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A Comparison of Soybean Agglutinin in Cultivars Resistant and Susceptible to *Phytophthora megasperma* var. sojae (Race 1)[1]

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ABSTRACT

The amount of soybean agglutinin (SBA) detectable by radioimmunoassay in seeds of resistant cultivars to *Phytophthora megasperma* var. sojae was approximately twice that of susceptible cultivars. SBA was preferentially released at earlier times (6–9 hours) and in higher amounts in the imbibate from resistant cultivars as compared to susceptible cultivars. The lectin in the imbibate was immunologically identical to the seed lectin, indicating little or no proteolysis had occurred, and was active in hemagglutination. Binding of fluorescein isothiocyanate-labeled SBA to mycelial cell walls could be abolished by adding *N*-acetyl galactosamine or galactose. Purified SBA at concentrations of 150 to 300 micrograms inhibited mycelial growth by 50%, and the imbibate from Govan (resistant) cultivar was more inhibitory than the imbibate from Shore (susceptible) cultivar. Removal of SBA from the imbibate by affinity chromatography abolished the inhibition of mycelial growth, but the inhibition could be recovered from the eluant containing lectin.

SBA[4] is a tetrameric glycoprotein found in high concentrations within the seed (12). Some investigators have also reported the presence of SBA by hemagglutination on root tissue (1), and one group has succeeded in purifying a lectin from the surface of 1-week-old root tissue (5). Although preliminary experiments reported specific SBA binding to *Rhizobium* strains known to nodulate soybean, others have not been able to establish a definitive role for SBA in initiating symbiosis (17). However, SBA, as assayed by hemagglutination, is released during the initial stages of germination in one soybean cultivar (3). If SBA serves as a protective agent to the germinating seedling, its location outside the seed would be consistent with a role in providing resistance to infection.

Many soybean cultivars are available which have resistance or susceptibility to certain plant pathogens including *Phytophthora megasperma* var. sojae, the causal agent of root rot (11). Although this fungus can infect soybean at all stages of development, it causes extensive root rot and damping off of pre- and post-emergence seedlings of susceptible varieties and reduction in seed yield of adult plants. Resistance to race 1 of the fungus is controlled by a single dominant gene, although the factor involved is unknown (11).

Our studies have centered on the possible role of SBA in preventing infection by *P. megasperma* var. sojae (race 1) based on quantitation of SBA content in resistant and susceptible seeds, release of SBA during imbibition of seeds of resistant and susceptible cultivars, and inhibition of fungal growth by SBA.

MATERIALS AND METHODS

Source of Material. Soybean cultivars were donated by J. Hartz Seed Co., Stuttgart, AK; Coker Seed Co., Hartsville, SC; Dr. H. Skipper of the Agronomy Department, Clemson University; and Dr. J. Harper of the Department of Agronomy, University of Illinois, Urbana, IL.

Sepharose 4-B and 1,4-butanediol diglycidylether were obtained from Sigma Chemical Co. FITC anti-rabbit IgG (goat) was purchased from Miles Biochemicals. [^{14}C]Formaldehyde was obtained from New England Nuclear, and Aqua-Sol was purchased from Anoroc Scientific. All other chemicals were of reagent grade and were not purified further.

Preparation of Seed Extracts. Seeds (20 g) were placed in a Salton quick food mill and ground to a fine flour, which was then added to 4 volumes of petroleum ether to remove fats and collected on a Büchner funnel after 15 min. Four volumes of cold PBSA (pH 7.2) was added, and the mixture was allowed to stand at 4°C overnight. After squeezing it through cheesecloth, the extract was centrifuged at 12,000*g* for 15 min to remove particulates. Seed extracts were used for quantitation of SBA content. Subsequent purification of SBA was carried out for comparison among cultivars. For purification, the extract was loaded onto an *N*-acetyl galactosamine-Sepharose column prepared with epoxide-activated gel (22). After absorbance had stabilized to less than 0.05 A_{280} units, SBA was eluted as a sharp peak with 0.1 M galactose in PBSA. After extensive dialysis against distilled H_2O, SBA was lyophilized and stored at 0°C.

Radioimmunoassay. Antibody to SBA was raised in female New Zealand white rabbits using a series of three weekly subcutaneous injections of 0.5 mg SBA emulsified in an equal volume of Freund's incomplete adjuvant. Preimmune sera was collected prior to injecting the antigen. Animals were bled at biweekly intervals for 2 months, and sera were pooled. Sera were preserved with the addition of thimerosol and phenol at final concentrations of 0.01% and 0.025%, respectively.

