Regeneration of Soybean Plants from Embryogenic Suspension Cultures Treated with Toxic Culture Filtrate of *Fusarium solani* and Screening of Regenerants for Resistance

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ABSTRACT

Jin, H., Hartman, G. L., Huang, Y. H., Nickell, C. D., and Widholm, J. M. 1996. Regeneration of soybean plants from embryogenic suspension cultures treated with toxic culture filtrate of *Fusarium solani* and screening of regenerants for resistance. Phytopathology 86:714-718.

Soybean embryogenic suspension cultures established from immature cotyledons of four cultivars were selected for resistance to a toxic culture filtrate of *Fusarium solani*, the causal agent of sudden death syndrome (SDS) of soybean. The embryogenic cultures were challenged with the fungal culture filtrates for 1 to 2 months. Many well-developed somatic embryos formed when the toxin-selected embryogenic cultures were incubated on regeneration medium; however, only a few plants were regenerated from three cultivars, whereas 98 plants were regenerated from cv. Jack embryos. The regenerants that survived were grown to maturity to obtain more seeds for screening plants for resistance to SDS. In the first experiment, the R_1 (187, first-selfed generation) and R_2 (225, second-selfed generation) plants of cv. Jack regenerants, resistant line PI520.733, and cvs. Jack, Great Lakes 3202, and Ripley were inoculated with a *F. solani* SDS isolate and rated on a 1 to 5 scale. The regenerants had significantly ($P < 0.05$) lower mean disease ratings than two of the three cultivars but did not differ from PI520.733. In the second experiment, R_3 plants (990 from cv. Jack and 53 from cv. Spencer) were screened for SDS resistance. The regenerants did not differ significantly from the other cultivars/lines in mean disease severity ratings, although a greater percentage of regenerants (Jack and Spencer R_3 combined) had disease severity ratings of 1 and 2 than did the soybean cultivars tested but not greater than PI520.733. The regenerants with lower disease severity ratings were grown to maturity to produce more seeds that will be used to screen plants for SDS resistance and for crossing to determine how heritable and useful the resistance is in these regenerants.

Sudden death syndrome (SDS) of soybean (*Glycine max* (L.) Merr.) is caused by some isolates of *Fusarium solani* (Mart.) Sacc. (26). Symptoms of SDS include root rot, crown necrosis, vascular discoloration of roots and stems, interveinal chlorosis and necrosis of leaves, and pod abortion (17). SDS has been reported in most major soybean production areas in the United States (16, 17). In east-central Illinois in 1993, SDS occurred in 46% of the soybean fields sampled, based on air and ground surveys (15). Yield reductions of up to 70% have been reported (17). Several hundred soybean cultivars and lines have been tested, but so far, none has been symptomless (12). However, marked differences in foliar symptom expression have been observed among cultivars, and the differences in SDS response are heritable (12,22,27,29). A cross of cvs. Ripley and Spencer indicated that a single dominant gene in Ripley controls resistance (29). Other reports have used molecular markers to study SDS resistance based on field reactions (4,18). Unfortunately, problems exist in screening plants for resistance in the field because disease levels vary greatly within and between fields and from year to year (15,27).

It has been possible, using in vitro selection to toxins produced by pathogens, to obtain somaclonal variants with disease resistance (31), and this method has been used in a variety of crop plants with different pathogen toxins. In maize, callus with T-cytoplasm was selected for resistance to a partially purified toxin of *Bipolaris maydis* (synamorph *Helminthosporium maydis*) race T (10). Plants regenerated after five or more cycles of recurrent selection were resistant to the toxin and pathogen (11). In potato, callus with resistance to culture filtrates of *Phytophthora infestans* were selected, and the resistance was not lost through regeneration (2). Protoplast-derived tobacco callus with resistance to toxins produced by *Pseudomonas syringae* pv. *tobacci* and *Alternaria alternata* were selected, and regenerated plants were resistant to both pathogens (30). Peach callus cultures, selected for insensitivity to a partially purified toxin of *Xanthomonas campestris* pv. *pruni*, were regenerated, and the resulting plants were resistant to the pathogen (13). In soybeans, plants regenerated from calli selected as resistant to the pathotoxic culture filtrate of *Septoria glycines* had delayed brown spot symptoms compared to nonselected plants (28). Culture filtrates of *F. solani* SDS isolates were toxic to soybean cotyledons, calli, and plants (20,21). A toxin from culture filtrates of a *F. solani* SDS isolate was further characterized and purified and was toxic to soybean calli and plants (19).

