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Evaluation of soybean resistance to *Phialophora gregata* culture filtrate in tissue culture

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Summary. Resistance to the fungal pathogen, *Phialophora gregata* (Allington and Chamberlain) W. Gams, the cause of brown stem rot (BSR) in soybean [*Glycine max* (L.) Merr.], is an important trait for cultivars grown in the northern USA. A novel tissue culture method was developed where ten soybean cultivars were differentiated on the ability of their excised cotyledons to remain green and initiate callus in a tissue culture medium containing *P. gregata* culture filtrate. Cultivar BSR classifications by the cotyledon method corresponded to greenhouse root-dip assay classifications in 80%, 100%, and 90% of the three *P. gregata* isolate treatments. Another method, employing pieces of somatic callus exposed to the culture filtrate, had a 70% average correspondence to the greenhouse results. Physiologic specialization was demonstrated in parallel in vivo/in vitro assays for the first time. These data suggest that the cotyledon method would accurately identify soybean lines resistant to certain aberrant or wild-type *P. gregata* isolates.

Key words: *Glycine max* (L.) Merr. – Brown stem rot – Pathotoxins – Physiologic specialization

Introduction

Brown stem rot (BSR) of soybean [*Glycine max* (L.) Merr.] is a disease of major importance in the north central USA. The causal fungus, *Phialophora gregata* (Allington and Chamberlain) W. Gams, invades through the roots and colonizes and discolors the pith and vascular tissues of the stem (Allington and Chamberlain 1948). Under conditions favorable to disease development, leaf symptoms appear as interveinal chlorosis and necrosis. *P. gregata* isolates have been broadly categorized as those which produce stem and leaf symptoms (Type I) and those which produce only stem symptoms (Type II) (Gray 1971). Seed yield losses from the disease have been estimated at between 12% and 38% (Gray 1972; Sebastian et al. 1986).

Resistance to the pathogen has become an increasingly important factor in midwest USA soybean breeding programs. Selection of breeding lines in the field for BSR resistance is hampered by high environmental variability (Hanson 1987; Sebastian et al. 1986).

Gray et al. (1987) developed a soybean tissue culture protocol to differentiate between calli derived from BSR-resistant or susceptible cultivars, by adding *P. gregata* culture filtrate containing phytotoxic metabolites (Kobayashi and Ui 1977) to a callus growth medium. The method is rapid but labor-intensive, and sensitive to the fungus culture filtrate concentration, size of calli, condition of calli, pathogen, and soybean strains employed.

The *P. gregata* filtrate also produces a genotype-specific wilting of detached soybean trifoliolates (Gray and Chamberlain 1975). The mother liquor extract of *P. gregata* culture filtrate inhibited the photosystem II complex and the cytochrome b/f complex in thylakoid membranes (Reeder et al. 1986).

Relatively few host-pathogen systems display corresponding results between intact plant response to the organism and callus or protoplast response to phytotoxins. Those systems which are amenable to in vitro screening differ widely for both host and pathogen species. Wheeler and Luke (1955) were the first to exploit such a system with a mass evaluation of oats for Victoria

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blight using the HV toxin produced by *Helminthosporium victoriae* Meehan and Murphy.

The objectives of this study were to: (i) develop an efficient evaluation method for BSR resistance, and (ii) determine the influence of pathogen isolate on the relationship of cultivar reactions from in vivo and in vitro evaluation assays.

Materials and methods

For this study, three *P. gregata* isolates (1, 2, and 11) were selected based on differences in their pattern of physiologic specialization on a differential host set (Willmot 1988). Ten soybean cultivars were evaluated for their response to the three pathogen isolates with three different protocols. Each experiment was a factorial arrangement in a completely randomized design.

The soybean line L78-4094 and plant introduction (PI) 437833 have the major genes for BSR resistance Rbs_1 and Rbs_2, respectively (Hanson et al. 1988). Plant introduction 437970 has the Rbs_3 gene. PI 437685D has a single dominant gene different than Rbs_1 and Rbs_2, but of unknown allelic relationship to the Rbs_3 gene in PI 437970 (Willmot 1988). 'Gnome 85' and 'Fayette' have shown intermediate to high levels of BSR resistance (Hanson 1987; Sebastian et al. 1986). 'Corsoy 79', 'Century 84', and 'Pioneer Variety P9271' (P9271) are BSR-susceptible. 'Hodgson 78' was chosen for its differential reaction to the isolates included.

