Selection and Regeneration of Soybeans Resistant to the Pathotoxic Culture Filtrates of *Septoria glycines*

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ABSTRACT

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Soybean plants resistant to a host-specific pathotoxic culture filtrate of *Septoria glycines* were regenerated by organogenesis from immature embryos of the cultivar BSR 201 and from mature seeds of three genotypes, BSR 201, Fayette, and L1615. When the progeny obtained from immature embryos were evaluated for brown spot disease resistance in the field, the R_2 (the second selfed generation of regenerated plants) and R_3 resistant plants did not develop disease symptoms until the R6 growth stage. The incubation period of *S. glycines* in the resistant plants was at least 5 wk longer than the incubation period in susceptible plants. The resistant plants differed from their parental plants in height, maturity, growth habit, and fertility. Resistance to *S. glycines* culture filtrate continued to segregate in unexpected ways after several generations of selfing. Among the resistant plants obtained from mature seeds, only progeny from BSR 201 inherited disease resistance, and these plants were of normal height. The resistant plants obtained from immature embryos and mature seeds matured later than the parent. This study showed that soybean plants with resistance to *S. glycines* can be selected from cultured cells of brown spot-susceptible cultivars using pathotoxic culture filtrates of *S. glycines*.

Since 1915, brown spot disease on soybean (*Glycine max* (L.) Merr.), caused by *Septoria glycines* Hemmi has been studied epidemiologically (13,14,16,17,21–23,26); however, soybean plants resistant to *S. glycines* have not been reported. There also has been no difference in pathogenicity on soybean noted with the isolates found to date (14). Therefore, it is necessary to find different approaches for obtaining soybean plants resistant to this pathogen.

When soybean leaves are infected with *S. glycines*, chlorosis spreads rapidly and eventually causes premature defoliation. MacNeil and Zalasky (18) observed plasmolysis in soybean epidermal cells prior to *S. glycines* penetration and also found that the growth of the fungus was restricted after penetration, but chlorosis spread rapidly. These results suggest that *S. glycines* produces a toxin that is an important factor in pathogenesis on soybean. Recently, a host-specific pathotoxin from culture filtrates of *S. glycines* has been purified, partially characterized as to its chemical nature, and shown to have a role in disease development (24).

Many plant pathogenic fungi produce host-specific pathotoxins that are primary determinants in pathogenesis and induce typical disease symptoms in the absence of the pathogen (7,10,27). Toxic culture filtrates and purified toxins have been used for in vitro selection and regeneration of disease-resistant plants (1–4,9, 11,12,25).

Because no resistance to *S. glycines* has been found in soybean by conventional methods (16), an alternative approach may be to use pathotoxic culture filtrates to screen tissue cultures for resistance to *S. glycines*. In this study, we describe the selection and regeneration of soybean plants resistant to pathotoxic culture filtrates of *S. glycines* and the evaluation of the regenerated plants for resistance to *S. glycines* in the field.

MATERIALS AND METHODS

Plant and pathogen. Eleven soybean (*G. max*) genotypes were tested to determine differences in plant regeneration and selection efficiency: BSR 201 (maturity group II), Corsoy 79 (II), Dawson (0), Fayette (III), L1615 (II), L8280 (III), Morsoy (00), PI 437833 (I), PI 84946-2 (IV), Sibley (0), and Williams 82 (III). An Illinois isolate of *S. glycines* (ATCC 38699) was used to produce the pathotoxic culture filtrate. Preparation of pathotoxic culture filtrates and plant bioassays were described previously (24). Inoculum was produced from potato-dextrose agar cultures of *S. glycines* (24). Cultures including agar were blended in 1 L of fresh tap water for 2 min and filtered through several layers of cheesecloth. Inoculum was adjusted to approximately 10^6 conidia per milliliter.

