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REGULATION OF NODULATION AND INTERSTRAIN COMPETITION IN SOYBEAN (*Glycine max* L., Merr.) / *Bradyrhizobium japonicum* SYMBIOSIS

Ph. D. THESIS

REGULACIJA NODULACIJE IN KOMPETICIJE MED SEVI BAKTERIJE *Bradyrhizobium japonicum* PRI FORMIRANJU SIMBIOZE S SOJO (*Glycine max* [L] Merr.)

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AI To investigate the mechanisms of regulation of nodulation and interstrain competition in B. japonicum/G. max symbiosis, soybean plants were inoculated sequentially with the same strain of B. japonicum or simultaneously with the two strains of different competitiveness. Plants were grown under different environmental conditions and nodule initiation and development was monitored from inoculation until nitrogen fixation was measurable. Nodule numbers and mass were correlated with leaf area and root length at the time of inoculation and with light intensity in the growth environment during the period of nodule development. Photosynthate partitioning to developing nodules and roots was evaluated by radioactive labeling. A root staining and a serological procedure were used to identify the earliest nodule structures and rhizobial strains within. Number of nodule primordia and number of functional nodules formed by the competing strains indicated that, interstrain competition pattern was determined during the earliest stages of the infection process, where the rate of infection and nodule initiation by a given strain apparently plays a crucial role. Subsequent nodule development was shown to be regulated by competition between early and late initiated nodules for current photosynthate and did not significantly affect the initial competition pattern. Ultimately, nodule number and mass per plant at harvest was dependent on plant photosynthetic potential.

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ABBREVIATIONS AND SYMBOLS / Okrajsave in simboli

E - early inoculation, early inoculated

D - delayed inoculation, delayed inoculated

U - uninoculated

dpm - disintegrations per minute

CD - cortical cell division center(s)

NP - nodule primordia

EN - emerging nodules

NO - functional, mature nodules

AR - acetylene reduction

ARA - acetylene reduction activity, acetylene reduction assay

LA - leaf area

RL - root length

HSD - honestly significant difference

FA - fluorescent antibody

FITC - fluoresceine iso-thio-cyanate

PAR - photosynthetically active radiation

PBS - phosphate buffer saline

PNS - plant nutrient solution

CHAPTER I

THESIS INTRODUCTION AND LITERATURE REVIEW

The symbiotic association between rhizobia and leguminous plants is formed through a complex sequence of interactions between the partners (for review see 21, 68) and culminates in the establishment of nitrogen fixing nodules. On the basis of present knowledge, three distinct stages of these interactions are recognized:

1. The preinfection stage, comprises the interactions in the rhizosphere/rhizoplane, controlling the attachment of bacteria to the root surface and initiation of infection. These interactions include: a) proliferation of rhizobia in the rhizosphere of their respective hosts and chemotaxis towards defined regions of the roots, stimulated by nutrients and phenolic compounds in the root exudate (7, 45); b) root colonization and adsorption to the root surface in a non specific (67, 83) or specific manner, mediated by plant lectin and bacterial surface polysaccharide molecules (5, 15, 41, reviewed in 25, 29); c) induction of bacterial nodulation (nod) genes by flavonoids released from legume seeds and roots (28, 29, 47, 57, 62, reviewed in 21, 68). Common nodABC genes are involved in generating extracellular factors, inducing root hair curling, branching, initiation of the infection threads and proliferation of centers of cortical cell division in the host plant. Another set of nod genes is responsible for host specific nodulation (hsn genes) and determines the host specificity among rhizobia. Expression of common and hsn nod genes is controlled by the interaction between the regulatory nod D gene and plant flavonoids (see 74 for compilation and 21, 68 for review on nod genes).

