**Project Title**: Spinach Breeding and Genetics

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**Abstract:**

Our emphasis is on problems facing the spinach industry in California, including coastal, desert, and interior valley. New or existing diseases, insects, or pathogens continue to appear or evolve to pose new challenges for growers, shippers, researchers, and the industry. Changes in production practices and marketing approaches also demand new genetic solutions. The spinach breeding and genetics program aims to incorporate valuable traits into spinach cultivars including resistances to downy mildew, Verticillium wilt, and Stemphylium leaf spot diseases, leafminer insect, and herbicides, and nutritional improvement in oxalic acid content. Horticultural traits, adaptation, and yield are also important. The most economical means of disease and insect control is through the use of genetic resistance. This is especially true for organic growers who must rely on a combination of plant resistance, organically certified pesticides and cultural practices to control diseases and insects. The use of resistant cultivars may reduce expenses for chemicals, energy, and labor associated with pesticide applications and minimize potential adverse effects of pesticide use. Historically, wilt disease caused by *V. dahliae* has not presented a problem in California spinach production because the crop is harvested well before the symptoms develop during the post-stem elongation (bolting) stage. However, infested spinach seeds introduce or increase inoculum in the soil for rotational crops such as lettuce. This investigation was designed to identify Verticillium wilt-resistant accessions in the USDA spinach germplasm collection against races 1 and 2 of *V. dahliae*, and to examine seed transmission of the pathogen in different spinach genotypes. In a seed health assay of 392 accessions of the collection, 21(5.4%) were positive for *V. dahliae*, and 153 (39%) were positive for *V. isaacii*. A

total of 268 accessions plus nine commercial cultivars were screened against one race 1 and two race 2 isolates from spinach in replicated greenhouse experiments. Plants were observed for disease incidence, severity, and seed transmission through plating on NP-10 medium and real-time quantitative polymerase chain reaction (qPCR). There was a wide range of variation in reactions among accessions against *V. dahliae* with disease incidence ranging from 0 to 100%. The two race 2 isolates differed in their virulence against spinach genotypes. Resistant accessions were identified against both races 1 and 2. Recovery of *V. dahliae* from seeds plated on NP-10 medium and qPCR results were highly correlated ($P = 0.00014$). Some accessions identified as resistant based on disease incidence showed little seed transmission. Even though lower wilt incidence and severity generally corresponded with lower seed transmission rates, there were exceptions ($r = 0.52$). Variation among plants within accessions was also observed. Nevertheless, the sources of resistance identified in this study are useful for spinach cultivar improvement. These results are encouraging and suggest that the development of Verticillium-resistant spinach cultivars is feasible. We also identified resistant varieties and conducted experiments to breed spinach for resistance to downy mildew, leafminers, and linuron herbicide.

**Objective. Screening for Resistance to Verticillium wilt in the USDA Spinach Germplasm Collection.**

*Verticillium dahliae* is a soilborne fungal pathogen that causes wilt diseases and devastating losses in many important crops (Klosterman et al. 2009). The resting structures produced by *V. dahliae*, known as microsclerotia, can survive in soil for up to 14 years (Wilhelm 1955), and in seed and plant debris for several years (Pegg and Brady 2002). The pathogen can be dispersed through soil, seed, vegetative material, farm equipment, water, and air (Pegg and Brady 2002). Two pathogenic races of *V. dahliae* have been identified in lettuce and tomato (Baergen et al. 1993; Hayes et al. 2011; Vallad et al. 2006), and both of these races can be recovered from infested spinach seeds (Short et al. 2014).

California accounts for over 60% of the spinach production in the U. S., valued at $233 million in 2013 (NASS 2013). Although Verticillium wilt has not been perceived as a problem for California spinach growers because disease symptoms normally appear following the stem elongation (bolting) stage (du Toit et al. 2005), it is now apparent that infected spinach seeds introduce or increase inoculum levels in the soil for rotational crops of lettuce (Short et al. 2014), but also likely for artichoke, strawberry, etc. A seed health assay revealed that 68 of 75 (91%) commercial seed lots produced in Denmark, Holland, New Zealand, and the United States were infested with *V. dahliae* with pathogen recovery rates ranging from 0.3 to 84.8% (du Toit et al. 2005). Similarly, Duressa et al. (2012) recorded seed infestation rates of up to 85% in commercial spinach seed lots. Spinach seeding rates are exceptionally high in California with 8.6 to 9.9 million seeds planted per hectare for the popular baby leaf production (Koike et al. 2011). The introduction of large amount of infected seeds coupled with the steep increase in spinach production in central coastal California since the 1990s has coincided with the appearance and spread of Verticillium wilt on lettuce, first discovered in the region in 1995 (Atallah et al. 2011; Subbarao et al. 1997). Fumigation with methyl bromide or other chemicals is effective, but is neither registered for use nor economically feasible for lettuce (Atallah et al. 2011). Crop rotation

has limited practicality, since most of the potential alternate crops are also susceptible to *V. dahliae*.