Regeneration of soybean plants resistant to the pathotoxic culture filtrates of *S. glycines*. Two sources of plant materials, immature embryo and mature seed, were used for selection of the pathotoxic culture filtrate-resistant plants. In the immature embryo selection system, 225 immature embryos (4–5 mm in length) of soybean (cv. BSR 201) were sterilized with 70% ethanol for 1 min, 15% (v/v) bleach (5.25% sodium hypochlorite) for 16 min, and rinsed five times with sterile distilled water. Culture filtrate-containing medium was prepared by dilution of 1 vol of filter-sterilized culture filtrate with 2 vol of soybean culture medium. Sterile immature embryos were placed on culture filtrate-containing water-agar with 40 μM 2,4-dichlorophenoxyacetic acid for 2 wk. Surviving embryos were transferred to the culture filtrate-containing Murashige and Skoog (MS) medium (20) with 20 μM benzylaminopurine (BAP). After this step, surviving organogenic calli were continuously transferred to the culture filtrate-containing MS medium with 1 μM BAP every 2–3 wk for at least seven selection cycles to select shoots resistant to the pathotoxic culture filtrates. The selected shoots were transferred to toxin-free MS medium containing 1 μM BAP and subcultured twice for evaluation of stability of the samples against the pathotoxic culture filtrates of *S. glycines*. Root formation was induced

by transferring the selected multiple shoots to the culture filtrate-containing MS basal medium. All tissue-culture samples were incubated at 25 C under 16/8 h photoperiod (180 μE m^{-2} s^{-1}) in the growth chamber. The selected regenerants were transplanted to sterile soil and grown in the greenhouse to produce seed.

In the mature seed selection system, cotyledonary nodes from more than 240 mature seeds, instead of immature embryos of the 11 genotypes, were used to increase the probability of selection and regeneration of disease-resistant plants using the selection procedure described above.

Evaluation of resistance to *S. glycines* in the progeny of regenerated plants. The immature embryo selection system produced three regenerated R_0 (the first regenerated plant) plants and four R_1 (first selfed generation) plants, designated B1, B2, B3, and C1, which were grown in the greenhouse. The three leaflets of the first trifoliate leaf of each R_1 plant were detached and bioassayed by placing 20 μl of the pathotoxic culture filtrates of *S. glycines* on the leaves to confirm resistance in the regenerants. The leaflet bioassay with the culture filtrates was done with the R_1 plants obtained from only the first selection system. The seeds of the R_2 and R_3 generations were planted in 4-m rows (90 seeds per row) in the field for evaluation of resistance to *S. glycines*. The cultivars Kenwood (Maturity Group II), Asgrow 2943 (II), Resnik (III), parental BSR 201 (II), and regenerated BSR 201 without toxin treatment were used as controls for evaluation of the resistance to the pathogen and comparison of maturity. The plants in the field were inoculated with *S. glycines* at the V3 growth stage (8) using a pressurized sprayer (5.6 kg/cm^2). Disease severity was rated using a modified Horsfall-Barratt (21) rating scale based on the proportion of diseased leaf tissue. Disease severity and plant growth stage (8) were evaluated at least once a week for 8 wk. Plant type (determinate or indeterminate), variation in plant height, shape of leaf, maturity, and male sterility also were recorded. Reactions of individual regenerants to *S. glycines* were classified into three groups based on percentage of diseased leaf area: resistant (0–6%), intermediate (6–25%), and susceptible (25–100%). Plants that exhibited resistance were harvested individually. The pathotoxic culture filtrate-resistant R_1 plants obtained by the mature seed selection system also were planted in the field and evaluated for their reactions to *S. glycines*.

RESULTS

Selection of soybean plants resistant to pathotoxic culture filtrates. More than 90% of the immature embryos and mature seeds did not grow or germinate on the selection medium with the pathotoxic culture filtrate at a 1:2 dilution. Most of the shoots obtained from organogenic calli died on the selection medium (Fig. 1A). In the immature embryo selection system, three R_0 soybean plants were regenerated from the organogenic callus and shoot cultures that survived on the selection medium with the pathotoxic culture filtrates. All of these regenerated plants were less than 10 cm tall (Fig. 1B). One of the R_0 plants (A) was sterile, another plant (B) produced three seeds, and the third plant (C) produced one seed.