2. The infection stage encompasses the interactions within the roots, controlling nodule initiation and subsequent nodule development until the

onset of nitrogen fixation. Nodule formation involves coordinated expression of rhizobial nod genes and plant symbiotic genes coding for nodule specific proteins, termed nodulins (12, 36, 37, reviewed in 21, 39). Even before the infection thread penetrates the root cortex, cortical cells begin to divide (79), giving rise to an initial nodule meristem. Further nodule ontogeny includes enlargement of the nodule tissue and differentiation of the vascular system connecting nodule meristem to the root vascular system. Infection thread ramifies intercellulary in the nodule meristem tissue, eventually penetrates the walls of adjacent plant cells and rhizobia multiplying in the infection thread are released into the host cytoplasm. Within the plant cells rhizobia become enclosed in plant derived peribacteroid membrane and differentiate into nitrogen fixing bacteroids. Infection and nodule initiation and development may vary in different legume species (for reviews see 25, 29, 63, 68, 76).

3. The nitrogen fixation stage encompasses interactions within the nodules associated with the onset and efficiency of nitrogen fixation. The synthesis of nitrogenase, the key enzyme converting atmospheric nitrogen into ammonia, is encoded by rhizobial nif or fix genes (63). Ammonia is then assimilated by plant enzymes and exported from nodules in the form of amides or ureides, depending on legume species (for reviews see 29, 32, 64, 65). Rhizobial strain and environmental factors affecting plant growth and nitrogen assimilation determine the efficiency of nitrogen fixation in nodules ((2, 3, 29, 51, 71, 72, 84).

A distinct physiological phenomenon, occurring in the infection stage, which appears to be strictly host controlled is a process termed autoregulation (reviewed in 21, 39, 68). By autoregulation the host plant controls the number of nodules formed on the roots and prevents

overnodulation. Autoregulation has attracted considerable research attention over the past 50 years, since the process appears responsible for competition between Rhizobium strains for nodule occupancy on the common host. However, the mechanism(s) of autoregulation remain(s) as yet unknown.

In his classical experiments with red clover Nutman (58 - 60) postulated physiological homology of lateral roots and nodules and envisaged the sites of lateral root initials as predetermined foci of infection (58). Studying the effect of delayed inoculation on nodulation (59), he concluded that number of nodules is determined by the number of these preinfection foci and therefore the increased nodule number obtained with delayed inoculation could be attributed to increased number of preinfection foci on a larger root system. Increased nodulation (60), following nodule and root tip excision, led Nutman to the proposal of the regulatory mechanism based on production of inhibitors by nodule and root meristems. According to his hypothesis, a significant increase in nodule number following the excision of root tips and nodules formed by the effective strain was due to the removal of the source of inhibitor. Consequently, ephemeral nodules formed by the ineffective strain do not produce considerable amount of inhibitor since their excision did not stimulate subsequent nodulation. Nutman (59) has also pointed out that only certain regions of the roots are susceptible to infection by rhizobia. Localized and transient susceptibility of legume roots to infection has been extensively studied by Bhuvaneshvari et al. (9, 11) and shown to be widespread among common legumes (10).

Recent studies on autoregulation employing time separated inoculations (i.e. early inoculation followed by delayed inoculation at various time intervals) on the intact or split - roots of soybean and alfalfa (see 21, 39, for review) and clover (69) have demonstrated systemic mechanism of the autoregulatory response. Two concepts of regulatory substances with regard to the site of their production were generated:

a) - a shoot derived inhibitor, production of which is induced by primary (early) inoculation and its effects manifested as a suppression of nodule development in the secondary (delayed) inoculated root portion (19, 61); b) - an inhibitor is produced at the site of primary inoculation - i.e. in the root - and then transported to developmentally younger regions of the root (19, 66) or, as the case may be, across the split-root system (e.g. 49).

Delves et al. (27) demonstrated nodulation may be controlled by shoot and root factors.

Kosslak (46), using a split root system, has tested regulation of nodulation over a variety of soybean cultivar/B. japonicum strain combinations. She observed suppression of nodule development on delayed inoculated root side even when a less competitive or ineffective strain was used as a primary inoculum. Maximum autoregulatory response was obtained with 4 - 7 day delay of secondary inoculation. Preexposure of young seedlings to a less competitive or ineffective (nod+, fix-) strain for 6 to 72 hours, indicated that, first nodules, formed by a less competitive strain inhibited nodulation by a more competitive strain (48).

