



Immunofluorescent localization of sainfoin lectin
by Sharon Jo Fitzwater Solomon

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
in Biochemistry

Montana State University

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Abstract:

Immunofluorescent staining procedures were developed and applied toward the localization of lectin in the leguminous plant Sainfoin (*Onobrychis viciifoliae*, Scop.). Tissue sections from root, seed, and nodule were microscopically examined with the "indirect" immunofluorescent staining technique. Specific lectin antiserum was produced in rabbit. Goat anti-rabbit immunoglobulin labelled with rhodamine isothiocyanate was used as the secondary stain.

Immunofluorescent stains with comparative histological stains showed lectin to be present in the cell wall area of the aleuron layer of the seed as well as in the protein matrix surrounding the starch granules. In the nodule, positive staining was found in the cell wall area of the outer cortical cells. Lectin was localized in the cell wall areas of the central vascular elements of 10, 20, and 30 day old root as well as in the cell wall area of the outer epidermis. There was no significant staining of the cortical parenchyma of root tissue in these age groups. In young (96 hr) root tissue, lectin was localized in the wall areas of all cells, including the outer epidermis. Cells near the extreme root tip also contained lectin localized in the cytoplasmic regions. In contrast, older tissue showed no cytoplasmic staining.

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by

SHARON JO SOLOMON

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ABSTRACT

Immunofluorescent staining procedures were developed and applied toward the localization of lectin in the leguminous plant Sainfoin (*Onobrychis viciifoliae*, Scop.). Tissue sections from root, seed, and nodule were microscopically examined with the "indirect" immunofluorescent staining technique. Specific lectin antiserum was produced in rabbit. Goat anti-rabbit immunoglobulin labelled with rhodamine isothiocyanate was used as the secondary stain.

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INTRODUCTION

Phytolectins can be defined as proteins or glycoproteins from plants that are capable of binding animal erythrocytes and other cells due to their specificity towards saccharide receptors present on the cell surface (1). This selective binding is presumably the basis of certain cell responses such as agglutination, lysis, mitosis, and induced contact regulation of growth in malignant cells (2). Therefore, lectins provide a fruitful area of study not only to the protein biochemist and plant physiologist but also to the immunologist, cell biologist and other researchers involved in cell phenomena.

Since the late 1800's lectins have been studied as model systems analogous to the antigen-antibody reaction and to investigate specificity of erythrocyte agglutination (3). In the 1940's Concanavalin A (Con A) was isolated and to date it has been the most thoroughly studied phytolectin. Edelman's group at Rockefeller University has recently established the primary sequence and the 3-dimensional, x-ray crystallographic structure including elucidation of the metal and saccharide binding sites (4). In the last decade lectin research has increased dramatically. It has been found that besides possessing specific erythrocyte agglutination properties, lectins bind sugars specifically and specifically precipitate polysaccharides and glycoproteins (5). Some lectins, such as Con A are mitogenic; that is, they convert resting lymphocytes into actively growing and dividing

blast-like cells (6). Some lectins are also capable of specifically agglutinating malignant cells (7) and are therefore used as probes to investigate cell surface changes during malignant transformation. The increased intensity of lectin research is evidenced by four recent reviews (8,9,10,11).

Phytolectins are easily obtainable by direct extraction and chromatography techniques (12) and in most instances have been isolated from legumes. However, they have also been found in other plants such as wheat (13) and more recently in some slime molds (14).

Although their biological and chemical properties are beginning to be investigated, the exact *in vivo* role or roles of plant lectins is still speculative. It has been postulated that they function in a plant protection system that counteracts soil bacteria or inhibits fungal polysaccharases (15). Another suggestion is that they are involved in sugar transport and storage and cell wall extension growth (16). It has been thought that possibly they serve in the attachment of glycoprotein enzymes in organized multi-enzyme systems (17) or that perhaps they in some way control cell division and germination in the plant (18). All of these hypotheses are under study and it may be that the role of lectin in nature is not related directly to biological properties observed in laboratories.

Recently some evidence has accumulated suggesting that lectins may be involved in the localization of rhizobia bacteria on the root

hairs of legumes prior to infection and subsequent nitrogen fixation. During this infection process the rhizobia bacteria enter the plant via invagination of the cell wall at the site of binding on the root hair (25). This progressive invagination is termed an infection thread and usually contains many actively replicating bacteria. The infection thread eventually reaches a large cortical cell in the epidermis of the root where the tip of the thread ruptures. The bacteria are released into the cortical cells which then enlarge to form a nodule. Although the infected cortical cell is often tetraploid, ploidy has not been conclusively shown to be a prerequisite for infection (26). Once in the nodule the bacteria differentiate to form a bacteroid which is capable of fixing nitrogen. The ammonia produced can be combined with the products of photosynthesis to yield those compounds essential to plant growth. This symbiotic relationship between the legume host and the rhizobia is specific. In other words, only certain species of rhizobia form this association with any one legume. Obviously there is a type of recognition system operating between legume and bacteria. Some researchers feel that plant lectins may be the mediators of that recognition. Kent and Hamblin from Cambridge and the University of Alabama correlated PHA (phytohemagglutinin, a lectin from red kidney bean) binding to rhizobia and its hemagglutination activity to their observation that young plant root hairs also were capable of binding erythrocytes (19). Bohlool and

Schmidt from the University of Minnesota have shown that soybean lectin carrying a fluorescent tag binds selectively to infective strains of *Rhizobium japonicum* (20). Wolpert and Albersheim from the University of Colorado have demonstrated a specific interaction between the O-antigen containing lipopolysaccharides of Rhizobia and the lectin of their legume host (21). Using an affinity chromatography technique, they were able to show that the lipopolysaccharide from a rhizobium surface interacted with lectin isolated from its normal host plant but not with other non-host lectins. Working with white clover, Dazzo and Hubbel from the University of Florida showed that antibodies produced to antigen on the root surface were cross-reactive with antigen on the surface of *Rhizobium trifoli* and conversely anti-*R. trifoli* was cross-reactive with antigen on the root surface (22). They also extracted a clover lectin capable of binding to root surface and agglutinating only infective *R. trifoli*. On the basis of this data, they proposed a model suggesting the preferential adsorption of infective vs non-infective cells of *R. trifoli* on the surface of clover roots by a cross bridging of their common surface antigens with a multi-valent clover lectin. More recently they have utilized quantitative microscope techniques to examine the adsorption of rhizobial cells to clover root hairs and demonstrate rhizobial selectivity for the natural host (23). They were also able to show that the presence of host lectin greatly increased the binding of infective bacteria.

Chen and Phillips at Indiana State University have developed a reproducible, quantitative technique for studying interactions between labelled lectins and rhizobia that suggests no relationship between lectin-rhizobium interactions and the capacity to infect a plant (24). Although this data does not completely support previously published work on rhizobium-lectin interactions, it doesn't disprove the idea that the rhizobium-host specificity occurs through a recognition mechanism. It merely points out that many bacteria that are non-infective to a particular legume are still capable of binding to the root and also that lectin binding to rhizobia is minimal and may be non-specific. Clearly, more research is required to fully explain the rhizobia-plant host recognition mechanism.

This current study is part of an interdisciplinary research program directed toward improvement of the legume, sainfoin (Onobrychis viciifolia), a forage and pasture crop which is unfortunately a poor nitrogen fixer. A lectin isolated from sainfoin was studied with the objective of determining its possible role in the rhizobia-host symbiosis. Localization of sainfoin lectin in the plant tissue was undertaken to help elucidate such a role. Because of the high degree of sensitivity and specificity of the antigen-antibody reaction, immunofluorescent localization was the technique chosen. If lectin does participate in rhizobia-host recognition, microscopic evaluation of root tissue sections treated with lectin specific immunofluorescent

stain should locate it on the outer surface of root tissue. The lectin's availability to interact with rhizobia, a critical prerequisite, was defined in this study by successful tissue localization.

In a larger sense, it is probable that phytolectins are multifunctional. The immunofluorescent technique was used to examine several tissues from sainfoin in addition to root in order to suggest other functions. Seed, nodule, and hypocotyl were among the other tissues examined and the location of lectin determined. Although localization can never completely define a function, it can serve as a valuable signpost to guide the investigator in further research. In this particular study it has answered certain specific questions as well as suggesting several new and interesting problems for investigation.

RESEARCH OBJECTIVES

The general objective of this study is to localize sainfoin lectin, via immunofluorescent techniques, in the seed, root, and nodule tissue of the plant. Specific objectives are:

- a. Development of appropriate histological methodology.
- b. Development of appropriate immunofluorescent staining procedures and instrumentation.
- c. Localization of the lectin in plant tissue as evidenced by photomicroscopy.

MATERIALS AND METHODS

Isolation and Purification of Sainfoin Lectin Antigen

Extraction. A simple phosphate buffered saline (PBS) extraction was used in the initial isolation process (36). Dehulled, finely ground sainfoin seeds were extracted at 4°C with constant stirring in PBS buffer for four hours. The extraction buffer was 0.01 M in phosphate, 0.15 N in NaCl, 0.01 M in ascorbate and 0.1 M in glucose. The buffer was titrated to a pH of 7.0. Sodium Azide, 0.025% w/v, was added to the buffer as a preservative. Following the initial extraction period, the extract was centrifuged at 10,000 rpm's for 20 minutes. The supernatant solution was collected and brought to 40% saturation with ammonium sulfate. This mixture was stirred for four hours at 4°C and then centrifuged. The supernatant solution was again collected and additional ammonium sulphate was added to bring the solution to 80% saturation. The mixture was stirred for four hours at 4°C and then centrifuged again. After this centrifugation, the precipitate was redissolved in 100 ml of PBS buffer. This buffer was the same as the extraction buffer except that the ascorbate was deleted and the glucose concentration was 0.25 M. The dissolved precipitate was dialyzed in the cold for 24 hours against frequent changes of buffer. The dialysis buffer was the same as the extraction buffer except that it contained no ascorbate or glucose.

Affinity chromatography. Since sainfoin lectin's saccharide specificity includes mannose (36), the lectin was isolated on an affinity chromatography column of mannose covalently attached to sepharose 6B via the divinyl sulphone technique of Porath and Fornstedt (27). A column of this matrix 1.5 cm in diameter and 2.0 to 4.0 cm in height was prepared and equilibrated with PBS buffer which was the same as the dialysis buffer. The dialyzed sample was centrifuged and the supernatant solution was applied to the column. The column was then washed with the PBS buffer used in equilibration until the O.D.₂₈₀ of the wash was 0.02 or below. Elution of the lectin from the column was performed with PBS buffer 0.25 M in glucose. The effluent was monitored spectrophotometrically. Fractions containing protein were pooled and dialyzed against PBS to remove the glucose prior to repurification.

Repurification. The lectin was repurified by repeating the affinity chromatography process. A small column 0.9 cm by 1.5 cm was prepared using virgin mannose-sepharose as the matrix. Fractions containing the protein were pooled and stored frozen in the PBS-glucose elution buffer at -20°.

Root lectin. Lectin from the root was isolated and purified in the same manner as seed lectin with the exception that fresh washed root tissue was homogenized in PBS containing glucose in a Waring

blender. The resulting supernatant was subjected to affinity chromatography as previously described.

Production of Specific Antisera to Sainfoin Lectin

Injection schedule. Antisera was prepared in the rabbit system. Primary immunization was carried out using 13.0 mg of purified seed lectin in 2.0 ml of PBS mixed with 2.0 ml of Freund's adjuvant and injected in 1.0 ml portions near the four axial lymph nodes. At week one the animal was immunized using 4.0 mg of purified lectin and Freund's incomplete adjuvant. At week two the animal was immunized again with 5.0 mg of purified lectin and Freund's incomplete. The animal was bled by cardiac puncture 10 days after the last injection. The blood was allowed to clot and then the serum fraction was separated from the cells by centrifugation. The serum was divided into 1.0 ml aliquots and stored in a Revco deepfreeze at -80°C .

Gel double diffusion. To establish antisera specificity and antigen purity gel double diffusion (29) was run with antisera vs purified lectin and antisera vs crude extract after affinity chromatography. The antigenic identity of seed lectin and root lectin was also compared by gel double diffusion with antisera to seed lectin. The gel double diffusion plates were set up using small petri dishes containing 1% agar. The wells held a sample volume of 10 microliters.

Diffusion was allowed to proceed 24 hours in a moist chamber, then the gels were washed with distilled water and photographed.

Titer. An Ouchterlony gel double diffusion (37) was carried out using serial dilutions of the antisera in the six outer wells of the plate and lectin antigen in the center well. Diffusion was allowed to proceed 24 hours at which time pattern development was complete.

Immuno-electrophoresis of seed and root lectin. Scheidiger's microtechnique for immuno-electrophoresis was used (29). A microscope slide of standard size was used for a gel support and 2.0 ml of buffered agar, pH 8.5 were poured on top giving a gel layer of about 1 mm thickness. Seed and root lectin (1 microliter) were applied to small circular wells 1 mm in diameter that were punched out of the gel. A longitudinal trench 40 mm x 2 mm was cut central and parallel to the long side of the slide at a suitable distance from the circular wells. An E-C Apparatus Corporation electrophoresis cell was filled with barbital buffer, pH 8.5, and the gels were electrophoresed two hours at a potential drop of 6 v/cm in the gel. At the end of this time 0.05 ml antisera was added to the central trough and double diffusion was allowed to proceed for 24 hours at which time pattern development was complete. The gels were stained with Coomassie blue for 15-30 minutes, destained for 5-10 minutes with acid alcohol and soaked for two 24

hour periods in distilled water. The stained gels were then photographed.

