Genotype Response of Soybean (*Glycine max*) Whole Plants and Hairy Roots to *Fusarium solani* f. sp. *glycines* Infection

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Abstract: *Fusarium solani* f. sp. *glycines*, a soilborne fungus, infects soybean roots and causes sudden death syndrome. The response of 13 soybean genotypes to *F. solani* f. sp. *glycines* infection was tested with potted greenhouse grown plants and with cultured hairy roots. The taproots of all genotypes grown in the greenhouse had dark brown lesions following inoculation. Foliar disease severity for greenhouse grown plants measured 21 days after planting was greatest for Peking, followed by Spencer, Ripley, P3981, Williams 82, Essex, Forrest, Iroquois, PI 520733, Hartwig, PI 567650B, Jack, and PI 567374. There were significant negative correlations between foliar disease severity and shoot length ($r = -0.422$, $P = 0.0018$), shoot weight ($r = -0.857$, $P < 0.0001$), root weight ($r = -0.732$, $P < 0.0001$), and total plant dry weights ($r = 0.855$, $P < 0.0001$). The taproot lesion length was not correlated with foliar disease severity indicating that soybean resistance may not be fully controlled at the root level. When cultured hairy roots were inoculated with *F. solani* f. sp. *glycines* mycelial plugs, the colony diameters after 10 days were significantly ($P = 0.05$) different among soybean genotypes ranging from 17 to 40 mm. Fungal colony diameters on hairy roots of Spencer and Peking were greater ($P = 0.05$) than on PI 567374 and PI 520733. In another experiment, following inoculation of Spencer and PI 567374 hairy roots with 10 μL of *F. solani* f. sp. *glycines* macroconidial suspensions, 10-day-colony diameters were 50 and 38 mm, respectively ($P = 0.05$). While there was generally a correlation between the growth of *F. solani* f. sp. *glycines* on the cultured hairy roots and the whole plant symptoms of the different genotypes, this was not always the case. The exceptions may be due to the fact that none of the genotypes showed clear root resistance even though they may show toxin resistance that would result in fewer foliar symptoms.

Key words: Fungal pathogen; Hairy roots; Soybean; Sudden death syndrome

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Soybean sudden death syndrome (SDS) is an economically important disease caused by the soilborne fungus *Fusarium solani* f. sp. *glycines* (Mart.) Sacc[1-3]. The pathogen colonizes soybean roots causing root rot and vascular discoloration of roots and stems. Even though the pathogen infects roots, the most conspicuous symptoms occur on leaves beginning with chlorotic mottling and proceeding to interveinal chlorosis, necrosis and defoliation[2-3]. SDS has become a widespread and consistent problem causing significant yield losses in many soybean growing areas[4-5]. Extensive soybean germplasm screening by inoculation of roots with *F. solani* f. sp. *glycines* infested sorghum grains in soil has identified some partially resistant genotypes based on foliar symptoms[6-7]. It was shown that the root infection of four soybean plant introductions (PI), PI 520733, PI 567374, PI 567650B, and PI 567659, did not differ ($P = 0.05$) in root lesion length in greenhouse experiments[8]. In another study based on foliar symptoms using aeroponic chambers, root lesion lengths of three partially resistant PIs (PI 520733, PI 567374, PI 567650B) and one susceptible soybean cultivar Great Lakes 3202 'differed ($P = 0.05$), but did not correlate with foliar disease or plant dry weights[9]. Some cultivars were reported to have significantly fewer colony forming units (CFU)/g of root tissue following inoculation[10]. In addition, it was shown that inoculum rate influenced selection of field resistance to SDS in the greenhouse[11]. Development of fast and reliable bioassay systems to study the soybean pathogen interac-

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tion and to evaluate the response of soybean genotypes to infection of *F. solani* f. sp. *glycines* could provide important information to breeding programs for selecting soybean genotypes resistant to *F. solani* f. sp. *glycines*. Most common assays on pathogen-infected roots include measurement of root lesions[12,8], determination of plant weights[13-15], and rating root severity based on root discoloration or necrosis[16]. These traditional methods have been used for several decades and provided data for root disease evaluation, but these methods are in general time-consuming and labor intensive.