Experiments of Pierce and Bauer (66) and Malik and Bauer (52) indicated autoregulatory response in young tap root between 6 - 15 hours after primary inoculation.

Microscopic analysis of nodule initiation and development in soybean by Calvert et al. (24) revealed that, regulation takes place during transition of infections into nodule primordia. Infections were defined as centers of subepidermal cortical cell divisions with associated infection threads. Cortical cell division centers without infection threads in the adjacent root hairs were called pseudo infections. On a single tap root they could identify up to 50 fold greater number of cortical division centers than the average number of mature nodules ultimately formed on the root. Similar observations were reported by Mathews et al. (55).

Microscopic analysis of double inoculation experiments (15 h apart) by Calvert et al. (24) showed that, 3 days after the second inoculation there was no evidence of suppression of either number or maturation of late induced infections. However, 7 days after the second inoculation development of infections in younger root regions was arrested at stages after nodule meristem formation but before the emergence of nodule primordia, which appear as bumps on the root surface.

Experiments with host and Rhizobium mutants have demonstrated, that nodulation phenotype, as well as, the intensity of autoregulatory response is controlled by plant and bacterial genotype (11, 55, 61) their interaction (42) and inoculant titer (66, 77). Furthermore, nodulation is affected by several physiological and environmental factors such as nitrate levels (53, 61) and light (49, 54). Malik et al. (54) observed inhibitory (nonphotosynthetic) effect of light on number of infections and stimulatory effect (photosynthetic) on subsequent development of those infections. Kosslak and Bohlool (49) demonstrated that, number of nodules on the early/delayed inoculated split-root system was proportional to the amount of light available to soybean plant for photosynthesis.

Direct dependence of nitrogen fixation in nodules on carbohydrate supply from photosynthesis (51, 84), as well as, selective partitioning of current photosynthate to effective and ineffective nodules (71) have been clearly demonstrated. Differential partitioning of labeled photosynthate to roots and nodules in early stages of the infection process has so far not been reported. Similarly, no direct experimental evidence of the nature or transport of the inhibitory substance(s) involved in regulation of nodulation has been obtained. Since in early developmental stages, nodules and bacteria are nutritionally completely dependent on the host plant, selective abortion of infections in soybean, observed by Calvert et al. (24) might be compared to selective abortion or abscission of flowers and fruits

in higher plants (17, 86), where the process is under nutritional and hormonal control.

Exogenously supplied phytohormones (IAA, GA3, ABA, CCC) were shown to inhibit nodulation depending on nitrogen supply to the plant (see ref 39 for review). Production of phytohormones by rhizobia has also been demonstrated (44, 75). Bauer et al. (6) showed that cytokinins can induce cortical cell divisions in the absence of rhizobia. The role of phytohormones in regulation of nodulation, however, remains unclear.

By contrast to autoregulation, which is generally recognized as a plant controlled response, determinants of the interstrain competition pattern on the common host are generally associated with the rhizobial strain attributes. It has been proposed (1) that, relative numbers of cells of homologous of rhizobia in soil or in the inoculum mixture determine relative numbers of nodules formed by competing strains. Whereas environmental factors affecting persistence (16,88) and performance (82) of rhizobia may influence relative numbers of rhizobial strains in soil, when the strains are added to roots in equal cell numbers, speed of nodule formation by competing strains may become the key determinant of the proportion of nodules occupied by each strain. Smith and Wollum (73) and McDermot and Graham (56) examined the relationship between nodulation rate and competitiveness of several strains and obtained somewhat inconclusive results.

Motility and chemotaxis, though not essential for infection, may provide competitive advantage to a strain colonizing the root surface (7, 23). The rate of infection and nodule formation may be influenced by the response of different strains to plant symbiotic signals in the root exudate (40). Plant and rhizobial symbiotic signals acting in concert and affecting nodule initiation and development may vary considerably with plant and rhizobial genotype or with the combination of the two (34, 40, 57, 81). How

the early host/strain molecular interactions affect interstrain competition is unknown.

Since the genetic and biochemical basis of autoregulation and interstrain competition remains unknown, further hystochemical and physiological studies may provide indirect evidence for the underlying mechanisms.

