Role of Lectins in Plant-Microorganism Interactions

III. INFLUENCE OF RHIZOSPHERE/RHIZOPLANE CULTURE CONDITIONS ON THE SOYBEAN LECTIN-BINDING PROPERTIES OF RHIZOBIA[1]

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ABSTRACT

The influence of rhizosphere/rhizoplane culture conditions on the ability of various rhizobia to bind soybean seed lectin (SBL) was examined. Eleven strains of the soybean symbiont, *Rhizobium japonicum*, and six strains of various heterologous *Rhizobium* species were cultured in root exudate of soybean (*Glycine max* [L.] Merr.) and in association with roots of soybean seedlings which were growing either hydroponically or in montmorillonite clay soil amendment (Turface). All 11 of the *R. japonicum* strains developed biochemically specific receptors for the lectin when cultured under these conditions, whereas six of the 11 did not develop such receptors when cultured in synthetic salts medium. Two cowpea strains also developed receptors for SBL. The other four heterologous strains of rhizobia gave no evidence of biochemically specific SBL binding in either synthetic salts media or rhizosphere/rhizoplane cultures. These results demonstrate that the environment provided by plant roots is an important factor in the development of specific lectin receptors on the cell surface of *R. japonicum*.

The ability of plants to respond to the presence of pathogenic or symbiotic microorganisms is an important aspect of their physiology. In order to respond appropriately, a plant must recognize a particular microorganism as a potential pathogen or symbiont. It has been suggested that the carbohydrate-binding plant proteins known as lectins might function in the recognition of microbial pathogens and symbionts by binding to characteristic carbohydrate receptors on the microbial cell surfaces (1, 3). Several recent studies have indicated that microorganism recognition mechanisms based on lectin (or agglutinin) binding may indeed be operative in a variety of plant species (3, 7, 8, 11, 12, 15–17, 20).

The results of studies on the role of soybean lectin as a determinant of host specificity in the soybean/*Rhizobium japonicum* symbiosis, however, have been variously supportive, contradictory, and enigmatic (2–5, 10, 13, 20). The initial study by Bohlool and Schmidt (3) indicated that SBL[3] bound to 22 of 25 strains of *R. japonicum*, the soybean symbiont. These authors reported that SBL did not bind to three of the *R. japonicum* strains or to any of 23 strains representative of various heterologous *Rhizobium* species. In three subsequent studies (4, 5, 10), however, it was reported that SBL bound to several heterologous strains and failed to bind to several homologous strains, with the result that no clear corre-

lation between SBL binding and host-specific infectivity could be discerned.

In a previous study (2) we provided evidence in confirmation of Bohlool and Schmidt's original observations (3) on the SBL-binding properties of more than a dozen homologous and heterologous *Rhizobium* strains. Furthermore, the ability of strains of *R. japonicum* to bind SBL in a biochemically specific manner was shown to depend greatly on the growth phase of the bacteria in artificial culture media (2). It appeared that the SBL receptors on *R. japonicum* were transient, rather than constitutive, components of the cell surface, and that the appearance of these receptors might be critically dependent on the growth environment of the bacteria. We suggested that those strains of *R. japonicum* which gave no evidence of lectin binding at any stage of growth in artificial media might develop SBL receptors under *in vivo* culture conditions. A study of the influence of root exudate and rhizoplane culture conditions on the development of SBL receptors by various strains of rhizobia is reported here.

MATERIALS AND METHODS

Chemicals and Plant Materials. Eriochrome black was obtained from ICN, Cleveland, Ohio, methyl green from Bio-Rad Laboratories, FITC and D-galactose from Sigma Chemical Company, and N-acetyl-D-galactosamine from Aldrich Chemical Company. Montmorillonite clay soil amendment ('Turface,' regular size) was purchased from Wyandotte Chemical Co. Seeds of soybean (*Glycine max* [L.] Merr. var. Beeson) were obtained from Dewine and Hanna Seed Co., Yellow Springs, Ohio, and seeds of pea (*Pisum sativum* var. Wando) from a local garden store. SBL was purified from defatted seed flour (Soya Fluff 200W, Central Soya Chemungy, Chicago, Ill.) by affinity chromatography, labeled with FITC, and repurified by affinity chromatography as described previously (2).