Preparation and Characterization of Rhodamine
Anti-rabbit IgG Conjugate

Preparation. Rhodamine-B isothiocyanate (mixed isomers) was purchased from Sigma Chemical Corporation, St. Louis, Missouri. Purified IgG fraction of anti-rabbit IgG produced in the goat was purchased from Miles Laboratories, Incorporated, Elkhart, Indiana. The purified anti-rabbit IgG was dialyzed into the reaction buffer, 0.2 M sodium carbonate, pH 9.6, prior to carrying out the reaction. Rhodamine-B isothiocyanate (10 micrograms per milligram of protein) was dissolved in 0.5 ml of DMSO and was added to the stirring, buffered protein solution in 3 aliquots at hour intervals at room temperature. The volume of DMSO used to solubilize the rhodamine did not exceed 10% v/v final concentration in the reaction mixture. The reaction was allowed to continue for 24 hours at 4°C. To stop the reaction, the conjugate solution was slowly titrated to pH 7.0 and was applied to a sephadex G-50 column 1.8 by 50.0 cm which had been equilibrated with 0.01 M PBS buffer 0.1 M in glucose. Unreacted dye was separated from conjugate on this column by molecular sieve filtration. The conjugate fraction was collected, dialyzed and stored frozen in small aliquots at -80°C.

Characterization. Absorption spectra of a standard solution (10 micrograms per milligram) of rhodamine and also of a sample of the conjugate were obtained on a Varian Techtron UV-Vis spectrophotometer model 635. Using the Lambert-Beer Law, a molar extinction coefficient for rhodamine was calculated from the absorbance maxima and known concentration of the standard solution of the free dye. The standard solution of free dye was prepared using DMSO and PBS as in the reaction procedure. Fluorescence excitation and emission spectra of the conjugate were obtained using a 500 mm Bausch and Lomb grating monochromator, a EMI 9558 QC photomultiplier tube, an Osram XBO 150 W xenon lamp and a Hewlett Packard 7030A x-y recorder. The molar rhodamine to protein ratio was calculated using a modification of the method of Wells et al. (30).

Tissue Preparation

Non-infected tissue. Sainfoin seedlings which had not been exposed to infective rhizobia bacteria were germinated and grown in 1 inch dialysis tubing planted in sterile vermiculite. The seeds were surface sterilized with Chlorox for 15 minutes prior to planting and were placed singly in the tubing at a depth of 1/2 inch below the surface of the vermiculite. One end of the tubing was left exposed approximately 1 inch above the vermiculite and left open to allow growth of the cotyledons. The seedlings were fed sterile Thornton's nitrogen

free liquid media (31), initially on the surface of the vermiculite and then from the bottom of the pot after the appearance of cotyledons. Samples of the root tissue from these seedlings were taken at 96 hours, 10 days, 20 days, and 30 days after planting.

Infected tissue. Samples of root and nodule were taken from 30 day old seedlings that had been grown in sterile vermiculite, watered with nitrogen free water and infected with a mixture of several strains of rhizobia known to infect sainfoin. The seedlings were inoculated 4-5 days after planting.

Seed. Seeds were dehulled and soaked in distilled water for 24 hours prior to sectioning.

Sectioning. Serial cross sections of all tissue examined except the seed were cut at 10 microns on a standard Universal cryostat-microtome (32). Seed sections of 10 microns were cut longitudinally on the same instrument. Tissue was frozen fresh, sectioned and affixed to standard microscope slides without the use of adhesive.

Fixation. All tissue sections were fixed 15 minutes in 95% ethanol after cutting and prior to staining.

Photomicroscopy

A Leitz Ortholux microscope equipped with a Ploempak vertical illuminator was used to evaluate and photograph the immunofluorescent stains. An HB 200 mercury vapor lamp was the energy source. All tissue sections were observed with transmitted darkfield illumination. The condenser was adjusted for maximum illumination of field. Spectra of various primary and secondary filters were compared. The filters chosen were a 546 wideband interference primary filter and a K580 secondary filter. Photographs were taken with Kodak High Speed Ektachrome film. The exposure time varied from 1 to 2 minutes depending upon the intensity of the stained sections. Exposure time for the controls was always the same as that used for the corresponding stained section.

Staining Procedures

Hematoxylin-eosin. Delafield's hematoxylin was used and the standard procedure for routine H&E histological staining was followed (33). Histological stains for each type of tissue examined were prepared. The tissue sections were stained in the filtered hematoxylin for 6 minutes, washed briefly in tap water, dipped in acid alcohol and washed in running tap water for 15 minutes. They were then placed in 70% ethanol for 5 minutes prior to counterstaining for 30 seconds with eosin. The tissue was then passed briefly through baths of 95%

ethanol, absolute ethanol and 3 successive baths of xylene. The sections were coverslipped with Permount mounting media immediately after the final xylene wash.

Immunofluorescent. A dilution series of both antisera and rhodamine conjugate was used in the initial staining to establish optimal concentrations. Dilutions of 1:100 v/v for the antisera and 1:50 v/v for the conjugate were found to offer the best staining and were then used routinely. Both antisera and conjugate were diluted with PBS, pH 7.0, 0.1 M in glucose and 0.3% in Triton X-100 (34). Staining was carried out in a moist chamber. The diluted antisera was applied directly to the tissue sections; approximately two drops per section. The sections were stained with antisera for one hour and then were washed five minutes in a solution of PBS, pH 7.0, 2% in Triton X-100. After washing, the sections were allowed to drain and then the conjugate was applied in the same manner as the antisera and allowed to stain for one hour. The sections were then washed again for five minutes in PBS, pH 7.0, 2% in Triton X-100. Following this wash they were rinsed in distilled water, allowed to drain, mounted and coverslipped. The mounting media was glycerol diluted 1:1 with PBS, pH 6.0 (35).

Controls

General staining. Sainfoin lectin was covalently attached to sepharose 6B by the cyanogen bromide activation procedure of Cuatrecasas (44). A micro-chromatography column, 0.5 cm by 1.0 cm, was poured using that matrix. Two similar columns were also prepared; one of methylated chymotrypsin covalently attached to sepharose 6B and one of sepharose 6B without a protein ligand attached. All three columns were equilibrated with PBS, pH 7.0. One ml of the antisera, diluted as in the immunofluorescent staining procedure, was applied to each column. After the sample had been absorbed, the columns were washed with several column volumes of PBS. One ml of conjugate, also diluted as in the staining procedure, was then applied to each column and allowed to penetrate the matrix. Again, the columns were washed with PBS. After extensive washing a sample of beads was taken from the top of each column, mounted in mounting media on microscope slides, and observed microscopically for fluorescence.

Tissue staining. Autofluorescence controls were run for each tissue examined by substituting PBS for antisera and conjugate in the staining process. Non-specific binding controls were run for each tissue examined by substituting normal rabbit serum (NRS) for specific antisera in the staining procedure. The control sections were

microscopically evaluated and photographed under identical conditions as the sections treated with the complete stain.

RESULTS AND DISCUSSION

Antigen-Antibody Characterization

Antigen preparation. Pure Sainfoin lectin was prepared by affinity chromatography using sepharose columns containing covalently linked mannose. Figure 1 shows that treatment of the column with glucose resulted in displacement of the lectin which emerged from the column as a single sharp peak. The yield of repurified lectin from 200 g of ground seeds was typically near 50 mg. Time required for the entire lectin preparation was 2-3 days. Unpublished characterization studies (36) have shown the lectin to be free of contaminating proteins. It is a glycoprotein containing 6 per cent carbohydrate and the molecular weight as established by gel filtration is 57,000 daltons. The molecular weight measured by sodium dodecyl sulfate polyacrylamide electrophoresis is 28,000. This antigen was used in the rabbit immunization program for elicitation of specific antibody. The purity of antigen and specificity of antibody obtained was demonstrated as described below.

Ouchterlony double diffusion. Figure 2 gives the result of a double diffusion experiment employing antisera in the center well. The outer wells contained samples of the crude extract before and after affinity chromatography and a sample of purified lectin. The purified lectin and the initial sainfoin extract formed a confluent precipitin

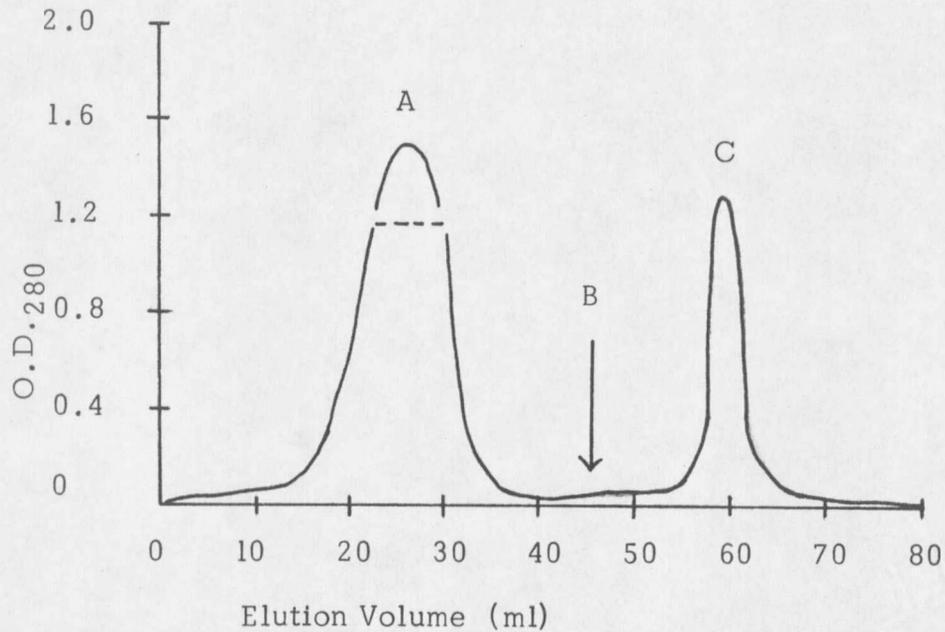


Figure 1. Elution of sainfoin lectin from mannose-sepharose affinity chromatography column, 1.5 cm by 4.0 cm. A - peak resulting from application of crude protein extract, B - application of elution buffer, PBS - Glu (0.5 M), C - peak resulting from elution of pure sainfoin lectin antigen.

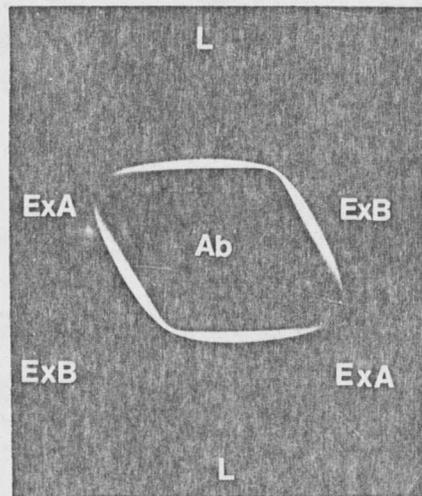


Figure 2. Gel double diffusion of crude extract before and after affinity chromatography and purified lectin vs specific antisera. ExA - extract after column, ExB - extract before column, L - purified lectin, Ab - specific antisera.

band. The extract after treatment with mannose sepharose showed no precipitin band. These results indicated that the antigen had been completely removed from the extract by affinity chromatography and further that there was no cross-reactivity between the antisera and other components of the extract. Lectin isolated from the root was shown to be immunologically indistinguishable from seed lectin as shown in Figure 3. A confluent, precipitin band formed between the seed and root lectin.

Immuno-electrophoresis. Results of immuno-electrophoresis of Sainfoin seed lectin and root lectin are shown in Figure 4. Single, identical precipitin arcs between antisera and antigen indicated that the antigens were homogeneous and immunologically indistinguishable. These data indicated that the antigen-antibody system was pure and monospecific. Additionally, lectin isolated from seed tissue and root tissue were shown to be identical. Antisera produced toward seed antigen could therefore be used for immunofluorescent localization of lectin in root tissue.

Antisera titer. The dilution of antisera still capable of giving a visible precipitin band when diffused against 1mg/ml antigen was 1:32.

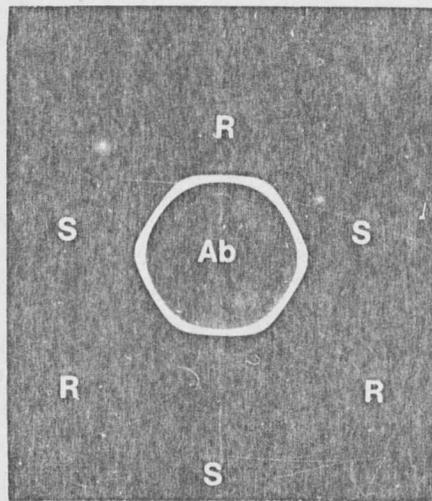


Figure 3. Immunological identity of sainfoin root and seed lectin as evidenced by gel double diffusion. S - seed lectin, R - root lectin, Ab - specific antisera.

