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Lectin Binding to the Root and Root Hair Tips of the Tropical Legume *Macroptilium atropurpureum* Urb.

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Ten fluorescein isothiocyanate-labeled lectins were tested on the roots of the tropical legume *Macroptilium atropurpureum* Urb. Four of these (concanavalin A, peanut agglutinin, *Ricinis communis* agglutinin I [RCA-I], wheat germ agglutinin) were found to bind to the exterior of root cap cells, the root cap slime, and the channels between epidermal cells in the root elongation zone. One of these lectins, RCA-I, bound to the root hair tips in the mature and emerging hair zones and also to sites at which root hairs were only just emerging. There was no RCA-I binding to immature trichoblasts. Preincubation of these lectins with their hapten sugars eliminated all types of root cell binding. By using a microinoculation technique, preincubation of the root surface with RCA-I lectin was found to inhibit infection and nodulation by *Rhizobium* spp. Preincubation of the root surface with the RCA-I hapten β-D-galactose or a mixture of RCA-I lectin and its hapten failed to inhibit nodulation. Application of RCA-I lectin to the root surface caused no apparent detrimental effects to the root hair cells and did not prevent the growth of root hairs. The lectin did not prevent *Rhizobium* sp. motility or viability even after 24 h of incubation. It was concluded that the RCA-I lectin-specific sugar β-D-galactose may be involved in the recognition or early infection stages, or both, in the *Rhizobium* sp. infection of *M. atropurpureum*.

Research in the last 10 years has disclosed the widespread biological importance of cell surface carbohydrates in the many processes involving specific cellular recognition (14, 21). These findings and the realization that relatively small changes in the structure of complex surface carbohydrates may lead to large effects on recognition specificity (17) and that more structural variation can be contained in complex carbohydrates, on a weight basis, than in either proteins or nucleic acids (14) have stimulated considerable interest in cell surface carbohydrates. Since plant-parasite and *Rhizobium* sp.-legume interactions represent well-defined examples of specific recognition, it has been suspected that surface carbohydrates on the cells of these organisms may be functionally involved.

Bohlool and Schmidt (6) reported a strong correlation between the in vitro binding of soybean seed lectin to *Rhizobium japonicum* cells and the ability of such strains to infect soybean roots. These observations were independently confirmed by Bhuvaneswari and co-workers (4), who also demonstrated that the soybean lectin haptens *N*-acetyl-D-galactosamine and D-galactose reversed lectin binding to *R. japonicum* cells. It was later demonstrated (16) that the soybean lectin haptens specifically inhibited attachment of *R. japonicum* cells to soybean roots. In early studies, a few infective strains of *R. japonicum* were found that did not bind soybean lectin (4, 6), but later work (3) demonstrated that the few non-lectin-binding *R. japonicum* strains in fact possessed lectin-binding properties when cultured in a soybean root exudate medium. However, the recent papers of Halverson and Stacey (9–11) have demonstrated a direct effect of lectin presence on nodulation. By using a mutant of *R. japonicum* (strain HS111) which is delayed in its ability to initiate nodulation in the emerging root hair zone, it was possible to change the nodulation profile of this strain HS111 on soybean roots by first preincubating the rhizobia in soybean root exudate or in a suspension of soybean lectin (11). Furthermore, this soybean lectin enhancement of strain HS111 nodulation of soybeans can be prevented by the addition of D-galactose, a hapten of soybean seed lectin (11).

Lectin-mediated attachment has also been implicated in clover-*R. trifolii* interactions and perfect correlations between in vitro lectin binding and strain infectivity have been described (7). The hapten 2-deoxyglucose was shown to specifically prevent lectin binding to bacterial cells and also prevented the attachment of *R. trifolii* cells to clover root surfaces. Significantly, the clover seed lectin originally used in binding studies has been shown to be present on the root hair surfaces where *Rhizobium* sp. attachment occurs (8). Similarly, soybean seed lectin has recently been localized to the site of *Rhizobium* sp. attachment to roots (15, 16).

Apart from their hypothetical involvement in recognition and specificity, lectins are also useful tools in biology because of their highly specific recognition of sugar molecules. By using lectins with differing affinities, a surface carbohydrate profile of any cell can be obtained and is thus useful for separating organisms that may differ by only a single surface sugar. This paper reports on the use of lectins to investigate the zone of emerging root hairs on siratro roots and *Rhizobium* sp. root hair interactions.