The removal of roots from soils and cleaning the roots often causes the lost of root materials that also may affect the accuracy of the data. The use of aeroponic systems is an alternative approach to overcome the limitation of destructive sampling. In this system, roots are suspended and misted with a nutrient solution. An aeroponic system was used to evaluate soybean taproots inoculated with *Phytophthora sojae*[17], to screen maize genotypes for resistance to *F. graminearum*[18], and to study the interaction of selected soybean genotypes to *F. solani* f. sp. *glycines*[19]. Plant hairy root cultures, induced by the soil bacterium *Agrobacterium rhizogenes*, have unique properties including fast growth, unlimited propagation in culture, and biochemical stability[19]. Hairy roots, induced by inserting a set of rol genes from A. rhizogenes, alter plant hormone levels and sensitivity[20]. Along with the rol genes, other genes carried on a binary vector can be inserted at a high frequency[2]. Hairy roots also show stable production of root specific metabolites for an indefinite number of passages in culture, and allow visual monitoring of root growth. Hairy root cultures have been used to monitor root pathogen interactions for cytological studies, enumeration of the pathogen, and biochemical analysis. For example, soybean hairy roots were used to propagate the soybean cyst nematode[21], analyze isoflavonoid accumulation upon treatment with *F. solani* on two genotypes of soybean[22], and modify phenolic metabolism through down regulation of chalcone synthase or isoflavone synthase[23]. Sugar beet hairy roots were used to study infection processes of obligate fungal parasites[24]. Hairy roots of *Convolvulus sepium* were used to study mycorrhizal fungi[24]. Cultured hairy roots provide an excellent opportunity to investigate fungal pathogen interactions with roots under readily observable and controlled conditions. Soybean hairy roots provide a potential model system to study the interactions of soybean roots and pathogens. The objectives of this study were to (i) analyze root and foliar severity of different soybean genotypes in whole plant inoculation tests; (ii) determine the growth of *F. solani* f. sp. *glycines* on hairy roots of different soybean genotypes; and (iii) compare the response of soybean genotypes to *F. solani* f. sp. *glycines* infection in the hairy root system with that of greenhouse inoculated plants.

1 Materials and methods

1.1 Whole plant inoculations

Thirteen soybean genotypes that had different levels of resistance or susceptibility to foliar disease severity[6,8,25] were used in this study. Inoculum preparation and whole plant inoculations were performed in a greenhouse as previously described[8,26]. Seeds were sown in Cone-Tainers (Pay Leach Cone-Tainers, Stuewe & Sons, Inc., Corvallis, OR) with steamed sand and soil mix (2∶1 v/v) and inoculated with fungal infested sorghum grains. Three cm^3 of infested sorghum grains was placed 2 to 3 cm below a soybean seed in each Cone Tainer. Non-infested sorghum grains were used as controls. The Cone-Tainers were placed in a greenhouse with a 14 h photoperiod and a light intensity of 300 mol photons $m^{-2} s^{-1}$ at 28℃ day and 22℃ night temperatures. Five plants in each of two replications (10 plants total) for each genotype were inoculated with *F. solani* f. sp. *glycines* isolate Mont-1. Treatments (genotypes) were arranged in a randomized complete block design. Foliar disease rating was recorded 21 days after planting using a 1 to 5 scale, where 1 = no foliar symptoms to 5 = most severe foliar symptom development with interveinal chlorosis and necrosis on foliage and/or dead plants[6] Shoot length, root length, total plant length (shoot tip to root tip), and the root lesion length on the taproot were measured 21 days after planting. Dry weights of shoots and roots were determined by drying plants in a 55℃ oven for 3 days after

recording the foliar disease symptoms and plant length. The data for plant shoots, roots and total dry weights were calculated as a percentage of the uninoculated control. The experiment was repeated once.