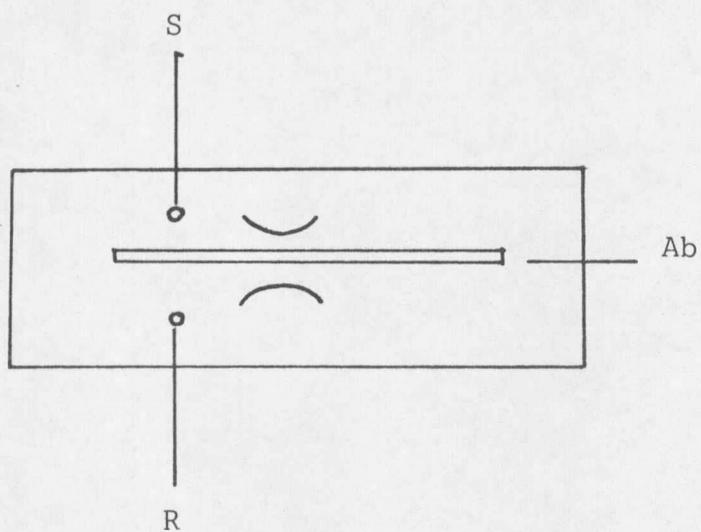


Figure 4. Immunoelectrophoresis of sainfoin seed and root lectin.
S - seed lectin, R - root lectin, Ab - specific antisera.

Preparation and Characterization of the Fluorescent
Antirabbit IgG Conjugate

Unsuitability of fluorescein conjugate. Rhodamine was the fluorophore chosen in this study because of tissue autofluorescence problems encountered with the fluorescein staining system. Figure 5 is a photoplate of stained sections and tissue controls evaluated with the fluorescein system. As is illustrated in this figure, blue light excitation, via a fluorescein BG 12 primary filter, produced tissue autofluorescence of the same color as the emitted light of the fluorescein dye. Use of a 495X interference filter in the primary position with a secondary K530 did not significantly alter the autofluorescence problem. It was found that autofluorescence of all the tissue examined in this study was negligible with green light excitation, wavelength 546, which is commonly used with the fluorophore rhodamine. Consequently, a rhodamine conjugate was prepared and used throughout the immunofluorescent staining assays.

Conjugation reaction. Figure 6 is a diagrammatic representation of the reaction used to prepare the rhodamine conjugate. The reaction is a simple nucleophilic displacement occurring between the isothiocyanate portion of the dye and the nucleophilic centers on the protein, i.e. amino groups. Rhodamine B isothiocyanate was found to be nearly insoluble in the pH 9.6 carbonate reaction buffer, therefore it was

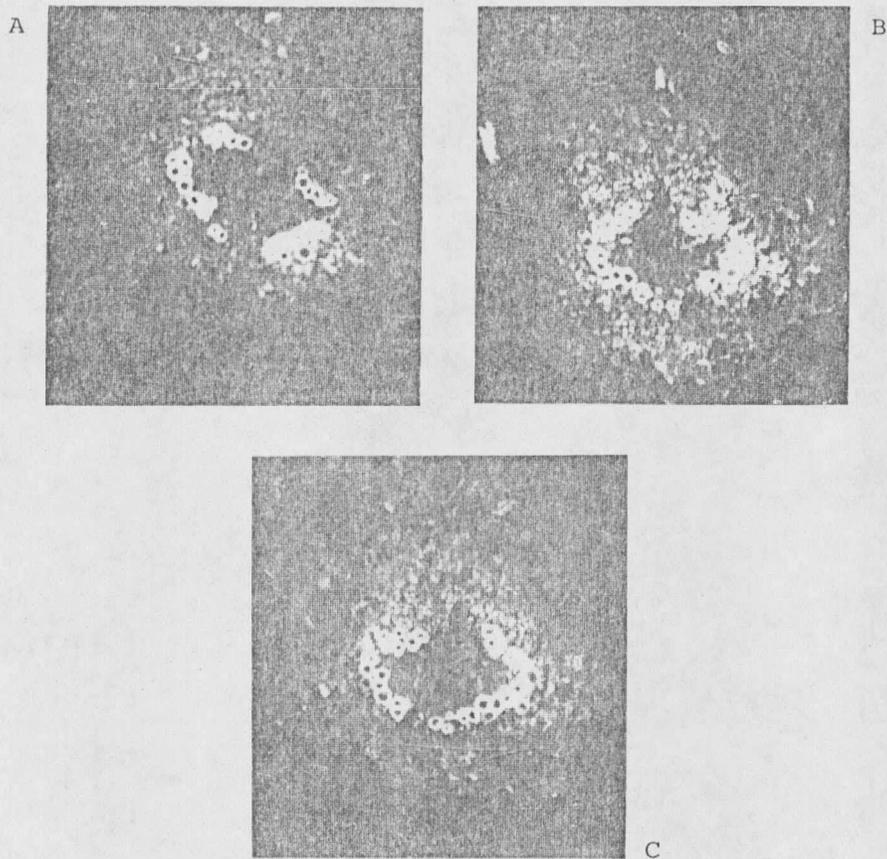
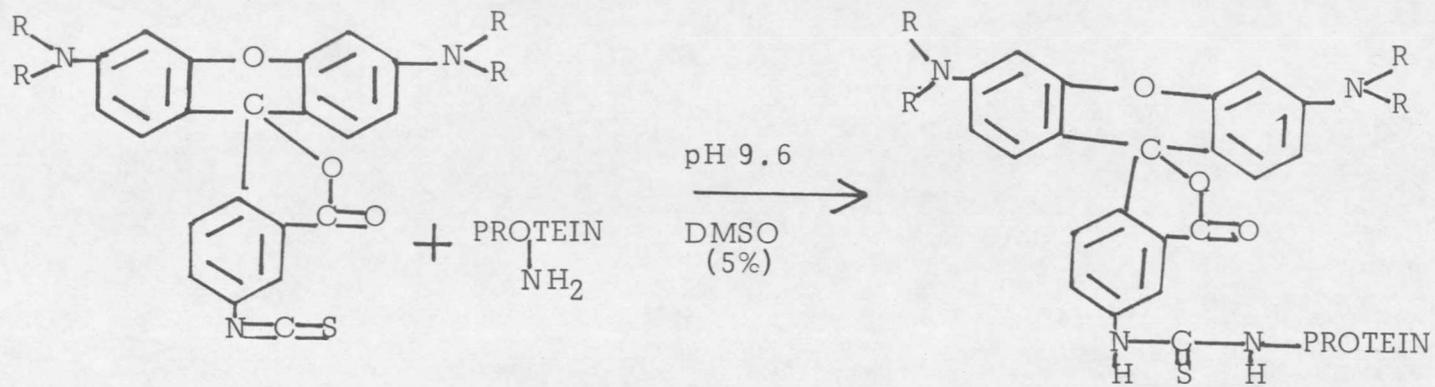


Figure 5. Autofluorescence of sainfoin root tissue 10 micron cross sections encountered with fluorescein isothiocyanate staining system. A - autofluorescence control, B - NRS control, C - fluorescein immunofluorescent stain, (100X).



RHODAMINE

CONJUGATE

Figure 6. Preparation for rhodamine-anti-rabbit IgG conjugate.

dissolved in a small amount of dimethylsulfoxide (DMSO) prior to reacting it with the buffered protein solution. An excess of dye was added in order to maximize labelling. Some precipitation did occur during the reaction, presumably due to protein denaturation, and this was removed by centrifugation following completion of the reaction.

Gel filtration. Unreacted dye was separated from the protein conjugate by molecular sieve gel filtration on a column of Sephadex G-50. The conjugated protein was delivered early in the elution process, within the first 15.0 ml of eluant. Further elution with large amounts of buffer removed the slower band of unreacted dye. If the conjugate prepared shows an undesirable amount of non-specific staining, it may be attributed to the presence of over-labelled globulin molecules. Those globulins carrying fewer rhodamine molecules retain the charge characteristics of native globulin and stain more specifically so it may be necessary to further purify the conjugate by chromatography on the anion exchanger DEAE cellulose (39).

Absorption spectra of rhodamine and rhodamine conjugate. The absorption spectrum of the standard solution of free rhodamine is illustrated in Figure 7. There is a large absorption peak at 560 nm, a smaller one at 350 nm and 260 nm, and a very large, sharp peak at 230 nm. The spectrum is typical of rhodamine dyes of mixed isomer form (39). Using this spectrum and the Lambert-Beer Law, a molar extinction

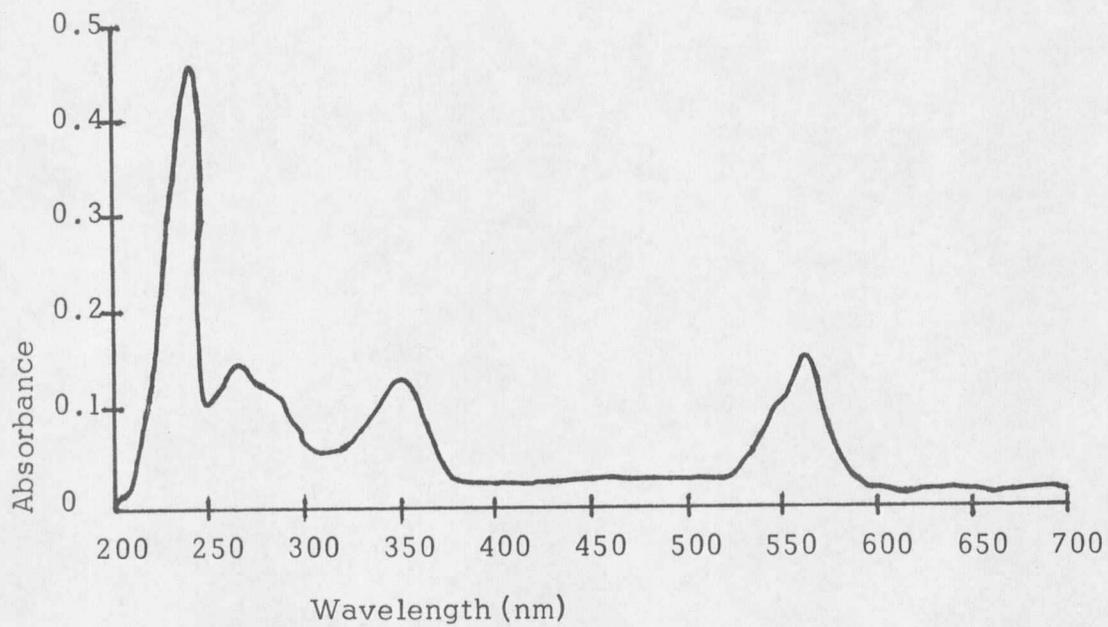


Figure 7. Absorption spectrum of rhodamine B isothiocyanate dye.

coefficient for this dye was calculated to be $23,043 \text{ M}^{-1} \text{ cm}^{-1}$ at 560 nm.

The absorption spectrum of the rhodamine conjugate is illustrated in Figure 8. There are major absorption bands at 560 nm, 350 nm, and 280 nm. The spectrum of the conjugate is very similar to the spectrum of the free dye with the exception of the large protein peak at 280 nm attributable to the antibody.

Coupling ratio of rhodamine conjugate. The coupling ratio for the rhodamine conjugate used throughout this study was 3.0, as determined spectrophotometrically. The concentration of rhodamine was calculated directly from the O.D. of the conjugate at 560 nm and the previously determined molar extinction coefficient. The protein concentration was calculated using a correction factor for rhodamine contribution at 280 nm (39). The molar fluorophore to protein ratio of fluorescent conjugates is extremely important in situations where non-specific staining may be a problem. An optimal ratio for rhodamine has not been conclusively established but it is generally accepted that the ratio should be near the fluorescein optimum of 2.0-5.0.

Excitation and emission spectra of rhodamine conjugate. The excitation and emission spectra for the conjugate are given in Figure 9A. Excitation maximum for the conjugate is at 560 nm. Maximum

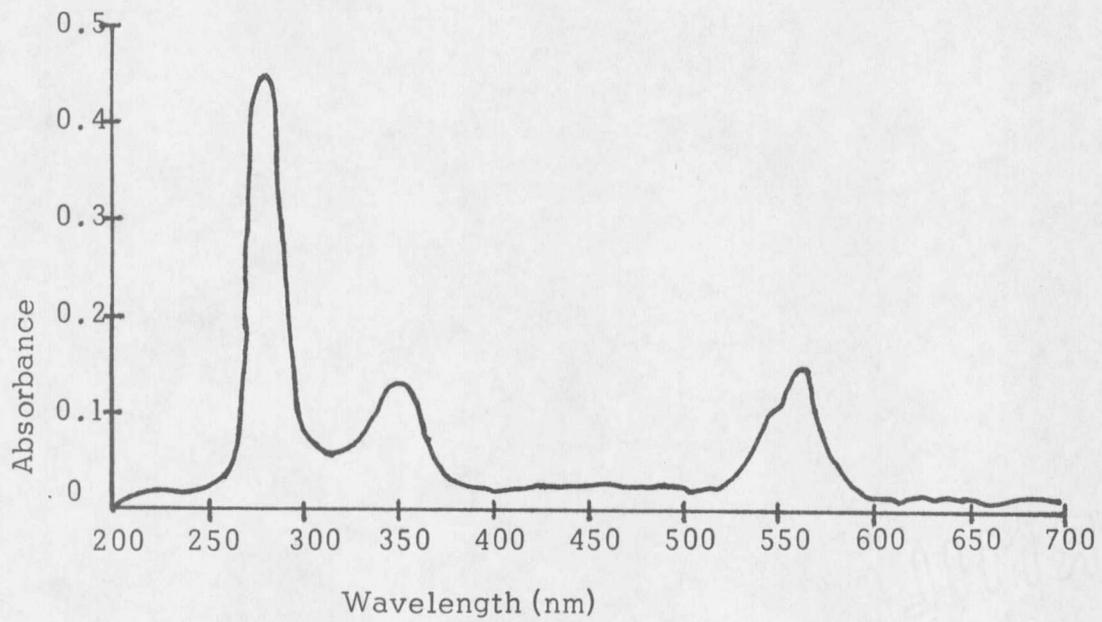


Figure 8. Absorption spectrum of rhodamine-anti-rabbit IgG conjugate.