[^{14}C]SBA with a specific activity of 2×10^6 cpm/mg was prepared using [^{14}C]formaldehyde via reductive methylation (18). The RIA protocol was as follows: to 0.5-ml polypropylene tubes was added in the following order, with vortexing between additions, varying amounts of PBSA, 50 μl diluted [^{14}C]SBA in PBSA, 1 to 50 μl of diluted sample or standard, and 50 μl of diluted antisera in PBSA. Tubes were incubated at 37°C for 1 h, then placed on ice for 15 min. Cold 10% PEG-6000 in PBSA was added for a final concentration of 4%. After storage at 4°C for 12 to 16

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[4] Abbreviations: SBA, soybean agglutinin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline, pH 7.2; PBSA, phosphate-buffered saline with 0.02% sodium azide, pH 7.2; RIA, radioimmunoassay; PEG-6000, polyethylene glycol-6000 mol wt; WGA, wheat germ agglutinin.

h, complexes were precipitated by centrifuging in a Gilson airfuge for 3 min, and 200 μl supernatant in 3 ml Aqua-Sol was counted. A standard competition curve with unlabeled SBA was constructed for each assay over the range of 0.1 to 1 μg SBA, and multiple dilutions of seed extracts were tested in duplicate so that several values would fall within the standard curve.

Seed Imbibates. Two g seeds from each cultivar were placed in 15% Clorox for 10 min, followed by rinsing in sterile H_2O, and then transferred into tissue culture tubes containing 5 ml of sterile H_2O. Aliquots (200 μl) were removed at specified time intervals and frozen for assay of SBA content by RIA and for protein content (13).

Electrophoresis. Polyacrylamide gel electrophoresis was performed with a 7.5% acrylamide separating gel at pH 8.5 with a glycine-ethanolamine buffer (2). Gels were stained with Coomassie brilliant blue for protein or were placed on Gel-Bond film (Marine Colloids) which was then overlaid with melted 1% agar. After agar had solidified, troughs were cut 15 mm from gels and either preimmune or postimmune sera was placed in the trough. Precipitation arcs were detectable after 24 h.

Fluorescence Microscopy. FITC-SBA was prepared according to standard procedures (19). *Phytophthora megasperma* was cultured directly on sterile glass slides containing a thin coating of V-8 juice agar and incubated in a sterile humidity chamber. After two days growth, FITC-SBA was added directly to the slides, allowed to incubate at room temperature for 20 minutes, in the absence or presence of 10 mM galactose or *N*-acetyl galactosamine, then slides were rinsed with PBS. Microscopic observations were carried out using illumination in the blue-violet region (350–500 nm) with a barrier filter at 540 nm in a Leitz 20 fluorescence microscope with Orthomat automatic camera attachment.

Inhibition of Fungal Growth. *P. megasperma* var. sojae (race 1) was provided by Dr. R. Keeling, United States Department of Agriculture Delta Experimental Station, Stoneville, Ms. Cultures were maintained on V-8 juice solid media (21) as well as in liquid culture (9). For growth inhibition experiments, varying concentrations of SBA were added to Petri dishes previously coated with V-8 juice agar. Fungal discs were obtained from dilute lima bean agar (1 g lima beans/L) plates using a sterile corkborer, and positioned at the center of the lectin or water-saturated agar plates. The area of mycelial growth was measured after 2 to 4 d growth by calculating areas from diameter measurements. Zoospore suspensions were prepared from 7- to 10-d-old mycelial discs which were then placed in dilute lima bean replacement liquid (1 g lima beans/L H_2O) for 72 h at 25°C. After chilling at 15°C for 8 h, liquid containing zoospores was removed and used as a source of inoculum. Typical preparations contained approximately 1×10^6 zoospores/ml. Zoospores (100 μl) were germinated at 25°C in 100 μl minimal media containing SBA (7–155 μg in 100 μl H_2O) or 100 μl H_2O.

