Identification of tolerance to *Fusarium* root rot in wild pea germplasm with high levels of partial resistance

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Fusarium root rot, caused by *Fusariumsolani* (Mart.) Sacc. f. sp. *pisi, (Fsp)* is a serious seed and root rot disease affecting pea growing areas throughout the world (1, 2, 3). The disease may damage peas produced in both dry land and irrigated fields, and has been reported to reduce yield between 30 to 57% in eastern Washington, U.S.A. and Canada (3, 4, 5). The pathogen infects the cotylendon region and spreads downward to the roots and upward to the crown. Early disease symptoms include reddish brown to black lesions on the roots that often expand and coalesce as the growing season progresses towards harvest. Mature plants may be severely stunted or die due to infection.

The commercial pea cultivars currently available have not been specifically bred for resistance to *Fsp.* However, several pea germplasm lines with partial resistance have been released (6, 7, 8, 9, 10), and resistant cultivars are being developed from these sources. In addition, new sources of partial resistance to *Fsp* have been identified in *Pisum sativum* ssp. *elatius* var. *pumilo* (11) and in 44 accessions from the *Pisum* Core Collection in 2003 (12). The partial resistance of these 44 accessions was characterized solely on root disease severity ratings from 0 to 5 (0 = no infection). Quantitative measurements of these 44 accessions comparing plant germination rates, plant height, fresh and dry foliage weight, and root dry weight between inoculated and non-inoculated plants of the same accessions were never assessed. Such comparisons would provide additional valuable information to characterize pea accessions that are not only highly resistant to root rot, but also tolerant to Fsp, with tolerance being defined as the ability of the infected plants to maintain normal growth (growth that is not significantly different from that of healthy plants when assessed under the same conditions) although infected by the pathogen.

The hypothesis of this research was that tolerant lines existed among the 44 *Pisum sativum* accessions that had previously been determined to have partial resistance to *Fusarium solani* f. sp. *pisi* (12). Identification of these lines would improve the selection of wild pea germplasm with both high partial resistance and tolerance to *Fsp* that could be used by pea breeders to select the most promising germplasm to be used to incorporate new genes into breeding programs to improve resistance to *Fsp.*

Materials and Methods

Pea seed of wild pea germplasm of forty-four accessions were obtained from the *Pisum* Core Collection (United States Department of Agriculture, Western Regional Plant Introduction Station, Pullman, WA, USA). These accessions had previously been determined to have some level of disease resistance to *Fsp* based on a 2003 survey of the *Pisum* Core Collection (12). The accessions were comprised of *Pisum sativum* (41 accessions), *Pisum sativum* subsp. *sativum* (2 accessions) and *Pisum sativum* var. *arvense* (1 accession). The accessions represented germplasm originating from fourteen countries (Table 1 provides information on selective pea genotypes from among the 44 accessions that demonstrated partial resistance and tolerance based on the present research).

The *Fsp* isolates F54, FS-01-B1, and F215, from our culture collection were used for inoculum in this study. These isolates originated from infected pea plants from three separate locations within Washington State, USA. Macroconidia of these isolates were mass produced following the below mentioned inoculum procedure and stored at 5°C in 10 ml test tubes containing 10 g of a sterile 1:1:1 (vol/vol/vol) soil:peat moss:perlite mixture (13). Inoculum was prepared for each isolate separately by transferring infested soil grains from the 10 ml test tubes onto peptone-pentachloronitrobenzene agar (PCNB) (14) in 9 mm Petri

Table i PI accession number, origin and flower/seed color of selective wild pea germplasm °1mwn tivmn iniimbim^m Pisum Core Collection as part of the Western Regional Plant Introduction Station located in Pullman, WA, U.S.A., in which the pisi.*