MATERIALS AND METHODS

***Rhizobium* sp. culture.** Stock cultures of strain NGR234 (13) were streaked onto Bergersen modified medium (BMM), and single colonies were used to inoculate 30 ml of BMM (2) in 125-ml culture flasks. These starter cultures were maintained on a rotary shaker (200 rpm) at 30°C until late log/early stationary phase (24 h). The optical density of this culture was determined and a volume equivalent to 1 ml of a 200-Klett-unit suspension was added to 50 ml of fresh BMM in a 250-ml culture flask. Inoculum cultures were grown to late log/early stationary phase in the same manner as the starter cultures and used undiluted (approximately 10^9 cells ml^{-1}).

Seed germination. *Macroptilium atropurpureum* (siratro) seeds were etched for 15 min with concentrated H_2SO_4,

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TABLE 1. Lectins tested on the siratro root surface

Lectin[a]	Source	Hapten[b]	Buffer[c]
CON-A	*Canavalia ensiformis*	α-L-Mannose/α-L-glucose	TRIS
DBA	*Dolichos biflorus*	GalNAc	PBS
GSA-I	*Griffonia simplicifolia*	α-D-Galactose	PBS
GSA-II	*Griffonia simplicifolia*	GlcNAc	PBS
MPA	*Maclura pomifera*	Galactose	PBS
PNA	*Arachis hypogaea*	D-Gal-β(1–3) GalNAc	PBS
RCA-I	*Ricinus communis*	β-D-Galactose	PBS
SBA	*Glycine max*	GalNAc or D-galactose	PBS
UEA-I	*Ulex europaeus*	α-L-Fucose	PBS
WGA	*Triticum vulgaris*	Sialic acid	PBS

[a] CON-A, Concanavalin A; DBA, *Dolichos biflorus* agglutinin; GSA, *Griffonia simplicifolia* agglutinin; MPA, *Maclura pomifera* agglutinin; PNA, peanut agglutinin; SBA, soybean agglutinin; UEA, *Ulex europaeus*, agglutinin; WGA, wheat germ agglutinin.

[b] GlcNAc, *N*-Acetyl-α-D-glucosamine; GalNAc, *N*-acetyl-α-D-galactosamine.

[c] PBS, Phosphate-buffered saline, pH 7.0; TRIS, 0.005 M Tris–0.001 M $CaCl_2$–0.015 M NaCl, pH 7.0.

washed thoroughly with tap water, surface sterilized with 12% sodium hypochlorite for 10 min, and washed with sterile distilled water five times. The seeds were left to soak for 30 min before germinating over 2 days in plates of BMM in 1.5% agar at 30°C. No contaminants were detected with the BMM and germination was >90%.

Growth of seedlings. Germinated siratro seeds with radicles 1 to 2 cm long were transferred aseptically to plastic growth pouches (Northrup, King & Co., Minneapolis, Minn.) which had been previously watered with 7.5 ml of half-strength Jensen's medium (5), three plants per pouch. The quantity of liquid medium was critical as the seedlings did not grow well in overwet conditions. Seedlings were maintained in a Conviron growth chamber at 85% relative humidity; 29°C day (13 h); 26°C night; photon flux density of 200 to 220 μmol m^{-2} s^{-1} at midday from Sylvania Gro-lux VHO cool white fluorescent tubes. The pouches were kept upright (roots kept dark with aluminum foil) and checked daily for watering requirements.

Spot inoculation procedures. Spot inoculation (19) involves the placing of a drop of inoculum (approximately 50 nl) on a specific region of the root (between the root tip and smallest emerging root hairs). To achieve this, a glass microelectrode, such as is used for neurological work, was araldited to a 19-gauge hypodermic, which in turn was attached to a 1-ml disposable syringe. Microelectrode inoculation needles were made from filament borosilicate glass (DO 1.2, DI 0.6), using a David Kopf vertical electrode puller (model 700c).

Plants were pouched and left to grow for 2 days until the root tips had reached halfway down the pouch. With a stereo dissecting microscope, the pouch face was cut and peeled back, exposing the roots. A map pin, bent for easy insertion, was put next to the intended spot inoculation area, pinned into the pouch paper. This prevented the pouch face from interfering with the inoculated area when the pouch face was taped back to its original position. A single or several anion-exchange resin beads (Bio-Rad Cl^-, 50 to 100 μm) were placed approximately halfway between the root tip and smallest emerging root hairs, using fine forceps. The beads transferred easily and adhered tenaciously to the root surface, even through fixation and embedding procedures. Control experiments showed that the bead did not affect the root surface or its ability to grow root hairs at that point. Inoculum was transferred to the surface of a single bead, using the bead as a guide to droplet size (generally 50-μm diameter).