RESULTS

Quantification of SBA Content. Seed extracts from susceptible, resistant, and nonnodulating cultivars were examined for SBA content using RIA (Table I). SBA contents varied considerably among cultivars (0.0019–0.0355 mg SBA/mg protein) as has been reported previously by other workers (17). When the means of resistant and susceptible groups are compared, there is approximately double the amount of SBA per mg protein in the resistant cultivars as compared to the susceptible cultivars. If SBA is

Table I. *Concentration of SBA in Soybean Seeds Quantitated by RIA*

Cultivars	mg SBA		
	/mg protein	*/g seed*	*/seed*
Resistant			
PI 200.544	0.0100	1.4769	0.5996
Pine del Perfection	0.0072	1.0121	0.1230
Pickett 71	0.0259	1.7675	0.2281
Davis	0.0120	1.5268	0.1985
H-24	0.0296	1.8277	0.1589
Bragg	0.0125	1.7411	0.2430
Lee 74	0.0245	2.9685	0.4304
Sohoma	0.0263	4.0910	0.5455
Govan	0.0263	2.5004	0.3018
Tracy	0.0115	0.6202	0.0913
Forrest	0.0335	2.7704	0.3292
Mean	0.0201 ± 0.0096	2.0278 ± 0.9836	0.2954 ± 0.1677
Susceptible			
Shore	0.0070	0.9131	0.1660
Rokusun	0.0123	2.1371	0.8848
Dorchstoy 2A	0.0094	1.3741	0.1963
Lee non-nod	0.0098	0.9065	0.0910
Peking	0.0019	0.0181	0.0013
PI 171.444	0.0061	0.5686	0.0434
Mean	0.0091 ± 0.0032	0.7561[a] ± 0.5020	0.0996[a] ± 0.0816
Susceptible/Resistant			
Ransom	0.0172	1.8325	0.3465
Nonnodulating			
Harosoy	0.0185	1.3100	0.2326
Clark	0.0250	1.8990	0.2301
Lee	0.0098	0.9065	0.0910

[a] Rokusun data excluded in determination of mean value.

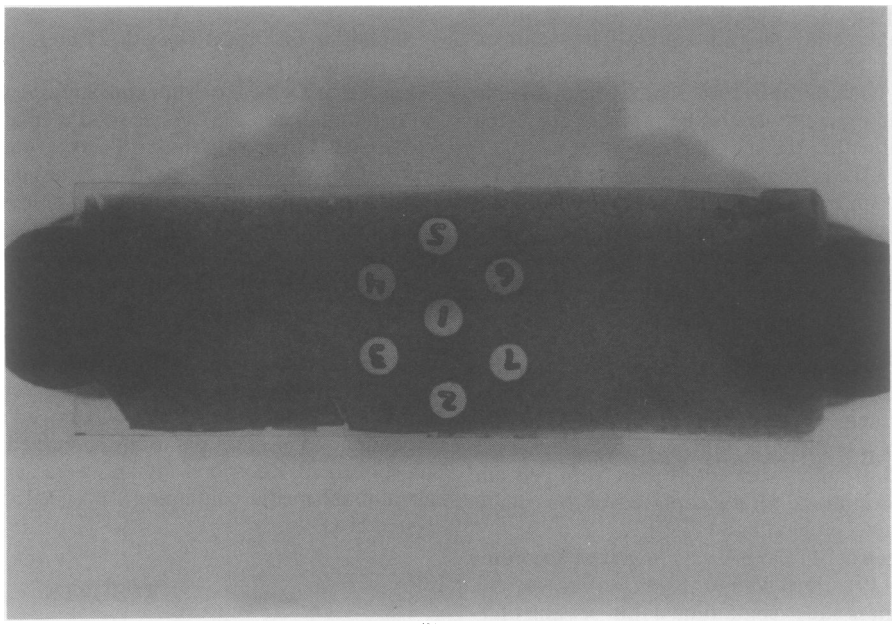


FIG. 1. Immunodiffusion of extracts of soybean cultivars. The wells contained 15 μl of the following seed extracts: 1, anti-SBA; 2, Govan; 3, Shore; 4, Sohoma; 5, Dorchstoy 2A; 6, Bragg; 7, PI 171.444.

involved in providing resistance to infection during germination, a more useful measure is SBA content per seed. When these data are compared on this basis for both resistant and susceptible cultivars, there is approximately 3 times as much SBA present in the resistant cultivar group. The susceptible cultivar Rokusun was not included in the determination for mean values in these experiments since it was clearly distinct from other cultivars in this group, as discussed later. However, in all cases, the SD of the resistant and susceptible group overlap.

Of the nonnodulating cultivars examined, only Lee had low levels of seed lectin similar to other susceptible varieties. Previous work has reported on the absence of SBA in varieties which are still able to nodulate (17). Nonnodulating Clark and Harosoy cultivars contained SBA at levels similar to those of resistant cultivars.