In the mature seed selection system, when cotyledonary nodes initiated from mature seeds were placed on the selection medium without culture filtrate, more than 60% of the explants of BSR 201, Fayette, and L1615 regenerated shoots. None of the Dawson and Morsoy and less than 15% of the other six genotypes regenerated shoots. When more than 240 mature seeds from each cultivar were placed on the selection medium with the pathotoxic culture filtrate, one (0.4%), three (1.3%), and four (1.7%) plants were regenerated from Fayette, BSR 201, and L1615, respectively. No plants were regenerated from the other cultivars on the selection medium.

Reactions in the progeny of regenerated plants to the pathotoxic culture filtrates. When detached leaves of a parental soybean plant (cv. BSR 201) were treated with the pathotoxic culture filtrate, browning appeared within 10 h. Once the area turned brown, the surrounding tissues began to show chlorosis that spread rapidly through the entire detached leaves within 24 h (Fig. 2). When

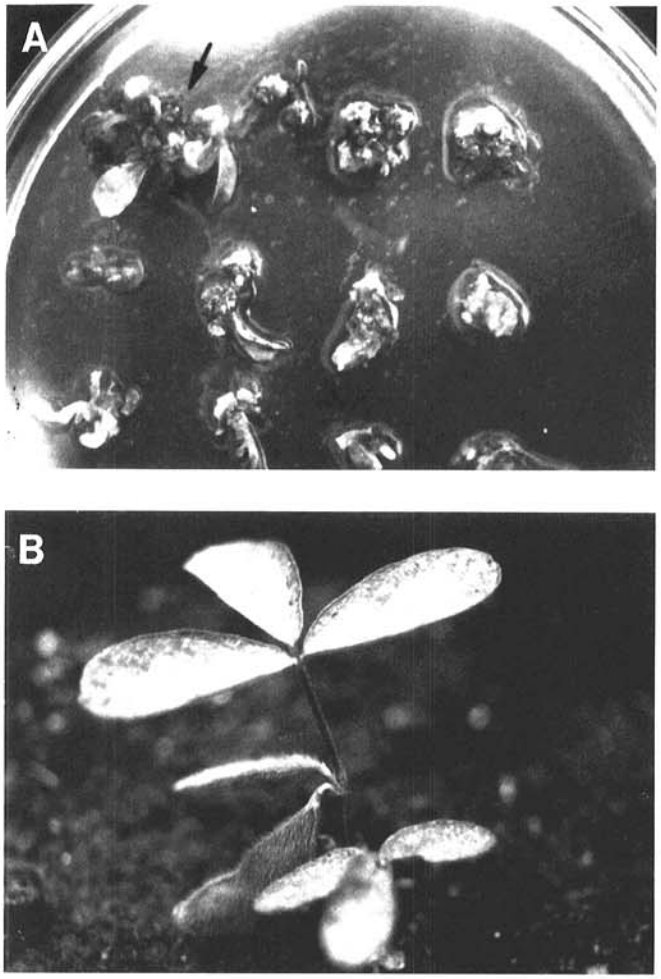

Fig. 1. Regeneration of toxin-resistant plants from immature embryos. **A,** In vitro reactions of shoots to *Septoria glycines* culture filtrate-containing medium at a 1:2 dilution. Most shoots died; the arrow indicates survivors. Pictures were taken 4 wk after soybean samples were placed on the culture filtrate-containing medium. Plate diameter is 9 cm. **B,** Toxin-resistant R_0 plants (*Glycine max* cv. BSR 201) selected and regenerated from immature embryos. The plants were less than 10 cm tall.