1.2 Induction and preparation of hairy root cultures

The procedures for the induction and preparation of hairy root cultures are as previously described[2]. Briefly, cotyledon explants from 4- to 5- day- old seedlings of 13 soybean genotypes (Table 1) were harvested and wounded with a scalpel previously dipped in an overnight culture of *A. rhizogenes* strain K599. Cotyledons were inoculated by uniformly wounding the abaxial face several times. They were cultured abaxial side up on filter paper immersed in sterile distilled water at 25℃ for 3 to 5 days. Hairy roots were produced from the wounded surface of the cotyledon explants inoculated at 25 ℃ in the dark for 3 – 4 weeks on MXB medium which contains MS[27] basal nutrient salts, B5 vitamins[28] and 3% sucrose (pH 5.7) solidified with 3 g L^{-1} Gelrite (Greil Bros. Crop., East Coast Division, Spotswod, N. J., USA) in Petri dishes (100 mm diameter, 25 mm deep). Carbenicillin disodium (500 g mL^{-1}) was used to inhibit the growth of *A. rhizogenes*. About 10 – 14 days after root emergence, 1- to 2- cm- long root tips were transferred to MXB medium without any antibiotics for two or three passages of two weeks each at 25℃ in the dark. The established bacterium- free hairy roots of the 13 soybean genotypes on antibiotic- free medium in petri dishes were used to test *F. solani* f. sp. *glycines* growth.

1.3 Inoculation of *F. solani* f. sp. *glycines* on hairy roots

A 4- mm- diameter mycelial plug from the margin of 2- week- old cultures of the *F. solani* f. sp. *glycines* isolate Mont – 1 grown on potato dextrose agar (PDA) was placed mycelial side down directly on top of 20- to 30- day- old hairy roots, as well as on PDA and MXB medium without hairy roots. Six replicated hairy root culture plates (9- cm diameter) from each genotype were used. In another experiment, 10 microliters of *F. solani* f. sp. *glycines* macroconidial suspension (10^3 mL^{-1}) was added at the center of the hairy roots of three soybean genotypes, Spencer, PI 567374, and Williams 82. Three hairy root culture plates from each genotype were tested. After inoculations, hairy root plates were randomly placed in an incubator at 25℃ in the dark. To evaluate the inoculated hairy roots, the colony diameter of *F. solani* f. sp. *glycines* either from culture plugs or macroconidial suspension was determined 10 days after inoculation. Colony diameter was determined by taking four measurements, one from each quadrant of an equally divided Petri dish. Three Petri dishes were used for each genotype. Both culture plug and macroconidial suspension inoculation experiments were done twice.

1.4 Determination of fungal CFU on regular roots and hairy roots

To determine the CFU from inoculated whole plants, three root samples in each of two replications were collected from a susceptible (Spencer) and a partially resistant (PI 567374) lines that were inoculated with *F. solani* f. sp. *glycines* 21 days prior to collection in the greenhouse. Roots were removed from soil, washed thoroughly with tap water, surface disinfected for 3 min in 0.5% NaOCl solution, and rinsed twice with sterile deionized water. Roots were blotted dry with sterile filter paper and weighed. Roots from two plants of each inoculated genotype were combined together and ground in 10 mL of sterile deionized water using a blender (Waring Co. Santa Monica, CA) at high speed for 1 minute. The homogenized root suspension was then diluted 10 and 100- fold with sterile deionized water and made up to 100 and 1000- fold final dilutions. For each dilution, 100 L samples were placed and evenly spread with a sterile glass rod on *F. solani* f. sp. *glycines* semi- selective medium[29]. All plates were incubated at 25℃ in the dark. The number of colonies formed on the semi- selective medium was counted 7 to 10 days after plating. This experiment was repeated once.

To determine the CFU from inoculated hairy roots, hairy root cultures from four plates (20- 30 day- old) of a susceptible (Spencer) and a partially resistant (PI 567374) soybean line were removed from the medium, washed with sterile distilled water three times, and

then two plates of roots were pooled together, transferred to new empty plates. Ten mL of macroconidial suspension prepared from a *F. solani* f. sp. *glycines* isolate Mont – 1 was added to the hairy roots on the plates. After incubation at 25℃ in the dark for 4 days, hairy roots were rinsed with sterile deionized water, blotted dry on autoclaved paper towels, and then surface – disinfected for 30 seconds with 0.5% NaOCl solution, rinsed with sterile distilled water three times and blotted dry with sterile filter paper. After determining the fresh weight, the hairy roots were placed in a blender cup (Cole- Parmer Instrument Company, Vernon Hills, IL, USA) in 10 mL sterile deionized water and homogenized at high speed (Waring Co. Santa Monica, CA) for 30 seconds. The homogenized hairy roots suspension was further diluted 10, 100, and 1000-fold with deionized water, and 100 L of the 100 and 1000 fold dilutions was placed and evenly spread with a sterile glass rod on *F. solani* f. sp. *glycines* semi-selective medium[29]. Plate incubation and CFU counts were performed as described in the whole plant experiments. This experiment was repeated once.