***Rhizobium* Cultures.** The sources, maintenance, and culture of the *R. japonicum* strains have been described (2). Strains of *Rhizobium* spp. (cowpea group) 6411e and 3G4b4 were obtained from Dr. J. D. Paxton, University of Illinois, Urbana. The heterologous *Rhizobium* strains were cultured in the synthetic salts medium (2) supplemented with biotin (0.5 μg/1).

Preparation of Inoculum. Cultures of *Rhizobium* strains grown in the synthetic salts medium for 72 hr were used as inoculum. The cultures were harvested by centrifugation at 20,000g for 5 min in sterile centrifuge tubes, washed twice with 30 ml of autoclaved phosphate-buffered saline (PBS, see ref. 2) and suspended in sterile, filtered, Jensen's medium (19). Bacteria in these suspensions were counted with a Petroff-Hauser bacterial counter and diluted with filtered Jensen's medium so that the suspensions contained 2×10^9 cells/ml. Aliquots of such suspensions were used to inoculate the synthetic salts medium, the root exudate media, the seedlings grown under hydroponic conditions, and the seedlings grown in Turface.

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[3] Abbreviations: FITC: fluorescein isothiocyanate; SBL: soybean lectin; PBS: phosphate-buffered saline.

Growth of Plants for Collection of Root Exudate and for Hydroponic Culture. Undamaged soybean seeds were surface-sterilized for 5 min in 0.1% $HgCl_2$, then rinsed and soaked in sterile distilled H_2O for 3 hr. The seeds were germinated on yeast extract mannitol agar (19) at 28 ± 1 C in the dark. Yeast extract mannitol agar was used to detect the growth of any seed-borne microorganisms, including rhizobia. After 72 hr, the seedlings that were free of any detectable microbial contamination were affixed to a sterile piece of Teflon by means of a rubber band and transferred to a test tube (30 × 300 mm) containing 15 ml of filtered Jensen's (N-free) solution. The rootlets of these seedlings remained immersed in the solution. The seedlings were maintained hydroponically in a growth chamber at 28 ± 1 C and 7,500 lux from fluorescent lamps (16-hr photoperiod). After 7 days, the Jensen's medium containing the root exudate from several plants was pooled, centrifuged at 20,000g for 15 min, and filtered through a Millipore membrane (0.1-μm pore size) to remove small particles of plant debris. The seedlings were then supplied with 15 ml of fresh Jensen's medium containing 0.5 ml of the inoculum suspension, prepared as indicated above. At least four plants were maintained for each strain of *Rhizobium* tested. Several uninoculated plants were also maintained as controls.

Plants and Cultures of Nodulation and Rhizoplane Studies. Aseptically germinated seedlings were planted in test tubes (30 × 200 mm) containing sterilized Turface to a depth of 5 cm. Before planting, 7 ml of Jensen's nutrient solution was added to each tube. These seedlings were inoculated at the time of planting with 1 ml of the inoculum suspension of a *Rhizobium* strain. The plants were maintained in the growth chamber as described earlier. Seven to 10 plants were maintained for each strain of *Rhizobium* tested. Of these, five to six plants were used for observations of rhizoplane cultures over a 7-day period. The remaining plants were grown for an additional 14 days and then checked for the presence of nodules. Uninoculated plants were used as controls for both studies.

***Rhizobium* Cultures in Root Exudate or Synthetic Salts Media.** Aliquots (100 μl) of the *Rhizobium* inoculum suspensions were added to test tubes (15 × 100 mm) containing either 2 ml of the synthetic salts medium or the filter-sterilized root exudates, or to 1.8 ml of the root exudate supplemented with 0.2 ml of 10-fold-concentrated synthetic salts medium. These cultures were incubated at 25 ± 1 C without shaking.