PI accession[a]	Origin	Flower/seed color[b]
PI125839	Afghanistan	p/p
PI125840	Afghanistan	p/p
PI175226	India	p/m
PI184128	Yugoslavia	p/p
PI198735	Afghanistan	p/p
PI220174	Afghanistan	p/p
PI220189	Afghanistan	p/p
PI222071	Afghanistan	p/p
PI222117	Afghanistan	p/p
PI223526	Afghanistan	p/p
PI223527	Afghanistan	p/p
PI226561	Ethiopia	p/p
PI227258	Iran	p/p
PI271119	China	p/p
PI166159	Nepal	p/p
PI257593	Ethiopia	p/p
Bolero	NA	w/np
DSP	NA	w/np

[a] *Two moderately resistant PI accessions (PI257593 and PI166159) and two susceptible pea cultivars (Bolero and Dark Skin Perfection) to Fusarium solani f. sp. pisi were used as standards in each test. Information on accessions was obtained from GRIN = Germplasm Resource Information Network, United States Department of Agriculture.*

[b] *m= mixed flower color, np = non-pigmented (green/white) seed coat, p = pigmented, w = white flower.*

plates. The plates were incubated for eight days at 23°C under continuous florescent cool white 34-watt lights. Following incubation, three plugs (4 mm in diameter) containing mycelia were transferred from the agar plates into 120 ml flasks containing Kerr's Medium (15). The flasks were placed on a shaker for seven days at 23°C under continuous florescent cool white light. Cultures of each isolate were then strained through one layer of cheesecloth and centrifuged at 2500 rpm for 5 minutes. The supernatant was poured off, and the spores were re-suspended using sterilized distilled water. The conidia concentrations for each isolate were determined using a hemacytometer, and the suspensions were adjusted to 1 x 10^6 conidia per/ml of water. Prior to inoculation, equal volumes of the isolates were combined into a single suspension and the seeds were immediately inoculated.

Fifty seeds of each pea genotype were placed in each of two 100 ml beakers to which 60 ml of a conidial suspension of *Fsp* were added to one beaker and 60 ml of sterilized distilled water were added to the other. The seed were soaked for 17 h at 25°C. The seed of each genotype soaked in sterilized distilled water were used as non-inoculated controls. Following the soaking period, ten wet seeds of each inoculated and non-inoculated genotype were planted in separate plastic trays (52.5 by 26.25 by 6.25 cm) filled with propagation grade-course perlite (Supreme Perlite Company, Portland, OR). Each tray contained three inoculated or three non-inoculated pea genotypes, each planted in the trays lengthwise in single rows with 10 plants per row. There were three replications of each genotype per test that were inoculated and non-inoculated, and each test was repeated one or two times for a maximum of three separate screening tests per genotype. Due to greenhouse space constraints, the forty-four accessions screened for *Fsp* resistance were divided into four tests labeled 1 through 4, with eleven different PI accessions screened per test. Multiple screenings of the same genotypes conducted at different times were identified as Test la, Test lb and Test lc. In addition to the eleven accessions screened per test, each test also contained two Fsp-susceptible pea cultivars (Dark Skin Perfection and Bolero) and two Fsp-moderately-resistant PI accessions (PI257593 and PI166159) as controls, except in Test la and lb, where only Dark Skin Perfection was used as a control.

The plants were grown in a greenhouse under natural sunlight, and supplemental lighting with 1000-watt metal halide lights used as needed to maintain a photoperiod of approximately 14 hours. The greenhouse temperatures for each test ranged from 15 to 28°C. The plants were watered uniformly as needed with approximately 500 ml of water per tray and fertilized uniformly with 500 ml of Miracle-Gro (24-8-16, N-P-K) (Marysville, OH) at a concentration of 4.93 ml Miracle-Gro per liter of water at 9, 12, 15, 18 and 21 days after planting.