1.5 Data analysis

The experimental data were analyzed by analysis of variance using GLM procedure (SAS version 8, SAS Institute, Cary, NC, USA). Data from the two runs of experiments were combined if the homogeneity of error variances and the genotype × experiment interaction were not significant. Treatment means were compared by least significant difference (LSD) at $P = 0.05$. A Pearson 's correlation coefficient was calculated among growth of *F. solani* f. sp. *glycines* on hairy roots, hairy root CFU, whole plant root CU, foliar severity, shoot, and root lengths, plant heights, taproot lesion length, shoot dry weights, root dry weights, and total plant dry weights using the SAS Pearson correlation procedure (PROC CORR).

2 Results

2.1 Pathogenicity tests and whole plant assays

Data from two experiments (trials) of pathogenicity tests were combined as the homogeneity of error variance and the treatment × experiment interaction was not significant. All 13 soybean genotypes showed SDS foliar symptoms after inoculation with *F. solani* f. sp. *glycines* in the greenhouse. The initial foliar symptoms included leaf mottling and mosaic 10 to 12 days after inoculation. The SDS foliar severity ratings ranged from 1.5 to 4.5 (Table 1) and the previously described partially resistant genotypes PI 567374, Jack, PI 567650B, Hartwig, and PI 520733 had the lowest foliar disease scores[8]. The known susceptible genotypes, Peking and Spencer, had foliar disease severity ratings of 4.5 and 4.2, respectively. The mean values of SDS foliar disease severity, shoot height, root length, plant height, taproot lesion length, shoot weight, root weight, and total plant dry weight indicated that there were significant ($P = 0.05$) differences among the soybean entries (Table 1). There were negative correlations between foliar disease severity and the shoot weight, root weight, total plant dry weights and plant height (Table 2). However, the taproot lesion length (percentage of the root length) was not correlated with foliar disease severity indicating that foliar symptoms are independent of root infection severity. Regression analysis of weight measurements to foliar severity was linear with shoot and total weights having higher R^2 values than root weights (Fig. 1).

2.2 Assays of *F. solani* f. sp. *glycines* growth on hairy roots

Growth of *F. solani* f. sp. *glycines* on hairy root cultures of the 13 soybean genotypes varied significantly ($P = 0.05$) and ranged from 17 to 40 mm in diameter 10 days after inoculation (Fig. 2). The diameter of fungal growth was greater on the hairy roots of genotypes, PI 567650B, Spencer, Peking, and Essex, than on PI 567374, Forrest, and PI 520733 (Fig. 2). There was an overall poor correlation between the fungal growth on the different hairy roots and the whole plant evaluations (Table 2). The growth of *F. solani* f. sp. *glycines* was much greater on PDA without hairy roots (46 mm in 10 days) than on MXB medium (19 mm in 10 days). Following inoculation of Spencer and PI 567374 hairy roots with 10 L of *F. solani* f. sp. *glycines* – macroconidial suspensions, the 4 – day- growth diameters were 50 and 38 mm, respectively ($P = 0.05$), confirming the results found with mycelial plug inoculation

where the fungus grew faster on Spencer than PI 567374 hairy roots (Fig. 2).