We used sequential inoculation of the split-root systems with the same strain or, simultaneous inoculation with two strains of different competitiveness, in soybean plants, grown under different environmental conditions to determine: 1) in what stage(s) of the infection process regulation of nodulation takes place; 2) how the host plant growth potential and root infectible area affect nodule initiation and development; 3) whether selective partitioning of current photosynthate to developing nodules and roots provides a regulatory mechanism, controlling nodule number and mass per plant; 4) whether the outcome of interstrain competition is determined during the early stages of root infection and nodule initiation or, during the process of nodule development.

CHAPTER II

EFFECT OF PLANT GROWTH PARAMETERS ON NODULATION OF THE SPLIT-ROOT SYSTEM OF SOYBEAN (Glycine max L., Merr. cv. D-68) BY Bradyrhizobium japonicum strain USDA 110.

ABSTRACT

Regulation of nodulation by the host plant (autoregulation) is well documented but the regulatory mechanism(s) are still unknown. Several studies indicated plant growth potential affects nodulation and nitrogen fixation in Rhizobium - legume symbiosis. To evaluate the effects of host plant growth parameters on nodule initiation and development we used early and delayed inoculation treatments of the split-root system of soybean and related the rate of nodule initiation and development on the opposite root halves to light intensity, leaf area and root length.

When inoculation of one side was delayed for 1, 2, 4, 8, 16, 32, 64 and 96 hours, nodule mass on delayed inoculated (D) side was significantly reduced when D side was inoculated 16 hours or more after early inoculated (E) side and nodule number on D side was significantly reduced by inoculation delay for 64 hours or more. Number of nodules per plant, 3 weeks after D inoculation, in the uninoculated/delayed inoculated treatment increased linearly (r=0.97) with inoculation delay (1 - 96 hours). Nodule numbers per plant were highly correlated with leaf area at the time of inoculation (r=0.98), whereas correlation with root length was less pronounced. Plants with larger leaf area at the time of inoculation also formed nodules faster. Nodule numbers per plant were directly proportional to the light intensity in the growth environment and nodule number on D side was always inversely related to nodule number on E side. When D side was inoculated 4 days after the E side

and the same experiment was done in the greenhouse (high light intensity) and in the growth room (low light intensity), nodulation on D side was suppressed by 32% in the greenhouse and by 74% in the growth room. When inoculation of D side was delayed until the onset of N_2 fixation in early nodules (14 days after E inoculation) only 23% suppression was observed under growth room conditions. Removal of E side at the time of inoculation of D side (14 days after E inoculation), significantly increased the number of nodule primordia, formed on the remaining root half compared to total number of nodule primordia on the intact E/D root system. However, the number of mature nodules per plant, 3 weeks after D inoculation, was essentially the same in both treatments.

These results suggest that number of initiated nodules (nodule primordia) per plant depends to a large extent on the host plant growth potential at the time of inoculation, whereas number and mass of mature nodules per plant is determined by the amount of light available to soybean plant for photosynthesis. Plant regulatory response, controlling nodule number and mass per plant, can be observed already with 16 h inoculation delay. Lower level of autoregulation, when second inoculation was delayed until the onset of nitrogen fixation in early nodules, indicates physiological changes within the nodulating plant according to factors limiting plant growth and nodule development.

INTRODUCTION

Establishment of nitrogen fixing nodules on the roots of legumes is a multistep process and plant and rhizobial attributes affect the numbers and mass of mature nodules formed on a particular host plant (11, 42, 61, 68, 72).

A regulatory mechanism (autoregulation, cf. 68) by which the host plant controls nodule development was first observed in red clover (58) and had since been demonstrated in other common legumes (18, 35, 49, 66, 69). Studies employing time separated double inoculations showed that, autoregulatory response in soybean occurs between 8 and 15 hours after primary inoculation (52, 66) and is maximal when early and delayed inoculation are separated by 4 to 7 days (49, 61).

