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. In this study, a wide range of genetic variation and sources of resistance to Verticillium wilt disease were found in the USDA spinach genebank. Eleven germplasm accessions showed no seed transmission of Race 1 and/or Race 2 isolates from spinach. Some accessions showed symptoms on plants but no seed transmission, while other accessions had seed transmissions without obvious plant symptoms. PI 175931 had no disease incidence, severity, seed infection, and pathogen copies in seeds when inoculated with the two races of pathogen. The susceptible controls and commercial cultivars exhibited high levels of

disease symptoms and seed transmission. These results are consistent with the results from previous preliminary screening experiments. NP-10 plating and quantitative PCR results were highly correlated. The spinach genotypes identified could potentially serve as source of resistance to Verticillium wilt disease. 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, heat stress, and linuron herbicide.

Objective 1. Screening for Resistance to Verticillium wilt in the USDA Spinach Germplasm Collection.

Procedures.

Plant materials. Sixteen accessions of USDA spinach germplasm collection were selected from preliminary screening to confirm their resistance to *Verticillium dahliae*. These accessions plus two susceptible and 9 cultivar controls were planted in 4 inoculated reps and 1 uninoculated rep in greenhouse to test their resistance to Race 1 and Race 2 isolates from spinach. In each replication, 8 seeds of each accession were planted in Sunshine Plug 5 Growing Mix in plastic transplanting trays (128 cells, 3 x 3 x 5 cm in length x width x height) in a greenhouse in winter to control day length.

Inoculations. In collaboration with Dr. Krishna Subbarao's lab, four replications were inoculated with a Race 1 isolate So302 from spinach, and four replications were inoculated with a Race 2 isolate So925 from spinach, while the other replication was used as uninoculated checks. 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 $2x10^6$ conidia/ml in sterile, distilled water. Seedlings were incubated for another week after 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 by supplemental lighting to promote bolting, as symptoms of Verticillium wilt on spinach mainly develop after bolting stage.

Evaluations. Starting from three weeks after 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 final rating, roots were cleaned 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 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. Roots, crown, and lower stems were placed on NP-10 medium and examined microscopically for development of conidiophores and/or microsclerotia of *V. dahliae*. 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. The seeds were observed under a microscope for microsclerotia and/or conidiophores and conidial characteristics of *V. dahliae*. In collaboration with Dr. Steve Klosterman's lab, the seeds were also analyzed with a real-time PCR assay by using primers derived from the β-tubulin gene of *V. dahliae* coupled with SYBR green dye.

Results and Discussion.

The Verticillium disease incidence (% diseased plants) varied greatly among the genotypes tested. There were also large variation in disease severity among different accessions and cultivars. These results suggest that there are significant genetic differences in Verticillium disease resistance among the genotypes.

An accession, PI 175931, had no disease incidence, severity, and seed transmission (as tested by NP-10 and quantitative PCR assays) when inoculated with a Race 1 isolate So302 from spinach (Table 1). Some accessions had disease incidence and severity, but showed little pathogen transmission through seeds. It seems the pathogen infected plants but could not enter seeds. On the other hand, NSL 81328 showed no disease symptom but had seed transmission.

Three accessions, PI 261789, PI 175931, and PI 181923, showed no disease incidence and severity, and had little seed transmission (as tested by NP-10 and quantitative PCR assays) when inoculated with a Race 2 isolate from spinach, So925 (Table 2). Although PI 303138 and PI 648945 showed disease symptoms, they had little seed transmission of the pathogen.

Disease incidence and seed transmission (as tested by NP-10 assay) of the accessions against the Race 1 isolate and the Race 2 isolate are listed in Table 3. PI 175931 and PI 179042 had no seed transmission of the pathogen against the two isolates. NSL 6092, PI 171861, and NSL 6097 had no seed transmission of the Race 1 pathogen, while PI 181923, PI 303138, and PI 648945 showed no seed transmission of the Race 2 isolate.