Binding Studies with FITC-SBL. Rhizobia growing in the hydroponic seedling cultures, root exudate media, and synthetic medium were collected by centrifugation at 20,000g for 5 min, washed once with PBS, and suspended in 500 μl of PBS. FITC-SBL (25 μl of a 3.0 mg/ml solution in PBS) was added to these cell suspensions. After 10-min incubation at room temperature, the cells were sedimented, washed once with PBS, and suspended in a small volume (200 μl) of PBS. A portion of this suspension was placed in a Petroff-Hauser counter and the binding of FITC-SBL to the bacteria was determined.

The lectin-treated bacteria were examined with both fluorescence and phase contrast optics in order to assure that any observed fluorescence was associated with individual bacteria or clumps of bacteria, and in order to estimate the proportion of cells in a sample that bound FITC-SBL. Three replicate determinations were generally made. In addition, the biochemical specificity of FITC-SBL binding to the bacteria was determined by hapten inhibition for each sample. In the hapten inhibition tests, a portion of the cell suspension labeled with FITC-SBL was washed twice with 100 mM galactose in PBS, resuspended in the galactose-PBS solution, and observed again to check the hapten reversibility of binding. In those instances where 100 mM galactose did not remove the bound FITC-SBL, the cells were washed again with 5 mM N-acetyl-D-galactosamine. N-Acetyl-D-galactosamine is also an effective hapten inhibitor of SBL binding (2). The binding of FITC-SBL to the bacteria was normally determined at 36, 60, 84, and 108 hr after inoculation. The sensitivity of the FITC-SBL-

binding assay was estimated by treating 250-μl aliquots of suspensions of 84-hr-old cultures of *R. japonicum* 3Ilb 138 in synthetic salts medium containing 1.4×10^9 cells/ml with 10-μl aliquots of FITC-SBL containing either 7.2, 0.72, or 0.072 μg of lectin. The number of fluorescing cells and the number of nonfluorescing cells were then determined for each sample. A Leitz ortholux II instrument, equipped with a Ploemopak 2.2 incident fluorescence illuminator, FITC-filter module H (2 × KP490/TK510/K515), and with 40 X/N.A. 1.3 and 100 X/N.A. 1.25 fluorescence objectives, was used for all microscopy.

Binding of FITC-SBL to Rhizobia Growing on Soybean Root Surfaces. Seedlings grown in Turface were collected 2 or 3 days after inoculation and washed with distilled H_2O to remove the adhering clay particles. The washed roots were dipped in a dilute solution of FITC-SBL (6 μg/ml) in PBS for 10 min, washed with PBS, and then counterstained with eriochrome black and methyl green according to the procedure of Schenk and Churukian (14) to reduce autofluorescence. Older portions of the root system were observed without this counterstaining as these tissues did not have strong autofluorescence.

RESULTS

Sensitivity of the FITC-SBL-binding Assay. Cell suspensions containing 3.5×10^8 cells treated with either 7.2 or 0.72 μg of FITC-SBL could be readily assayed for FITC-SBL-positive cells. The fluorescence of individual cells could easily be detected, and the proportion of FITC-SBL-labeled cells in the population reliably determined (approximately 57%). Cell suspensions treated with less than 0.72 μg of FITC-SBL (0.072–0.36 μg), however, could not be effectively assayed since the fluorescence of individual cells, although detectable, bleached more rapidly than the cells could be reliably brought into focus and counted.

Assuming that essentially all of the added FITC-SBL in these experiments bound to the bacteria, and that the lectin bound only to 57% of the cells in the population, it appears that the binding of approximately 2×10^3 FITC-SBL molecules/cell is required for reliable quantitation. For comparison, previous experiments with tritium-labeled SBL (2) (using SBL concentrations [140 μg/ml] comparable to the concentration of FITC-SBL used in the binding assays described in this paper [150 μg/ml]) indicated that approximately 1.4×10^6 molecules of SBL bind/FITC-SBL-positive cell, and that about 90% of the added lectin binds to the bacteria. The lower limit of reliable detection thus appears to be approximately $\frac{1}{1,000}$ of the binding normally encountered and described in this paper as maximum fluorescence.