Bhuvaneswari et al. (9, 10) demonstrated localized and transient susceptibility of legume roots to rhizobial infection but, Pierce and Bauer (66) showed that limited nodulation at high inoculum doses (10^6 to 10^9 rhizobia/root) cannot be explained by limited root infectible area. However, increased nodulation, observed with delayed primary inoculation, had been attributed to larger root system in older plants (58, 59).

Kosslak and Bohlool (49), used different levels of shading to demonstrate plant photosynthetic potential as an important determinant of the extent of nodulation. Malik et al. (54) also found that photosyntheticaly active light stimulated the development of already initiated nodules.

It is still not clear what determines the nodulation threshold in a particular strain-host combination and what stages of the infection process are principally affected by autoregulation. In a series of experiments, we used various time intervals between primary and secondary inoculation of the split-root system of soybean to evaluate autoregulation in the early and late stages of the infection

process and examined the effect of infectible root area and plant photosynthetic potential on nodule initiation and development.

MATERIAL AND METHODS

Growth systems : Two variants of a split-root procedure, described by Singleton (70) were used:

1) A split-root growth system, shown in Figure II-1 was used in the time course experiment. Two PVC columns, supported by PVC couplers were taped together. Bottoms of the columns were sealed with Parafilm and columns filled with dry horticultural vermiculite. Plastic elbows (90 deg. angle, 1/2" diam.) with a planting hole drilled in the angle center were used to direct split roots into columns. Drainage tubes were inserted 0.5 cm above the bottom in each column. The PVC parts were sterilized prior to assembly with 2.5% sodium hypoclorite and rinsed with H_2O.

2) A split-root growth system shown in Figure II-2 was used in all other experiments described in this chapter. Two growth pouches (Northrup King Co.) were stapled together at the top. Planting troughs were separated from the wicks along the existing perforation and the wicks were shortened by 1.5 cm by folding. A vertical cut 1.5 cm long (Fig. II-2 -D) was made through both bags in the middle of the top edge of the pouches. A single trough (Fig. II-2 -B) was passed through the cut so that one half of the trough was in each bag. A strip (half of the second trough - Fig. II-2 -A) was inserted into each bag to make a capillary connection between the trough (Fig. 11-2 -B) and the main wick (Fig. 11-2 -C) and at the same time direct root growth into the bags.

Planting procedure: Seeds of soybean (Glycine max L., Merr.) cv. D-68 (T.E. Carter, Dept. of Crop Science, NC State University at Raleigh) were surface sterilized with 2.5 % NaClO for 5 minutes, rinsed 7 times with sterile H_2O, imbibed for 4 hours and then sown hillum down in moist, sterile horticultural vermiculite. Approximately 48 hours after sowing, uniform

seedlings (1.5 to 2.5 cm radicle) were selected, the tips of the radicles cut off and seedlings planted into the center of the trough or into a hole in the elbows packed with wet vermiculite. Elbows were planted in a layer (approx. 5 cm deep) of sterile vermiculite. After planting, pouches and elbows were covered with transparent polyethylene film to provide sufficient humidity for lateral root growth under growth room conditions. Three to 5 days after planting lateral roots had grown a few cm into pouches or emerged from the elbows. At that point, seedlings were selected for uniformity and roots trimmed to the same number of lateral roots per side; strip connectors between the trough and the wick were removed and the elbows were attached to the PVC columns containing vermiculite moistened with 50 ml N-free plant nutrient solution (PNS). Tops of the columns were sealed with Parafilm. Seedlings in pouches received 30 ml PNS at planting and subsequently, PNS level was maintained at 1-3 cm from the bottom of the pouch with half strength PNS. Plants in PVC columns were irrigated every other day with half strength PNS via microtubing inserted through the top Parafilm cover. Concentrations of nutrients in PNS were: 0.58 mM $CaSO_4 \cdot 2H_2O$, 0.5 mM K_2HPO_4, 0.25 mM $MgSO_4 \cdot 7H_2O$; and concentrations of micronutrients (added as preformulated Hawaiian Horticulture Mix) were 51 µM Mg, 97 µM S, 40 µM B, 0.6 µM Co, 2.9 µM Cu, 33.3 µM Fe, 10 µM Mn, 0.5 µM Mo, 9.4 µM Zn.