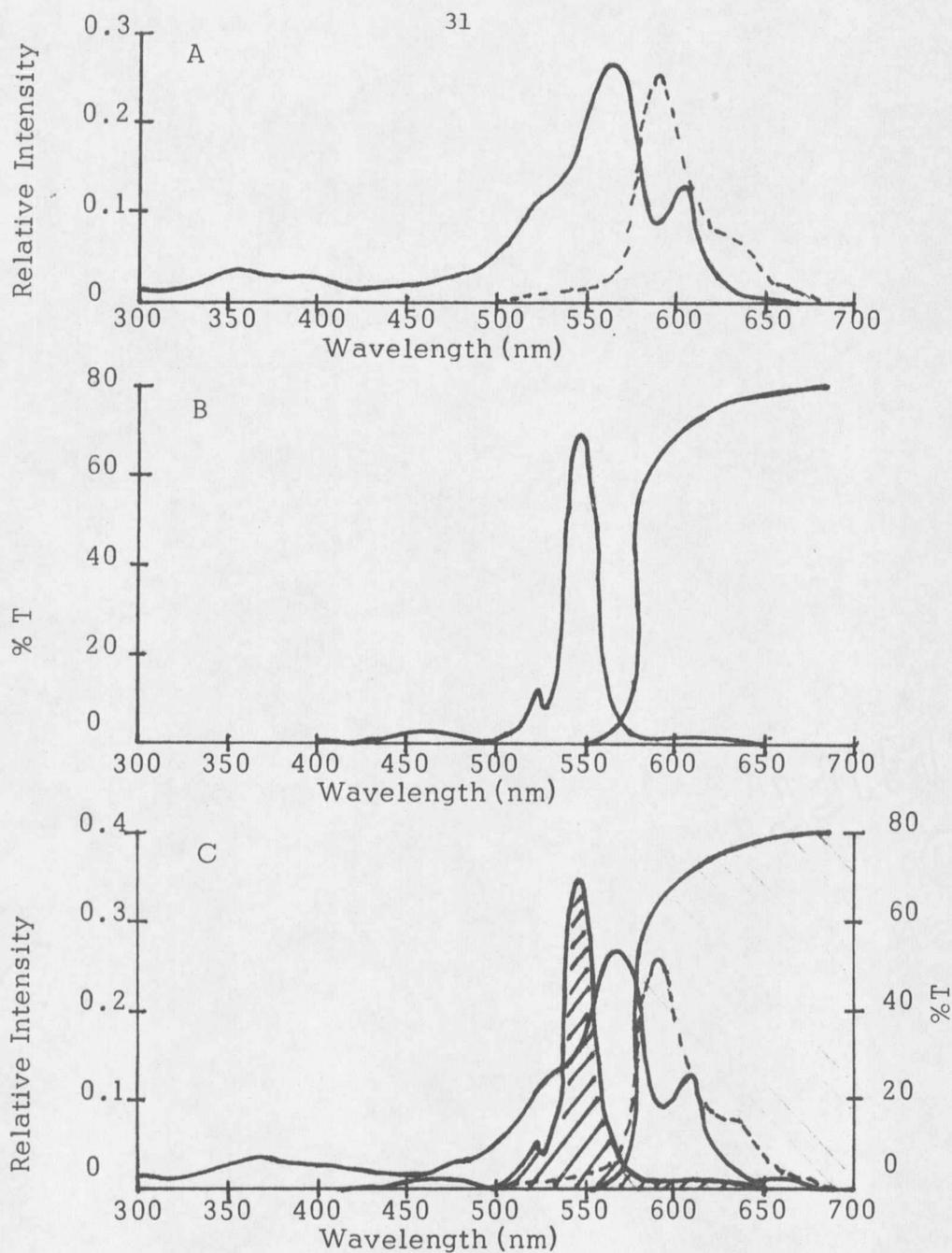


Figure 9. A. Excitation and emission spectra of rhodamine conjugate.
 B. Transmission spectra of 546 nm primary filter and K580 secondary filter.
 C. Combined graph of A & B.

fluorescence emission for the conjugate is at 590 nm. These spectra are characteristic of rhodamine B isothiocyanate conjugates (41).

Filter System for Rhodamine Fluorescence Microscopy

A filter system to optimize the fluorescence spectral characteristics of the rhodamine conjugate was established via a 546 nm interference filter in the primary position and a K580 cut-on filter in the secondary position. Transmission spectra of these two filters are illustrated in Figure 9B. Figure 9C is a combined graph of the excitation and emission spectra of the conjugate and the transmission spectra of the filter system, giving a complete picture of the fluorescence system used in this study. Primary or excitation filters are placed between the light source and the object and are selected to pass those wavelengths that produce fluorescence in the specimen. Interference primary filters are most efficient in that they allow isolation of narrow band spectra. The secondary or cut-on filters are placed between the specimen and the observer and are selected to remove excitation radiation not absorbed by the specimen but transmit fluorescent light being emitted by the specimen.

Tissue Preparation

Germination. Sainfoin seedlings were germinated in dialysis tubing in sterile vermiculite to help insure sterility of the seedlings

that were not to be infected with rhizobia and to minimize loss of lectin that may be present on the surface of sainfoin root tissue.

During initial preparation, seedlings were germinated in Thornton's nitrogen free media. After three weeks the media that the seedlings were grown in was examined for the presence of lectin. This was done immunologically by gel double diffusion. Lectin was found to be present and immunologically identical to that isolated from seed and root tissue. It was not clear if lectin had been exuded by the root or if it was extracted from the surface, however, germination in dialysis tubing was chosen for subsequent work.

Seedlings infected with rhizobia bacteria were germinated and grown in greenhouse pots containing sterile vermiculite. A drawback to this approach was that the root tissue had to be washed briefly in distilled water to remove adhering pieces of vermiculite that might damage the microtome blade during sectioning. It is possible that some loss of surface lectin may have occurred during washing.

Seed preparation. Dehulled sainfoin seeds were soaked in distilled water for 24 hours prior to cutting. Soaking was necessary to obtain adequate frozen sections at 10 microns thickness. Attempts to section unsoaked seed tissue were not successful due to the dry, brittle nature of the tissue. Soaking the seed tissue also made possible

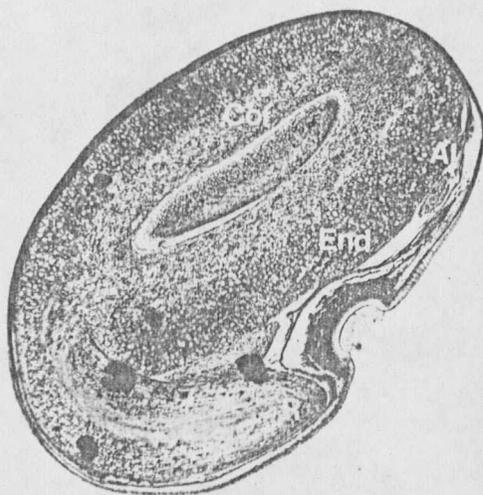
the microscopic examination of the initiating radicle prior to its actual emergence through the seed coat.

Frozen sectioning. Using standard procedures for operating the cryostat microtome, it was possible to cut sections of all the tissue examined at 10 microns. In general, frozen sections are not as anatomically distinct or as artifact free as paraffin embedded sections. However, this technique does offer several very important advantages. With frozen sectioning there is no risk of denaturing tissue antigen or introducing autofluorescence with organic solvents and heat as in the paraffin process. Also, frozen sectioning is a much quicker and more convenient process since embedding is completely bypassed and the tissue can be fixed and stained immediately without first clearing and rehydrating. Frozen sectioning is the most efficient and acceptable method of preparing tissue for immunofluorescent staining.

Tissue Staining

Hematoxylin-Eosin. Photomicrographs of Hematoxylin-Eosin (H&E) stains of the seed and nodule are given in Figure 10. Figure 11 is a series of H&E stains of root tissue with a diagram illustrating the area of the root from which each section was taken. Histological stain on each type of tissue examined was done in order to establish a reference for interpretation of the immunofluorescent stains. H&E

A



B

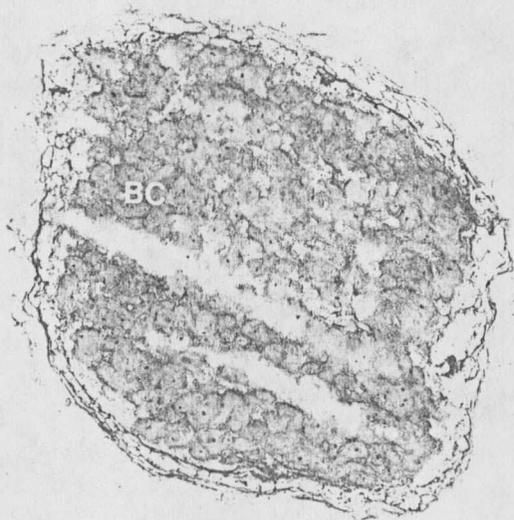


Figure 10. A. Histological stain of sainfoin seed, 10 micron longitudinal section (14X). Cot - cotyledons, End - endosperm, Al - aleuron layer, R - root radicle. B. Histological stain of nodule, 10 micron cross section (45X). BC - central core containing bacteroids, C - outer layers of cortical cells.

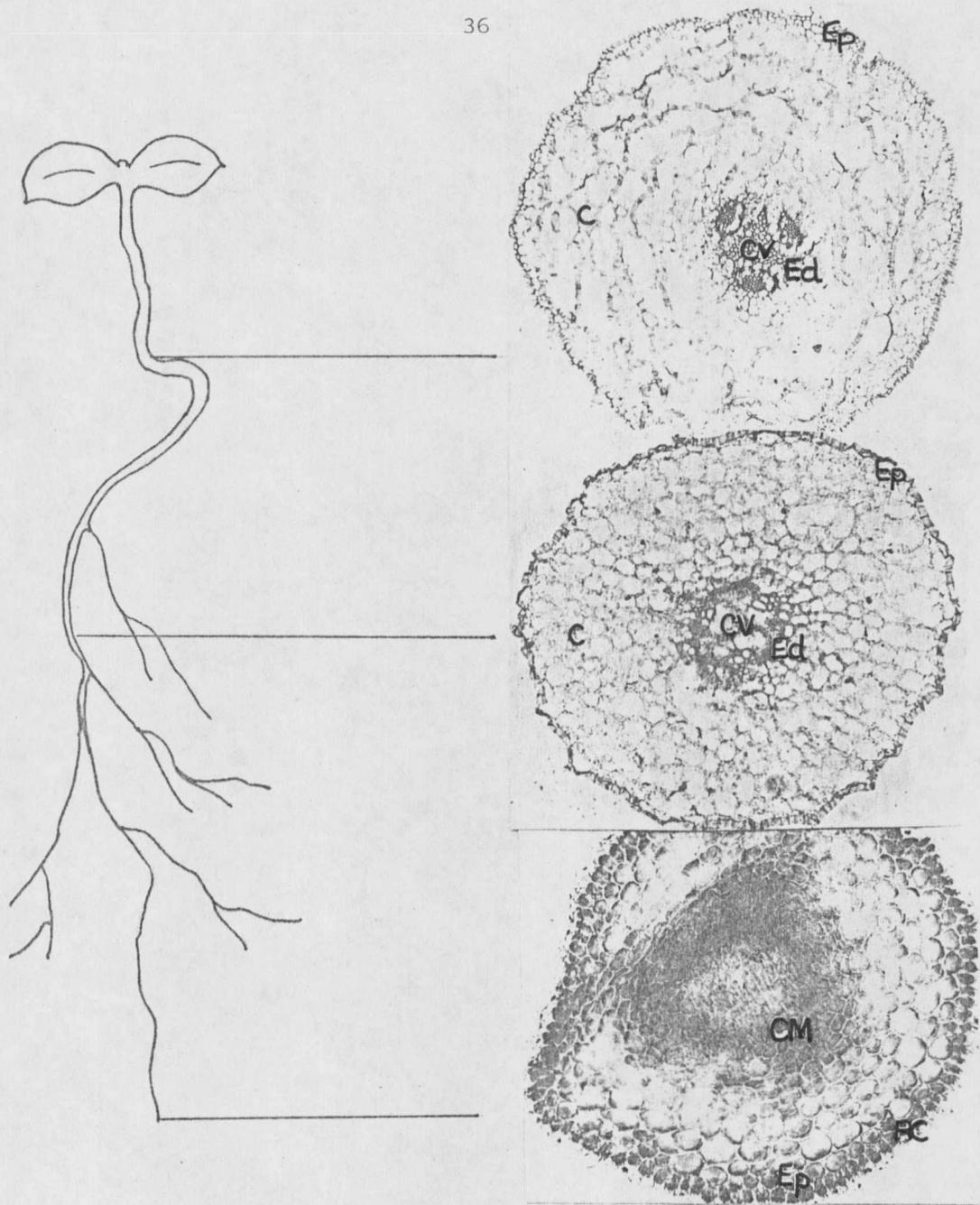


Figure 11. Histological stains of sainfoin root and hypocotyl tissue. Median root (90X), root tip (175X), hypocotyl (50X). Ep - outer epidermis, Ed - endoderm, CM - central meristem, CV - central vascular bundle, C - cortex, RC - root cap cell.