The most economical means of Verticillium wilt management is through host resistance. Resistance (*R*) genes against *V. dahliae* race 1 have been identified in cotton, sunflower, tomato, and lettuce (Fick and Zimmer 1974; Hayes et al. 2011; Schaible et al. 1951; Zhang et al. 2011). Cross-pathogenicity of the race 1 isolates on different hosts, such as tomato and lettuce, has been demonstrated (Maruthachalam et al. 2010). So far, only partial resistance to race 2 has been found in tomato (Baergen et al. 1993) and lettuce (Hayes et al. 2011).

One recent study has examined resistance to Verticillium wilt in spinach (Villarroel-Zeballos et al. 2012). They screened 120 spinach accessions from the USDA spinach collection and 10 commercial cultivars against Verticillium wilt. No accession was immune (completely resistant) to the disease, and there did not appear to be qualitative or major gene resistance to *V. dahliae* in the germplasm screened. Despite spinach crops being unaffected by Verticillium wilt, seed from resistant cultivars may reduce the quantity of *V. dahliae* microsclerotia introduced into the soil when the crop is planted, and increase seed yields in spinach seed producing regions.

There is also concern about the introduction of race 2 pathogen on spinach seeds, as disproportionate numbers of race 2 isolates of *V. dahliae* have been identified among isolates recovered from infested spinach seed. A race-specific PCR assay identified 96% of isolates as race 2 from among the 340 *V. dahliae* isolates recovered from spinach seeds produced in Chile, Denmark, the Netherlands, and the United States (Short et al. 2014). With the deployment of race 1 resistance in lettuce cultivars (Hayes et al. 2011), it is expected that the proportion of race 2 will increase in California soils under the genetic selection pressure. For this reason, identification and development of spinach germplasm with resistance to race 2 isolates are necessary. Except for the 120 accessions evaluated by Villarroel-Zeballos et al. (2012), the majority of USDA spinach germplasm collection has remained untested for resistance to Verticillium wilt. This investigation was undertaken to: 1) identify Verticillium wilt-resistant genotypes in the USDA spinach germplasm collection; 2) observe the responses of spinach varieties to race 1 and race 2 isolates of *V. dahliae*; and 3) examine the seed transmission of *V. dahliae* in spinach genotypes.

**Procedures.**

**Plant materials**. Seeds of the USDA spinach germplasm collection were provided by the North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa. The 120 accessions used in a previous screening experiment (Villarroel-Zeballos et al. 2012) were excluded from the current investigation. Twenty seeds from each of the accessions were plated on NP-10 medium in petri dishes as previously described (Maruthachalam et al., 2013), and following incubation on laboratory benches for 10 days ($24 \pm 1^{o}C$), examined under a microscope for morphological characteristics typical of *Verticillium* species (Inderbitzin et al. 2011). A total of 21 accessions were positive for *V. dahliae* and were also excluded from the experiment. The remaining 268 accessions plus 9 commercial cultivars were included in initial screening in the greenhouse using a race 2 isolate So 923. From this initial screen, 12 putative resistant, 2 susceptible, and 2 commercial cultivars were selected for further testing, including four resistant accessions identified in a previous study (Villarroel-Zeballos et al. 2012). In all tests, genotypes were planted in four inoculated replications and one uninoculated replication in a

greenhouse in a randomized complete block design. In each replication, eight seeds of each accession were planted in Sunshine Plug 5 Growing Mix (Sun Gro Horticulture, Agawam, MA) in plastic transplanting trays (128 cells, 3 x 3 x 5 cm in length x width x height) in a greenhouse in the winter to control day length with supplemental lighting. All experiments were repeated once to confirm the results.