Plants in pouches were grown in the growth room under photosynthetically active radiation (PAR) 300-400 µE/m^2/sec, 18 h photoperiod and temperature range 22 - 28°C. Plants in columns were grown in the greenhouse under PAR 1350-1600 µE/m^2/sec, approximately 13 h photoperiod and temperature range 18 - 37°C.

Inoculation treatments: Bradyrhizobium japonicum strain USDA 110 (TAL 102, obtained from the Niftal Project collection) as peat based inoculant was used in all the experiments described in this chapter. Peat inoculant was suspended in N-free PNS so that 2.5 x 10^7 cells was applied per root side

- in 2 ml of inoculum per pouch and in 30 ml of inoculum per PVC column. Cell density in PNS was determined by drop plate method (43).

Half root portions were first inoculated (early inoculation) 8 days after planting, when the roots had grown to the bottom of the pouches or columns and at least one trifoliate leaf had emerged on the shoot. Inoculation of the other root half was delayed as indicated in the results for the individual experiments, following the same inoculation procedure as for early inoculation.

Harvest: Plants were harvested as indicated for the individual experiments in the results section. Shoots, roots and nodules were separated and dried at 65°C prior to weighing.

Measurements of leaf area and root length: Length and width of individual leaves was measured at 24 h intervals and leaf area calculated as a surface of elypse. Net increase in length of tap and lateral roots was measured at the same intervals as leaf area. Each time, root tips were marked with the pen on the surface of the pouches, using different pen colors for successive markings.

Counts of nodule primordia and early nodules: Emerging nodule primordia and nodules in pouches were counted on the colony counter.

Statistical analysis: Data were analyzed by Duncan's multiple range test, paired t-test, Tukey's HSD test and by regression where applicable, using SYSTAT statistical package (87). At least four replicates per treatment were included in the analysis.

Determination of nitrogenase activity: To detect the beginning of N_2 fixation, extra plants for each treatment were tested for nitrogenase activity at 24 h intervals from 7 to 10 days after inoculation. Half root systems were placed into 100 ml test tubes. Tubes were injected through a serum stopper with 5 ml acetylene and ethylene production was determined by gas chromatography (Varian 940 GC).