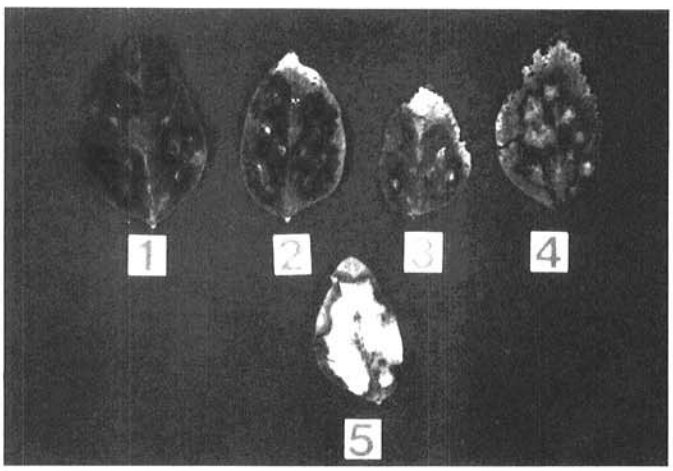

Fig. 2. Soybean leaf (R_1 generation, cv. BSR 201) bioassay with culture filtrate of *Septoria glycines*. The first trifoliate leaves are from the four R_1 plants (plants 1–4) and the parent, *Glycine max* cv. BSR 201, plant (plant 5). The resistant plants were regenerated from immature embryos using the immature embryo selection system. The parent, BSR 201, detached leaf showed browning and chlorosis within 24 h. The detached leaves of the resistant plants showed browning 36 h after treatment with the pathotoxic culture filtrates, but chlorosis did not develop.

leaves of the four R_1 plants selected from the immature embryo selection system were treated with the pathotoxic culture filtrates, some browning occurred within 36 h, but chlorosis did not occur on the detached trifoliate leaves (Fig. 2).

Reactions in the progeny of regenerated plants to *S. glycines* in the field. Seeds from the four R_1 plants in the immature embryo selection system were planted in a 12-row plot in the field, and 89% of the seeds germinated to produce R_2 plants. The progeny of plant B (B1, B2, and B3) produced 61 disease-resistant plants that exhibited no symptoms until the R6 growth stage when inoculated with *S. glycines* at the V3 growth stage. Disease severity of these resistant R_2 plants was less than 10% at harvest. The parental plants started showing symptoms 1 wk after inoculation and lost most leaves by harvest. The remaining R_2 plants were classified into intermediate or susceptible groups. The progeny of plant C produced no disease resistance in the R_2 generation. However, R_2 plants from both plants B and C classified as intermediate or susceptible groups produced some disease-resistant plants in the R_3 generation. In the R_2 generation, all short plants were resistant and were less than 40 cm tall with few (four to six) branches. In the R_3 generation, the height of all resistant plants (approximately 50 cm tall) was between that of the normal soybean plants and the R_2 resistant plants. Some short plants were, however, susceptible to the pathogen. The number of seeds harvested from the resistant plants ranged from one to 67 seeds per plant in the R_2 generation and from one to 300 seeds per plant in the R_3 generation. No phenotypic variation was found in the intermediate and susceptible plants in the R_2 generation. However, variation in plant maturity, growth habit, and fertility was observed in the R_3 generation (Table 1). Most short and disease-resistant plants belonged to maturity group III, whereas the parent, BSR 201, belonged to group II.

Eight soybean plants resistant to the pathotoxic culture filtrates were regenerated from three genotypes using the mature seed selection system: one plant from Fayette, three from BSR 201, and four from L1615. These R_0 resistant plants were normal in height, and their progeny showed no phenotypic differences. All R_1 plants were susceptible to brown spot disease in the field. However, brown spot disease-resistant plants have been found only on some of the progeny of cultivar BSR 201 in the R_6 generation (S. M. Lim, *unpublished data*). The disease symptoms on these disease-resistant plants progressed very slowly, and the plants matured later than did the BSR 201 parent.

DISCUSSION

We have reported that *S. glycines* produces a host-specific toxin when grown in Septoria medium (24). The culture filtrates induced the typical brown spot disease symptoms on soybean leaves and cotyledons and inhibited the growth of soybean calli (24). In this study, we selected and regenerated soybean plants resistant to the pathotoxic culture filtrate of *S. glycines*. The selected regenerants produced progeny resistant to the brown spot disease in terms of having a longer incubation period for *S. glycines* than the susceptible plants. Plants were regenerated from only three of 11 soybean genotypes when the mature seed selection system was used.