Modified greenhouse method cultures of *P. gregata* were initiated from agar plugs from stem agar plates (Allington and Chamberlain 1948) initiated from inocula stored at −70 °C in 40% glycerol in order to avoid genetic changes in pathogenicity. From the colony borders of *P. gregata* plates, four 1 cm^2 agar plugs were added to flasks containing soybean seed broth (80 g of a susceptible cultivar seed l^{-1} water-steamed, strained and autoclaved). Stationary liquid cultures were incubated for 21–24 days at 22.5 °C, blended at high speed for 75 s, and diluted to 1.2×10^6 conidia and mycelial fragments ml^{-1}. Methyl cellulose (400 centipoise viscosity) was added at 0.75% (w/v).

Seeds were germinated in sand in 10-cm diameter plastic pots. Seedlings at the early unifoliolate stage were removed, roots were rinsed in water, and groups of five uniform, healthy plants selected, blotted with a paper towel and dipped in 50 ml of inoculum. The excess inoculum was poured into 15-cm diameter steam-sterilized clay pots containing a steam-treated 1:1 sand to topsoil mix, and inoculated seedlings were transplanted.

Plants were maintained under 15 h photoperiod at an average of 600 μE m^{-2} s^{-1} at midday and 18°–24 °C. Each pot received 150 ml of water twice daily. Weekly fertilization was with 150 ml of a solution providing 98 mg N, 89 mg P_2O_5, 85 mg K_2O, 0.12 mg chelated Cu, Mn and Zn, 0.05 mg B and 0.24 mg chelated Fe l^{-1}. Measurements were made after 5–5½ weeks on leaf and stem symptom severity as percent tissue discolored. Three pots of five plants were included. The sampling error was tested before pooling with experimental error as 15 replicates. Isolates and cultivars were treated as fixed effects in the analysis of variance of leaf and stem symptom severity reactions (Table 1).

Modified callus method (Gray et al. 1987): Stem extract-low glucose broth was prepared from 20 g green, young soybean stems of a susceptible cultivar blended at high speed for 60 s, passed through a milk filter, rinsed and diluted with distilled, de-ionized water to 1 l with 10 g glucose added. Increments of 100 ml were dispensed into 250 ml Erlenmeyer flasks, covered, and autoclaved. Cultures of *P. gregata* were initiated from three 0.5 cm^2-stem agar plugs. Stationary cultures were incubated for 19 days at 22 °C in the dark.

Table 1. Greenhouse root-dip assay analysis of variance of percent leaf and stem symptom discoloration by three *Phialophora gregata* isolates on ten soybean cultivars

Source of variation	Significance of F test		
	d.f.[a]	Leaf	Stem
Isolate	2	**	**
Cultivar	9	**	**
Isolate × cultivar	18	**	**
Error	419		

** Significant at $P < 0.01$

[a] Fifteen replications in a completely randomized design

The crude culture filtrate was prepared under low light intensity in covered glassware in order to avoid photodegradation of the toxic metabolites (Gray et al. 1987). The cultures were passed through three layers of Whatman no. 1 filter paper to remove mycelia and then passed through a 5.0 μm Millipore filter. The filtrates were adjusted to pH 5.8 with 0.1 N NaOH and conductivities were determined. Finally the filtrates were sequentially passed through 0.8 and 0.45 μm Millipore filters to prepare for overnight storage at 4 °C covered with foil. For control treatments, uninoculated broth was filtered as above and diluted to 80% of its original concentration in order to equalize the conductivity to that of the culture filtrate and avoid treatment differences due to the osmotic potential of the medium.

To initiate callus, soybean seeds were surface-sterilized and grown for 10 days in a growth chamber (16 h day^{-1} of 90 μE m^{-2} s^{-1}) using Murashige and Skoog (1962) basal salts agar medium (MS) without hormones. Epicotyl sections were then cut and placed on a modified MS medium denoted 4MSII (Barwale et al. 1986), excluding indole-3-acetic acid (IAA) and kinetin and containing 2.36 μM alpha-naphthaleneacetic acid (NAA) and 6.66 μM 6-benzylaminopurine (6BAP).

A different modified MS agar medium, that also excluded IAA and kinetin and included 44.4 μM 6BAP and 0.455 μM 2,4-dichlorophenoxyacetic acid (2,4-D), was used to assay the callus. Ingredient concentrations were adjusted in order to accommodate dilution with one part culture filtrate to five parts modified MS molten agar medium at 42 °C after autoclaving and cooling. The mixture was poured in 20 ml increments into 60 × 15 mm petri dishes previously marked in radial sections to separate the calli of ten cultivars. Three replicates (plates) for each pathogen isolate and six replicates of the control uninoculated broth-treated plates were included.