The pea plants were harvested 25 days after planting, which allowed sufficient time for root rot symptoms to develop, and disease resistance was characterized based on the following comparisons between inoculated and non-inoculated plants of each accession: l) percent germination of seed, 2) root disease

severity (RDS), 3) foliage fresh weight, 4) foliage dry weight and 5) root dry weight. RDS values were assessed using a 0 to 5 scale: 0 = no infection; l = 1-10% infected root area (IRA); 2 = 11-25% IRA; 3 = 26-50% IRA; 4 = 51-80% IRA; 5 = 81-100% IRA (16). The foliage and the root dry weights were obtained by placing root and foliage samples in individual paper bags and placing the samples in a dryer at 45°C for 72 hours before weighing.

The greenhouse screening technique used to identify *Fsp* resistance in these tests is a refined method developed by Dr. John Kraft based on a previously published technique (16). This screening method has been used successfully to identify Fsp-resistant pea lines screened under greenhouse conditions, that are also Fsp-resistant when screened in the field (7, 8).

The general linear model (PROC GLM) in SAS (SAS Institute Inc., Cary, NC) was used to analyze the disease and plant parameters that were measured. Mean pair-wise comparisons among accessions were made using Fisher's Least Significant Difference Test ($P < 0.05$). A PROC RANK procedure in SAS was also used to rank accessions from l to ll according to their RDS rating within each trial of each test. A rank of "i" was assigned to the accession with the lowest disease rating and an "ll" to the accession with the highest rating.

Results

In tests l, 2, 3 and 4 and their repeated screenings (a to c), the mean percent germination was not significantly different ($P > 0.05$) between inoculated and non-inoculated plants of the same accession in two or more screenings tests. However, the mean percent germination of the inoculated seed of both susceptible controls, Dark Skin Perfection and Bolero, was significantly less ($P < 0.05$) than the non-inoculated seed of these cultivars in 10 of 10 and 8 of 8 screenings, respectively, in which they acted as susceptible controls in tests l to 4 for all screenings (data not shown due to lack of significant difference in two or more test among the accessions).

The roots of all non-inoculated control plants for all the screening tests were free of root rot and were rated as zeros according to the RDS scale. In Tests la, lb and lc, the mean RDS of accession PI125839 was numerically less than all other inoculated accessions and was significantly less ($P < 0.05$) than the severity of nine or more accessions in trials la and lb (Table 2). In Tests 2a, 2b and 2c, accessions PI184128 and PI198735 consistently demonstrated significantly lower ($P < 0.05$) mean RDSs than six other inoculated accessions screened in two or more of these tests (Table 2). In Tests 3a and 3b, accessions PI220174, PI220189, PI222071 and PI222117 consistently demonstrated the lowest mean RDSs in both tests that were either significantly ($P < 0.05$) or numerically less than five or more other inoculated accessions (Table 2). In Test 4a and 4b, the mean RDS of accession PI271119 was either numerically or significantly less ($P < 0.05$) than 9 or more of the inoculated accessions screened for both tests (Table 2).

The mean plant height, foliage fresh weight, foliage dry weight and root dry weight of the inoculated and non-inoculated plants were not significantly different ($P > 0.05$) from each other in two or more screenings for only eight (PI125839, PI125840, PI175226, PI220174, PI223526, PI223527, PI226561 and PI227258) of the 44 pea accessions including the controls (Table 3, only shows genotypes demonstrating no significant differences in growth parameters assessed between inoculated and non-inoculated genotypes).

Discussion

Previous research identified the 44 *Pisum* accessions assessed in the present study as having some partial resistance to *Fsp* based on low RDS ratings (12). This resistance was identified based on a single test, where two replications of 10 seed were used to screen each accession (12). Therefore, in the present study, more highly replicated screening tests were used to further characterize consistent resistance to *Fsp* root rot among these 44 wild *Pisum sativum* accessions. The present study identified accessions PI125839,

Table 2. PI accession number, screening test and root disease severity of selective wild pea germplasm from the Pisum Core Collection in Pullman, WA, USA demonstrating resistance to Fusarium solani f. sp. pisi when seed of forty-four accessions were inoculated and assessed 25 days after planting for root disease severity in repeated tests (Only the most consistently resistant PI accessions in the trials of each test are represented in the table).

