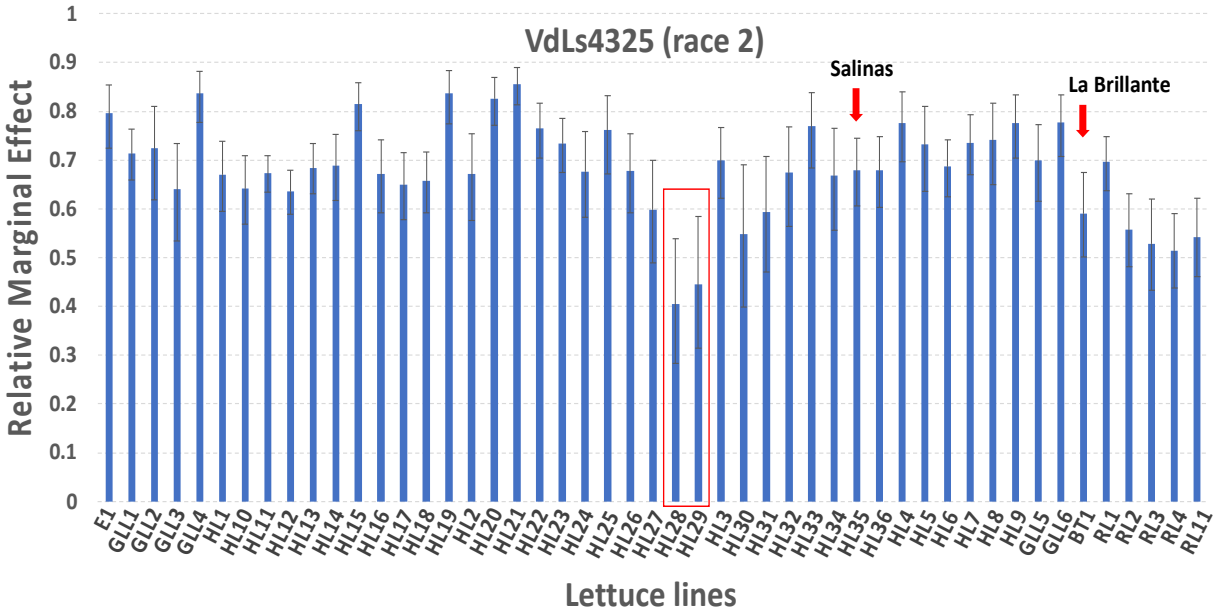
There was a significant ($P < 0.0001$; Table 2) interaction between isolate and cultivar, suggesting that Verticillium wilt severity on individual cultivars was dependent on the *V. dahliae* isolate inoculated (Figure 2). Except for the Batavia type La Brillante (BT1) and the two crisphead lines HL27 and HL28, all other lines showed root vascular discoloration against the race 1 isolate (VdLs16). Median disease rating ranged from 2 to 5, and RME ranged from 0.13 (BT1) to 0.88 (HL19) on VdLs16 inoculated plants (Figure 2A, Table 4). Disease severity levels were similar to those observed with VdLs17. However, race 1-tolerant lines such as BT1, HL27, HL28 with median VDS scores of 0.0, 2.0 and 2.0, respectively, against VdLs16, showed extensive root discoloration with median VDS score of 4.0, 4.0 and 3.5, when inoculated with VdLs17 (Figure 2B). The RME values on the 49 lettuce lines ranged from 0.51 (RL2) to 0.81 (HL1) (Figure 2B, Table 4). The race 2 isolate VdLs4325 caused extensive root discoloration in all lines save for HL28 (RME 0.40, median VDS 2) and HL29 (RME 0.44; median VDS 2) (Figure 2C, Table 4). Overall, in response to VdLs4325, the RME ranged from 0.40 (HL28) to 0.85 (HL21) with median disease ratings from 2 to 5. The race 3 isolate VdCv4323 from watermelon, was the least virulent

among all four isolates and caused little to no root discoloration in most lines (Figure 2D, Table 4). The RME ranged from 0.24 (GLL1) to 0.59 (HL2) with median disease rating ranges from 1 to 3.5.

Figure 2. Relative marginal effects (RME) of disease severity on different lettuce lines challenged with: A. Race 1 isolate VdLs16; B. VdLs17 (undefined race); C. Race 2 isolate VdLs4325; and D. Race 3 isolate VdCv4323