**Pathogen inoculations.** Two race 2 isolates from spinach, So 923 and So 925, and a race 1 isolate So 302 were used in the inoculated experiments. Seedlings were inoculated at 3, 4, and 5 weeks after sowing by saturating the soil in each plug tray well with a 3-ml suspension containing $2 \times 10^6$ conidia $ml^{-1}$ in sterile, distilled water. Seedlings were incubated for another week after the last inoculation and then transplanted into 0.5-liter (16 oz) foam-insulated cups filled with a pasteurized sand: potting soil mixture (3:1, vol/vol). One week after transplanting, day length was extended to 19 hr $day^{-1}$ by supplemental lighting to promote bolting, as symptoms of Verticillium wilt on spinach mainly develop after bolting stage.

**Disease evaluations.** Starting from three weeks after the last inoculation, severity of symptoms were rated weekly using a scale of 0 to 4: 0 = no symptoms, 1 = lower leaves with patches of yellow areas or wilting, 2 = middle leaves with patches of yellow areas or wilting, 3 = upper leaves with patches of yellow areas or wilting, and 4 = all leaves died. After the final rating, roots were washed free of sand and cut longitudinally to evaluate disease severity as the % brown discoloration of vascular tissue in the roots, crown, and lower stem, characteristic of Verticillium wilt. The growth period (from planting to death of all leaves) of the inoculated plants was compared with the uninoculated control. To confirm the presence of the pathogen, *V. dahliae* was re-isolated from diseased tissue by placing roots, crown, and lower stems on NP-10 medium following surface sterilization (1% bleach solution for 1 min), and examined microscopically for development of conidiophores and/or microsclerotia of *V. dahliae*.

**Seed transmission.** To examine the seed transmission of the pathogen, mature seeds from each plant were harvested separately and assayed for *V. dahliae* by plating 20 seeds on NP-10 medium and incubating on laboratory benches for 10 days. The seeds were then observed under a microscope for microsclerotia and/or conidiophores and conidial characteristics of *V. dahliae*. The seeds were also analyzed with a real-time quantitative polymerase chain reaction (qPCR) assay by using primers derived from β-tubulin of *V. dahliae* (Duressa et al., 2012). All of the qPCR assays were performed as described previously (Duressa et al. 2012) with SYBR green dye, and β-tubulin standard curves for copy number calculation, with the exception that only 20 seeds per sample were ground for testing in this current study.

**Statistical analysis.** The highest weekly disease severity ratings for each plant were used in the statistical analysis. Disease severity data were analyzed by a nonparametric analysis, Friedman's Test for the two-way classification (Steel and Torrie, 1980). Percentage data of diseased plants (incidence) and seed infection (determined by NP-10 test) were subjected to $\sin^{-1}\sqrt{Y}$ transformation (Steel and Torrie, 1980) before being analyzed by analysis of variance (ANOVA) using the general linear models (GLM) procedure of JMP Version 10 (SAS Institute, Cary, NC). Genotype was considered a fixed effect with replication as a random effect. For comparisons among genotypes, least significant differences (LSD) were calculated with a Type I ($\alpha$) error rate of $P = 0.05$. Correlation and regression analyses of seed infection and qPCR data were also carried out by using the Fit Y by X function of JMP.

## Results

The race 2 isolate So 923 of *V. dahliae* was used in the preliminary screening of 268 accessions and 9 commercial cultivars to select 12 putative resistant accessions for further testing. All cultivars tested were susceptible to the isolate (data not shown). Two of the cultivars, Tarpy and Polar Bear; two susceptible accessions, PI 648942 and PI 648948; and four resistant accessions from a previous screening of the collection (Villarroel-Zeballos et al. 2012); were included as controls in the tests beyond the preliminary screening. There was a wide range of variation among genotypes in response to inoculations with the *V. dahliae* isolates (Table 1 and 2). Disease incidence ranged from 0 to 100% and severity varied from 0 to 3.

One accession from the Netherlands, PI 303138, showed no disease (0% incidence and mean severity of 0) when inoculated with the race 2 isolate So 923 (Table 1). Accessions PI 176774, PI 179042, NSL 6092, and NSL 6097 also exhibited low disease incidence and severity in response to isolate So 923. In contrast, the susceptible controls (PI 648942 and PI 648948) and cultivars (Polar Bear and Tarpy) all had high levels of disease symptoms (Table 1).

Against the race 2 isolate So 925, however, PI 303138 exhibited symptoms, with 35% and 25% incidence in tests 1 and 2, respectively (Table 1). No accession was immune to isolate So 925. PI 179588, NSL 6097, and NSL 81328 showed low disease incidence and severity. These three accessions also had low disease ratings when inoculated with So 923.