To verify that seed extracts did contain SBA capable of reacting in RIA, the following experiments were conducted. Samples of seed extracts were eluted through an affinity column and the effluent was tested by RIA. Most (90%) of the previous reactivity was abolished by this procedure. SBA from each seed extract could be removed from the column by eluting with 0.1 M galactose. Immunodiffusion analysis was performed on seed extracts and purified lectin, and all samples showed immunological identity (Fig. 1). Also, seed extracts and purified lectin from various cultivars were analyzed by gel electrophoresis and compared to a SBA standard. A protein band migrating similarly to standard SBA was detectable, and one precipitation arc corresponding to the position of SBA was obtained using immunoelectrophoresis (data not shown).

Release of SBA during Imbibition. Previous investigators have reported the release of SBA from seeds as measured by hemagglutination (11). Therefore, we decided to determine the amount of SBA released with time in relation to known resistance or susceptibility to root rot.

Although most cultivars were examined, only representative data are presented in Figure 2. Govan had an average value of SBA typical of that present in resistant seeds (26.3 ng SBA/mg protein), while Shore had average SBA values typical of the susceptible group (7 ng SBA/mg protein). Both Govan and Shore showed similar rates and amounts of protein released during imbibition (Fig. 2A), but Govan released an earlier and higher level of SBA (Fig. 2B). Between 6 and 9 h of imbibition, a large amount of SBA was found in the imbibate from Govan, while no SBA from Shore was detectable with RIA during the same time interval. When the amount of SBA per mg protein is calculated, it is evident that SBA is a preferential release product for Govan (Fig. 2C). This finding is significant since high levels of lectin would be present in the microsphere surrounding the germinating seedling. All susceptible cultivars examined, with the exception of Rokusun, released SBA with a pronounced lag and reduced amounts in comparison to resistant cultivars.

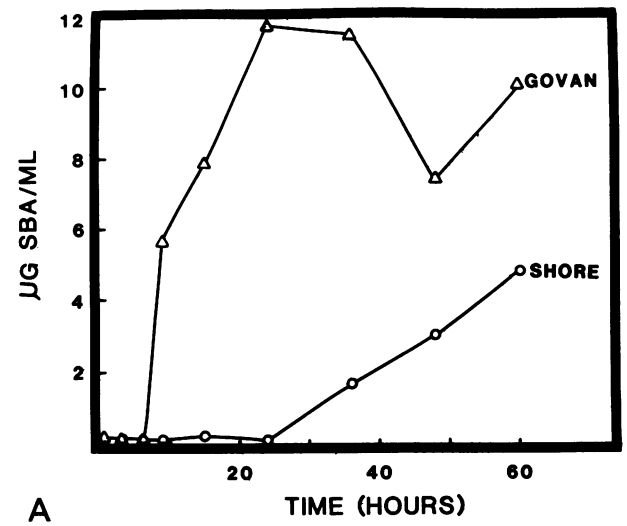
The cultivar Rokusun was an exception to the typical profile seen for other susceptible cultivars. Not only did Rokusun have a high SBA content in seeds (see Table I), it also released massive amounts of SBA during imbibition (Fig. 3) as compared to the resistant cultivar Govan. Rokusun, an edible variety of soybean, possesses a low pectinaceous and cellulosic cell wall and is easily ruptured by the addition of aqueous solvents. During these experiments, Rokusun seeds disintegrated in comparison to Govan which maintained their ultrastructure. Rather than a timed release, most of the seed lectin was dissipated quickly into the surrounding liquid.

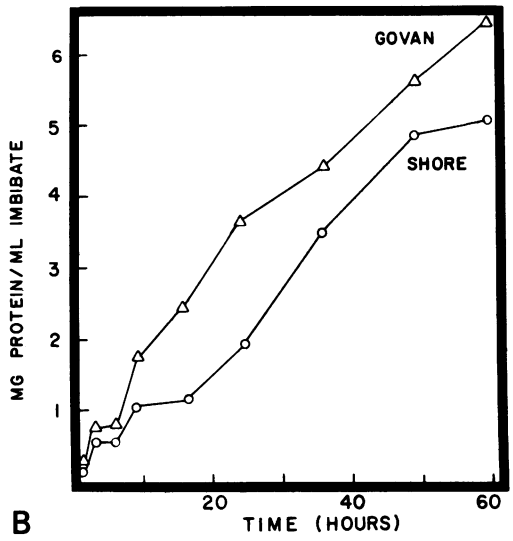
Verification of Intact SBA in Imbibate. Although the previous experiments detected antibody reactive material by RIA in imbibates, this result did not establish whether the imbibate contained native SBA or fragments which still possessed immunological determinants.