C


VdLs4325 (race 2)
Salinas
La Brillante
Relative Marginal Effect
Lettuce lines

D

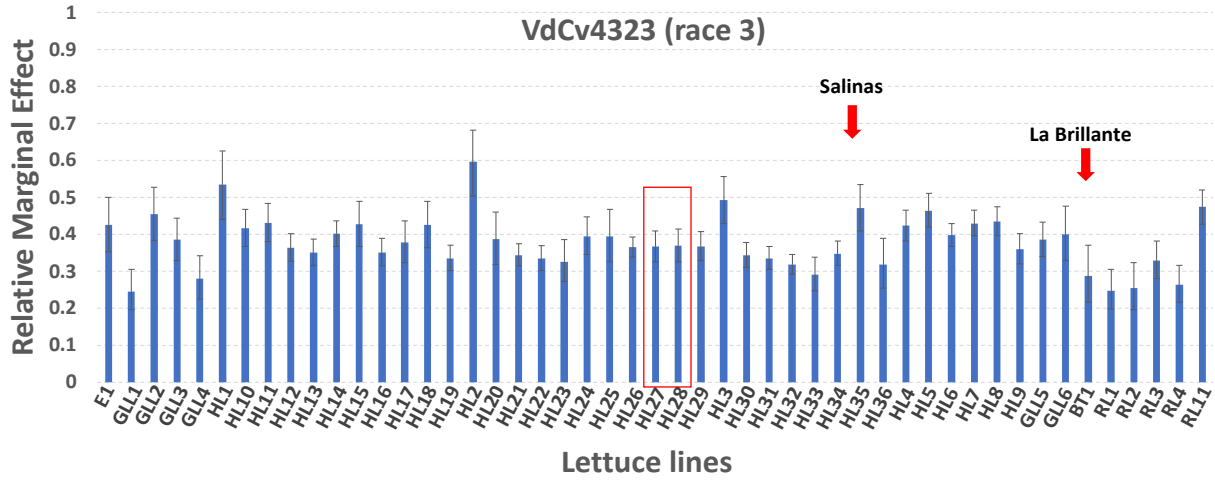

VdCv4323 (race 3)
Salinas
La Brillante
Relative Marginal Effect
Lettuce lines

Table 4. Median, mean, minimum (Min) and maximum (Max) disease severity and relative marginal effects (RME) along with 95% confidence intervals for Verticillium wilt severity ratings obtained from the evaluation of the response of 48 lettuce lines and endive against three isolates representing the three races of *Verticillium dahliae* along with another virulent isolate from lettuce in two independent experiments

Lettuce type/endive	Disease Severity				RME	95% Confidence Interval	
	Median	Mean	Min	Max		Lower	Upper
BT1	1.0	1.7	0.0	5.0	0.34834	0.29923	0.40107
HL28	2.0	1.9	0.0	5.0	0.36324	0.31882	0.41023
HL27	2.0	2.1	0.0	5.0	0.39359	0.34896	0.44016
RLL1	2.5	2.3	0.0	5.0	0.40887	0.36901	0.45002
RL2	3.0	2.2	0.0	5.0	0.40913	0.36362	0.45632
HL31	2.0	2.4	0.0	5.0	0.44568	0.39343	0.49922
HL29	2.5	2.4	0.0	5.0	0.44677	0.3937	0.50114
RL1	3.0	2.4	0.0	5.0	0.44836	0.39784	0.50002
HL30	2.0	2.4	0.0	5.0	0.44876	0.39396	0.50489
HL36	3.0	2.5	0.0	5.0	0.44983	0.40182	0.49883
RL4	3.0	2.5	0.0	5.0	0.45116	0.39908	0.50437
HL23	3.0	2.5	0.0	5.0	0.45746	0.40569	0.51021
HL25	2.0	2.5	0.0	5.0	0.46055	0.40887	0.51312
GLL1	3.0	2.5	0.0	5.0	0.46489	0.41212	0.51849
RL3	3.0	2.6	0.0	5.0	0.4708	0.41847	0.52381
HL26	3.0	2.6	0.0	5.0	0.47146	0.42179	0.52173
GLL3	3.0	2.6	0.0	5.0	0.47719	0.42696	0.5279
HL24	3.0	2.6	0.0	5.0	0.47784	0.42499	0.53122
HL34	3.0	2.7	0.0	5.0	0.48356	0.42954	0.53798
HL32	3.0	2.7	0.0	5.0	0.48819	0.4343	0.54236
HL17	3.0	2.7	0.0	5.0	0.49173	0.43761	0.54605
HL18	3.0	2.8	0.0	5.0	0.49675	0.4451	0.54846
GLL2	3.0	2.8	0.0	5.0	0.50584	0.45329	0.55826
HL10	3.0	2.8	0.0	5.0	0.50626	0.45629	0.5561