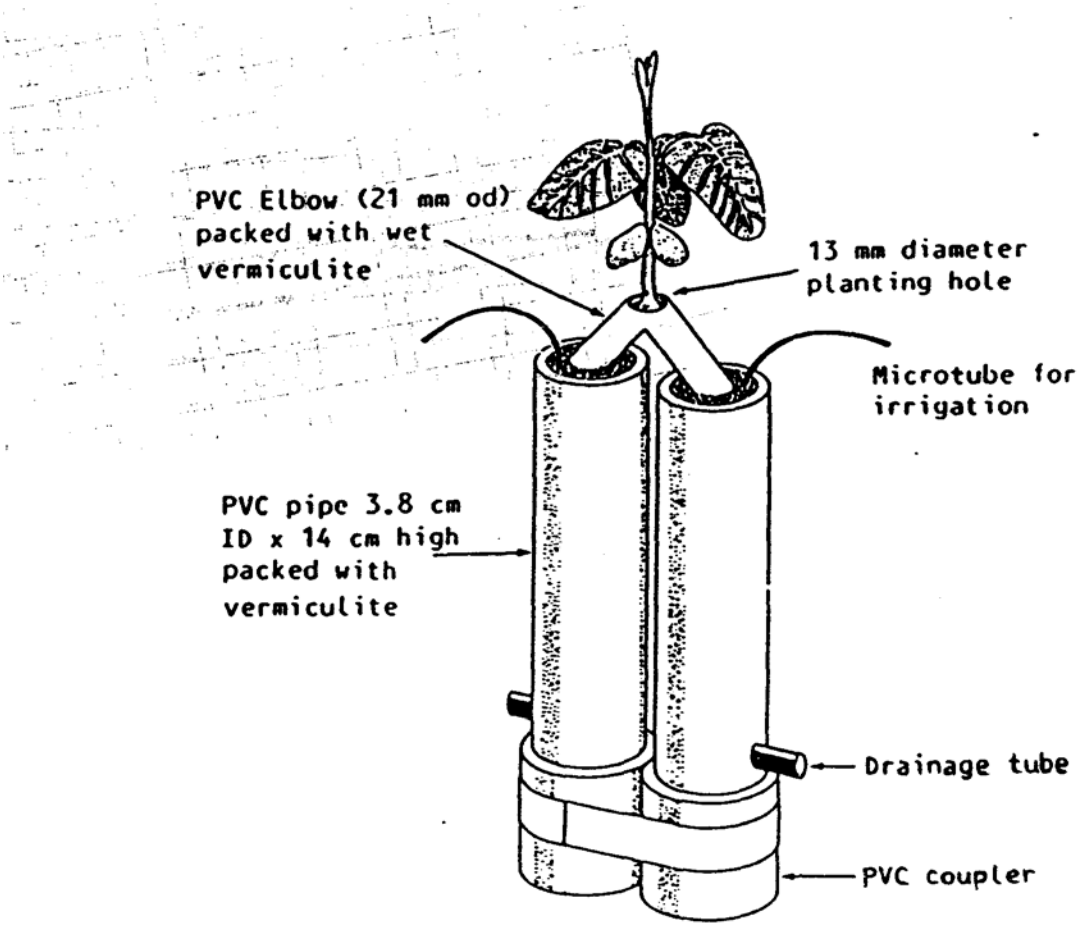


Figure II-1. Diagram of the split-root growth system consisting of PVC columns; (designed by Singleton 1984).