Lectin probes. All lectins (Table 1) were purchased from E-Y Laboratories, Inc., Los Angeles, Calif. Lectins were supplied labeled with fluorescein isothiocyanate (FITC) as 1 mg of lectin in 1 ml of buffer and had been tested for purity by disc gel electrophoresis prior to dispatch. Lectins were diluted to 200 μg ml^{-1}, divided into 0.5-ml samples, and maintained at −20°C.

Roots were incubated in the dark in 200, 100, or 50-μg ml^{-1} solutions of lectin for 30 min at room temperature. For hapten controls, lectins were preincubated for 20 min in the appropriate 0.2 M solution before application to the root surface.

Inhibition experiment. Plants were spot inoculated with a large volume (ca. 300 nl) of either 100 or 200 μg of *Ricinus communis* agglutinin I (RCA-I) lectin ml^{-1}, or a 0.2 M solution of the RCA-I hapten sugar β-D-galactose, or a preincubated mixture of RCA-I lectin and hapten. This was incubated for 1 h, and then a small inoculum (ca. 50 nl, containing approximately 500 to 1,000 *Rhizobium* sp. cells) of strain NGR234 was spot inoculated at the same position. Flood- and spot-inoculated controls and uninoculated controls were used.

Light microscopy and FM. After lectin incubation, plant material was either unrinsed or rinsed three times with appropriate buffer and mounted in buffer on a microscope slide. For fluorescence microscopy (FM), material was observed with a mercury vapor source and filters to provide excitation at 365- and 495-nm main wavelengths, which are appropriate for the proper excitation of FITC.

Observations were made with a Nikon Optiphot with an EF epifluorescence attachment and Nikon Fluor objectives and were recorded on Ektachrome 400 daylight diapositive film. For light microscopy, material was observed either with bright field or by Nikon differential interference contrast attachment (Nomarski optics).

RESULTS

Lectin binding. FITC has a bright green fluorescence and could be reliably distinguished against the faint blue autofluorescence of the siratro root surface. Of the lectins tested, only RCA-I bound to root hair tips (Fig. 1 to 4). Tips of all root hairs showed binding of RCA-I regardless of their maturity or position on the root surface relative to the root tip.

The RCA-I lectin also bound to sites at which root hair emergence could only just be detected by differential interference contrast when observing the surface of whole root tissue (Fig. 1). When such tissue was sliced longitudinally, the early stage of root hair development and the nature of RCA-I lectin binding in such examples was apparent (Fig. 2). The application of high levels of RCA-I (200 μg ml^{-1}) resulted in large clumps of fluorescing material at the root hair tips and small amounts of clumping over the hair (Fig. 3). However, at 50 μg ml^{-1}, RCA-I bound smoothly over the root hair tip region only (Fig. 4).

Concanavalin A, peanut agglutinin, RCA-I, and wheat germ agglutinin bound to the exterior of root cap cells (Fig. 5) and to the regions between epidermal cells along the elongation zone (Fig. 6). All other lectins tested showed no binding to any part of the root surface. When these lectins were preincubated with their haptens (Table 1) and applied to the siratro root surface, no FITC fluorescence was observed. RCA-I was also tested against *N*-acetylgalactosamine, the hapten for the more toxic RCA monomer