For each cultivar, eight 1 mg pieces of friable callus were taken from the initiation plates and placed on each randomly designated plate section under diffuse incandescent light. Plates were sealed, placed in plastic boxes lined with coarse paper, and placed in the growth chamber at 25 °C.

Visual ratings for necrosis were made at 4 and 7 days on a scale of 0 (none) to 9 (100% necrotic). Ratings were also made in 16 days for percent survival of the pieces and fresh weight. Ratings were analyzed as the difference from the mean of the uninoculated broth checks for the respective cultivars. The experiment was repeated in like manner (Table 2).

Cotyledon method: culture filtrate from the aforementioned *P. gregata* isolates and aseptic seedlings of the soybean cultivars

Table 2. Callus method combined analysis of variance of the difference from control broth treatments in 16-day fresh weight gain of callus for ten soybean cultivars in response to culture filtrates of three *Phialophora gregata* isolates

Source of variation	d.f.	Significance of F test
Experiment (E)	1	NS
Replication/E	4	–
Isolate (I)	2	**
E × I	2	**
Cultivar (C)	9	**
E × C	8	**
I × C	18	**
E × I × C	16	*
Error	108	–

* Significant at $P < 0.05$
** Significant at $P < 0.01$
NS, not significant

Table 3. Cotyledon method combined analysis of variance of the composite score of the differences from control broth treatment of ten soybean cultivars for cotyledon color and degree callus initiation in response to culture filtrates of three *Phialophora gregata* isolates

Source of variation	d.f.	Significance of F test
Experiment (E)	2	**
Replication/E	6	–
Isolate (I)	2	NS
E × I	4	NS
Cultivar (C)	9	**
E × C	18	**
I × C	18	*
E × I × C	36	NS
Error	174	–

* Significant at $P < 0.05$
** Significant at $P < 0.01$
NS, not significant

were produced as described for the callus method. The medium contained MS basal salts with 1.0 μM 2,4-D, 1.0 μM kinetin, 5.0 μM 6BAP, and no agar. The medium was autoclaved and aliquots of 0.85 ml were added to sterile, 20 ml scintillation vials. An equal volume of culture filtrate or control broth was added to each vial and swirled before positioning a cotyledon on its abaxial surface with a 1 cm excision wound at the basal end. Three replicates of each isolate × cultivar treatment were included and six replicates of each cultivar in control broth. Vials were capped and incubated in the dark for 8 days at 22.5 °C followed by 8 days at 24 °C in the lighted growth chamber under a coarse paper cover. Visual scores were then assigned on a linear scale for cotyledon color (0 = chlorotic or necrotic to 9 = fully green) and for the degree of healthy callus initiation (0 = none to 9 = over 45 mm^3). Scores were expressed as the difference from the respective cultivar control broth treatment mean. In addition, a composite score of the sum of the differences from controls in color and callus initiation was analyzed. The experiment was repeated twice. Combined analysis was performed after testing for homogeneity of variance. Experiments were considered random while all other variables were treated as fixed (Table 3).

Cultivar means within each method and isolate were compared by Fisher's protected least significant difference test. A probability level of 0.10 was set as proposed by Carmer (1976).

Cultivars within each method and isolate were then classified as resistant (R) if their mean was statistically like the more resistant cultivars, or susceptible (S) if their mean was the same as the more susceptible cultivars and distinct from the resistant class (Table 4). The in vitro parameters most consistent with the greenhouse results were chosen. The percentage of agreement of the classification decisions between greenhouse and in vitro reactions was tabulated in order to compare the efficiency of each in vitro method for selection purposes.

Results and discussion

The greenhouse evaluation displayed highly significant cultivar, isolate, and isolate × cultivar effects (Table 1). The results were consistent with previous experiments (Willmot 1988) which duplicated many of these treatments, with the exception that isolate 2 was not virulent on PI 437833 as before (Table 4). Cultivar stem and leaf symptom ratings by the greenhouse method corresponded for all three isolates (Table 4). For the cotyledon method, the composite score of the sum of the differences from control means for color and callus initiation corresponded more closely with greenhouse leaf symptom results than either component score alone. Selection based on this composite score corresponded for 27 of 30 (90%) cultivar decisions over the three isolates. The cotyledon method had the highest correspondence of classification with leaf symptom responses (100%) with aberrant isolate 2, compared to isolates 1 and 11 (80% and 90% agreement, respectively).