To verify the presence of SBA, the following tests were conducted. Aliquots (50 μg) of 48- and 60-h imbibates from Govan and Shore were run on disc gel electrophoresis, then stained for protein or tested using immunoelectrophoresis. Although many bands were present, there was a major band migrating with a similar mobility to purified SBA (Fig. 4). When examined for immunochemical reactivity, only one precipitin arc was seen in the location corresponding to SBA. Hemagglutination assays also verified the presence of active lectin in the imbibate and titers correlated well with the quantity detectable by RIA.

Imbibition experiments were also conducted in the presence of phenyl methylsulfonyl fluoride or streptomycin, and little or no change in amount of SBA in the imbibate was observed.

Interaction of SBA with *P. megasperma*. The ability of SBA to bind to *P. megasperma* hyphae was tested using FITC-SBA. When tissues were examined, bright areas of yellow-green fluorescence were seen on hyphal cell walls indicating binding of FITC-SBA (Fig. 5A). Control sections incubated with buffer alone had background fluorescence due to the agar coating (Fig. 5B), while sections incubated with FITC-SBA in the presence of 10 mM galactose had diminished fluorescence (Fig. 5C). Binding of FITC-SBA was also reduced in the presence of 10 mM *N*-acetyl galactosamine or Hog A + H substance (1 mg/ml), which are carbohydrate haptens for SBA.





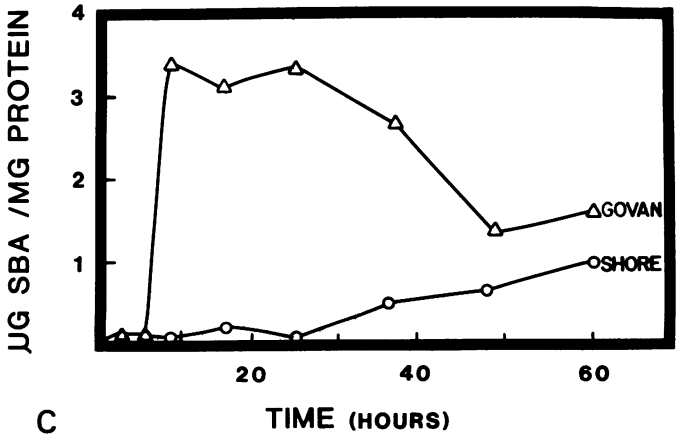


FIG. 2. Assay of Govan and Shore seed imbibates with time. A, Release of SBA with time; B, release of protein with time; C, Release of SBA per mg protein with time. The quantity of SBA in imbibates was determined on 25 to 50 μl of sample using RIA. (△), Govan; (○), Shore.

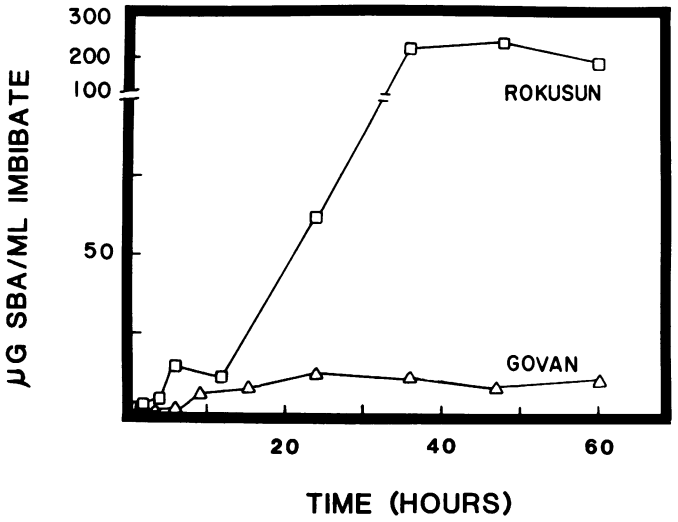


FIG. 3. Assay of SBA in Rokusun and Govan imbibates with time (□), Rokusun; (△), Govan.