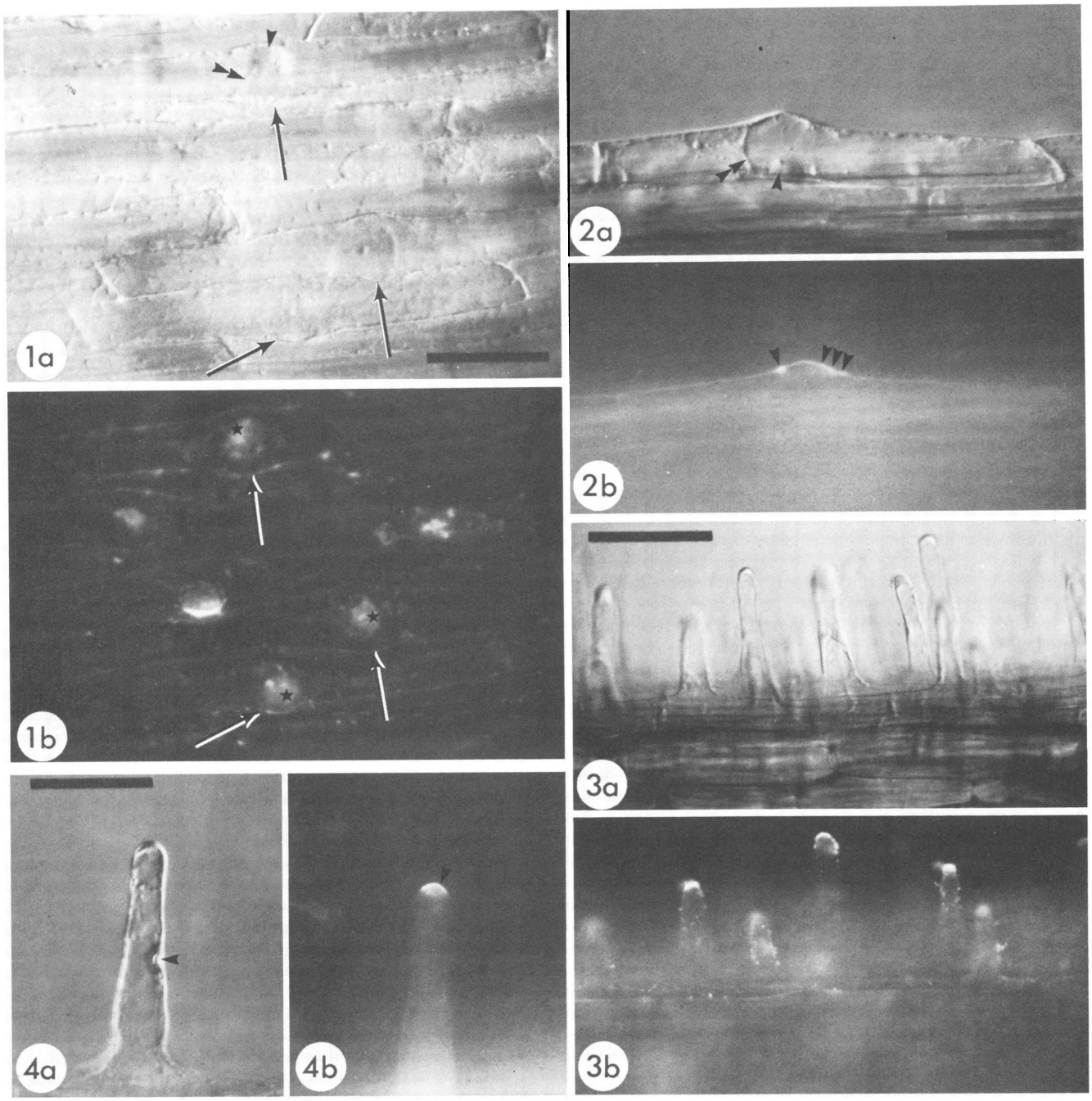

FIG. 1. Surface view of a region between the root tip and emerging root hairs. RCA-I lectin (100 μg ml^{-1}) binds to sites of hair emergence (stars in b) at a stage when such sites are not easily detected by other means (examples arrowed). The nucleus (arrowhead) and a cytoplasmic strand (double arrowhead) can be observed in one of the sites. Magnification, ×400; bar, 50 μm. (a) Nomarski optics; (b) FM, U/420.

FIG. 2. View of emerging root hair from a longitudinal section. There appears to be uneven binding of the RCA-I lectin (100 μg ml^{-1}) (arrowheads in b). Arrowhead in (a) indicates nucleus; double arrowhead indicates cytoplasmic strand. Magnification, ×400; bar, 50 μm. (a) Nomarski optics; (b) FM, U/420.

FIG. 3. RCA-I lectin (200 μg ml^{-1}) binding to root hair tips. Magnification, ×200; bar, 100 μm. (a) Nomarski optics; (b) FM, U/420.

FIG. 4. RCA-I lectin (50 μg ml^{-1}) binding to an individual hair (arrowhead in b). The binding to the tip appears to be smooth and evenly distributed. Arrowhead in (a) indicates nucleus. Magnification, ×400; bar, 50 μm. (a) Nomarski optics; (b) FM, U/420.

(RCA-I is the less toxic dimer form). *N*-Acetyl-galactosamine did not prevent binding of the RCA-I lectin to the root surface.