Binding of FITC-SBL to Rhizobia Grown in Synthetic Salts Medium. Cells of *R. japonicum* strains 3Ilb 46, 3Ilb 83, 61A72, 61A76, 61A93 and W 505 failed to bind detectable amounts of FITC-SBL at any stage of growth in the synthetic salts medium under still culture conditions (Table I). Strain 127F17 of *R. phaseoli*, strain 102F71 of *R. meliloti*, strain 96B9 of *R. lupini*, strain 162P17 of *R. trifolii* and cowpea strain 6411e also failed to bind FITC-SBL at any stage of growth in the synthetic medium. The remaining *R. japonicum* strains, and cowpea strain 3G4b4, had SBL-positive cells in the early, mid-, and late log phases of growth in the synthetic salts medium under still culture conditions. Over 50% of the cells in log phase cultures of these latter strains bound the lectin (Table I). The binding of FITC-SBL to these cells was reversible by the addition of the sugar hapten galactose. The labeled lectin did not bind to any cells in stationary phase cultures of these strains. These results from still cultures of *Rhizobium* strains in the synthetic salts medium agree closely with the results from earlier shake culture experiments (2).

Binding of FITC-SBL to Rhizobia Cultured in Root Exudate Media. *R. japonicum* strains 3Ilb 31, 110, 135, 138 and 140 and the heterologous strain 3G4b4 of *Rhizobium* sp. (peanut isolate) bound FITC-SBL when grown in root exudate media (Table I), as they did when grown in synthetic salts medium or in root

Table I. *Binding of FITC-SBL to Rhizobia Cultured in Synthetic Medium, in Soybean Root Exudates, or in Association with Soybean Seedling Roots*

Strains of rhizobia were cultured in the synthetic salts medium, in root exudate medium, or in association with the roots of soybean seedlings growing either hydroponically or in Turface clay soil amendment as described under "Materials and Methods." The binding of FITC-SBL to rhizobial cells from these cultures and the ability of rhizobial strains to nodulate soybean (var. Beeson) were determined as described under "Materials and Methods." Rhizobial cultures which bound the lectin are indicated in the table by a + followed by the approximate percentage of cells in a given culture which were SBL-positive. Percentages of SBL-positive cells in rhizoplane cultures were not determined. The results given in the table are for cultures examined 36 hr after inoculation (or between 48 and 72 hr for Turface rhizoplane cultures). The reversibility of FITC-SBL binding with the sugar hapten galactose was demonstrated for each culture characterized as SBL-positive.

Strain No.	Host	FITC-SBL-Positive Cells in:				Nodulation of Soybean
		Synthetic medium	Root exudate	Hydroponic seedling culture	Rhizoplane (Turface)	
R. japonicum	Soybean					
311b 31[a]	"	+ (>50%)	+ (>50%)	+ (>50%)	+	+
311b 46	"	−	+ (1–5)	+ (1–5)	+	+
311b 83	"	−	−	+ (1–5)	+	+
311b 110	"	+ (>50)	+ (>50)	+ (>50)	+	+
311b 135[a]	"	+ (>50)	+ (>50)	+ (>50)	+	+
311b 138	"	+ (>50)	+ (>50)	+ (>50)	+	+
311b 140	"	+ (>50)	+ (>50)	+ (>50)	+	+
61A72	"	−	+ (1–5)	+ (1–5)	+	+
61A76	"	−	+ (1–5)	+ (1–5)	+	+
61A93	"	−	+ (1–5)	+ (1–5)	+	+
W 505	"	−	+ (1–5)	+ (1–5)	+	+
R. phaseoli 127F17	Kidney bean	−	−[b]	−[b]	−	−
R. meliloti 102F71	Alfalfa	−	−	−	−	−
R. trifolii 162P17	White clover	−	−	−[b]	−	−
R. lupini 96B9	Lupin	−	−	−	−	−
R. sp. 3G4b4	Peanut	+ (>50)	+ (>50)	+ (>50)	+	−
R. sp. 6411e	Cowpea	−	−	−	+	−

[a] Slimy colonies selected for testing (see ref. 2).