Two spinach accessions were identified that exhibited either no disease or low disease severity ratings in response to the race 1 isolate So 302. Accession PI 30318 showed no disease symptoms following inoculation with isolate So 302 (Table 2), similar to the response observed following inoculation with the race 2 isolate So 923 (Table 1). NSL 6092 also displayed low disease incidence and severity ratings against these isolates (Table 2). In contrast, the susceptible controls and cultivars exhibited high disease ratings (Table 2).

The use of quantitative PCR is often employed to analyze quantities of fungal pathogens *in planta* (Klosterman 2012). In this study, there was a significant correlation ($P = 0.00014$) between quantification cycle (Cq) values obtained by qPCR detection of the β-tubulin fragment from *V. dahliae* (pathogen DNA copy number) and percent seed infected with *V. dahliae* from twenty spinach cultivars or accessions tested (Figure 1).

The percentage of seed infected with *V. dahliae*, as determined by plating seeds on NP-10 medium, and pathogen DNA copy number, derived from qPCR of the *V. dahliae* β-tubulin target, are presented in Table 3. These values are representative of two independent measures of the pathogen transmission through seeds. When inoculated with the race 2 isolate So 923, the accessions PI 176774, PI 179042, and NSL 6097 showed little seed transmission of the pathogen. Against another race 2 isolate, So 925, accessions PI 175931, PI 261789, PI 303138, and PI 648945 exhibited low seed infection. Inoculated with a race 1 isolate So 302, the accessions PI 179042, PI 175931, PI 261789, NSL 6092, PI 648942, and NSL 6097 exhibited no seed infection on NP-10 medium and low pathogen copy number. Accessions PI 175931, PI 179042, PI 261789, and NSL 6097 showed little seed infection against both a race 1 and a race 2 isolate of *V. dahliae*.

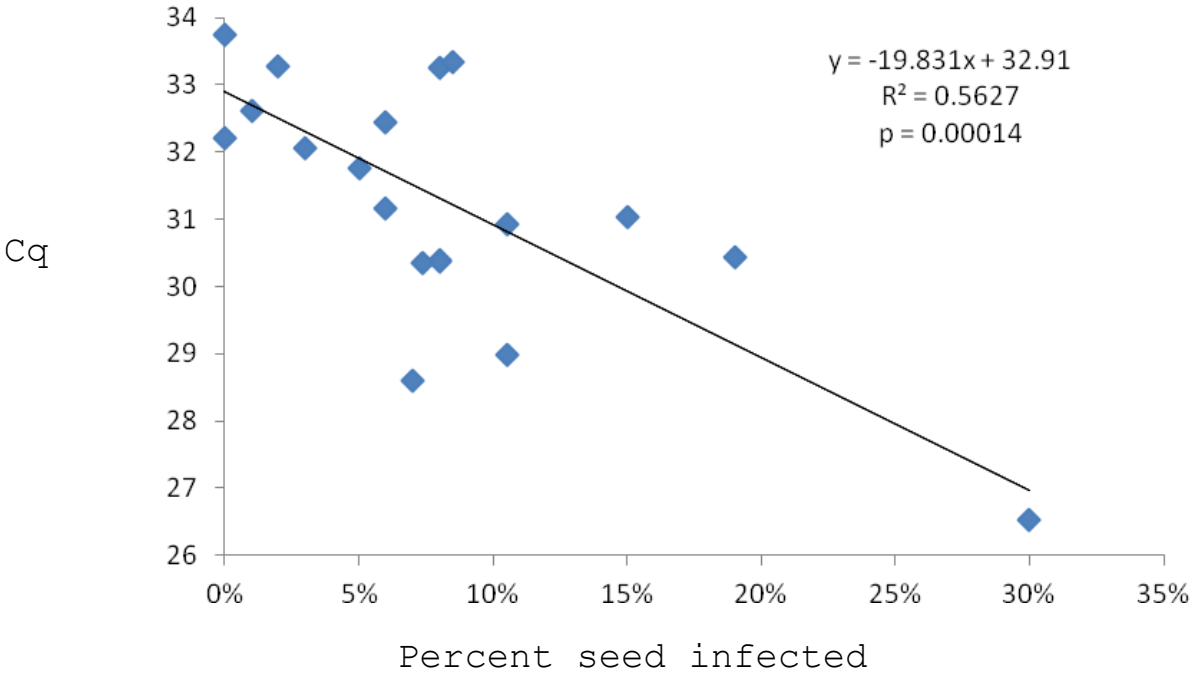


Figure 1. Correlation between Cq values obtained by real-time quantitative PCR detection of the *Verticillium dahliae β-tubulin* gene and percent infected seed with *V. dahliae* from twenty spinach cultivars or accessions. A lower Cq value indicates increased DNA detection from the pathogen, *V. dahliae*. No outliers were removed. The p value indicates a significant relationship between the two variables.