One difference in cultivar BSR classification arose from the normally resistant PI 437833 reacting as susceptible to the aberrant isolate in 1 in vivo, but resistant in the cotyledon method. Indeed, isolate 1 was included in the study for having virulence against the Rbs_2 gene (Willmot 1988). In view of the resistance of PI 437833 in most cases (Willmot 1988; Hanson et al. 1988), differences in BSR reaction may reveal resistance not reflected with exposure of the intact plant to the pathogen. Such resistance may complement other sources of resistance to provide multirace and/or a higher level of resistance.

Cultivars that are resistant in vivo but susceptible in vitro may indicate a latent in vivo susceptibility. For example, Fayette and L78-4094 were classified as resistant according to the greenhouse leaf symptom parameter, but susceptible according to the cotyledon method – with isolates 1 and 11, respectively. Such differences between evaluation methods may highlight genotypes that may be susceptible under increased disease pressure

Table 4. Comparison of brown stem rot evaluation methods for the reaction of ten soybean cultivars to three *Phialophora gregata* isolates

Isolate and Cv.	Screening method							
	Greenhouse[a]				Cotyledon		Callus	
	Leaf (%)	class[b]	Stem (%)	class	CCC[c] (score)	class	fw[d] (mg)	class
Isolate 1								
L78-4094	9	R	14	R	1.57	R	−24	R
PI 437833	25	S	35	S	0.31	R	−118	S
PI 437970	22	R	28	R	2.33	R	−24	R
PI 437685D	14	R	22	R	1.62	R	−99	S
Gnome 85	9	R	14	R	−0.94	R	−97	S
Fayette	0	R	4	R	−3.90	S	−20	R
Hodgson 78	12	R	21	R	−0.50	R	−99	S
Corsoy 79	33	S	37	S	−4.30	S	−108	S
Century 84	26	S	33	S	−3.42	S	−79	R
P9271	32	S	32	S	−1.74	S	−139	S
LSD (0.10)	8		14		3.01		61	
Isolate 2								
L78-4094	7	R	14	R	−0.54	R	−68	R
PI 437833	10	R	12	R	2.31	R	−104	R
PI 437970	17	R	19	R	3.11	R	−94	R
PI 437685D	11	R	14	R	0.51	R	−97	R
Gnome 85	6	R	10	R	−0.10	R	−97	R
Fayette	16	R	13	R	0.10	R	−56	R
Hodgson 78	28	R	26	R	0.39	R	−135	S
Corsoy 79	44	S	41	S	−3.30	S	−121	S
Century 84	34	S	33	S	−1.31	S	−82	R
P9271	42	S	30	S	−4.07	S	−185	S
LSD (0.10)	11		9		2.33		46	
Isolate 11								
L78-4094	32	R	37	R	−1.99	S	47	R
PI 437833	12	R	17	R	0.98	R	−118	S
PI 437970	13	R	22	R	2.33	R	19	R
PI 437685D	1	R	6	R	2.96	R	−28	R
Gnome 85	21	R	36	R	0.23	R	−16	R
Fayette	34	R	42	R	−0.12	R	43	R
Hodgson 78	77	S	71	S	−0.61	S	1	R
Corsoy 79	66	S	71	S	−0.97	S	−44	S
Century 84	55	S	59	S	−3.76	S	40	R
P9271	48	S	45	S	−2.41	S	−68	S
LSD (0.10)	12		14		2.55		61	

[a] Rating based on percent leaf and stem tissue discoloration
[b] Brown stem rot reaction class: R = resistant, S = susceptible
[c] Composite score of difference from respective controls for cotyledon color and callus volume
[d] Difference in 16-day fresh weight gain from control treatments

where a higher level of toxin is produced by the pathogen in the plant and tissue is killed in advance of colonization. In preliminary studies, genotypes that are normally BSR resistant reacted as intermediate or susceptible in vivo and in vitro where inoculum or filtrate treatments were excessive.

In the analysis of variance for the cotyledon method (Table 3), some variation for the reaction of cultivars over isolates and between experiments was measured. The main effect of experiments was also significant – possibly due to small differences in the activity of the culture filtrate. The reaction classifications of the cultivars were nevertheless consistent over experiments. Moreover, the interaction of isolate and cultivar was consistent over the three experiments.