[b] Hapten-*irreversible* binding.

exudate medium supplemented with synthetic salts medium. Over 50% of the cells in root exudate cultures of these strains bound FITC-SBL. In addition, strains 311b 46, 61A72, 61A76, 61A93, and W 505 of *R. japonicum*, which failed to bind the lectin when grown in synthetic salts medium, did bind the labeled lectin when grown in the root exudate medium. The proportion of cells that bound FITC-SBL in cultures of these strains was considerably lower (1–5%) than the proportion of SBL-positive cells (50–80%) in cultures of those strains that bound the lectin when grown in the synthetic salts medium. The number of cells that bound FITC-SBL did not increase significantly when these strains were cultured in root exudate medium supplemented with synthetic salts medium. Of the 11 *R. japonicum* strains tested, only 311b 83 failed to bind detectable amounts of lectin in the root exudate media. Cells of *R. phaseoli* 127F17 bound FITC-SBL, but the binding was not reversed either by galactose or by N-acetyl-D-galactosamine. Except for cowpea strain 3G4b4, none of the other heterologous strains of rhizobia tested bound SBL when cultured in media containing root exudate.

Binding of FITC-SBL to Rhizobia Cultured in the Presence of Soybean Seedlings Growing Hydroponically. All of the strains of *R. japonicum* tested bound FITC-SBL when cultured in the presence of a soybean seedling growing hydroponically (Table I). The proportion of FITC-SBL-positive cells recovered from the hydroponic seedling cultures of the various *Rhizobium* strains was found to be very similar in each instance to the proportion observed in the root exudate cultures. Strain 3G4b4 of *Rhizobium* sp. was the only heterologous strain of *Rhizobium* tested that showed hapten-reversible FITC-SBL binding when grown under these conditions. Although cells of *R. phaseoli* 127F17 and *R. trifolii* 162P17 bound FITC-SBL when cultured in the presence of hydroponically growing soybean seedlings, the binding was not hapten-reversible. In order to determine the minimum time required for lectin-binding cells to appear, cultures of *R. japonicum* strains 61A72 and 61A76 were harvested at 4-hr intervals prior to the normal 36-hr growth

period. FITC-SBL-positive cells appeared within 16 hr after inoculation in cultures of these strains.

Binding of FITC-SBL to Rhizobia Present on the Rhizoplane of Soybean Seedlings. When roots of Turface-grown seedlings which had been inoculated with *R. japonicum* strains 311b 31, 110, 135, 138, 140, or cowpea strains 6411e and 3G4b4 were dipped in a dilute solution of FITC-SBL as described under "Materials and Methods," numerous, very bright fluorescent spots were observed on the surface of the root (Table I). Such fluorescent spots were also observed on the root surfaces of seedlings inoculated with strains 311b 46, 311b 83, 61A76, 61A72, 61A93, and W 505 of *R. japonicum*, although their frequency was considerably lower. The fluorescent spots were often located on root hairs and root cap cells. The size, shape, and position of the fluorescent spots generally appeared to correspond to an individual bacterium or to clumps of bacteria. However, it was technically very difficult to unequivocally resolve individual bacteria on the root surface and to determine whether all of the observed FITC-SBL fluorescence was associated with bacteria. The fluorescent spots were completely removed by washing the FITC-SBL treated roots in 100 mM galactose, whereas washing the roots in the PBS had no effect. The fluorescent spots were not observed on the root surfaces of either uninoculated seedlings or on the roots of seedlings inoculated with *R. phaseoli, R. trifolii, R. meliloti* or *R. lupini.*

Binding of FITC-SBL to Rhizobia Cultured in the Root Exudate or Rhizoplane or Pea Seedlings. In order to determine whether the rhizosphere/rhizoplane environment of a non-host legume could also influence the development of *R. japonicum* lectin receptors, strains 61A76, 61A72, and 311b 138 were cultured in media containing pea root exudate and in the presence of hydroponically-grown and Turface-grown pea seedlings. The methods and conditions employed were the same as those described for the experiments with soybean seedlings. When cultured in the presence of pea roots or root exudates, all three strains tested bound FITC-SBL in a manner qualitatively and quantitatively similar to

that observed for cultures grown in the presence of soybean roots or root exudates.