One prerequisite for a successful in vitro selection system is the establishment of a plant regeneration system. For soybeans, cotyledonary node segments, cultured primary leaf tissue, calli derived from immature embryos via organogenesis (1,5,32), and immature embryos via somatic embryogenesis (1,25) have been used. A system for initiating and maintaining early-stage, highly embryogenic tissue cultures of soybean from immature cotyledons also

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has been developed (6). This tissue can develop into rapidly growing, maintainable, embryogenic suspension cultures (8) and can be used for soybean transformation to produce fertile plants with hygromycin and insect resistance (7,24). There have been no reports of use of this system to produce disease-resistant soybean plants. In this study, we used soybean embryogenic suspension cultures to select for resistance to a toxic fungal culture filtrate produced by *F. solani*, regenerated plants from surviving embryogenic cultures, and tested the regenerants for their reactions to *F. solani*.

MATERIALS AND METHODS

Preparation of fungal culture filtrate. *F. solani* isolates 269 (originating from Arkansas) and Monticello (originating from Illinois), both known to cause SDS, were provided by L. E. Gray (University of Illinois at Urbana-Champaign). Isolate 269 was grown in a modified Septoria medium (19). Cultures were grown in 2-liter flasks, each containing 400 ml of culture medium, or 500-ml flasks, each containing 100 ml of culture medium. The liquid medium was inoculated with a conidial suspension to 1×10^4 conidia per ml of medium and incubated without shaking in the dark at 23°C for 12 days. The cultures were filtered through Whatman No. 1 filter paper (Clifton, NJ) and a 0.22-μm Millipore membrane (Bedford, MA) and stored at 4°C.

Embryogenic callus culture. Soybean cvs. Asgrow A3427, Chamberlain, Jack, Ripley, and Spencer were grown in the field at the University of Illinois at Urbana-Champaign South Farm in 1993. Pods were collected when the seeds were 3 to 4 mm long. The pods were surface-sterilized for 20 min in a 20% solution of 0.525% NaOCl containing 0.05% Tween 20. After five rinses in sterile distilled water, the immature seeds were removed from the pods, and the seed coats were removed under sterile conditions. The embryo axes were excised, and pairs of immature cotyledons were separated and placed on MX20 and MX40 media containing Murashige and Skoog (MS) salts (23), B5 vitamins (9), 6% sucrose, and 20 (MX20) or 40 (MX40) mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter and 0.8% agar at pH 5.7 to induce embryogenic callus (6). Cultures were incubated at 28°C for 16/8 h light/dark with a light intensity of 30 μE m^{-2} s^{-1}. The induced embryogenic calli were transferred to 30 ml of liquid medium in 125-ml flasks. The liquid medium consisted of MS salts, B5 vitamins, 5 mg of 2,4-D per liter, 6% sucrose, and 15 mM glutamate at pH 5.7 (8) and is referred to as FG medium after J. J. Finer and the addition of glutamate. Cultures were kept at 28°C with a 16/8 h light/dark photoperiod with a light intensity of 30 μE m^{-2} s^{-1} and agitated at 130 rpm. The embryogenic cultures were subcultured every 4 weeks on the same fresh medium.

Selection. To determine the toxicity of the culture filtrates, cv. Jack embryogenic suspension cultures were treated with different concentrations of fungal culture filtrate of isolate 269 adjusted to pH 5.8 with 5 N HCl and 1 N KOH and filtered through a sterile 0.22-μm Millipore membrane. Either 0, 1, 2, 4, or 7 ml of fungal culture filtrate was added to 30 ml of FG medium in 125-ml flasks with duplicate flasks. Flasks were capped and sealed and incubated for 2 weeks under the conditions previously described.