PI accession #[a]	Screening test[b]	Root disease severity[d] Replicate a[c]	Replicate b	Replicate c
PI125839	1	0.92 (1)[9]	1.13 (1)[10]	1.42 (1)[3]
PI166159	1	...	1.64**[e]	2.43**
PI257593	1	...	2.32**	2.47**
Bolero	1	...	3.05**	3.34**
DSP	1	NG	4.50**	3.00**
RDS-Range	1	*1.18-2.04*	*1.38-2.32*	*1.44-2.49*
PI184128	2	1.58 (1)[6]	1.57 (1)[6]	2.32 (7)[4]
PI198735	2	1.82 (4)[6]	1.67 (2)[6]	1.93 (2)[6]
PI166159	2	...	2.27**	2.32*[f]
PI257593	2	...	2.93**	2.29*
Bolero	2	3.88 a	3.88**	3.5**
DSP	2	2.4 b	NG**	2.67**
RDS-Range	2	*1.59-3.05*	*1.67-3.91*	*1.74-3.69*
PI220174	3	1.47 (5)[3]	1.42 (1)[4]	...
PI220189	3	1.36 (1)[4]	1.53 (4)[3]	...
PI222071	3	1.37 (2)[4]	1.47 (3)[3]	...
PI222117	3	1.38 (3)[4]	1.46 (2)[3]	...
PI166159	3	1.76**	2.01**	...
PI257593	3	1.73**	2.24**	...
Bolero	3	3.56**	3.90**	...
DSP	3	3.60**	3.50**	...
RDS-Range	3	*1.42-1.97*	*1.65-2.53*	...
PI271119	4	1.36 (1)[4]	1.48 (2)[4]	...
PI166159	4	1.69*	1.71*	...
PI257593	4	2.78**	2.11**	...
Bolero	4	4**	3.75**	...
DSP	4	4.17**	NG**	...
RDS-Range	4	*1.50-2.68*	*1.38-2.31*	...

[a] Italizied names and PI accessions represent two moderately resistant (PI257593 and PI166159) and two susceptible (Bolero and Dark Skin Perfection) controls to ***Fusarium solani*** f. sp. ***pisi***. DSP and Bolero are commercial pea cultivars. RDS-Range = provides the range of the root disease severity values of the other PI accessions screened within a given screening test, not including the most resistant accessions already provided in the table. DSP = Dark Skin Perfection.

[b] Forty-four accessions were divided into four screening tests (1 to 4) and 11 accessions were screened per test. Tests were not screened at the same time but on different dates spaced at two-week intervals. Seed of inoculated plants and non-inoculated plants of each accession were treated with 1 x 10^6 macroconidia of ***F. solani*** f. sp. ***pisi*** and sterile distilled water, respectively. Roots of non-inoculated plants were free of infection.

[c] Example = 0.92 (1)[9] 0.92 = mean root disease severity value of the accession within a given trial. (1) = resistant rank among the 11 accessions screened per test based on mean root disease severity, with a rank of "1" being the most resistant accession and "11" being the least resistant accession to Fusarium root rot according to a PROC RANK procedure in SAS. [9] = Signifies that the mean root disease severity value of the accession was significantly less than ($P < 0.05$) the root disease severity value of 9 of the 11 accessions screened within a test according to a Fisher's LSD test. "..." = not included in the trial. NG = inoculated seed did not germinate due to infection by ***Fusarium solani*** f. sp. ***pisi*** based on comparisons with the non-inoculated water controls.

[d] Root disease severity was based on a 0 to 5 scale, "0" = no infection, 1 = 1-10% infected root area (IRA); 2 = 11-25% IRA; 3 = 26-50% IRA; 4 = 51-80% IRA; 5 = 81-100% IRA. Roots of non-inoculated controls of each accession were clean in all the tests and rated as zeros for their root disease severity.