**Table 1.** Mean disease incidence and severity for selected accessions of the USDA spinach germplasm collection and commercial cultivars in repeated tests inoculated with two Race 2 isolates of *Verticillium dahliae* So 923 and So 925 from spinach. Means with the same letter in a column are not significantly different at $P < 0.05$ using LSD test after $\sin^{-1}\sqrt{Y}$ transformation of data.

| | | So 923 | | | | So 925 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Incidence % | | Severity | | Incidence % | | Severity | |
| Genotype | Origin | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 | Test 1 | Test 2 |
| PI 648948 | China | 90.5 a | 91.7 a | 2.7 | 3.0 | 92.9 ab | 90.0 a | 2.7 | 2.7 |
| Tarpy Enza | 61.1 ab | | 31.7 cde | 3.0 | 2.8 | 75.0 abcd | 61.0 abcd | 2.9 | 2.3 |
| Polar Bear | Rijk Zwaan | 47.2 bc | 63.5 bcd | 2.6 | 2.8 | 58.4 bcdef | 72.9 ab | 2.6 | 2.7 |
| PI 175931 | Turkey | 36.1 bcd | 61.3 bcd | 2.4 | 1.8 | 70.2 abcde | 0.0 h | 2.1 | 0.0 |
| NSL 81328 | Maryland, U.S. | 33.3 bcde | 0.0 g | 0.5 | 0.0 | 19.7 defg | 10.4 fgh | 2.0 | 0.6 |
| PI 648942 | China | 31.0 bcde | 77.8 ab | 1.7 | 2.6 | 100.0 a | 73.2 abc | 2.9 | 2.8 |
| PI 648945 | China | 27.8 bcde | 63.5 bcd | 1.9 | 1.9 | 65.1 bcdef | 31.3 defg | 1.8 | 1.4 |
| PI 181923 | Syria | 26.4 bcde | 25.0 efg | 1.5 | 0.6 | 45.9 cdef | 0.0 h | 2.5 | 0.0 |
| PI 204735 | Turkey | 19.4 bcde | 15.3 efg | 1.0 | 1.0 | 25.0 fg | 16.1 fgh | 1.3 | 2.3 |
| NSL 6087 | New York, U.S. | 16.7 bcde | 28.9 def | 1.7 | 1.9 | 28.6 cdefg | 59.5 bcde | 3.0 | 2.1 |
| NSL 6097 | Minnesota, U.S. | 16.7 cde | 4.2 fg | 0.3 | 1.0 | 0.0 g | 11.3 fgh | 0.0 | 1.0 |
| PI 179588 | Belgium | 16.7 cde | 21.7 cdef | 0.7 | 3.0 | 12.5 fg | 4.2 gh | 2.0 | 0.8 |
| PI 171861 | Turkey | 14.3 cde | 12.2 efg | 1.2 | 2.0 | 42.9 cdef | 12.5 fgh | 2.8 | 1.0 |
| PI 179042 | Turkey | 14.3 cde | 0.0 g | 1.0 | 0.0 | 0.0 g | 43.8 bcdef | 0.0 | 2.1 |
| NSL 6092 | New York, U.S. | 8.3 de | 0.0 g | 1.0 | 0.0 | 50.0 bcdef | 41.3 bcdef | 2.7 | 1.7 |
| PI 167194 | Turkey | 0.0 e | 30.0 cdef | 0.0 | 2.7 | 30.0 efg | 4.2 gh | 1.0 | 0.5 |
| PI 176774 | Turkey | 0.0 e | 15.3 efg | 0.0 | 1.7 | 66.7 abcdef | 33.3 cdefg | 2.5 | 2.0 |
| PI 303138 | Netherlands | 0.0 e | 0.0 g | 0.0 | 0.0 | 34.8 cdef | 25.0 efgh | 2.8 | 0.5 |
| PI 163309 | India | ---- | 38.9 cde | ---- | 1.3 | 79.5 abc | 19.3 fgh | 2.9 | 1.1 |
| PI 261789 | France | ---- | 45.3 bcde | ---- | 2.4 | 46.5 cdef | 0.0 h | 3.0 | 0.0 |