Table 1 Mean values of two experiments for foliar severity, plant height and weight, root length and weight, taproot lesion length and shoot weight of soybean genotypes 21 days after being inoculated with *Fusarium solani* f. sp. *glycines*

Soybean entry	Genotype reaction[a]	Maturity group	Foliar ratings[b]	Plant length[c]	Root length (%)[d]	Lesion length (%)[e]	Shoot weight (%)[d]	Root weight (%)[d]	Total weight (%)[d]
Essex	S	V	3.6	221	62	36	21	18	20
Forrest	PR	V	2.5	287	74	34	38	37	38
Hartwig	PR	IV	2.4	242	71	28	43	45	43
Iroquois	PR	III	2.5	308	92	23	41	52	44
Jack	PR	II	2.1	250	73	30	52	44	49
Peking	S	II	4.5	232	69	26	16	16	16
Pionee 3981	S	III	3.8	272	83	31	26	23	25
PI 520733	PR	III	2.4	301	78	36	44	33	41
PI 567374	PR	IV	1.5	292	89	40	59	53	57
PI 567650B	PR	IV	2.3	283	86	42	42	34	40
Ripley	PR	IV	3.9	237	76	26	18	20	19
Spencer	S	IV	4.2	257	80	27	25	29	26
Williams 82	S	III	3.7	253	82	29	19	17	18
LSDf (0.05)			0.8	60	20	14	14	17	13

[a]S: susceptible; PR: partially resistant[6,25]; [b]Foliar severity based on a 1 to 5 scale where 1 corresponds to no visual symptoms and 5 to over 80% of the foliage affected[6]; [c]Measured from shoot tip to root tip; [d]Percentage of the control without *F. solani* f. sp. *glycines* inoculation; [e]Taproot lesion length as percentage of the root length; [f]Fisher's protected least significant difference.

Table 2 Pearson's correlation coefficients values and significance for growth of *Fusarium solani* f. sp. *glycines* on hairy roots, and foliar severity, taproot lesion length, shoot height and weight, root length and weight for soybean plants inoculated with *F. solani* f. sp. *glycines* in the greenhouse

Variable	Foliar ratings[a]	Shoot height[b]	Root length[b]	Plant length[b]	Lesion length[c]	Shoot weight[b]	Root weight[b]	Total weight[b]
Fungal growth[d]	0.122	0.118	−0.020	−0.145	0.078	−0.102	−0.163	−0.124
	(0.388)	(0.404)	(0.889)	(0.304)	(0.583)	(0.474)	(0.248)	(0.381)
Foliar ratings	1.000	−0.556	−0.233	−0.417	−0.200	−0.857	−0.732	−0.855
		(<0.001)	(0.098)	(0.002)	(0.155)	(<0.001)	(<0.001)	(<0.001)
Shoot height		1.000	−.0.332	0.607	0.111	0.779	0.648	0.773
			(<0.016)	(<0.0001)	(0.432)	(<0.001)	(<0.001)	(<0.001)
Root length			1.000	0.84929	−0.127	0.361	0.386	0.382
				(<.001)	(0.370)	(0.009)	(0.005)	(0.005)
Plant length				1.000	−0.046	0.587	0.540	0.596
					(0.749)	(<0.001)	(<0.001)	(<0.001)
Lesion length					1.000	0.176	−0.010	0.127
						(0.212)	(0.945)	(0.371)
Shoot weight						1.000	0.806	0.984
							(<0.001)	(<0.001)
Root weight							1.000	0.899
								(<0.001)

[a]Based on a 1 to 5 scale where 1 corresponds to no visual symptoms and 5 to over 80% of the foliage affected (Hartman et al., 1997); [b]Percentage of the control without *F. solani* f. sp. *glycines* inoculation; [c]Taproot lesion length as percentage of the root length; [d]Diameter of *Fusarium solani* f. sp. *glycines* on hairy roots.

2.3 Determination of fungal CFU in roots

To determine if the fungus had penetrated into the hairy roots, fungal colony forming units (CFU) were measured in homogenates of surface disinfected ground hairy roots collected 4 days after inoculation. The mean $\log_{10}$ CFU values of two experiments from inoculated hairy roots of Spencer and PI 567374 were 3.15 ± 0.18 and 3.03 ± 0.18 per gram fresh weight roots, respectively. These values were not significantly different. When the $\log_{10}$ CFU values were determined for roots following whole plant inoculation, Spencer and PI 567374 had 3.70 0.19 and 3.64 ± 0.23 CFUs perg-

ram fresh weight 3 weeks after inoculation, respectively. There was no correlation ($P = 0.05$) between the number of whole plant inoculated root CFUs and the hairy root CFUs in these two soybean lines.