Each 125-ml flask containing 30 ml of FG medium was initially inoculated with approximately 1 g of embryogenic cultures and 7 ml of fungal filtrate (19% vol/vol). Embryogenic suspension cultures were grown on this selective medium for 2 weeks, and the surviving embryogenic clumps from each flask were pooled and transferred every 2 weeks into the same fresh selective medium for a period of 1 to 2 months. In each selection, the embryogenic cultures were treated with the same batch of culture filtrate. Because the toxicity of culture filtrates may have varied from batch to batch within the multiple cyles of selection, the reaction of surviving embryogenic cultures was compared with the reaction of previously unselected embryogenic cultures (control). An attempt to regenerate surviving embryogenic cultures was made only when the same batch of culture filtrate caused greater than 95% mortality in the controls.

Plant regeneration. After selection, embryogenic cultures were transferred to FRG (Finer regeneration germination) medium containing MS salts, B5 vitamins, 6% maltose, and 0.2% Gelrite at pH 5.7 (7) at 23°C with a 16/8 h light/dark cycle with a light intensity of 30 μE m^{-2} s^{-1}. After 3 to 4 weeks, the elongated, well-developed embryos were placed in unsealed petri dishes in a desiccator at 85% relative humidity controlled by a saturated KCl solution. The desiccation time varied from 3 to 12 days depending on embryo conditions. After desiccation, the embryos were transferred to FRSG medium (7) that contained 3% sucrose in place of maltose in FRG medium. After 3 to 4 weeks, plantlets with elongated roots and shoots were transferred to test tubes containing modified tissue culture medium (23) until they were 10 cm tall. These plants were transferred to a sterilized 1:1 sand/soil mix in peat pots that fit into Magenta boxes and were covered with a plastic bag. After 1 or 2 weeks, the plastic bags were removed, and the plants were transferred to a 10-cm-diameter pot containing a sterilized soil mix. For the first 42 plants, the pots were kept in a screenhouse in the field. Regenerants 43 to 78 were kept in growth chambers at 28°C with a 23/1 h light/dark cycle at a light intensity of 300 μE m^{-2} s^{-1}. After flowering, plants were moved to the greenhouse to obtain seeds.

Progeny testing. Fifty milliliters of clean sorghum seeds was soaked in distilled water in a 250-ml Erlenmeyer flask overnight, drained, and autoclaved at 121°C for 20 min. After cooling overnight, three 1-cm^2 agar pieces containing fungal mycelia of the two isolates, Monticello and 269, were added to separate flasks of sterilized sorghum and incubated under continuous fluorescent light (60 μE m^{-2} s^{-1}). The flasks were shaken once daily for 2 weeks.

Plants were inoculated with *F. solani*-infested sorghum seeds in 8 × 12-cm trays. Pasteurized soil in the trays was marked into three 8-cm-long furrows spaced 3 cm apart. Five milliliters of infested sorghum seeds (inoculated with *F. solani* 2 weeks earlier) was evenly distributed in each furrow and covered with 2 cm of soil. Three furrows were marked directly above the inoculum, and 5 to 10 soybean seeds were sown per furrow and covered with 2 cm of soil.

In the first screen, 18 trays were inoculated with isolate 269. Twenty seeds each of cvs. Great Lakes 3202, Jack, and Ripley; resistant line PI520.733; and 187 R_1 seeds from 15 R_0 regenerants and 245 R_2 seeds from 19 R_1 regenerants from cv. Jack were planted over the inoculum in trays and placed in growth chambers at 25°C with a 12/12 h light/dark cycle with a light intensity of 300 μE m^{-2} s^{-1}. Plants were watered twice per day. Three and five weeks after planting, disease severity ratings were recorded based on a 1 to 5 scale: 1 = no foliar symptoms; 2 = mild symptoms on unifoliolate leaves but not trifoliolate; 3 = mottling and mosaic symptoms on both unifoliolate and trifoliolate leaves; 4 = interveinal chlorosis on unifoliolate and trifoliolate leaves; and 5 = interveinal necrosis and drying of both unifoliolate and trifoliolate leaves. Cv. Ripley has SDS resistance (29); PI520.733 and cv. Jack also have some level of resistance (G. L. Hartman, *unpublished data*); and cv. Great Lakes 3202 is susceptible (22).