E	3.0	2.8	0.0	5.0	0.51459	0.45944	0.56937
HL3	3.0	2.9	0.0	5.0	0.51756	0.46615	0.56859
HL33	3.0	2.8	0.0	5.0	0.51927	0.46065	0.57735
GLL6	3.0	2.9	0.0	5.0	0.52137	0.46473	0.57743
HL16	3.0	2.9	0.0	5.0	0.52189	0.46413	0.57904
HL12	3.0	2.9	0.0	5.0	0.52264	0.46902	0.57571
HL11	3.0	2.9	0.0	5.0	0.52323	0.4711	0.57483
HL13	3.0	2.9	0.0	5.0	0.52629	0.47119	0.58073
GLL5	3.0	2.9	0.0	5.0	0.53198	0.47549	0.58763
HL22	3.0	2.9	0.0	5.0	0.53294	0.47517	0.58981
HL6	3.0	3.0	0.0	5.0	0.53559	0.48158	0.58875
GLL4	3.0	2.9	0.0	5.0	0.53659	0.47513	0.5969
HL14	3.0	3.0	0.0	5.0	0.53971	0.48376	0.59464
HL20	3.0	3.0	0.0	5.0	0.54727	0.48841	0.60478
HL35	4.0	3.1	0.0	5.0	0.54844	0.49436	0.60135
HL5	3.0	3.1	0.0	5.0	0.55415	0.49864	0.60827
HL9	4.0	3.1	0.0	5.0	0.55579	0.49716	0.61285
HL7	3.0	3.1	0.0	5.0	0.55668	0.50035	0.61152
HL21	4.0	3.1	0.0	5.0	0.56475	0.50457	0.62299
HL2	4.0	3.2	0.0	5.0	0.56556	0.50951	0.61992
HL8	4.0	3.2	0.0	5.0	0.57154	0.5136	0.62751
HL4	4.0	3.2	0.0	5.0	0.57781	0.52017	0.63332
HL1	4.0	3.2	0.0	5.0	0.58075	0.52179	0.6374
HL15	4.0	3.2	0.0	5.0	0.58163	0.52196	0.63891
HL19	4.0	3.2	0.0	5.0	0.58575	0.522	0.64664

Combined effect of all races on each breeding line/cultivar

Considering the overall response of each lettuce line to all three races and VdLs17 together, the race 1-resistant La Brillante, (BT1, RME 0.35) exhibited the most tolerant response to the four isolates (Table 4). In contrast, the crisphead lettuce HL19 (RME 0.59) was the most susceptible among the lines in its response to the four isolates. Forty-five percent of crisphead lettuce lines showed extensive root discoloration against all isolates. Among other lettuce types, green leaf lettuce (GLL2, GLL4, GLL5 and GLL6), and Endive (E1) exhibited high wilt severity. The crisphead lettuce lines HL27 (RME 0.36) and H28 (RME 0.39) exhibited the same levels of

resistance as La Brillante (BT1). The red leaf lettuce (RLL1, RME 0.41) and romaine lettuce (RL2, RME 0.41) also were resistant to Verticillium wilt (Table 4). In summary, the Batavia type (La Brillante) still serves as the source of high tolerance relative to other lettuce types (RME: BT 0.35, RL 0.45, GLL 0.51, HL 0.51, CLL 0.51, RLL 0.53). The response of Endive, GLL, and HL types were comparable and not significantly different ($P > 0.05$, Table 5).

Table 5. Orthogonal contrasts of Verticillium wilt severity caused by different isolates of *Verticillium dahliae* among different lettuce types

Contrasts	Numerator DF	Denominator DF	*F* Value	*P* > F(DDF)
Endive vs GLL	1	149	0.26	0.609
Endive vs HL	1	120	0.28	0.5945
Endive vs BT	1	193	53.92	<.0001
Endive vs RL	1	164	12.35	0.0006
Endive vs RLL	1	222	22.32	<.0001
GLL vs HL	1	823	7.14	0.0077
GLL vs BT	1	109	90.15	<.0001
GLL vs RL	1	667	31.58	<.0001
GLL vs RLL	1	161	36.59	<.0001
HL vs BT	1	85	126.81	<.0001
HL vs RL	1	369	83.9	<.0001
HL vs RLL	1	122	59.9	<.0001
BT vs RL	1	123	35.54	<.0001
BT vs RLL	1	185	8.83	0.0034
RL vs RLL	1	183	5.95	0.0156

Discussion

This study was undertaken to identify potential sources of resistance against the newly identified effector-based races 2 and 3 of *V. dahliae* by screening 48 lettuce breeding lines and cultivars including an endive line. Understanding the genetic interaction between these *V. dahliae* strains and lettuce is complex and critically important, as it depends on the race of the pathogen localized in a field.

In this study, the three races of *V. dahliae* caused varying degrees of vascular root discoloration in lettuce. The race 2 isolate VdLs4325 caused higher root discoloration overall relative to isolate VdCv4323 (race 3) from watermelon, which was the least virulent among all three of the races and VdLs17. Because the isolate VdCv4323 was derived from watermelon, it may have reduced virulence on lettuce as isolates of *V. dahliae* may not be as virulent as they are on their original host (Bhat and Subbarao 1999). Additionally, genetic analyses of the race 2- and race 3-lettuce interactions may yield insights into cultivar-specific responses associated with susceptibility and resistance. These areas will be further explored in future research. Though none of the breeding lines/cultivar were completely resistant to the three *V. dahliae* races, two tolerant lines HL28 and HL29 identified in this study can serve as sources of race 1 and race 2 resistance in lettuce and can be deployed in Verticillium wilt resistance breeding programs. We also hypothesize that HL28

and HL29 could be used as differential cultivars to differentiate race 1 and 2 from race 3 and utilized to map the genetics governing partial resistance.

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