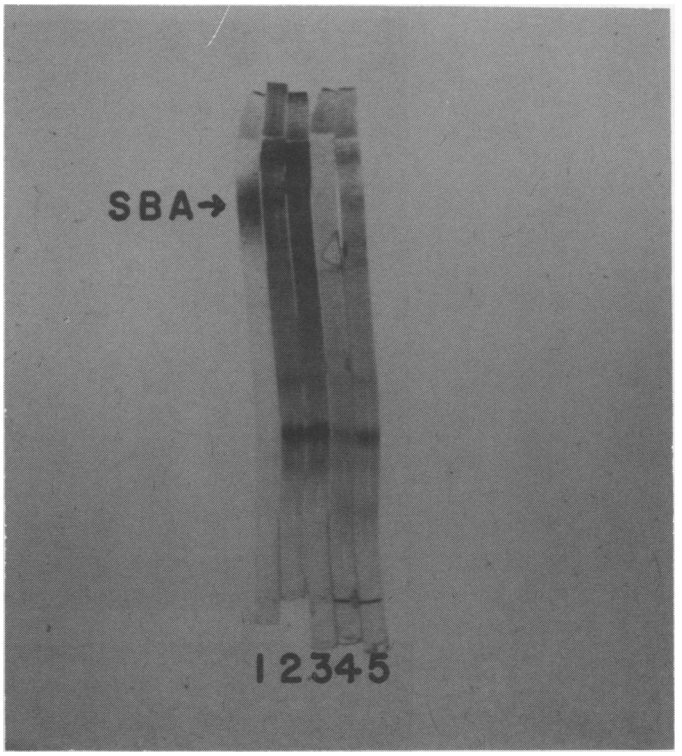


FIG. 4. Disc gel electrophoresis of SBA and imbibates. Native 7.5% gels were electrophoresed for 4 h at 3 mamp/tube and were stained for protein. Gels contained the following samples: 1, 50 μg SBA; 2, 48-h Govan imbibate (50 μl–265 μg protein); 3, 60-h Govan imbibate (50 μl–315 μg protein); 4, 48-h Shore imbibate (50 μl–220 μg protein); 5, 60-h Shore imbibate (50 μl–255 μg protein).