For the callus method, the difference in fresh weight gain from control treatments was the parameter most closely corresponding to greenhouse leaf symptom re-

sults. The level of selection decision correspondence between the parameters was 70%, averaged over isolates (60%, 80%, and 70% for isolates 1, 2, and 11 respectively). The cotyledon method had a higher level of correspondence with greenhouse leaf or stem symptoms than the callus method did for all three isolates.

In the callus method combined analysis of variance (Table 2), all main effects except experiment and all interactions were significant. The interaction of isolate and cultivar changed between the two experiments in time reflecting the expected variability of the test.

It was not expected that all in vitro treatments would correspond to in vivo responses. Apparently, some specific host-pathogen recognition mechanisms operate at leaf, stem, cotyledon, and callus tissue levels while others, e.g., morphological characteristics, do not. The present study shows a strong correspondence between in vivo genotypic responses to *P. gregata* isolate inoculation and exposure to culture metabolites in vitro. Moreover, physiologic specialization of isolates 1 and 2 was reflected at the whole plant and tissue culture levels.

Preliminary experiments for this study and those of Gray (personal communication) revealed that isolates which reduced the culture pH from 7.1 to below 5.0 were considerably more aggressive in the intensity of symptoms produced in vivo, and more effective against susceptible cultivars in vitro than isolates which were less reductive. Culture filtrates in the present study ranged from pH 4.20–4.68 before adjustment to pH 5.80. Gray et al. (1987) used the 2,3,5-triphenyl tetrazolium chloride dye reduction assay to show that calli of BSR-susceptible cultivars had lower metabolic electron transport capacity (a measure of cell viability) than calli of resistant cultivars after exposure to *P. gregata* culture filtrate. Nonpathogenic or Type II (non-defoliating) isolates did not differentiate genotypes of calli on the basis of cell reductive capacity or viability after exposure to the culture filtrate toxin(s).

For the cotyledon method, level of aggressiveness in vivo was not a good indicator of suitability of an isolate. The three isolates used in this study produced similar ranges in composite color/callus initiation score response in the cotyledon method despite the fact that isolate 11 was far more aggressive in vivo than isolated 1 or 2. However, in a preliminary replicated experiment, isolate 11 provided better differentiation of cultivars in the cotyledon method than did the less aggressive isolate 10 (Willmot 1988).

Certain isolates in a companion study (Willmot 1988) caused leaf tissue discoloration to a greater extent than stem tissue discoloration. Generally, stem symptoms exceed leaf symptoms in severity (Sebastian et al. 1986). Possibly, these aberrant isolates are over-producers of pathotoxins that would impart a competitive advantage over other soil micro-organisms for early substrate colonization (Bruehl et al. 1969). Such isolates may be superior for in vitro assays.

In the cotyledon and callus methods, cultivars differed in their response to the broth control treatment. For this reason, pathotoxin treatments were expressed as the difference from the mean of their respective cultivar control response. Any modified protocol which would nearly equalize control treatment reponses across cultivars would eliminate the need for control treatments for each entry in an evaluation assay. A better cytokinin media complement may achieve more uniform quality of callus and rate of initiation in control treatments while promoting toxin treatment responses that correspond on whole plant and tissue culture levels. Tobacco callus of resistant cultivars can react as resistant or susceptible to *Phytophthora parasitica* var. *nicocianae* depending on the cytokinin level of the medium (Haberlach et al. 1978; Helgeson et al. 1972).

Under the present protocol, the cotyledon method is slightly more labor-intensive than the greenhouse method, but far less labor-intensive than the callus method and more repeatable. The cotyledon method requires fewer days to complete in much less space than the greenhouse method. The main disadvantage is the requirement of sterile conditions. If callus initiation were ample in media without added sugar, then the assay may be amenable to non-sterile conditions with a bacteriostat and fungistat present in the media. Considerable time could also be saved if Millipore filtration of the fungal culture could be forgone. Gray and Chamberlain (1975) found that autoclaving did not decrease the effectiveness of the culture filtrate for inducing a differential wilting of excised soybean leaflets.

In summary, this is the first report where physiologic specialization has been demonstrated with parallel in vivo/in vitro assays. Time was saved by treating with the culture filtrate at the callus initiation phase rather than treating transferred, proliferating callus – as in past studies of this nature. The BSR reaction classification of 90% of the ten cultivar treatments (over three isolates) in the cotyledon method evaluation corresponded to classifications made from the greenhouse evaluation. The correspondence was 100% with one isolate. *P. gregata* isolates that are aggressive in vivo and that provide clear separation of standard cultivars in the expected manner in the cotyledon method, should be suitable for selection of BSR resistant breeding lines from heterogeneous populations.

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