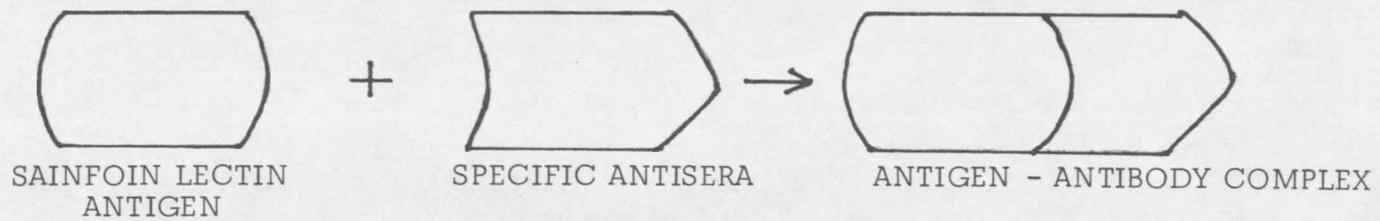
stain was found to provide adequate differentiation of anatomical structures in the plant tissue examined.

Immunofluorescent. Figure 12 is a diagrammatic representation of the "indirect" immunofluorescent staining technique used in this study. This technique was used to identify a known antigen in tissue, in this case sainfoin lectin, by using whole serum containing unlabelled specific antibody as the primary reagent. This specific antibody reacts with lectin antigen in the tissue to form an antigen-antibody complex. In the next step the tissue is washed and exposed to a different antibody which was labelled with rhodamine and was prepared specifically against the type of antibody used as the primary reagent. Thus, the third, labelled component is added and the complex is rendered fluorescent.

Figure 13 shows a series of photomicrographs of sepharose bead controls that were used to evaluate the "indirect" staining process. After being subjected to the complete staining process the sepharose beads with sainfoin lectin covalently attached exhibited the bright orange fluorescence characteristic of rhodamine, when observed microscopically. The sepharose beads with a non-antigenic protein attached showed a very dim non-distinct gold fluorescence indicating a small amount of non-specific staining. Sepharose beads without a protein ligand attached showed no detectable fluorescence. This type of model

INDIRECT IMMUNOFLUORESCENT STAINING METHOD

STEP ONE:



STEP TWO:

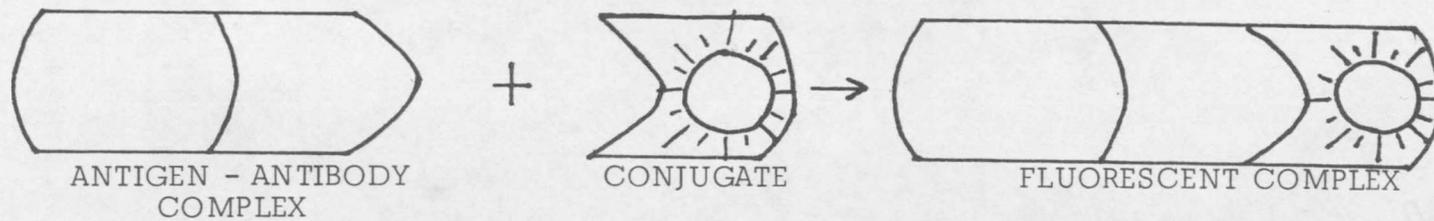


Figure 12.

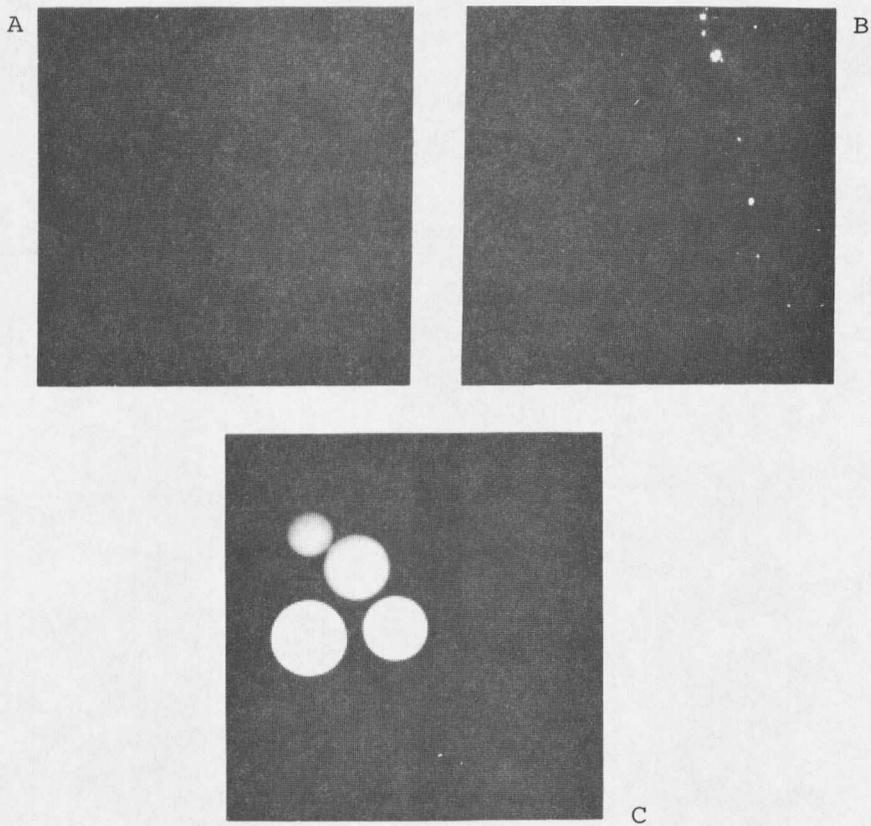


Figure 13. Sepharose bead model system of indirect immunofluorescent staining using rhodamine conjugate. A - sepharose beads with no protein ligand, B - sepharose beads with non-antigenic protein ligand, C - sepharose beads with lectin covalently attached, (100X).

system was used as an indication of staining efficiency and was not intended as a substitute for autofluorescence and NRS tissue staining controls.

Sainfoin seed. Photomicrographs of immunofluorescent stains of 10 micron longitudinal sections of sainfoin seed are shown in Figure 14. A comparative histological stain is given in Figure 10. The primary areas of fluorescent staining in the seed are the cell wall areas, the protein matrix enclosing the starch granules in the endosperm and cytoplasm of the cells of the endosperm. The cell wall area of the aleuron layer stained more heavily than the cell wall area of the endosperm. Most of the staining in the endosperm appeared to be associated with the matrix enclosing the starch grains. Also in the endosperm there appeared to be a slight amount of ground, or cytoplasmic staining which may have been due to stored protein. The autofluorescence control showed only slight gold fluorescence of the starch grains. The NRS control showed gold fluorescence of the starch as well as some very dim orange fluorescence associated with the cell wall area of the aleuron layer. However, the non-specific staining did not approach the intensity of the sections stained with specific antisera. It appeared that lectin might be stored in the seed and also serve a structural role in the cell wall areas and protein matrix surrounding

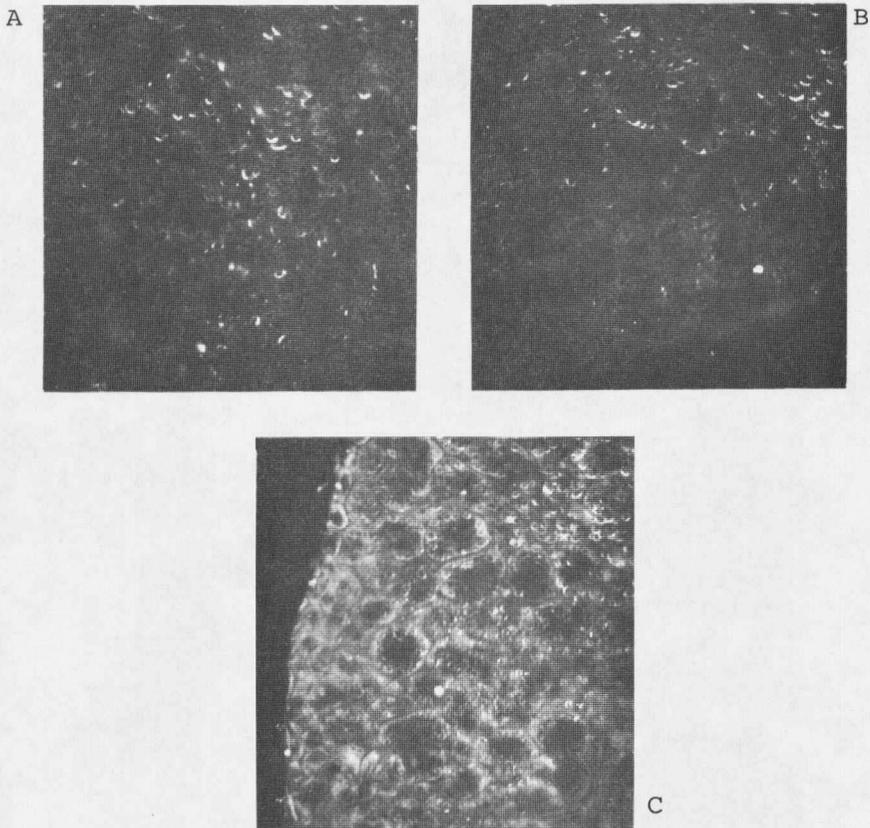


Figure 14. Immunofluorescent stain of sainfoin seed tissue 10 micron longitudinal sections. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

the starch granules. This latter suggestion seems most probable in view of the carbohydrate binding properties of lectin.

Sainfoin root nodule. Figure 15 is a photoplate of immunofluorescent stains of 10 micron cross sections of sainfoin root nodule. A comparative histological stain is given in Figure 10. The central, rhizobium infected zone formed the greatest portion of the nodule tissue. This area was composed of enlarged cortical parenchyma type cells filled with rhizobia bacteroids. The thin walls of these cells showed some slight specific staining. The bacteroids themselves did not stain; however, this was not easily discernible since a slime layer surrounds the bacteroids and stained non-specifically as evidenced by the NRS tissue control. Approximately 5 layers of loosely packed parenchyma cells surrounded the central infected core. Some of these cells showed wall thickening and appeared scleroid in nature. In general, the cell wall areas of the cells in this area stained very brightly and specifically indicating the presence of lectin in or near the wall. Only these outermost layers of cells showed heavy specific staining in the nodule tissue.

Non-infected sainfoin root tissue. Non-infected root tissue of varying ages exhibited the most interesting fluorescent staining patterns of any tissue examined. Figures 16, 17, 18, 19, 21, and 22 are a series of photomicrographs of immunofluorescent stains of 10, 20,

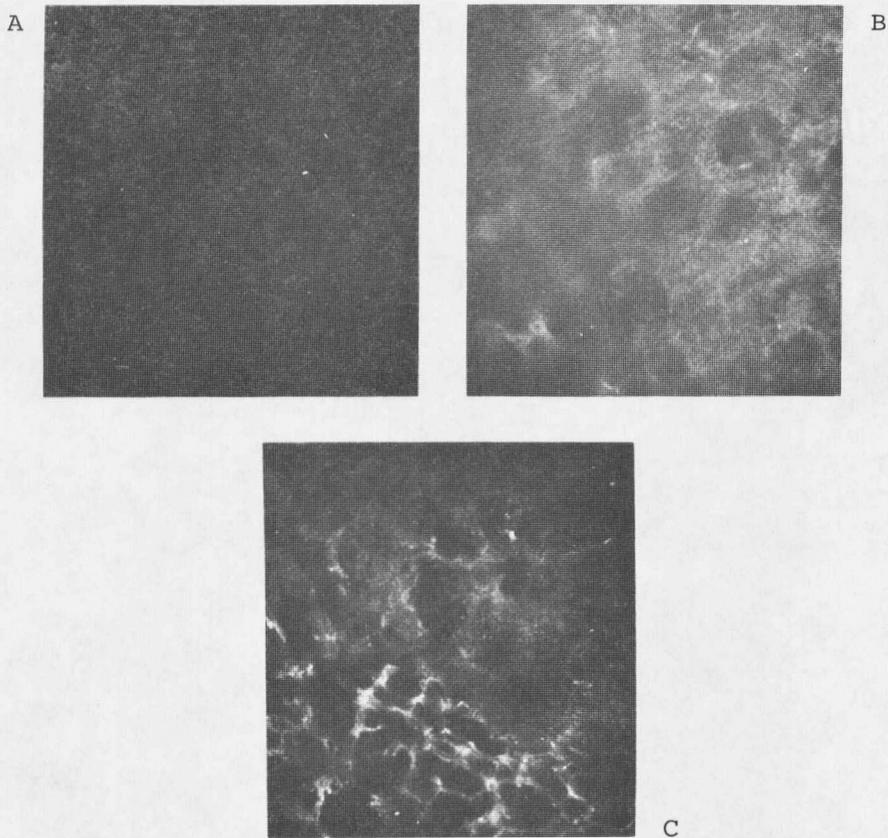


Figure 15. Immunofluorescent stain of sainfoin nodule tissue 10 micron cross section. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

