Correlation between infection by *Rhizobium leguminosarum* and lectin on the surface of *Pisum sativum* L. roots

Department of Plant Molecular Biology, Botanical Laboratorium, University of Leiden, Nonnensteeg 3, NL-2311 VJ Leiden, The Netherlands

**Abstract.** The lectin on the surface of 4- and 5-d-old pea roots was located by the use of indirect immunofluorescence. Specific antibodies raised in rabbits against pea seed isolectin 2, which cross-react with root lectins, were used as primary immunoglobulins and were visualized with fluorescein- or tetramethylrhodamine-isothiocyanate-labeled goat antirabbit immunoglobulin G. Lectin was observed on the tips of newly formed, growing root hairs and on epidermal cells located just below the young hairs. On both types of cells, lectin was concentrated in dense small patches rather than uniformly distributed. Lectin-positive young hairs were grouped opposite the (proto)xylematic poles. Older but still-elongating root hairs presented only traces of lectin or none at all. A similar pattern of distribution was found in different pea cultivars, as well as in a supernodulating and a non-nodulating pea mutant. Growth in a nitrate concentration which inhibits nodulation did not affect lectin distribution on the surface of pea roots of this age. We tested whether or not the root zones where lectin was observed were susceptible to infection by *Rhizobium leguminosarum*. When low inoculum doses (consisting of less than $10^6$ bacteria $\cdot$ ml$^{-1}$) were placed next to lectin-positive epidermal cells and on newly formed root hairs, nodules on the primary roots were formed in 73% and 90% of the plants, respectively. Only a few plants showed primary root nodulation when the inoculum was placed on the root zone where lectin was scarce or absent. These results show that lectin is present at those sites on the pea root that are susceptible to infection by the bacterial symbiont.

**Key words:** Epidermis (root) – Lectin (localization, root nodulation) – *Pisum* (lectin nodulation) – *Rhizobium* – Root hair.

**Introduction**

Plant lectins, able to recognize specific polysaccharide sequences, have been proposed to determine the specificity of the *Rhizobium*-legume symbiosis (Bohlool and Schmidt 1974; Dazzo and Hubbell 1975). The validity of this theory partly depends on the demonstration of legume lectins at the sites of infection. For the majority of the Leguminosae, root hairs have been shown to be the sites of infection (for a review, see Dart 1974). Lectins have been found to be localized at the tips of growing root hairs of *Trifolium repens* (Dazzo et al. 1978) and *Lotononis bainesii* (Law and Strijdom 1984). The location of lectin-positive hairs and the susceptibility of *Lotononis* to infection by its specific bacterial symbiont might be correlated (Law and Strijdom 1984). Pea root lectin has been observed on root hairs (Gatehouse and Boulter 1980; Kato et al. 1981), as well as on the surface of the primary pea root (Gatehouse and Boulter 1980). However, these authors did not specify the part of the pea root used in their experiments, nor did they try to establish a correlation between the presence of lectin and infection by *Rhizobium leguminosarum*.

We used indirect immunofluorescence to verify the location of lectin on the root surface of 4- and 5-d-old pea seedlings. A specific seed isolectin-2 rabbit antibody, which cross-reacts with root cell-wall and root-slime lectins, was used as primary antibody (Díaz et al. 1984). Seed isolectin 2 shows...
the same electrophoretic mobility as pea root-slime lectin (Kijne et al. 1983). As secondary antibodies, we used either fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRIC)-labeled goat antirabbit immunoglobulin (Ig)G in order to unequivocally distinguish between the specifically labeled lectin and the intrinsic fluorescence of plant tissues when excited with blue or green light. The effect of nitrate on the location of lectin

the root surface was assessed after the seedlings were grown in the presence of 20 mM NO₃⁻, a condition known to inhibit nodulation of the pea by *R. leguminosarum* (Diaz et al. 1984). Lectin was located on the roots of different pea cultivars and some of their nodulation mutants. Using a low-dose spot inoculation we tested whether or not there is a correlation between susceptibility to infection by *Rhizobium leguminosarum* and lectin presence on the pea root surface.

**Material and methods**

**Plant material.** *Pisum sativum*, cv. Finale and Rondo were purchased from CEBECO, Rotterdam, The Netherlands; Finale was used to standardize all experiments. Seeds of the non-nodulating Rondo NOD 3 mutant (Jacobsen and Feenstra 1984) were kindly provided by Dr. E. Jacobsen, University of Groningen, The Netherlands. The French pea cultivar Frisson and its non-nodulating mutant F4 58 were a generous gift of Dr. A. Messager, Station d’Amélioration des Plantes, Dijon Cedex, France

Nodulation by seed inoculation. Nod + Fix + *Rhizobium leguminosarum* strains, listed in Table 1, were used to inoculate surface-sterilized pea seeds. Cultivation of bacteria, seed inoculation, and growth conditions of the plants have been previously described (Diaz et al. 1984). Nodules were counted when the plants were 21 d old.