**Table 2.** Mean disease incidence and severity for selected accessions of the USDA spinach germplasm collection and commercial cultivars in repeated tests inoculated with a Race 1 isolate of *Verticillium dahliae* So 302 from spinach. Means with the same letter in a column are not significantly different at $P < 0.05$ using LSD test after $\sin^{-1}\sqrt{Y}$ transformation of data.

| | Incidence % | | Severity | |
|---|---|---|---|---|
| **Genotype** | **Test 1** | **Test 2** | **Test 1** | **Test 2** |
| PI 648948 | 85.6 a | 55.4 abcd | 2.9 | 2.1 |
| PI 648942 | 76.2 a | 78.5 a | 2.9 | 2.3 |
| PI 648945 | 72.6 ab | 48.6 abcde | 2.0 | 2.0 |
| Tarpy 70.2 abcd | 51.1 abc | 2.4 | 2.8 | |
| Polar Bear | 69.4 abc | 74.3 ab | 2.9 | 2.6 |
| NSL 81328 | 66.7 abcd | 0.0 g | 2.7 | 0.0 |
| PI 261789 | 59.0 abcde | 6.3 fg | 2.5 | 0.8 |
| PI 175931 | 52.4 abcdef | 0.0 g | 2.5 | 0.0 |
| PI 176774 | 52.4 abcdef | 33.6 bcdef | 1.7 | 1.5 |
| NSL 6097 | 36.7 bcdefg | 16.7 efg | 1.8 | 1.0 |
| PI 163309 | 36.2 bcdefg | 16.4 efg | 1.7 | 0.8 |
| PI 179042 | 34.9 bcdefg | 8.3 fg | 1.7 | 1.0 |
| PI 167194 | 33.3 bcdefg | 38.9 bcdef | 1.6 | 1.3 |
| PI 204735 | 27.8 cdefg | 13.3 defg | 1.8 | 2.0 |
| PI 171861 | 27.0 bcdefg | 4.2 fg | 2.8 | 0.5 |
| PI 179588 | 26.2 bcdefg | 6.3 fg | 3.0 | 0.8 |
| PI 181923 | 24.6 defg | 0.0 g | 1.3 | 0.0 |
| NSL 6092 | 11.1 fg | 0.0 g | 0.3 | 0.0 |
| NSL 6087 | 10.3 efg | 31.7 cdefg | 2.0 | 1.2 |
| PI 303138 | 0.0 g | 0.0 g | 0.0 | 0.0 |

**Table 3.** Mean seed infection % (tested on NP-10 plates) and pathogen DNA copy number in seed (determined by real-time quantitative PCR) for selected accessions of the USDA spinach germplasm collection and commercial cultivars inoculated with a race 1 isolate (So 302) and two race 2 isolates (So 923 and So 925) of *Verticillium dahliae* from spinach. Means of percentage was analyzed after $\sin^{-1}\sqrt{Y}$ transformation of data. Means with the same letter in a column are not significantly different at $P < 0.05$ using LSD test.