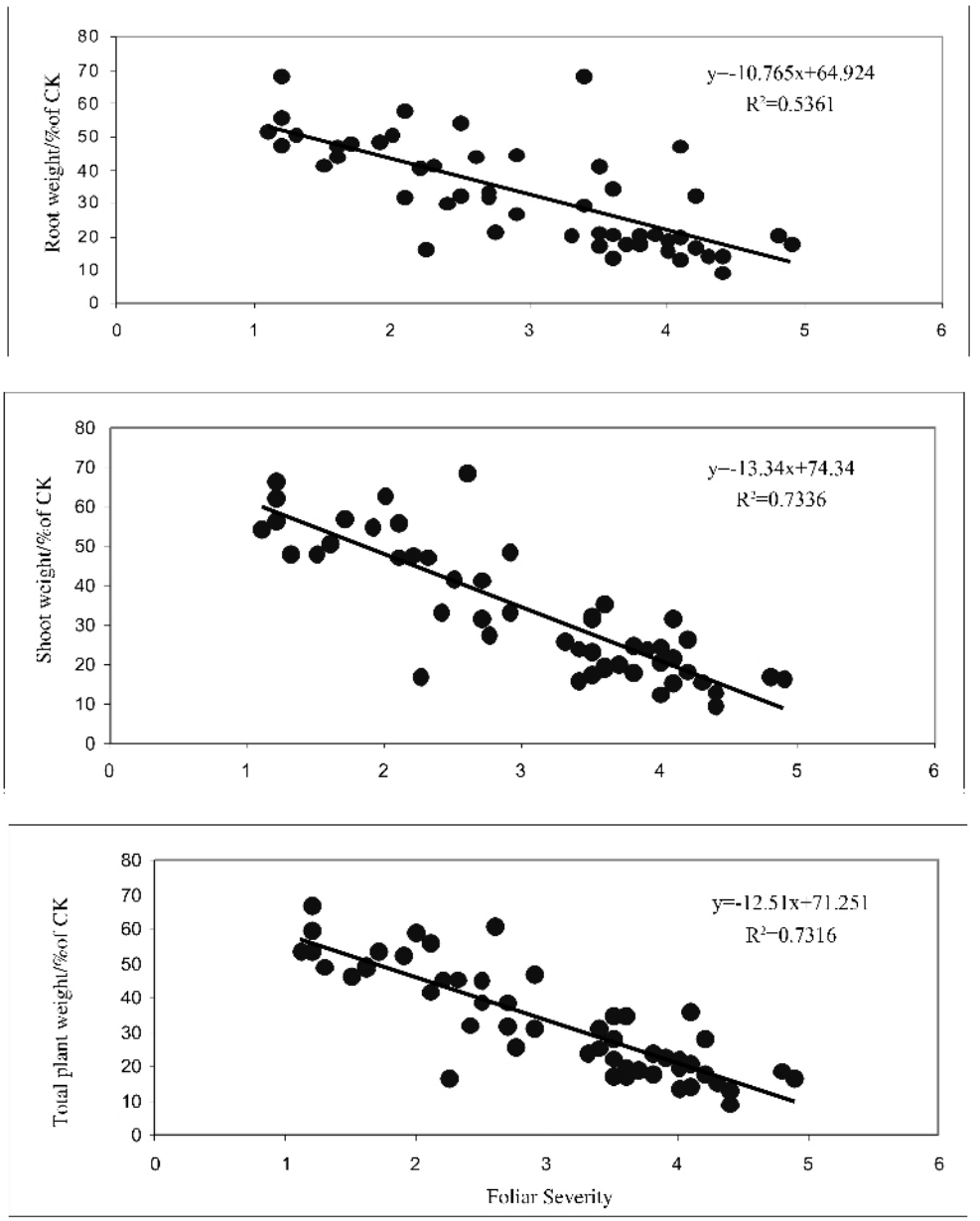

Fig. 1 Regression analysis between soybean sudden death syndrome foliar severity and shoot, root, and total plant dry weights (percentage of the control without inoculation of *Fusarium solani* f. sp. *glycines*) measured 21 days after planting in the pathogenicity tests in the greenhouse.