In the second screening, the same set of cultivars and PI520.733 were used as in the first screening; 53 R_3 regenerants from cv. Spencer and 975 R_3 regenerants from cv. Jack were tested under greenhouse conditions. Data were recorded 3 weeks after inoculation based on the 1 to 5 disease severity scale.

RESULTS

Induction of embryos. Immature cotyledons from all the cultivars, except Ripley, formed somatic embryos on both MX20 and MX40 media. When 100 mg of early-stage somatic embryogenic tissue was transferred to liquid FG medium, the tissue grew to about 0.5 to 1 g in 1 month in 0.1- to 5-mm clumps that usually

broke apart due to the physical agitation of shaking. After visual selection of good quality globular-stage cultures for 1 or 2 months, continuous embryogenic cultures were established.

Selection. When the cv. Jack embryogenic cultures were incubated with fungal culture filtrates, the percent survival decreased as the concentration of fungal culture filtrate in FG liquid medium increased (Fig. 1). At a culture filtrate concentration of 19%, approximately 95 to 99% of the embryogenic cultures died. Selection experiments were carried out with cvs. A3427, Chamberlain, Jack, and Spencer, with a total of 40, 40, 80, and 40 flasks, respectively (Table 1). For each selection, the embryogenic cultures went through two to five cycles of selection in 19% culture filtrate. For each selection, the number of flasks of the pooled surviving cultures decreased from 10 to 20 flasks to 1 to 2 flasks after selection.

Plant regeneration. Many well-developed somatic embryos formed when the toxin-selected embryogenic cultures were incubated on FRG medium for 1 month. The well-developed embryos varied in size, and the desiccation time varied from 4 days for embryos that were 3 to 4 mm long to 10 days for embryos longer than 5 mm. Dried embryos were yellow to slightly brown, but 3 to 4 days later they turned green when placed on the FRSG medium. Shoots emerged first, followed within a month by roots. For cv. Jack, 2- to 3-cm-tall plantlets were transferred to a test tube containing MS medium without growth regulators, where the plantlets grew to 5 to 10 cm in 2 to 4 weeks. Regeneration among the cultivars was generally quite low (Table 1), and the total number of plants regenerated was in large part a reflection of the amount of tissue that survived culture filtrate selection. However, many of the regenerants were stunted in MS medium and did not develop (Table 1). The plantlets that developed well were transferred to sterilized soil. The first 41 plantlets, which had developed under a 16-h photoperiod, were only 10 to 20 cm tall when transferred to a screenhouse in the field. They grew slowly, remained short, and produced only one to two seeds per plant. Seeds from the plants sown in the greenhouse appeared to be normal in appearance and growth. Another set of R_0 regenerants (plant numbers 42 to 78), initially grown in the growth chamber under long day lengths, grew much taller than the first 41 regenerants and produced 3 to 63 seeds per plant.

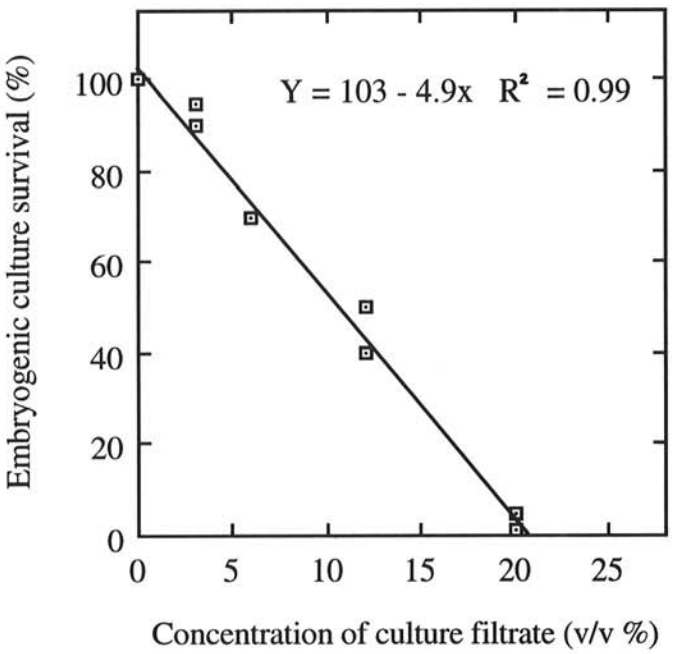

Fig. 1. Survival of soybean cv. Jack embryogenic cultures grown in different concentrations of culture filtrates of *Fusarium solani*-amended liquid medium for 2 weeks.