Previous analyses indicated that there are likely variable amounts of pathogen DNA per seed when comparing multiple seed lots (Duressa et al. 2012). The results of the qPCR herein also 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. Nevertheless there was a significant correlation ($p = 0.00014$) 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 the twenty spinach cultivars or accessions (Figure 1). Moreover, 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 conclusion, eleven germplasm accessions showed no seed transmission of Race 1 and/or Race 2 isolates from spinach. Some accessions showed symptoms on plants but no seed transmission, while other accessions had seed transmissions without obvious plant symptoms. PI 175931 had no disease incidence, severity, seed infection, and pathogen copies in seeds when inoculated with the two races of pathogen. The susceptible controls and commercial cultivars exhibited high levels of disease symptoms and seed transmission. These results are consistent with the results from previous preliminary screening experiments. NP-10 plating and quantitative PCR results were highly correlated. The spinach genotypes identified could potentially serve as source of resistance to Verticillium wilt disease. These results are encouraging and suggest that the development of Verticillium-resistant spinach cultivars is feasible.

Table 1. Verticillium disease incidence and severity, seed infection % (tested on NP-10 plates), and pathogen copy number in seed (determined by quantitative PCR) of selected accessions of the USDA spinach germplasm collection inoculated with a Race 1 isolate So302 from spinach.

Genotype	Incidence %	Severity 0 – 3	NP-10 positive %	Pathogen qPCR copy#
PI 175931	0.0 E	0.0 E	0.0 B	0.0
NSL 6092	0.0 E	0.0 E	0.0 B	2.3
PI 171861	4.2 E	0.5 DE	0.0 B	2.0
PI 261789	6.3 DE	0.8 CDE	0.0 B	3.2
PI 179042	8.3 DE	1.0 BCDE	0.0 B	2.3
NSL 6097	16.7 CDE	1.0 BCDE	0.0 B	2.6
PI 648942	78.5 A	2.3 ABC	0.0 B	5.6
NSL 6087	31.7 BCDE	1.2 ABCDE	0.0 B	25.3
NSL 81328	0.0 E	0.0 E	10.0 AB	78.3
Polar Bear	74.3 A	2.6 A	20.0 A	1,965.1

Table 2. Verticillium disease incidence and severity, seed infection % (tested on NP-10 plates), and pathogen copy number in seed (determined by quantitative PCR) of selected accessions of the USDA spinach germplasm collection inoculated with a Race 2 isolate So925 from spinach.

Genotype	Incidence %	Severity 0 – 3	NP-10 positive %	Pathogen qPCR copy#
PI 261789	0.0 E	0.0 C	0.0 B	0.0
PI 175931	0.0 E	0.0 C	0.0 B	1.5
PI 181923	0.0 E	0.0 C	0.0 B	18.7
PI 303138	25.0 DE	0.5 BC	0.0 B	0.0
PI 648945	31.3 CDE	1.4 AB	0.0 B	2.0
PI 179042	43.8 BCD	2.1 AB	0.0 B	56.7
Polar Bear	72.9 AB	2.7 A	14.6 A	1.3

Table 3. Verticillium disease incidence, seed infection % (tested on NP-10 plates), and pathogen copy number in seed (determined by quantitative PCR) of selected accessions of the USDA spinach germplasm collection inoculated with a Race 1 and a Race 2 isolate from spinach.

	Race 1 (So 302)			Race 2 (So 925)		
Genotype	Incidence %	NP-10 Positive %	Pathogen Copy #	Incidence %	NP-10 Positive %	Pathogen Copy #
PI 175931	0.0 E	0.0 B	3.2	0.0 E	0.0 B	0.0
PI 179042	8.3 DE	0.0 B	2.3	43.8 BCD	0.0 B	56.7
NSL 6092	0.0 E	0.0 B	2.3	41.3 BCDE	26.2 ABC	308.3
PI 171861	4.2 E	0.0 B	2.0	12.5 DEFG	5.8 BC	73.2
NSL 6097	16.7 CDE	0.0 B	25.3	59.5 ABC	36.3 AB	838.4
PI 181923	0.0 E	1.7 B	1.6	0.0 E	0.0 B	18.7
PI 303138	0.0 E	8.3 AB	24.2	25.0 DE	0.0 B	0.0
PI 648945	48.6 ABC	3.2 B	10.6	31.3 CDE	0.0 B	2.0
Polar Bear	74.3 A	20.0 A	1,965.1	72.9 AB	14.6 A	1.3