| | Race 2 So 923 | | Race 2 So 925 | | Race 1 So 302 | |
|---|---|---|---|---|---|---|
| **Genotype** | **NP-10+%** | **Copy#[a]** | **NP-10+%** | **Copy#** | **NP-10+%** | **Copy#** |
| Tarpy 68.3 a | 436.1 b | 26.6 abc | 56.8 cde | 8.3 efgh | | 202.0 cd |
| PI 648948 | 65.5 ab | 883.1 a | 12.5 abc | 0.0 e | 39.3 ab | 392.0 bc |
| PI 163309 | ---- | ---- | 12.2 abc | 188.4 c | 4.0 fgh | 31.8 d |
| Polar Bear | 35.7 abc | 78.3 cd | 14.6 abc | 1.3 e | 48.7 a | 1098.1 a |
| PI 648942 | 34.4 bcd | 135.3 c | 21.3 abc | 0.0 e | 0.0 h | 5.6 d |
| PI 175931 | ---- | ---- | 0.0 c | 1.5 e | 0.0 h | 0.0 d |
| PI 167194 | 25.0 bcde | 7.4 d | 40.0 a | 22.3 de | 19.5 cde | 268.8 cd |
| PI 181923 | 21.0 cdef | 38.4 cd | 0.0 c | 18.7 de | 1.7 gh | 1.6 d |
| PI 303138 | 16.7 cdef | 91.3 cd | 0.0 c | 0.0 e | 13.0 cdef | 15.3 d |
| NSL 6092 | 7.5 cdef | 70.5 cd | 26.2 abc | 308.3 b | 0.0 h | 1.6 d |
| PI 204735 | 5.7 def | 9.1 d | 22.4 abc | 2.2 e | 41.0 a | 630.3 b |
| NSL 81328 | 5.0 def | 6.6 d | 33.3 ab | 7.1 e | 10.0 efgh | 78.3 cd |
| PI 648945 | ---- | ---- | 0.0 c | 2.0 e | 3.2 fgh | 10.6 d |
| NSL 6087 | ---- | ---- | 36.3 ab | 838.4 a | 20.4 bcd | 210.0 cd |
| PI 179042 | 1.7 def | 0.0 d | 19.2 abc | 84.9 cde | 0.0 h | 2.3 d |
| NSL 6097 | 0.4 ef | 5.3 d | 17.2 abc | 163.0 cd | 0.0 h | 2.6 d |
| PI 179588 | ---- | ---- | 20.8 abc | 42.9 cde | 6.3 fgh | 2.7 d |
| PI 171861 | ---- | ---- | 5.8 bc | 73.2 cde | 27.8 abc | 86.0 cd |
| PI 176774 | 0.0 f | 0.0 d | 8.8 bc | 156.9 cd | 11.3 defg | 24.4 d |
| PI 261789 | ---- | ---- | 0.0 c | 0.0 e | 0.0 h | 3.2 d |

[a] Copy# refers to the pathogen DNA copy number as determined by qPCR, using a standard curve with the *V. dahliae β*-tubulin gene fragment as described (Duressa et al. 2012).

**Discussion**

In a previous screening of the USDA spinach germplasm collection (Villarroel-Zeballos et al. 2012), 21 of 130 (16%) accessions were reported as infested or infected with *V. dahliae, V. tricorpus,* or *Gibellulopsis nigrescens* (formerly known as *V. nigrescens*). Even though there was a major revision of the taxonomy of the genus *Verticillium* in 2011 (Inderbitzin et al., 2011), the authors used the names of the old taxa in their article (Villarroel-Zeballos et al. 2012). We have recently shown that (Short et al. *in press*) the majority of isolates previously characterized as *V. tricorpus* actually belong to the newly erected species *V. isaacii* (Inderbitzin et al. 2011) and thus, we report the results using the new taxonomy in this paper. In our seed health assay of 392 accessions in the collection, 21 (5.4%) were positive for *V. dahliae*, and 153 (39%) were positive for *V. isaacii*. The seed infection of the 21 accessions demonstrated that these accessions were susceptible to *V. dahliae,* and were therefore excluded from further testing. *Verticillium isaacii* is a weak pathogen on lettuce and artichoke, and potentially can reduce the symptoms caused by *V. dahliae* on lettuce, probably due to the competition between the two species (Qin et al. 2008).

In this current study, no spinach accession was immune to both race 2 isolates. The results suggested that accessions may be resistant to one race 2 isolate, but susceptible to another race 2 isolate (Table 1). Thus different race 2 isolates may differ in their virulence against spinach genotypes. Thus it is important to use more than one isolate in resistance screening against a given race of the pathogen.

Although the *V. dahliae* isolates from spinach seeds are predominately race 2, the pathogen population in the Salinas Valley, is currently composed mostly of race 1 (Short et al. 2014). This is especially important considering that the Salinas Valley represents the major leafy vegetable production region in California, and that *Verticilllium dahliae* is cross-pathogenic on most of these leafy vegetables ( ). Fresh market salad, bunched, and processing spinach crops are harvested 21-50, 32-62, and 48-90 days after planting in California, respectively (Koike at el. 2011). *Verticillium dahliae* hyphae can colonize spinach root cortical tissues both intra and intercellularly by 2 wk after inoculation (Maruthachalam et al. 2013). Although *V. dahliae* may not produce foliar symptoms on production spinach, susceptible cultivars can increase the inoculum in the soil. Therefore, it is also desirable to have resistance to race 1 of *V. dahliae* so that soil inoculum levels are not augmented with each new crop of spinach produced from infested seeds.