TABLE 1. Regeneration of R_0 plants of four soybean cultivars from selected embryogenic cultures resistant to culture filtrate of *Fusarium solani*

Cultivar	No. of flasks[x]	No. of regenerated plants: Died in MS[y]	Died in soil[z]	Survived	Sterile plants
Asgrow A3427	40	18	1	1	0
Chamberlain	40	1	7	2	1
Jack	80	25	8	65	0
Spencer	40	6	2	1	0

[x] Number of flasks started with selection.
[y] Murashige and Skoog medium (23) without growth regulators.
[z] Plants transferred to sterilized soil.

Progeny testing with *F. solani*. In the first screening 3 weeks after inoculation, 13% of the R_1 and R_2 plants had no SDS symptoms, whereas cv. Jack plants all had at least some symptoms. Most of the plants at the final rating had a disease severity score of 5, but a greater percentage of regenerants (R_1 and R_2 combined) had disease severity ratings of 1 and 2 than did the soybean cultivars tested (Table 2). The regenerants had significantly ($P < 0.05$) lower mean disease ratings than two of the three cultivars but did not differ from resistant line PI520.733. In the second screening, the majority of plants had disease severity ratings of 3 and 4 (Table 3). As in the first screening, a greater percentage of regenerants (cvs. Jack and Spencer R_3 combined) had disease severity ratings of 1 and 2 than did the soybean cultivars tested but not greater than PI520.733 (Table 3). The mean disease severity ratings did not differ among entries in the second screening.

DISCUSSION

The crude culture filtrate of *F. solani* SDS-causing isolates was used in our study as a selective agent for producing plants resistant to *F. solani*. A previous study showed that the response of soybean calli to crude fungal culture filtrate correlated with SDS severity of inoculated greenhouse-grown plants (20). Crude culture filtrates to select for resistance have been used for selection of alfalfa cell lines resistant to culture filtrate of *F. oxysporum* f. sp. *medicaginis* (3,14) and the selection of soybean calli resistant to culture filtrate of *S. glycines* (28). Others also have reported the use of a purified or partially purified toxin as a selective agent (10,13,30).

Several reports describing in vitro selection and testing of regenerated plants indicate that, at least in some cases, selected resistance is heritable, e.g., the resistance obtained in maize (11) and resistance shown in the R_1 generation obtained from tobacco calli

TABLE 2. Percentage of plants in each of five sudden death syndrome (SDS) severity ratings and mean severity for soybean regenerants obtained from cv. Jack embryogenic cultures (R_1 and R_2), resistant line PI520.733, and cvs. Jack, Great Lakes 3202, and Ripley 5 weeks after inoculation with *Fusarium solani*

Line/Cultivar	No. of plants	% Plants at each disease severity rating[y]: 1	2	3	4	5	Mean SDS severity[z]
Jack R_1	187	1	9	22	30	38	3.8 a
Jack R_2	225	2	4	21	24	49	4.1 ab
Jack	20	0	10	15	75	0	4.7 bc
Great Lakes 3202	20	0	0	0	0	100	5.0 c
Ripley	20	0	0	0	0	100	5.0 c
PI520.733	16	0	13	13	25.0	25.0	4.2 a–c

[y] 1 to 5 scale: 1 = no foliar symptoms; 2 = mild symptoms on unifoliolate leaves but not trifoliolate; 3 = mottling and mosaic symptoms on both unifoliolate and trifoliolate leaves; 4 = interveinal chlorosis on unifoliolate and trifoliolate leaves; and 5 = interveinal necrosis and drying of both unifoliolate and trifoliolate leaves.
[z] Numbers followed by the same letter are not significantly ($P < 0.05$) different in a student's t test ($t = 2.02$).

resistant to a toxin (30). In the case of brown spot of soybean, testing of the R_1 to R_3 generations for resistance to *S. glycines* indicated that the resistance was heritable (28). In our study, a range of SDS severity ratings was observed in the R_1 to R_3 regenerants. Further genetic analysis is needed to confirm that the resistance observed in the regenerants with lower severity ratings is heritable and stable. The screening assay used in our study was rather severe, because cv. Ripley and PI520.733, which are considered resistant, had fairly high severity ratings. Even under this severe screening assay, a few of the regenerants had lower ratings, which could potentially provide new sources of resistance.