Inhibition experiment. Because of the implications of specific sugars being involved in recognition or attachment (or both) of *Rhizobium* spp. to the root hair tip, and in light of the results of Hinch and Clarke (12), where *Ulex europaeus* agglutinin lectin prevented attachment of *Phytophthora* sp. zoospores to *Zea mays* root in the elongation zone, an inhibition experiment was conducted to establish

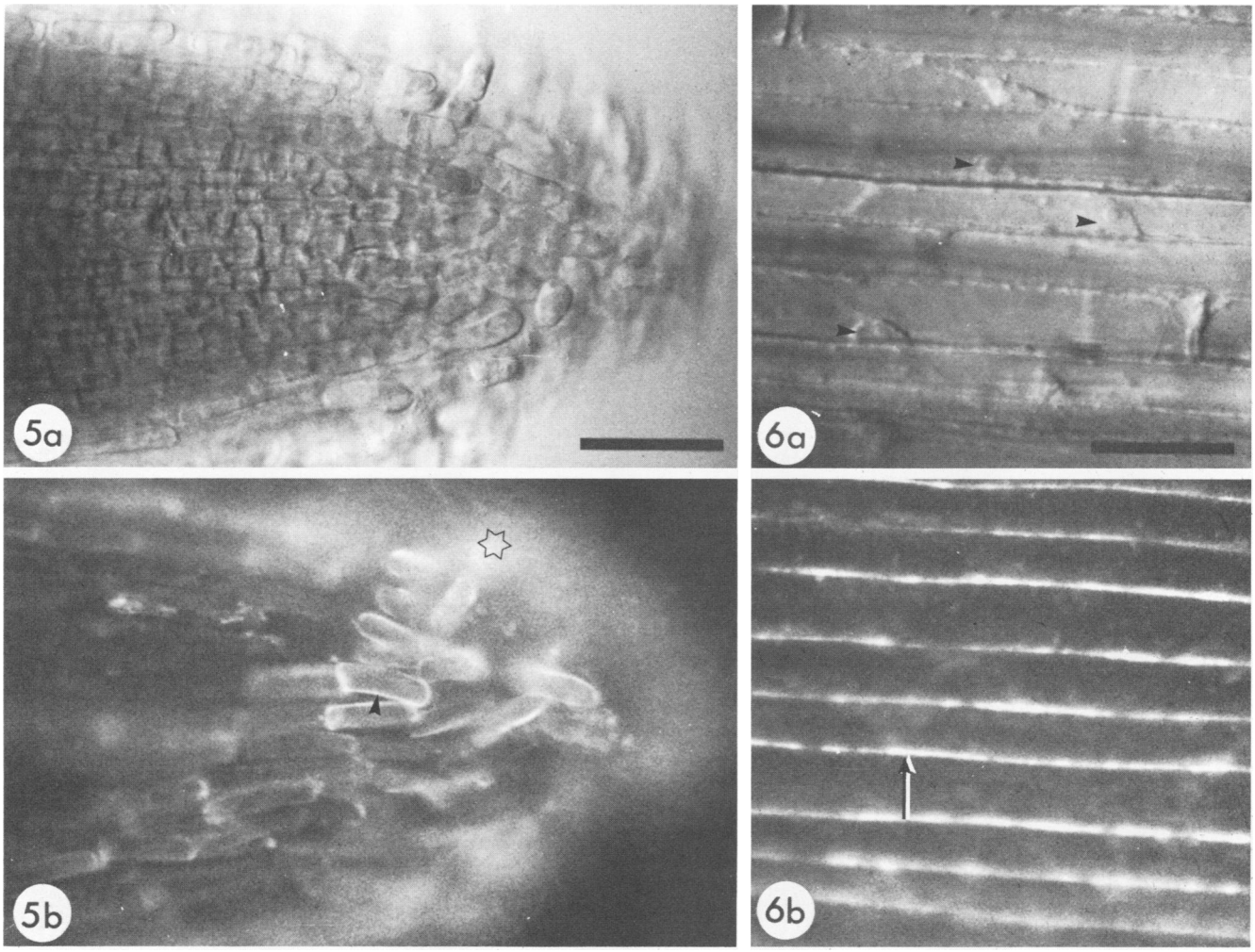

FIG. 5. RCA-I lectin (200 μg ml^{-1}) binding to siratro root cap slime (star in b) and exteriors of root cap cells, whose outline is clearly defined by the lectin. Magnification, ×200; bar, 100 μm. (a) Nomarski optics; (b) FM, U/420.