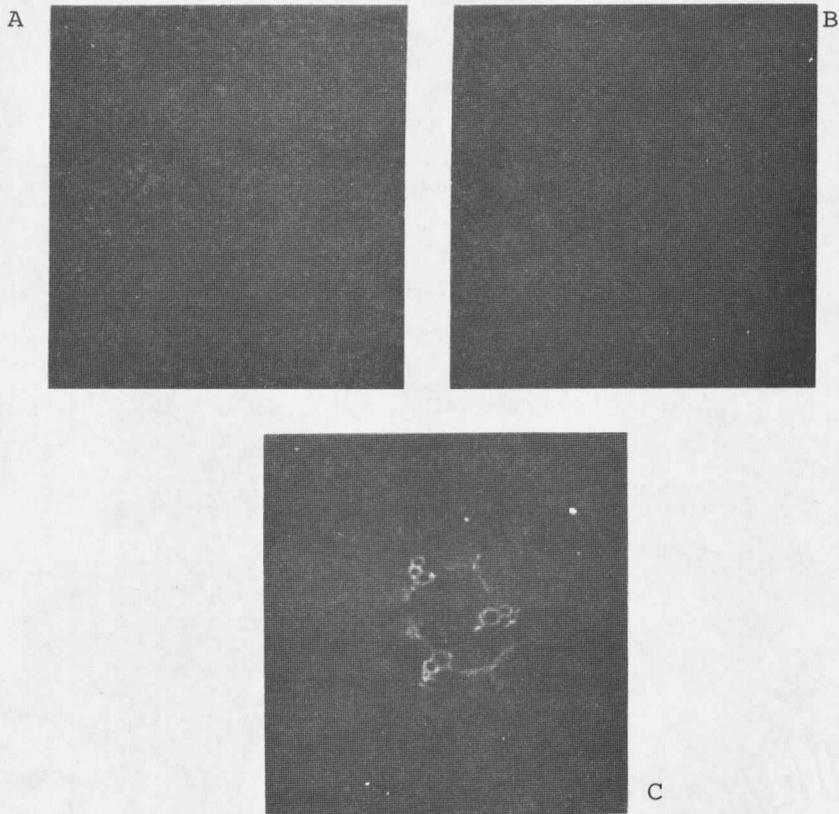


Figure 16. Immunofluorescent stain of sainfoin root tip 10 micron cross section, age 10 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

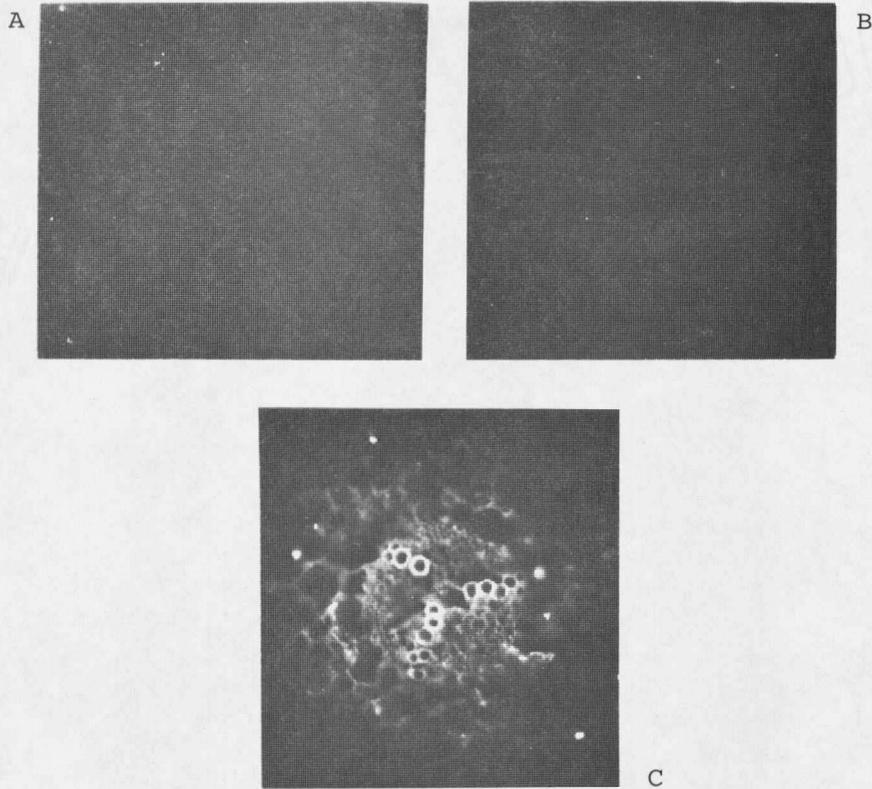


Figure 17. Immunofluorescent stain of sainfoin median root 10 micron cross section, age 10 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

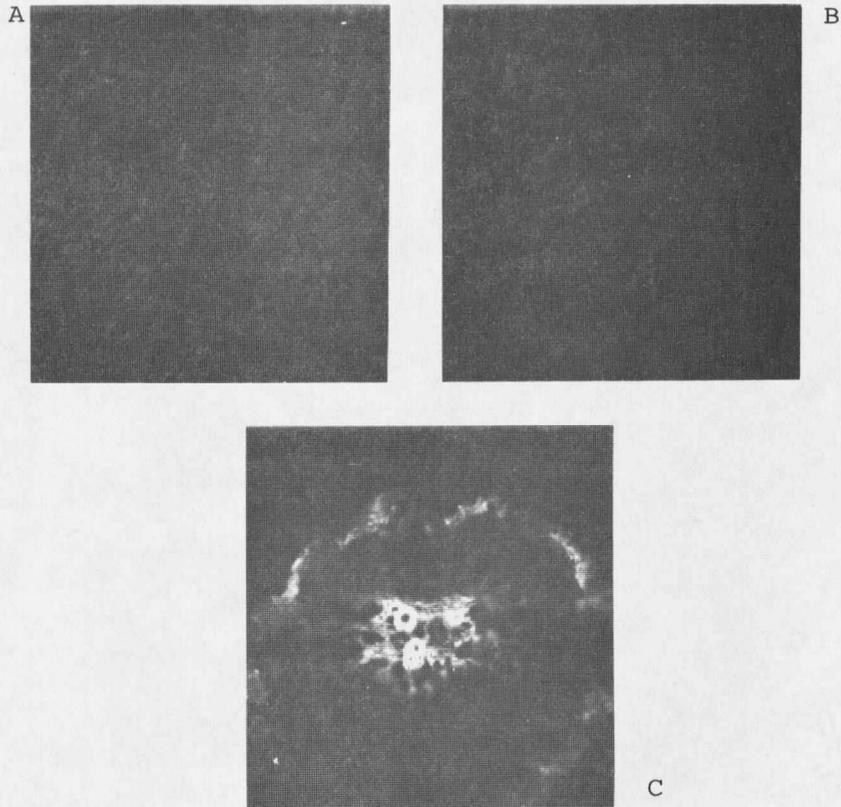


Figure 18. Immunofluorescent stain of sainfoin root tip 10 micron cross section, age 20 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

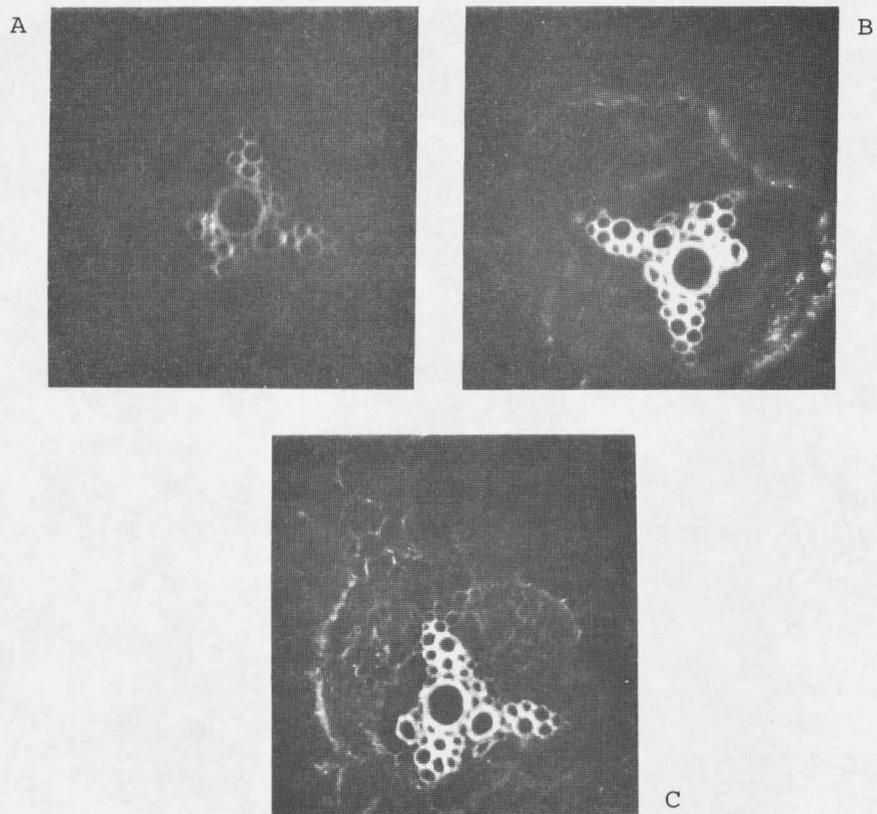


Figure 19. Immunofluorescent stain of sainfoin median root 10 micron cross section, age 20 days. A - autofluorescence control, B - NRS control, C rhodamine stain, (108X).

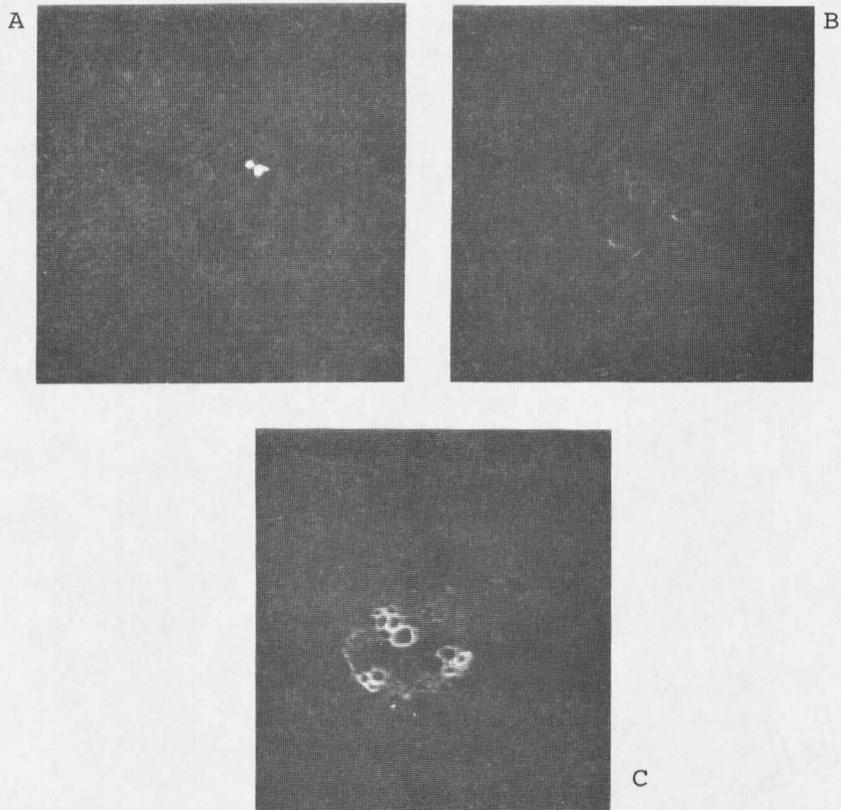


Figure 20. Immunofluorescent stain of sainfoin root tip 10 micron cross section, age 30 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

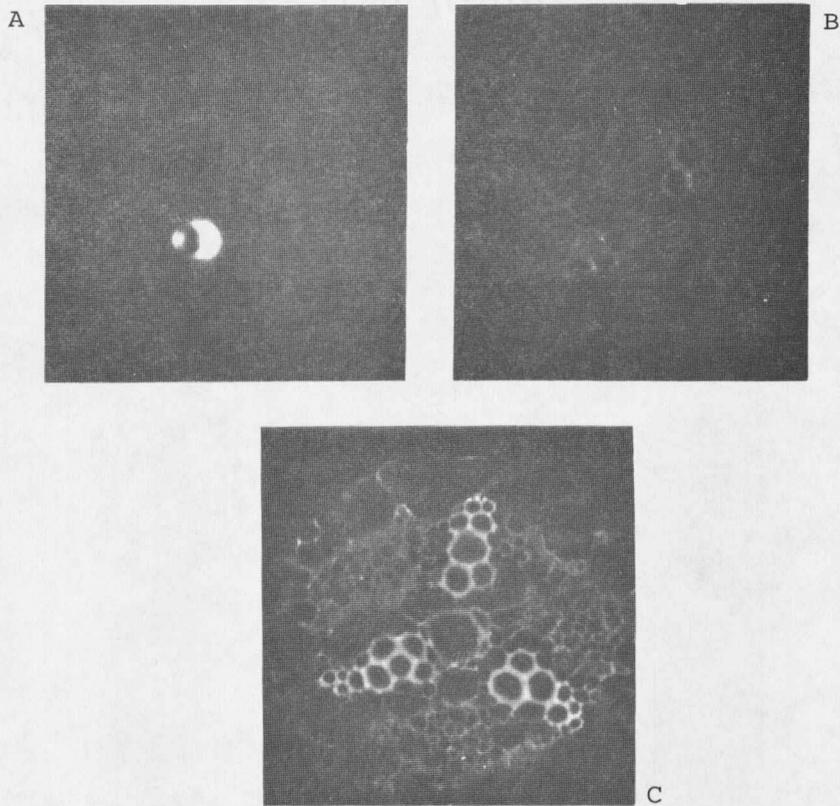


Figure 21. Immunofluorescent stain of sainfoin median root 10 micron cross section (central area of section), age 30 days.
A - autofluorescence control, B - NRS control,
C - rhodamine stain, (108X).