In vitro selection can be done in several ways. One method is one-step selection, in which a lethal concentration of the selective agent is used; another method is stepwise selection, in which the concentration of the selective agent is increased gradually until it reaches a lethal concentration (31). In our study, a one-step selection was used in each selection. This method also was used in the selection of protoplast-derived tobacco calli resistant to toxins (30) and short-term selection of alfalfa cell lines resistant to a toxin (14). Stepwise selection also has been used successfully (10, 13); however, the selection period tends to be longer. The longer selection time may allow for more genetic changes to occur, such as changes in the ploidy level, e.g., resistant selections of alfalfa from long-term culture were octoploid and hexaploid rather than normal tetraploids (14).

In our study, retreatment of recovered embryogenic cultures with the culture filtrate resulted in the death of some of the selected cultures (H. Jin, *unpublished data*). This may indicate that the selection pressure was not high enough to guarantee the selection of cultures completely resistant to the culture filtrate. However, we found in an earlier study that a longer period of selection decreased the fertility of the regenerants (H. Jin, *unpublished data*). Thus, to produce resistant plants, a combination of appropriately timed in vitro selection and greenhouse screening is necessary.

Reactions of soybean plants to *F. solani* infection can vary within and among cultivars (12,21,22,27), indicating that differences in resistance to SDS may already exist in some soybean genotypes under some conditions. The use of tissue culture offers a greater opportunity to select disease resistant plants because millions of cells, all of which have the potential for plant regeneration, can be exposed to a selective agent. It also is possible to screen regenerated plants from unselected cell cultures to identify resistant somaclonal variants (14). However, selection of the cultures with fungal culture filtrate should increase the frequency of resistant plants. This method could quickly produce resistant plants from susceptible commercial cultivars, which would shorten the period of time needed to improve disease resistance. New types of resistance also might be produced. The embryogenic suspension culture grows in liquid medium, providing high medium-to-tissue contact, so selection is more rigorous, and growth is faster than that of cultures on solid medium. The majority of cells in these cultures are competent to form embryos and plants (6). These embryogenic cultures require some skill for their initiation, and some cultivars, like Ripley, did not form embryogenic cultures, whereas others, like Jack, formed cultures easily. Further work with embryogenic cultures should provide the opportunity for selection and regeneration of more soybean cultivars with disease resistance. Likewise, additional studies of the regenerants are needed to determine if indeed stable, heritable, and useful resistance can be produced.

TABLE 3. Percentage of plants in each of five sudden death syndrome (SDS) severity ratings and mean severity for soybean regenerants obtained from different cv. Jack embryogenic cultures (R_3), resistant line PI520.733, and cvs. Jack, Great Lakes 3202, and Ripley 21 days after inoculation with *Fusarium solani*

Line/Cultivar	No. of plants	% Plants at each disease severity rating[z]					Mean SDS severity
		1	2	3	4	5	
Jack R_3	990	1	17	32	44	6	3.4
Spencer R_3	53	3	11	14	25	0	3.2
Jack	11	0	27	27	45	0	3.2
Great Lakes 3202	32	0	6	34	59	0	3.5
Ripley	34	3	15	24	53	6	3.5
PI520.733	33	0	27	45	27	0	3.0

[z] 1 to 5 scale: 1 = no foliar symptoms; 2 = mild symptoms on unifoliolate leaves but not trifoliolate; 3 = mottling and mosaic symptoms on both unifoliolate and trifoliolate leaves; 4 = interveinal chlorosis on unifoliolate and trifoliolate leaves; and 5 = interveinal necrosis and drying of both unifoliolate and trifoliolate leaves.

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