In qPCR, the Cq value is inversely proportional to the amount of input DNA (lower Cq = higher amount of DNA). Thus, the highest percentage of seed infection, 30% for PI 167194, was associated with the lowest Cq value (higher amount of pathogen DNA). Conversely, one of the two accessions with the lowest percent of seed infection (PI 175931), as determined by NP-10 plating, was associated with the highest Cq (lower amount of pathogen DNA). The finding that Cq values > 32 were always associated with < 10 % infected seed indicates that this particular value could be useful for screening purposes, to quickly identify those spinach accessions with lower amounts of pathogen DNA. In some instances, there is variable amount of pathogen DNA per individual seed when comparing multiple seed lots, as suggested previously (Duressa et al. 2012). The results of the qPCR analyses herein further support this conclusion. For instance, seeds collected from PI 163309 exhibited an infection percentage of 7% and a Cq value of 28.60. On the other hand, seeds collected from NSL 6087 were 19% infected, with an associated Cq value of 30.43.

The reduced leaf symptoms, as measured by disease incidence and severity in this experiment, may help increase spinach seed yield. Perhaps even more importantly, elimination or reduction of spinach seed infection with *V. dahliae* may prevent or reduce the introduction of new inoculums to regions where susceptible alternate host crops are produced, such as in central coastal California. Although lower disease incidence and severity generally corresponded to lower levels of pathogen seed transmission (correlation coefficient between percent disease incidence and results from NP-10 plating was 0.52), they were not always linked (Table 1-3). For example, PI 303138 and PI 648945 had relatively high disease incidence when inoculated with the race 2 isolate So 925, but showed low seed transmission as determined by NP-10 plating and qPCR analyses. In contrast, PI 303138 did not display disease symptoms in response to the race 2 isolate So 923, but exhibited seed infection.

Four resistant controls from a previous screening of the collection (Villarroel-Zeballos et al. 2012), PI 163309, PI 175931, PI 261789, and PI 648945(Ames 26243), all displayed relatively high disease incidences and severity against the three isolates of *V. dahliae* in our tests (Table 1 and 2). This discrepancy may be due to the different virulence of the isolates used in the screens. However, PI 175931 and PI 261789 showed almost no seed infection and pathogen DNA copy of a race 1 and a race 2 isolate (Table 3). PI 648945 also had little seed transmission of the pathogen. The results confirm that these accessions have a certain level of resistance that prevented the pathogen from entering seeds.

This study completes the screening of all accessions of the USDA spinach germplasm collection against *V. dahliae*. Resistant accessions were identified, with resistance against both races 1 and 2 of *V. dahliae*. These accessions can be used as sources of resistance in spinach cultivar development. As in a previous germplasm screening (Villarroel-Zeballos et al. 2012), variation in disease symptoms was observed among plants within accessions. This is probably due to the fact that all accessions in the USDA spinach germplasm collection are open-pollinated populations and heterogeneous in their genetic makeup. The results of this study further provide an opportunity to improve the resistance to *V. dahliae* in spinach through selection and breeding.

**Other Research Projects:**

**Downy Mildew** Crosses were made among cultivars with different DM resistant genes to combine their resistances. We planted downy mildew-susceptible spinach cultivar 'Viroflay' in different spacing in the field to test the infection and spread of the pathogen in conditions of natural infection. We found that one to three-inch plant spacing all resulted in high disease incidence and severity. These results will help us conduct spinach selection nursery for downy mildew resistance in the field.

**Leafminer** A recurrent selection method was used to increase the level of resistance to leafminers. Six populations of western and oriental types of spinach were planted in the field at USDA-ARS station in August 2014. Plants were selected for fewer leafminer stings or mines, and were transplanted into isolators to produce seeds for selection next year.

**Herbicide Resistance (with Steve Fennimore's Group)** We screened spinach germplasm for resistance to Linuron herbicide in the field. After the herbicide spray, surviving resistant plants were transplanted into isolators to produce seeds for future testing .

**Publications relevant to this project in 2014-15:**

Simko, I., R. J. Hayes, B. Mou, J. D. McCreight. 2014. Chapter 4. Lettuce and spinach. In: S. Smith, B. Diers, J. Specht, and B. Carver (Eds.) Yield Gains in Major U.S. Field Crops. CSSA Special Publications 33. p. 53-86. American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc.

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