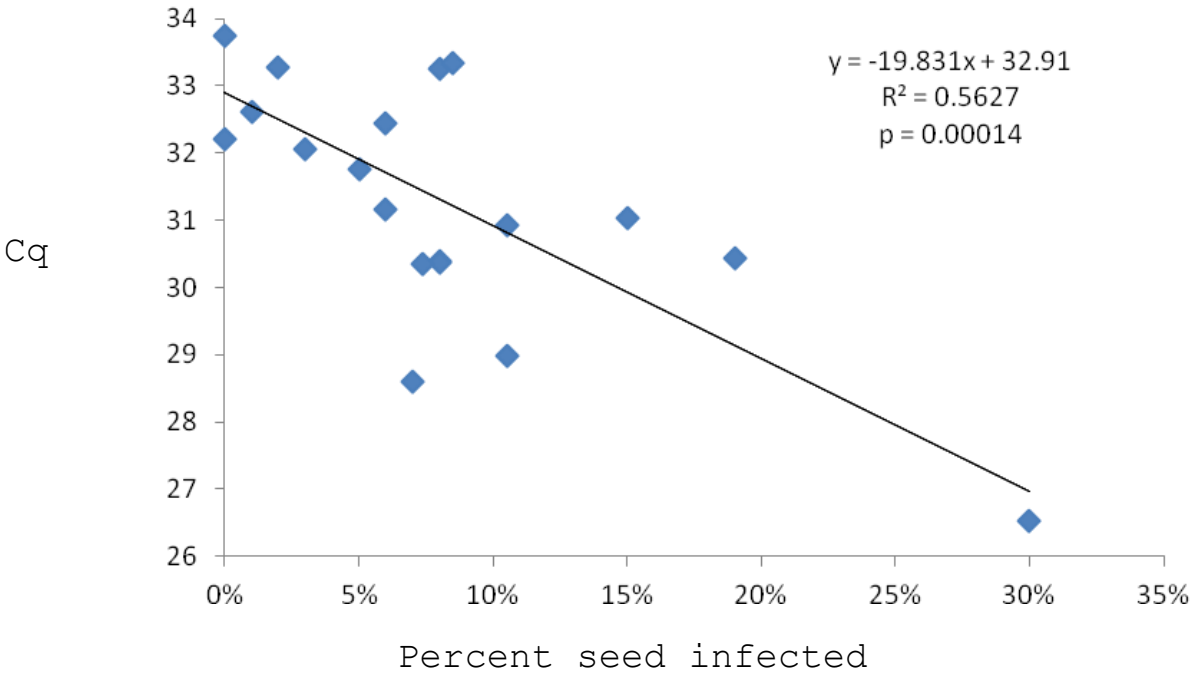

Figure 1. Correlation between Cq values obtained by real-time quantitative PCR detection of the *Verticillium dahliae β-tubulin* gene and percent infected seed with *V. dahliae* from twenty spinach cultivars or accessions. A lower Cq value indicates increased DNA detection from the pathogen, *V. dahliae*.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

Other Research Projects:

Downy Mildew (with Steve Koike) Methods of pathogen propagation were tried and modified. We are collecting and increasing inoculums of different downy mildew strains for germplasm screening and resistance breeding. Crosses were made among 15 cultivars with different DM resistant genes to combine their resistances.

Leafminer A recurrent selection method was used to increase the level of resistance to leafminers in 5 populations of different leaf types. Plants with fewer leafminer stings or mines were selected and transplanted into isolators to produce seeds for further rounds of evaluation and selection.

Heat Tolerance Over 400 spinach varieties were germinated under high temperatures in growth chambers. Genotypes with tolerance to seed thermo-dormancy were identified.

Herbicide Resistance (with Steve Fennimore's Group) We screened spinach germplasm for resistance to Linuron herbicide in the field. After the herbicide spray, surviving plants averaged 8.5% for 20 breeding populations and averaged 4.0% for 15 commercial cultivars. Resistant plants were transplanted into isolators to produce seeds for future testing .

Publications relevant to this project in 2013-14:

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.

Mou, B. 2013. Spinach. in: Wehner, T. C. and Mou, B. (Ed.) Vegetable cultivar descriptions for North America, List 27. HortScience 48: 268-269.

Maruthachalam, K., S. J. Klosterman, A. G. Anchieta, B. Mou, K. V. Subbarao. 2013. Colonization of spinach by *Verticillium dahliae* and effects of pathogen localization on the efficacy of seed treatments. Phytopathology 103: 268-280.