DISCUSSION

This study indicates that growth in a rhizosphere/rhizoplane environment is apparently required in order for some strains of *R. japonicum* to develop specific SBL receptors. All 11 of the *R. japonicum* examined here developed specific SBL receptors when cultured in association with soybean roots. It is significant that six of these 11 strains were previously reported to lack the ability to bind SBL when cultured in artificial media (2–5). These results suggest that all strains of *R. japonicum* are likely to possess the genetic potential to develop biochemically specific receptors for the lectin, although this potential may be expressed only under the environmental growth conditions encountered in close proximity to plant roots. Evidence from studies of microbial pathogens of animals indicates that new cell surface components develop under *in vivo* growth conditions, and that these new components may be involved in the infectivity of the microorganisms (18). It is of interest, therefore, to find that the soybean symbiont, *R. japonicum*, responds to a rhizosphere/rhizoplane environment by synthesizing new cell surface components which may be related to its ability to infect and symbiotically associate with a host plant.

The disappearance of SBL receptors from the cell surfaces of *R. japonicum* during certain growth phases in artificial culture media (2), and the inability of several *R. japonicum* strains to develop specific SBL receptors when cultured in artificial media (2–5, Table I), may explain why a species-specific surface antigen for *R. japonicum* has not been discovered by immunological techniques (6, 9).

With the exception of the two strains of *Rhizobium* spp. belonging to the 'cowpea miscellany', the strains representative of heterologous *Rhizobium* species gave no evidence of biochemically specific lectin binding, regardless of whether these were grown in artificial media or in rhizosphere/rhizoplane cultures (Table I). This suggests that in contrast to *R. japonicum*, these heterologous species do not have the genetic capacity to synthesize specific receptors for SBL. Further testing of heterologous strains, particularly those that have been previously reported to bind FITC-SBL, is needed to confirm this suggestion.

The binding of SBL to *Rhizobium* strains of the "cowpea miscellany" does not appear to be correlated with their ability to nodulate soybean. Brethauer (4) reported that 3 of the 15 cowpea strains which he examined were able to bind significant quantities of highly purified SBL. This binding was shown to be hapten-reversible, and thus biochemically specific. All 15 of these cowpea strains were reportedly able to nodulate cowpea, but unable to nodulate soybean variety Harosoy. Our experiments with two of these three strains, 6411e and 3G4b4, confirm Brethauer's report (4) that both strains bind purified SBL in a hapten-reversible manner and indicate that neither strain is able to nodulate soybean variety Beeson. These results indicate that either the binding of SBL to the cowpea strains is irrelevant and coincidental to their interactions with soybean, or that the lectin-mediated recognition of these strains by soybean, if it occurs, does not result in nodu-

lation. Further studies are needed to distinguish between these possibilities.

The development of biochemically specific SBL receptors by three strains of *R. japonicum* in pea seedling root exudate or rhizoplane cultures indicates that the development of the receptors by these strains is not a response elicited exclusively by the host plant. There may, of course, be considerable variation among *R. japonicum* strains with respect to the range of plant species which provide suitable rhizosphere/rhizoplane environments for the elicitation of SBL receptor development.

Although the SBL-binding properties of *R. japonicum* strains cultured in a rhizosphere/rhizoplane environment correlate very well with the ability of these rhizobia to nodulate soybean, it would be premature to conclude that the role of SBL in determining the host specificity of *R. japonicum* is fully established. The significance of SBL binding to some *Rhizobium* strains of the cowpea miscellany is still uncertain and requires careful investigation. Furthermore, it remains to be shown that the soybean seed lectin, or a related, *R. japonicum*-binding root lectin, is present in soybean roots at appropriate times and locations (13).

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