[e] ** = the mean root disease severity value of the control (PI166159 in this case) is significantly greater ($P < 0.05$) than the ***Fsp***-resistant PI accession(s) listed in the table for a given trial according to a Fisher's LSD test.

[f] * = the mean root disease severity value of the control (PI166159 in this case) is significantly greater ($P < 0.05$) than only one of the ***Fsp***-resistant PI accession(s) listed in the table for a given trial according to a Fisher's LSD test.

PI184128, PI198735, PI220174, PI220189, PI222071, PI222117 and PI271119 as accessions that consistently demonstrated low RDS values (mean RDS values between 0.92 and 2.32) in two or more screening tests (Table 2). Six of these eight accessions originate from Afghanistan (PI125839, PI198735, PI220174, PI220189, PI222071 and PI222117) and one accession each from China (PI271119) and Yugoslavia (PI184128). Thus, the most Fsp-resistant wild pea germplasm based on RDS values is represented by only three countries. The high number of Fsp-resistant accessions originating from Afghanistan may indicate a unique source of genetic resistance to *Fsp* that is specific to the wild germplasm from that country. My lab

in collaboration with Dr. Norman Weeden at Montana State University screened a mapping population developed to identify the genes/QTLs (Quantitative trait loci) associated with the observed resistance to *Fsp* in the Afghanistan accession PI220174. Preliminary results from this research indicated that the major QTL associated with *Fsp* resistance in PI220174 overlapped the *A* locus on linkage group II which is the gene associated with purple flowers (17). The seed of purple-flowered cultivars are not popular for human consumption due to flavor and pigment issues, calling into question the practical use of the Afghanistan *Fsp* resistance if the gene or genes associated with the resistance are the same genes associated with purple-flowered varieties that are currently not marketable for human consumption. However, despite these apparent obstacles, no mapping populations have been developed to assess whether the resistance to *Fsp* originating from PI accessions from China (PI271119) or Yugoslavia (PI184128) are associated with different or similar genomic regions as those observed in the Afghanistan accessions and whether they may be alternative sources of resistance.

Resistance to a pathogen may result in a fitness cost to the host to maintain that resistance. Therefore, the ability of an infected plant to maintain similar growth as that of a non-infected plant grown under the same conditions, is a good indicator of the tolerance of that plant to a particular pathogen. Eight (PI125839, PI125840, PI175226, PI220174, PI223526, PI223527, PI226561 and PI227258) of the 44 accessions screened in this study demonstrated high levels of tolerance to *Fsp*, since the mean height, foliage fresh weight, foliage dry weight and root dry weight of the inoculated plants was not significantly different from non-inoculated plants of the same accessions in repeated tests. Five of these eight accessions originate from Afghanistan (Table 3). The other three accessions originate from Iran (PI227258), Ethiopia (PI226561) and India (PI175226). These tolerant lines may have additional genes

Table 3. PI accession number, mean plant height, foliage fresh weight, foliage dry weight and root dry weight of selective wild pea germplasm from the ***Pisum*** Core Collection in Pullman, WA, USA of which the seed was either inoculated with ***Fusarium solani*** f. sp. ***pisi (Fsp)*** or not inoculated (water treated) in repeated tests and plants were assessed 25 days after planting. Germplasm presented in the table represents tolerant accessions to ***Fsp*** with no significant differences ($P > 0.05$) between the inoculated and non-inoculated seed in repeated tests for all the parameters measured according to a Fisher's LSD test. A total of forty-four accessions were screened.