Although disease resistance was definitely inherited through the R_3 generation of the selected regenerants, the trait was initially found only in short plants. In the R_2 generation, only short plants were resistant to *S. glycines*, but in the R_3 generation, not all short plants were resistant. Also, the plants in the R_2 and R_3 generations still segregated for disease resistance, and some intermediate and susceptible plants in the R_2 generation produced some resistant progeny. The R_3 resistant plants were taller and appeared stronger than the R_2 resistant plants. Disease-resistant plants that were taller than the parental plants also were found in the R_6 generation but at a low frequency (S. M. Lim, *unpublished data*). These results indicate that it is possible to select disease-resistant plants with normal soybean height in later generations. However, most of the disease-resistant progeny obtained by the first selection system were shorter than the parental plants until the R_6 generation. It is also clear that the inheritance pattern is not simple.

This approach to generating disease resistance raises a question about whether the trait is due to a mutation or epigenetic variation (5,15). The above results support the conclusion that the disease resistance was inherited. Many plant species have been selected for disease resistance using culture filtrates (2,3) or a purified toxin (25). The progeny of the selected regenerants exhibited variation in plant height, morphology, and fertility in the R_2 and R_3 generations. These variations could have been due either to long-term exposure to the toxic culture filtrates or to somaclonal variation (4,6,15,19). In addition, we used crude culture filtrate of *S. glycines* for selection, and it is possible that there could be more than one active principle present. Selection of disease-resistant plants without other apparent mutations has been accomplished in rice by short-term exposure of calli to *Helminthosporium oryzae* toxin followed by regeneration on toxin-free medium (25). These brown spot-resistant rice plants exhibit disease resistance through the R_3 generation without other mutations. We have partially purified *Septoria* toxin, and the relative toxicity was increased up to 17,000-fold in the purification (24). For future studies, we would use the purified toxin instead of crude culture filtrates for selection to possibly reduce the creation of other mutations.

TABLE 1. Variation noted in progeny (R_3 generation) of *Septoria glycines* pathotoxic culture filtrate-resistant soybean plants regenerated from immature embryos observed in the field

		Maturity group[c]			Height and resistance[c]		Plant type[c]	
Source[a]	Resistance[b]	I	II	III	Short + R[d]	Short + S[e]	DT[f]	MS[g]
B1	R	...	2	10	12	...	...	...
	I	206	42	3	...	8	...	...
	S	327	154	...	...	8	...	...
B2	R	...	4	15	19	...	...	...
	I	307	72	3	...	14	3	1
	S	550	38	8	...	8	...	1
B3	R	...	3	11	14	...	...	...
	I	182	70	8	...	15	3	1
	S	471	80	2	...	18	1	2
C1	R	...	1	10	11	...	...	...
	I	67	24	5	...	7	1	...
	S	228	15	3	...	5	...	3

[a] R_1 plants (first selfed generation).
[b] R_3 plants were classified as resistant (R), intermediate (I), and susceptible (S) based on disease severity after inoculation with *S. glycines*.
[c] Each value represents the number of plants that were collected at harvest.
[d] Short and resistant plants.
[e] Short and susceptible plants.
[f] Plants exhibiting determinate stems.
[g] Male sterile plants.

In this study, we can conclude that the sensitivity of the cultured cells to the pathotoxic culture filtrates of *S. glycines* is correlated with the susceptibility of the soybean regenerants to the pathogen, and the selection system using the pathotoxic culture filtrates of *S. glycines* can produce brown spot disease-resistant soybean plants. However, the resistance did not segregate in certain genetic patterns that would fit Mendelian segregation ratios during the earlier generations. Further studies of the progeny are needed to better understand the inheritance and stability of the selected *S. glycines* resistance.

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