Inhibition of Fungal Growth by SBA. The ability of SBA to

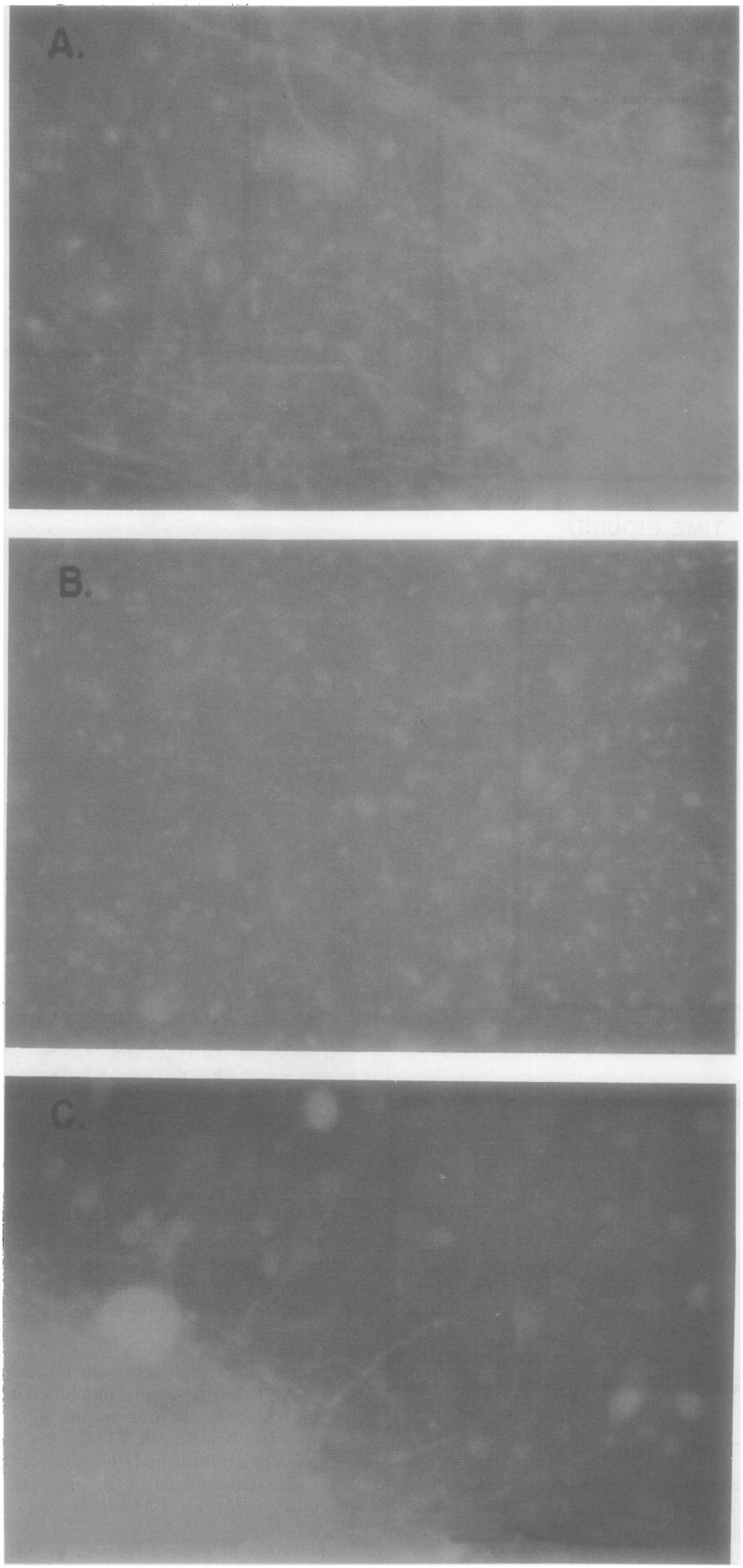


FIG. 5. Fluroescence of *P. megasperma* hyphae with FITC-SBA. Tissues were incubated with FITC-SBA for 15 min in the presence or absence of hapten, rinsed with PBS, and examined. (× 200). A, FITC-SBA alone; B, PBS alone; C, FITC-SBA and 10 mM galactose.

Table II. *Inhibition of Mycelial Growth of P. megasperma by SBA and Imbibates*

Sample	Area of Growth	Inhibition
	cm^2	%
A.[a]		
100 μl H_2O	8.04	
311 μg SBA/100 μl H_2O	3.80	52.8
156 μg SBA/100 μl H_2O	4.15	48.3
78 μg SBA/100 μl H_2O	7.56	6.2
B.[b]		
100 μl H_2O	4.34	
48 h Govan imbibate (0.78 μg SBA/100 μl)	3.63	16.3
48 h Shore imbibate (0.30 μg SBA/100 μl)	4.71	0
Govan effluent (3.76 μg protein/100 μl)	4.52	0
Shore effluent (2.65 μg protein/100 μl)	4.52	0
Govan eluant (0.59 μg SBA/100 μl)	3.46	20.1
Shore eluant (0.39 μg SBA/100 μl)	3.80	12.4

[a] Recorded at 72 h.
[b] Recorded at 48 h.

inhibit mycelial growth on solid culture media was tested using SBA purified by affinity chromatography. When SBA was filter sterilized, normally over 60% of protein adhered to the filter. Over 50% inhibition of growth was observed in the presence of 150 to 300 μg filter-sterilized SBA (Table IIA). At concentrations lower than 90 μg, only slight inhibition was noted. The filter sterilization procedure may partly inactivate or dissociate SBA, and the observed inhibition may be due only to active SBA still present.

The ability of the 58-h seed imbibates to inhibit mycelial growth was also tested (Table IIB). Govan imbibate was 30% more effective in retarding fungal growth as compared to Shore imbibate.

Similar results were obtained when SBA or imbibate was added to liquid cultures of mycelia, and growth was scored by dry weight measurements of mycelial mats (data not shown). To verify that SBA in the imbibate was responsible for inhibition of growth, a 0.5-ml aliquot was passed over the affinity resin and unadsorbed material was collected. The adsorbed material was subsequently eluted with 0.1 M galactose. Both the adsorbed and unadsorbed pools were dialyzed extensively against distilled H_2O and concentrated by ultrafiltration using an Amicon YM-10 membrane. After correcting for differences in absorbance, both Govan and Shore material were filter sterilized and tested for their ability to inhibit mycelial growth. The unadsorbed material from both Govan and Shore imbibates was ineffective in inhibiting growth, but the galactose-eluted material from each cultivar reduced mycelial growth, although Govan was more effective.