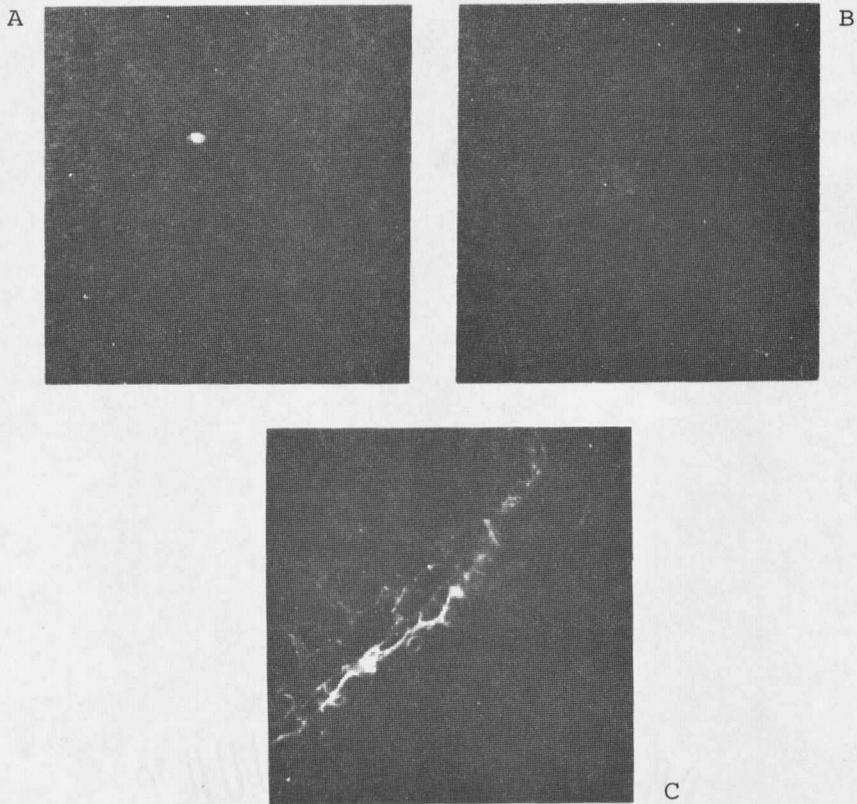


Figure 22. Immunofluorescent stain of sainfoin median root 10 micron cross section (outer wall area of section), age 30 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

and 30 day old root tissue. A distinct staining differential existed between actively growing differentiating portions of the root tissue and those areas such as the cortex which were not actively growing and were unspecialized. In the older tissue (10 day, 20 day, and 30 day), the vascular elements found within the endosperm stained very brightly. In general, this fluorescence was confined to the cell wall area. The wall areas of the cells of the endoderm were also highly fluorescent and the cell wall areas of the mature xylem were brightly stained. In contrast, the large parenchyma cells of the cortex stained very little or not at all. The small amount of staining that was present in some cortical cells was confined to the cell wall area. Tissue sections taken from the primary root halfway between the root tip and hypocotyl were termed medial root and exhibited the same general staining patterns as tissue sections of the root tip taken approximately 2 mm above the quiescent center. The only obvious difference was the extent of maturation of the vascular bundle. There were no significant differences in staining patterns between 10 day, 20 day, and 30 day old root tissue.

A very dramatic staining differential was found to exist between 96 hour and 10 day old tissue. Median root sections from 96 hour tissue showed heavy staining in or near the walls of cells throughout the entire tissue section as illustrated in Figure 23. Wall areas of the cortical parenchyma cells stained as brightly as wall areas of the

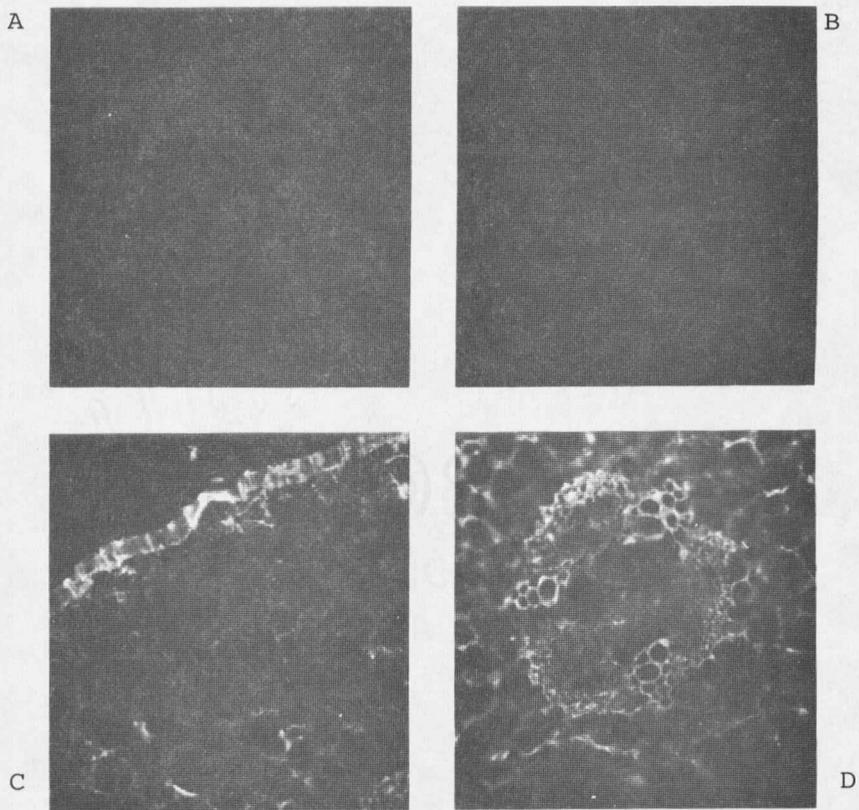


Figure 23. Immunofluorescent stain of sainfoin median root 10 micron cross section, age 96 hours. A - autofluorescence control, B - NRS control, C - rhodamine stain of outer wall area, D - rhodamine stain of central area, (108X).

endosperm pericycle and phloem and xylem elements. There may have been some cytoplasmic staining contributing to this pattern since the cytoplasm of these cortical cells is confined to a narrow strip just inside the cell membrane and the center of the cell is, in fact, a large vacuole. Sections of 96 hour root tip, as illustrated in Figures 24 and 25, also showed a very interesting staining pattern distinctly different from that observed in older tissue. Not only were all cell wall areas of the tissue section stained, but also there was definite staining of the cytoplasm throughout the section. Ground or cytoplasmic staining was readily apparent in this meristematic tissue of young root tip since the cells are engorged with large amounts of metabolically active cytoplasm.

There was one staining characteristic that was common to all the root tissue examined regardless of age. Every section exhibited the same bright specific staining associated with the wall area of the outer epidermis.

Infected sainfoin root tissue. All immunofluorescent stains of infected sainfoin root tissue exhibited the same staining pattern as those of non-infected root.

Hypocotyl tissue. Photomicrographs of immunofluorescent stains of sainfoin hypocotyl are given in Figures 26 and 27. Lectin in sainfoin hypocotyl is found in or near the cell walls and in fact shows a staining pattern virtually identical to that of mature root tissue.

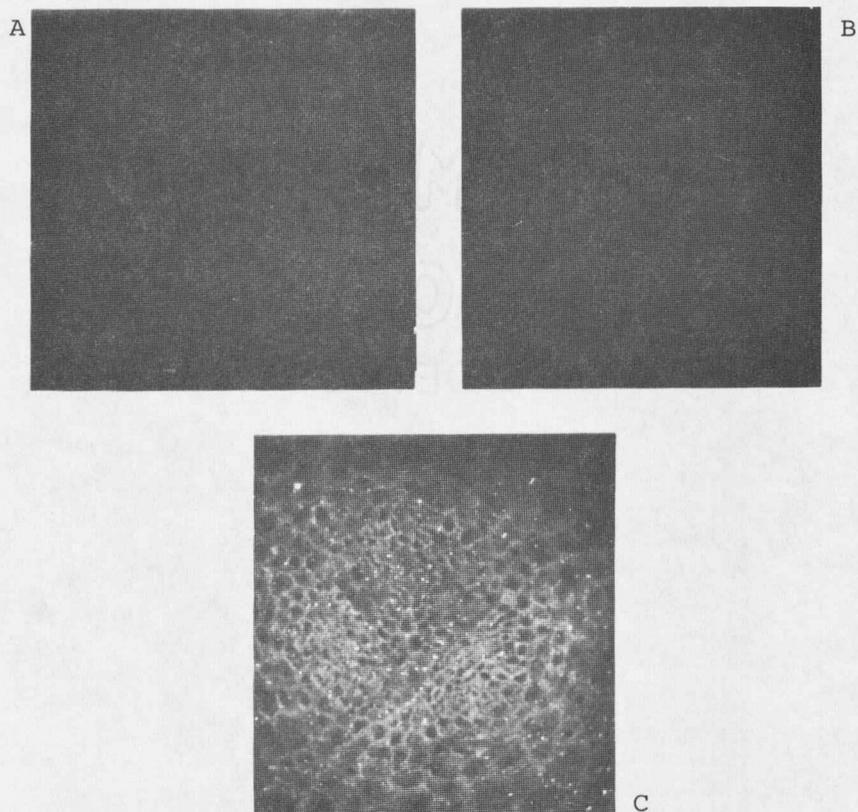


Figure 24. Immunofluorescent stain of sainfoin root tip 10 micron cross section (central area of section), age 96 hours. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

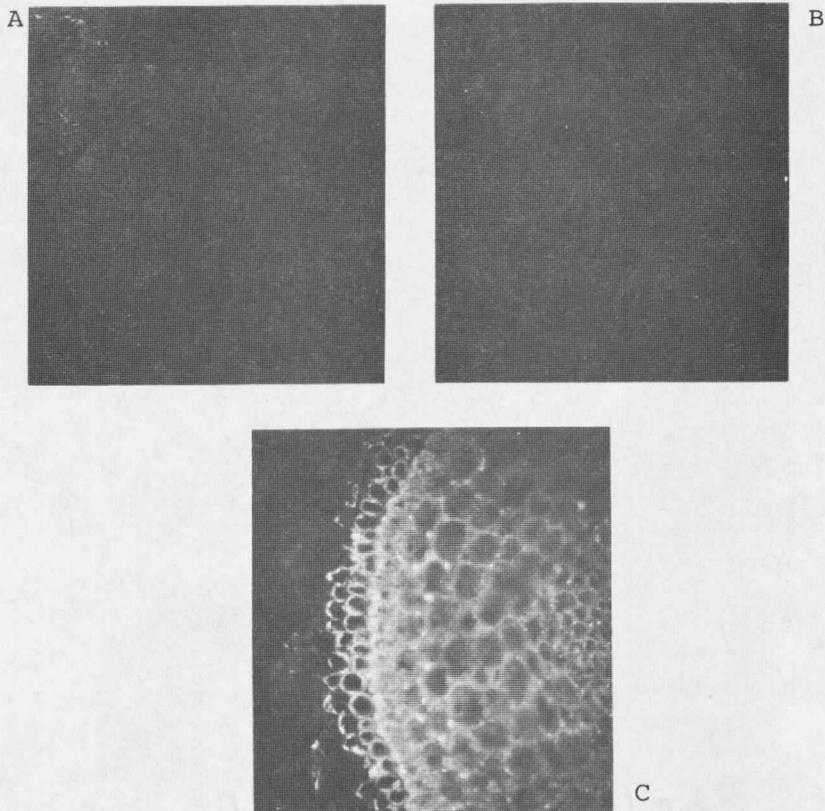


Figure 25. Immunofluorescent stain of sainfoin root tip 10 micron cross section (outer wall area of section), age 96 hours. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

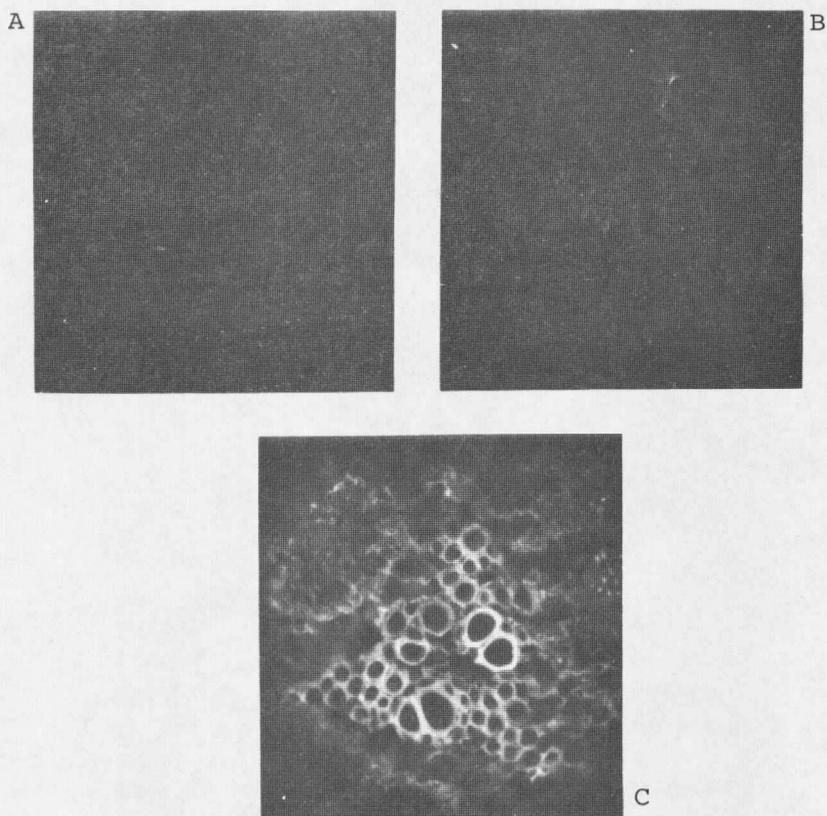


Figure 26. Immunofluorescent stain of sainfoin hypocotyl 10 micron cross section (central area of section), age 30 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