FIG. 6. RCA-I lectin (200 μg ml^{-1}) binding to the region between the root epidermal cells (arrow in b), probably to root cap slime remaining there. Arrowheads indicate epidermal cell nuclei. Magnification, ×400; bar, 50 μm. (a) Nomarski optics; (b) FM, U/420.

whether RCA-I binding to root hair tips prevented infection by *Rhizobium* spp. by saturating such hypothesized recognition or attachment sites. The results are presented in Table 2. Preincubation of the root with RCA-I lectin at either 100 or 200 μg ml^{-1} prevented nodulation by the wild-type strain NGR234 in almost all plants tested. Preincubation of the root surface with β-D-galactose did not prevent nodulation, nor did a preincubated mixture of RCA-I and the hapten prevent nodulation. Light microscopic observations showed that the lectin did not prevent root hair growth. No root hair curling was observed in inoculated areas preincubated with the lectin. RCA-I did not appear to affect living strain NGR234 cells; even when incubated overnight in a concentration of 200 μg ml^{-1}, NGR234 cells still appeared actively mobile when viewed under differential interference contrast. This was not Brownian movement.

DISCUSSION

The results show that the binding of different lectins to various parts of the root surface insinuate the presence of sugars for which each lectin is known to be specific. The binding to the regions between epidermal cells in the elongation zone is probably due to the presence of root cap slime remaining in those parts.

The lectin RCA-I binds to the domes of root hair tips and to the sites of emerging hairs, suggesting the presence of β-D-galactose. Preincubation of these lectins with their haptens prevented binding to the root surface, demonstrating that the observed binding activity was not nonspecific. Of these results, the binding of RCA-I lectin to root hairs is highly interesting. Preincubation of the infectible zone of siratro with the lectin RCA-I prevented infection by the wild-type strain under spot inoculation conditions. It is possible that the RCA-I-specific sugar β-D-galactose at the site of emerging hairs may normally act as a target for rhizobia, enabling them to establish on the hair-forming site even before the hair has fully emerged, and that incubation of the lectin saturated available β-D-galactose-binding sites, preventing the attachment and subsequent curling and infection by *Rhizobium* spp. As RCA-I is specific to β-D-galactose, and would therefore be unlikely to bind to other sites (and did not bind nonspecifically), the prevention of specific attachment and infection by strain NGR234 by saturation of β-D-galactose sites on the root hair tip surface

TABLE 2. Nodulation of siratro with or without preincubation of RCA-I lectin

Conditions	No. of plants nodulated (Nod^+)	No. of plants not nodulated (Nod^-)	Plant death during expt
Preincubation with RCA-I followed by spot inoculation with NGR234			
RCA-I, 100 $\mu g\ ml^{-1}$	5	54	1
RCA-I, 200 $\mu g\ ml^{-1}$	6	51	3
NGR234			
Spot inoculated	55	3	2
Flood inoculated	59		1
Uninoculated		28	2

suggests that this sugar is involved in an initial infection stage.

These results add to the growing body of information which indicate a critical role for lectins in the initial stages of root hair infection of legumes (1). While the precise mechanism of this role is still unknown, clearly the reaction must be at the very early stages of the interaction of the emerging root hair and the invading *Rhizobium* cells.

Recent studies on the infection of soybean (18, 20) and the infection of siratro (R. W. Ridge and B. G. Rolfe, J. Plant Physiol., in press) have shown that, in these plants at least, hairs are affected and infected as they arise from the trichoblast. If the *Rhizobium* spp. are to attach and cause the curling of root hairs as they arise from the surface, recognition molecules would need to be expressed at the root surface, just ahead of tip growth. Such a molecule could be β-D-galactose.

It is critical that more be known as to the nature, distribution, and sugar-binding properties of lectins in legume roots and of the relationships between root and seed lectins. Even with a highly specific recognition system functional at the nodulation site, other events must be required to communicate the success of the recognition and to signal the next step toward entry into the hair cell and subsequently the root tissue. At a preliminary, but equally critical, stage, it has been shown that the plant excretes signal compounds or special substrates that induce the expression of the nodulation genes of *Rhizobium* bacteria (M. A. Djordjevic, J. Plazinski, C. L. Sargent, P. R. Schofield, J. M. Watson, R. Innes, P. Kuempel, H. C. Canters-Cremers, and B. G. Rolfe, 9-07, 6th Int. Symp. Nitrogen Fixation, Corvallis, Ore., 1985).

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