Cross-reactivity of seed and root lectins. The lectin of all cultivars and mutants was extracted from dry seed meal (50 g) with buffer E (50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) with 150 mM NaCl, 10 mM MgCl₂, 10 mM CaCl₂ and 0.02% NaN₃, pH 8.2) in a 1:10 meal weight to buffer volume ratio. The extract was kept on ice during the first 2 h and the pH was repeatedly adjusted to 8.2. After this period, the pH did not change and the extraction was continued at 4°C for 16 h. The extract was centrifuged for 1 h at 21,000g at 4°C. The supernatant was placed on ice and its pH decreased to 3.0 with dilute HCl and kept at this value for 10–12 min. The precipitate formed was pelleted by 15 min centrifugation as described above and discarded. The pH of the supernatant fluid was increased to 7.3 and (NH₄)₂SO₄ was added to 55% saturation. The protein was pelleted by 30 min centrifugation as described. The pellet was dissolved in buffer E and extensively dialyzed against the same buffer. The dialyzed protein was applied to a Sephadex G-75 column (2.5 cm diameter, 12 cm long, Pharmacia, Uppsala, Sweden) previously equilibrated with buffer E, at an flow rate of 20 ml h⁻¹. The column was considered free of unbound protein when the absorption of the eluant at 280 nm had reached the same value as that of buffer E. Pea lectin was eluted with 0.2 M glucose in buffer E devoid of the magnesium and calcium ions. All 280-nm-absorbing fractions were pooled, extensively dialyzed against water and lyophilized. The agglutination-inhibition test of human erythrocytes and isoelectric focusing of pea seed lectin have been previously described (Kijne et al. 1980; van der Schaaf 1983, respectively). Ouchterlony double diffusion in 1.3% agarose in Na-veronal buffer pH 8.2, was performed with 1:1 (v/v) diluted antiisolectin-2 crude rabbit serum and 0.1 mg ml⁻¹ lectin (25 µl per well). The cross-reactivity of root homogenates of 5-d-old pea seedlings (cultivar Frisson and mutant F4 58) was tested using an enzyme-linked immunosorbent assay (ELISA) (Diaz et al. 1984).

**Root-lectin labelling.** Surface-sterilized pea seeds were imbibed for 18 h with water and planted in sterilized coarse gravel wetted with a mineral nutrient solution devoid of NO₃⁻ (Raggio and Raggio 1956) or supplied with 20 mM KNO₃, as required. The growth plates were wrapped in aluminium foil and placed in a chamber at 20°C and 70% humidity. When the seedlings were 4 or 5 d old, roots were excised and thoroughly washed with water. Only straight, similarly developed roots were chosen. Washed roots were incubated with goat serum diluted 1:20 (v/v) with TS buffer (20 mM Tris with 100 mM NaCl, pH 8.2) for 20 min at 30°C. This goat serum did not precipitate with 0.1 mg ml⁻¹ (25 µl) pea seed lectin in a previously performed Ouchterlony double-diffusion test. In some experiments, roots were mildly fixed with 4% paraformaldehyde in 100 mM NaH₂PO₄-NaH₂PO₄ buffer, pH 7.4, 100 mM NaCl, for 20 min at room temperature, and the excess of fixative was washed away.

Examination of the primary roots of 4- or 5-d-old pea seedlings with a dissection microscope revealed that four zones or regions could be easily distinguished. Accordingly, roots were sectioned in zone 1, which corresponds to the mitotisatic and elongation zones; zone 2, which is the region of root-hair emergence; zone 3, where the longest root hairs are found; and zone 4, which is the oldest part of the root showing no root hairs (see Fig. 2).

Root sections (four or five per treatment) were placed in air-dried plastic tubes (PPN, 39.5 mm long, 7.3 mm diameter; Flow Lab, Irvine, Scotland) coated with bovine serum albumin (BSA; fraction V; Sigma, St. Louis, Mo., USA). For coating, the tubes were incubated overnight at 4°C with BSA, 5 mg ml⁻¹ in 0.1 M Na₂CO₃-NaHCO₃ buffer, pH 9.6. Controls for autofluorescence and specific binding of the labeled antibody were incubated with a 1:40 dilution of goat and rabbit (lectin) nonimmune sera, respectively, in TS buffer for 30–40 min at 30°C, gently shaking in a water bath. Sections to be tested for lectin localization were incubated with 250–300 µl of specific pea-seed isolectin-2 rabbit antibody (2 mg ml⁻¹ in TS buffer). This volume completely covered the test sections. Washings included five subsequent refreshments of TS, incubation of the sections with fresh buffer for 5 min and repeats of these operations four times before the secondary antibodies were added.

The excess of washing buffer was discarded and the sections were incubated either with FITC- or TRIC-labeled goat anti-rabbit IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands; approx. 0.6 µg ml⁻¹ in TS buffer) for 30–40 min, under the same conditions as for the primary antibody. The controls for autofluorescence were incubated with a 1:40 dilution of (lectin) nonimmune goat serum in TS. Washings were performed as described.

After completion of the labeling procedure, each section was placed under a dissection microscope and strips of epidermis were obtained with a pair of straight-type tweezers (numbers 4 or 5; Dumont et Fils, Montinez, Switzerland). These strips, which were about 1 cm in length and 2–15 epider-