PI accession	Test	Plant height (cm)[a]	Foliage fresh wt. (g)	Foliage dry wt (g)	Root dry wt. (g)
125839	la	11.98/11.95	0.599/0.531	0.098/0.075	0.083/0.083
125839	lb	22.67/25.32	0.850/0.961	0.104/0.105	0.082/0.086
125839	lc	12.20/11.87	0.706/0.679	0.094/0.086	0.146/0.161
125840	la	15.74/17.17	0.465/0.542	0.078/0.084	0.067/0.073
125840	lb	29.86/31.91	0.722/0.764	0.090/0.090	0.063/0.056
125840	lc	17.58/15.56	0.643/0.578	0.091/0.073	0.108/0.113
175226	la	25.54/26.32	0.974/0.995	0.156/0.135	0.094/0.087
175226	lb	42.94/44.59	1.320/1.278	0.151/0.151	0.082/0.073
175226	lc	25.59/24.43	1.125/1.142	0.149/0.139	0.138/0.152
220174	3a	3l.95/3l.3l	0.782/0.800	0.089/0.089	0.074/0.069
220174	3b	28.02/29.10	0.716/0.722	0.091/0.079	0.088/0.076
223526	3a	30.90/30.82	0.754/0.673	0.086/0.078	0.062/0.067
223526	3b	27.80/28.91	0.648/0.676	0.085/0.083	0.076/0.072
223527	3a	28.72/29.11	1.089/0.957	0.114/0.100	0.122/0.089
223527	3b	24.87/27.53	0.893/0.962	0.11l/0.112	0.113/0.120
226561	3a	37.27/36.66	l.46/l.40l	0.147/0.143	0.116/0.107
226561	3b	35.93/38.86	1.469/1.592	0.186/0.184	0.126/0.132
227258	3a	33.68/35.20	0.687/0.717	0.078/0.082	0.063/0.061
227258	3b	29.85/32.01	0.653/0.696	0.086/0.088	0.066/0.061

[a] The first number followed by a "/" is the mean value of the inoculated plants followed by the mean value of the non-inoculated plants for the specified parameter. Seed of inoculated plants and non-inoculated plants of each accession were treated with 1 x 10^6 macroconidia of ***F. solani f.*** sp. ***pisi*** and sterile distilled water, respectively. Roots of non-inoculated plants were free of infection.

that are different from the genes providing partial resistance to *Fsp* root infection that allow them to tolerate root infection by this pathogen while maintaining similar growth as non-infected plants, thus

these plants are not demonstrating a fitness cost for Fsp-resistance. The plant growth of PI accessions 125839, 125840 and 175226 in test lb demonstrated significantly greater growth measurements than in tests la or lc (Table 3). The reasoning for the differential in growth is not understood but could have been due to more favorable greenhouse growing conditions experienced in test lb.

The mean percent germination of all accessions screened in this study was not significantly different ($P > 0.05$) between the inoculated and non-inoculated seed of the same accessions in two or more screenings in each test, indicating that the 44 accessions were resistant to seed rot or pre-emergence seedling damping off by *Fsp* under our screening conditions. Forty-three of the forty-four accessions screened have pigmented seed coats, with PI244121 (non-pigmented seed) being the only exception. Peas with pigmented seed coats are believed to be more resistant to seed rot or seedling damping off caused by *Fsp* than peas with non-pigmented seed coats (16). This theory was supported by the present results, since the mean percent germination of the inoculated seed of Bolero, DSP and PI244121, all having non-pigmented seed coats, was significantly less ($P < 0.05$) than the non-inoculated seed of these same pea lines in 8 of 8, 10 of 10, and l of 2 screening tests, respectively. There were only three incidences among the tests where the mean percent germination of an accession having pigmented seed was significantly less than the non-inoculated seed of the same accession (data not shown).

Future research will look to identify the genes/QTLs identified in this study that confer both partial resistance and tolerance to Fsp. A major emphasis will be placed on further evaluation of Fsp-resistant genes present in the wild Afghanistan pea accessions in addition to the potential new sources of resistance/tolerance from wild pea germplasm from Yugoslavia, China, Iran, Ethiopia and India. Efforts will be made to develop pea cultivars that will incorporate these resistances/tolerances and be used successfully throughout pea growing regions to manage Fusarium root rot.

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