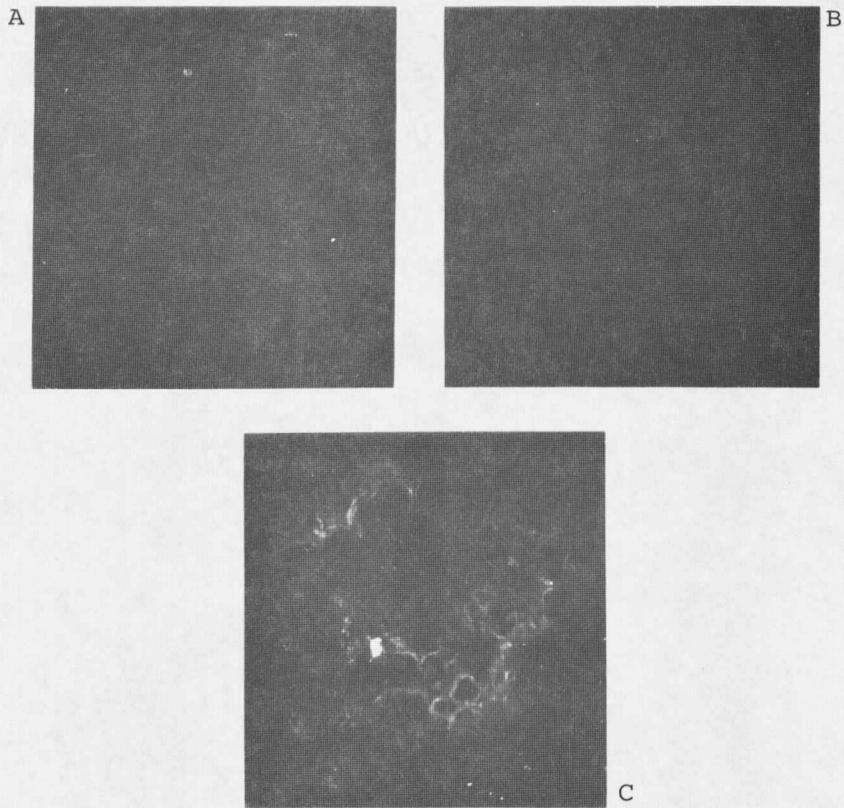


Figure 27. Immunofluorescent stain of sainfoin hypocotyl 10 micron cross section (outer wall area of section), age 30 days. A - autofluorescence control, B - NRS control, C - rhodamine stain, (108X).

Correlation with Current Literature

Histochemical localization of lectin in plant tissue using fluorescent techniques has been attempted only once in the literature. Australian researchers Clarke, Knox and Jermyn have described fluorescent localization of the lectins Concanavalin A and Phytohemagglutinin from jack and red kidney bean, respectively (41). They used FITC-labelled, non-specific, glycoprotein (immunoglobulins) to localize the lectin. Lectin presence was defined by specific sugar inhibition of binding resulting in the disappearance of stain. With this technique they found Con A and PHA to be present in the cell cytoplasm of 48 hour old, free-hand razor blade sections of cotyledon tissue from each type of plant.

In work reported in this thesis, several sainfoin tissues were examined, using thin tissue sections, and lectin was detected by the specific, indirect, immunofluorescent staining procedure. Staining was evaluated directly by fluorescence microscopy. Young, actively growing root tip of sainfoin did exhibit cytoplasmic staining as did the cotyledon tissue examined by Clarke, Jermyn and Knox. However, examination of other tissues from sainfoin indicated that sainfoin lectin is also found in or near the cell walls of growing, differentiating tissue. Although the data presented in this thesis does not contradict the observations of Clark et al., it represents expanded and improved

methodology through the use of immunospecific fluorescent staining and microtome tissue sectioning.

Staining of the cell wall area in sainfoin root and hypocotyl tissue is generally consistent with recent biochemical isolation data from the literature. Glaser and Kauss have isolated proteins from the cell walls of mung bean hypocotyl tissue and have shown that those proteins exhibit lectin properties (42). Bowles and Kauss have shown lectins to be present in extracts of isolated membranes of endoplasmic reticulum, dictyosomes and plasma membranes from rapidly extending hypocotyl of Phaseolus aureus as well as in cell wall extracts from the same tissue (43). They proposed that the lectins recovered in isolated membranes could be both membrane components and secretory substances carried within the membrane vesicles for deposition at the cell surface. Although immunofluorescent staining is not sensitive enough to allow identification of intracellular membrane systems, the staining patterns of young actively growing sainfoin root tissue described in this thesis are consistent with such a hypothesis since cell wall areas and cytoplasm of the tissue showed specific staining.

Sainfoin lectin has also been localized by immunofluorescence on the outer surface of root tissue. Although there have been indirect indications of surface lectin in other systems as described by Kent and Hamblin (19), Bohlool and Schmidt (20), and Wolpert and Albersheim (21)

this is the first study to directly localize lectin on the surface of root tissue. The detection of lectin on the outer surface of root was supported in this thesis by the fact that it was also isolated from root tissue and was shown to be present in liquid media used to germinate sainfoin seedlings. Although these data say little about possible recognition mechanisms between legume hosts and infecting rhizobia, the results are consistent with lectin involvement in specificity of interaction. The presence of lectin on the root surface is required with model systems such as the one proposed by Dazzo and Hubbell (22). They proposed that lectin molecules link the bacterium to the root hair surface by mutual binding of common receptors on the two surfaces. This specific role of lectin in rhizobial recognition has been recently challenged, however, by the data of Chen and Phillips (24) who see no specificity between lectin and infective bacteria.

SUMMARY AND CONCLUSIONS

A phytolectin was isolated from the seeds of the legume Sainfoin (Onobrychis viciifoliae, Scop.). The lectin was isolated by extraction in phosphate buffered saline followed by affinity chromatography on a column of mannose covalently bound to sepharose. Pure lectin was obtained by repeating the affinity chromatography process.

The purified lectin was used as antigen to produce specific antisera in the rabbit system. Gel double diffusion and immunoelectrophoresis were used to ascertain the purity of the Sainfoin lectin antigen and the specificity of the antibody. These techniques were also used to show that lectin isolated from root tissue and lectin isolated from seed tissue were immunologically indistinguishable.

The "indirect" immunofluorescent tissue staining method was used. This method employed specific antisera as the primary stain followed by the fluorescent conjugate prepared against the specific antibody as the secondary stain. The more traditional system using fluorescein isothiocyanate conjugate and blue light excitation produced a large amount of tissue autofluorescence. Therefore, a conjugate containing the fluorophore rhodamine B isothiocyanate coupled to anti-rabbit IgG produced in the goat was prepared. The spectral characteristics of the conjugate accommodated the use of a 546 nm interference filter in the primary position and a K580 cut-on filter in the secondary position. Use of the 546 nm exciting light significantly

reduced the amount of tissue autofluorescence and resulted in easily interpretable stains. The "indirect" rhodamine staining process was successfully tested on a model system of sepharose beads with sainfoin lectin covalently attached.

Sainfoin seedlings grown aseptically and sainfoin seedlings that had been infected with rhizobia bacteria were germinated for use in the tissue localization study. Samples of root tissue were taken at 96 hours, 10 days, 20 days, and 30 days after planting. Immunofluorescent stains and comparative histological stains were conducted on sections of seed, nodule, and the four age groups of root. Frozen sections of 10 microns of each tissue were used in the staining processes. Frozen sectioning was preferred since induced tissue autofluorescence and denaturation of antigen by the use of heat and organic solvents were eliminated.

Sainfoin lectin was localized by immunofluorescence in the cell wall area of the aleuron layer of the seed as well as in the protein matrix surrounding the starch granules. In the nodule, positive staining was found in the cell wall areas of the outer layer of the cortical cells. There was no staining of the central, bacteroid-containing core. Lectin was localized in the cell wall areas of the central vascular elements of 10, 20, and 30 day old root as well as in the cell wall area of the outer epidermis. In the young (96 hour) root tissue, lectin was shown to be present in the wall areas of all cells including

the outer epidermis. Cells near the extreme root tip also contained lectin localized in the cytoplasmic regions. In contrast, older tissues showed no cytoplasmic staining.

Intercellular localization of lectins via indirect immunofluorescent staining has not been previously described in the literature. Use of rhodamine as the primary fluorophore in histochemical studies of plant tissue is also new methodology. However, the overall staining patterns observed in Sainfoin tissue are generally consistent with current literature dealing with the presence and conjectured physiological roles of phytolectins.

LITERATURE CITED

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1. Watkins, W.M., and W.T.J. Morgan. 1952. Nature, 169, 825.
2. Sela, B.A., H. Lis, N. Sharon, and L. Sachs. 1970. Biology, 3, 267.
3. Landsteiner, K., Ed. 1945. The Specificity of Serological Reactions, Cambridge, Mass: Harvard University Press.
4. Wang, J.L., B.A. Cunningham, M.J. Waxdal, and G.M. Edelman. 1975. J. Bio. Chem., 250, 1490.
5. Goldstein, I.J., W.M. Galbraith, A. Misaki, and C. Samanen. 1972. Abstr. Int. Symp. Carbohyd. Chem., 30.
6. Naspitz, C.K., and M. Richter. 1968. Progr. Allergy, 12:1.
7. Bezkorovainy, A., G.F. Springer, and P.R. Desai. 1971. Biochemistry, 10, 3761.
8. Sharon, N., and H. Lis. 1972. Science, 177, 949.
9. _____, and _____. 1973. Ann. Rev. Biochem., 42, 541.
10. Cohen. E., Ed. 1974. Ann. N.Y. Acad. Sci., 234.
11. Leiner, I.E. 1976. Ann. Rev. Plant Phys., 27, 291.
12. Ginsburg, V., Ed. 1972: Methods in Enz.-Complex Carbohydrates Part B, 28, 313.
13. Aub, J.C., C. Tieslau, and A. Lankester. 1963. Proc. Nat. Acad. Sci. U.S.A., 50, 613, J.C. Aub, B.H. Sanford, M.N. Cote, ibid. 1965, 54, 396.
14. Frazier, W.A. 1976. Trends in Biochem. Sci., 1:6, 130.
15. Albersheim, P., and A.J. Anderson. 1971. Proc. Natl. Acad. Sci. U.S.A., 68, 1815.
16. Glaser, C., and H. Kauss. 1974. FEBS Letters, 45:1, 304.
17. Albersheim, P., and J.S. Wolpert. 1976. Pl. Physiol., Supplement item 416, 79.

18. Sharon, N., and H. Lis. 1972. Science, 177, 949.
19. Hamblin, J., and S.P. Kent. 1973. Nature New Biology, 245, 28.
20. Bohlool, B.B., and E.L. Schmidt. 1974. Science, 185, 269.
21. Wolpert, J.S., and P. Albersheim. 1976. Biochem. and Biophys. Res. Comm., 70:3, 729.
22. Dazzo, F.B., and D.H. Hubbell. 1975. App. Micro., 30:6, 1017.
23. _____, C.A. Napoli, and D.H. Hubbell. 1976. App. and Env. Micro., 32:1, 166.
24. Chen, A.T., and D.A. Phillips. 1976. Physiol. Plant., 38, 83.
25. Napoli, C.A., and D.H. Hubbell. 1975. App. Micro., 30, 1003.
26. Child, J.J., and T.A. LaRue. 1976. Proceedings of the 1st International Symposium on Nitrogen Fixation, vol. 2, 447.
27. Porath, J., and N. Fornstedt. 1975. FEBS Letters, 57:2, 187.
28. Weir, D.M. 1973. Immunochemistry, vol. 1.
29. _____. 1973. Immunochemistry, vol. 1.
30. Wells, A.F., C.E. Miller, and M.K. Nadel. 1966. App. Micro. 14:2, 271.
31. Thornton, E.K. 1930. Ann. Bot., 44, 385.
32. Sheehan, D.C., and B.B. Hrapchak. 1973. Theory and Practice of Histotechnology, 40.
33. _____, and _____. 1973. Theory and Practice of Histotechnology, 40.
34. Hartman, B.K. 1973. J. of Histochem. and Cytochem., 21:4, 312.
35. Hiramoto, R., and M. Hamlin. 1965. J. Imm., 95, 214.
36. Hapner, unpublished data.

37. Ouchterlony, Ö. 1948. Ark. Kemi Mineral. Geol., 26 B, 1-9.
38. Cherry, W.B., M. Goldman, and T.R. Caski. 1960. U.S. Public Health Service Pub., 729.
39. Goldman, M. 1968. Fluorescent Antibody Methods.
40. Brandtzaeg, P. 1975. Ann. N.Y. Acad. Sci., 254, 35.
41. Clarke, A.E., R.B. Knox, and M.A. Jermyn. 1975. J. Cell Sci., 19, 157.
42. Kauss, H., and C. Glaser. 1974. FEBS Letters, 45:1, 304.
43. Bowles, D., and H. Kauss. 1976. Biochim. et Biophys. Acta, 443, 360.
44. Quatrecasas, P. 1970. J. Bio. Chem., 245:12, 3059.