Since WGA has been shown to inhibit germination of spores of *Trichoderma viride* (15), we tested the ability of SBA to retard germination of zoospores of *P. megasperma*. After incubating with 7 to 155 μg SBA for 20 h at 25°C, microscope slides were prepared and stained with safranin for visual observation of zoospores. No inhibition of germination was detected in comparison to controls, although the germ tubes appeared somewhat smaller. Cultures were also incubated with [^{14}C]leucine to monitor growth of germinating zoospores in the presence of SBA. At concentrations between 38 and 155 μg SBA, 15% to 29% inhibition of growth measurable as [^{14}C]leucine incorporation into 10% TCA-insoluble material was observed, while at lower concentrations no inhibition was detectable.

DISCUSSION

As yet, direct evidence for lectin involvement as a host response to pathogen invasion has not been demonstrated. Several criteria must be met for lectins to be considered as protective agents against infection. First, the lectin must be present in an active form at the site of invasion. Second, the concentration of lectin present must be sufficient to cause at least a static effect on further growth of the pathogen. Thirdly, the lectin must bind to the cell walls or membranes of the pathogen and prevent growth of the infecting organism. Whether lectin acts alone or in concert with other molecules has yet to be demonstrated. Alternatively, lectin may bind to a product of the pathogen, such as a glycosidase, which facilitates infection. Finally, resistant cultivars should contain high concentrations of lectin at the appropriate site, while susceptible cultivars should have low levels of lectin or inactive form present.

If lectins are involved in providing resistance to infection, these proteins may act in concert with other compounds in the same plant which are also capable of inhibiting microbial growth. Legumes such as soybean and lentils release active protease inhibitors during germination which may retard microbial proteases (23). Phytoalexins, such as glyceollin in soybeans, have been shown to increase in resistant plants during infection (4, 7, 10) and are thought to function as general inhibitors of pathogenic invasion (for review, see 9).

All three types of components, lectins, protease inhibitors, and phytoalexins have broad specificity. For example, those lectins characterized to date, with the possible exception of *Phaseolus aureus* lectin, have a primary specificity for one type of sugar, although other carbohydrates are also bound with lesser affinity (6). Soybean agglutinin binds with high specificity to *N*-acetyl galactosamine, but galactose, methyl-galactose, melibiose, raffinose, and stachyose also bind with reduced affinities (8). Most of the binding specificities of lectins are for common carbohydrate determinants found on the outer surfaces of microorganisms.

In this paper, supporting evidence for a role of lectins as protective agents has been presented. The mean values of SBA content within seeds indicates a difference between cultivars resistant and susceptible to *P. megasperma*, although the variance within each group does not indicate a strong correlation of increased SBA content in resistant cultivars. More importantly, seed lectin in those resistant cultivars tested is transported sooner and at higher concentrations into the surrounding media during germination than in susceptible cultivars. The lectin released is active as detected by hemagglutination, and proteolysis of secreted lectin was not detected. Binding to SBA to mycelial cell surfaces was inhibitable with carbohydrate haptens for SBA. In fungal growth studies, purified SBA was able to retard mycelial growth at high concentrations as well as slightly retard growth of germinating zoospores. The imbibate from Govan, a resistant cultivar, showed slight inhibition of mycelial growth while the material from Shore, a susceptible cultivar, was ineffective. Evidence in support of lectin involvement in the retardation of mycelial growth was the removal of the inhibiting factor from the imbibate by an affinity resin and recovery of inhibition of growth in the galactose eluted material.

It is possible that lectin from resistant and susceptible cultivars possess slightly different binding affinities which could account for the observed differences in inhibition. Also, the imbibates might contain other factors such as protease inhibitors which were removed during the column adsorption, dialysis, concentration, or filter sterilization but could contribute to the observed inhibition of mycelial growth with the untreated imbibate. However, the adsorbed SBA peaks from the imbibates were capable of retarding

growth, indicating that lectin is at least a factor in the inhibition found in the unadsorbed imbibate.

The actual mechanism by which SBA could cause growth inhibition is not known at this point. The binding of WGA to *Trichoderma viride* and subsequent inhibition of growth appears to be similar, but the inhibition was present at lower concentrations of lectin (14). A recent study on the distribution of WGA has reported on the presence of the lectin in the rhizosphere of young plants (15), a location similar to that found in the seed imbibates in this study. Although lectins as protective agents has yet to be conclusively demonstrated, these studies suggest that lectins may be involved in the resistance mechanism.

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