3 Discussion

An ideal way to evaluate root resistance in soybean to *F. solani* f. sp. *glycines* would be to use the hairy root system because it is fast, easily observable, and soil-free. In our study, CFU of *F. solani* f. sp. *glycines* were detected in surface disinfected ground hairy roots 4 days after inoculation indicating that the fungus had colonized the hairy roots. While the colony diameter of *F. solani* f. sp. *glycines* on known susceptible genotype Peking and Spencer was higher than on the partially resistant genotypes Forest, PI 520733, PI 567374, and Ripley ($P = 0.05$), there was no overall correlation between growth of *F. solani* f. sp. *glycines* on hairy roots and disease severity recorded on whole plants in the greenhouse. This lack of correlation in

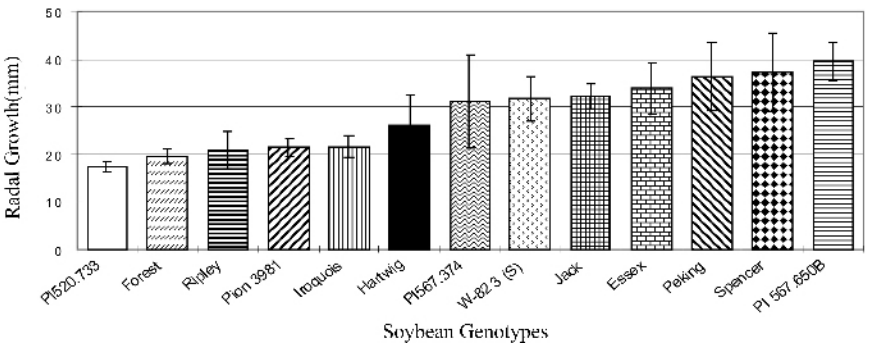

Fig. 2　Growth diameters with standard deviation of *Fusarium solani* f. sp. *glycines* on hairy roots of 13 soybean genotypes 10 days after inoculation

some cases could indicate that soybean plant resistance is not fully controlled at the root level. This conclusion is supported by the data showing that foliar symptoms do not correlate with taproot lesion length (Table 2) or very well with root weight (Fig. 1). Likewise fungal CFUs in both whole plant roots and hairy roots were not different with the susceptible genotype Spencer and the partially resistant genotype PI567374. It has also been shown that there are genotypic differences in the foliar symptom response to *F. solani* f. sp. *glycines* toxin-containing culture filtrates using the cut seedling assay where the roots are cut off and the diluted culture filtrate is taken up directly by the seedlings[8,30]. PI 567650B, one of the more resistant genotypes at the whole plant level (Table 1), had the highest fungal colony growth on the hairy roots (Fig. 2), and when tested in the cut seedling assay for toxin resistance, the area under the disease progress curve value of the SDS foliar severity was lower than three of five soybean genotypes tested including the partially resistant PI 520733[8]. When whole plants were inoculated, there were negative correlations between foliar symptoms and shoot weight, total plant weight and plant height. Foliar disease severity was also negatively correlated with root weight, but not the root lesion length (percentage of the root length) indicating that foliar symptoms are controlled at least in part by something other than amount of root infection. This is likely due to foliar susceptibility to the *F. solani* f sp. *glycines* toxin or toxins that can vary considerably between different genotypes[8,30]. The advantages of the hairy culture system include the speed and ease of infection of roots of different genotypes and the ability to visually monitor the fungal colonization of the roots. In our studies, fungal growth on hairy roots can be easily visualized and monitored, but the fungal growth did not correlate well with plant susceptibility measured by foliar symptoms. Hairy roots do however have the potential to make a good model system to measure the root resistance to infection of *F. solani* f. sp. *glycines* or other fungal pathogens. This might be especially important for testing genes that can easily be introduced and be expressed in the hairy roots. Genes that cause resistance at the hairy root level could then be inserted into soybean plants to produce cultivars with root resistance that should also result in foliar resistance since fungal penetration and growth should be limited.

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