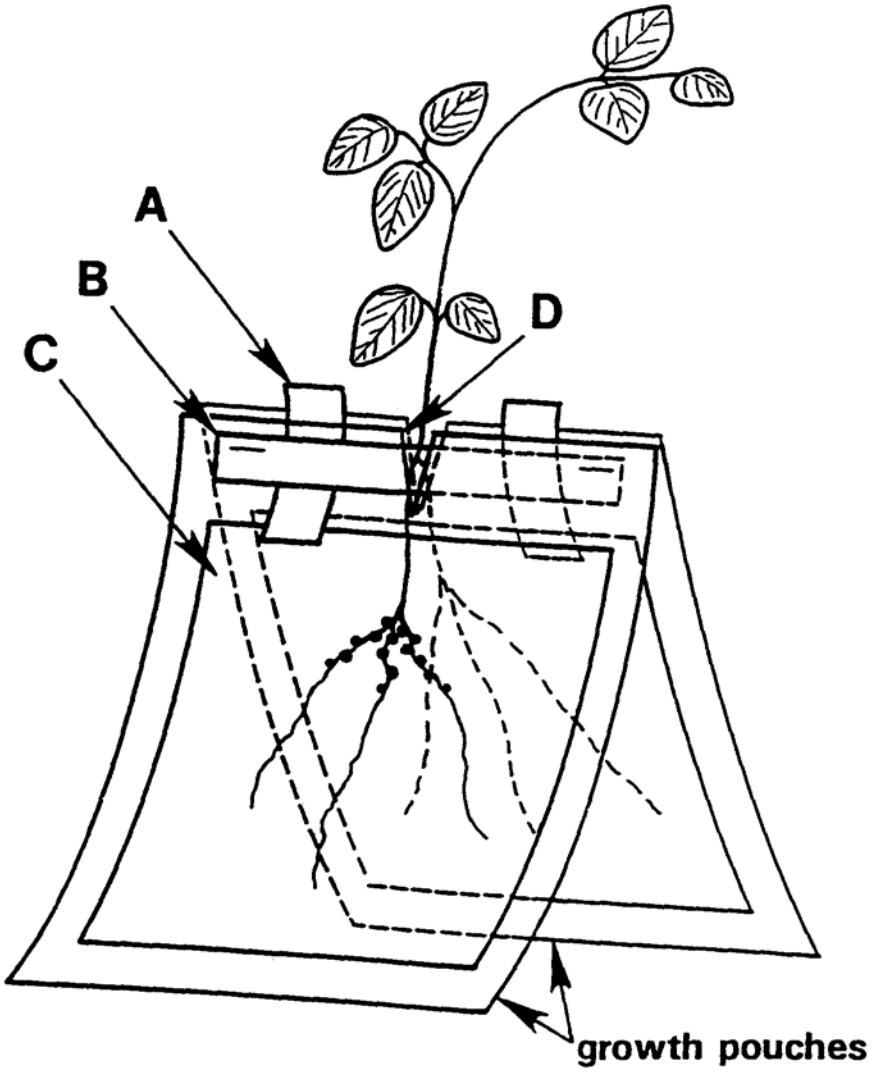


Figure II-2. Diagram of the split-root growth system consisting of two growth pouches stapled together:

A - strip connecting the planting trough - B and the main wick - C;
D - vertical cut through both pouches to allow the passing of the planting trough from one pouch into another.

Figure II-3. Type of nodulation observed on the early/delayed (E/D) inoculated and uninoculated/delayed inoculated (0/D) split-root system, when D side was inoculated 64 hours after E side.

RESULTS

Time course of inoculation delay and suppression of nodulation on delayed inoculated root side.

Inoculation delay for 16 hours or more significantly reduced nodule weight on delayed inoculated (D) side compared to early inoculated (E) side, whereas nodule numbers on D side were not significantly affected until 64 hour inoculation delay (Figure II-4). In the uninoculated/delayed inoculated treatment nodule numbers increased progressively with inoculation delay, while nodule weight at harvest decreased slightly with inoculation delay. Shoot weight in the uninoculated/delayed inoculated treatment was also reduced compared to early/delayed inoculated treatment (Figure II-5 B), while root weight was not significantly affected by the inoculation treatments (Figure II-5 A).

Correlation between leaf area, root length and nodulation

Nodule scores 3 weeks after delayed inoculation provide no insight into the pattern of nodule development on the early and delayed inoculated root side. Therefore, another experiment was set up to monitor nodule emergence and subsequent development during 2 weeks after inoculation. Nodule numbers were related to root infectible area (root length) and leaf area from the time of inoculation until emergence of first visible nodules (nodule primordia). Number of nodules per plant at the time of their emergence (5 days after inoculation) and at harvest (14 days after inoculation) was better correlated with leaf area than with root length (Table II-1, Figure II-6 A, B). Rate of nodule development was also much higher correlated with leaf area during first 5 days after inoculation than with root length during the same period (Table II-1, Figure II-7 B).

Effect of delayed inoculation on nodulation before and after the start of nitrogen fixation in early nodules.

In plants grown in pouches (Figure II-2), nitrogenase activity (acetylene reduction) in nodules on E side was first detected 10 days after inoculation. To evaluate the effect of nitrogen fixation on regulation of nodulation we used 4 and 14 day inoculation delay and monitored nodule development on the early and delayed inoculated side over 21 days from delayed inoculation.

When D side was inoculated 4 days after E side, nodule numbers on E and D side increased on successive scoring dates (Table II-II) but calculated suppression of nodulation on D side (Table II-II), for each scoring date, diminished significantly. When D side was inoculated 14 days after E side, suppression of nodulation on D side was considerably reduced (Table II-III), compared to treatment where D side was inoculated 4 days after E side (Table II-II).

Removal of E side at the time of D inoculation, significantly increased the number of nodule primordia formed on the remaining root half (D side), compared to total number of nodule primordia on the intact E/D split-root system. However, at harvest, plants with only half root system (D side) had essentially the same number of mature nodules as plants with both root halves attached (Table II-III).

Effect of light intensity on nodulation of the early/delayed inoculated split-root system.

When plants with early/delayed inoculated split-root system, using 4 day inoculation delay, were grown under high (greenhouse) and low (growth room) light intensity, number of nodules per plant was directly proportional to the amount of photosyntheticaly active radiation in the environment (Table II-IV). Furthermore, suppression of nodulation on D side was much more pronounced under low light intensity.

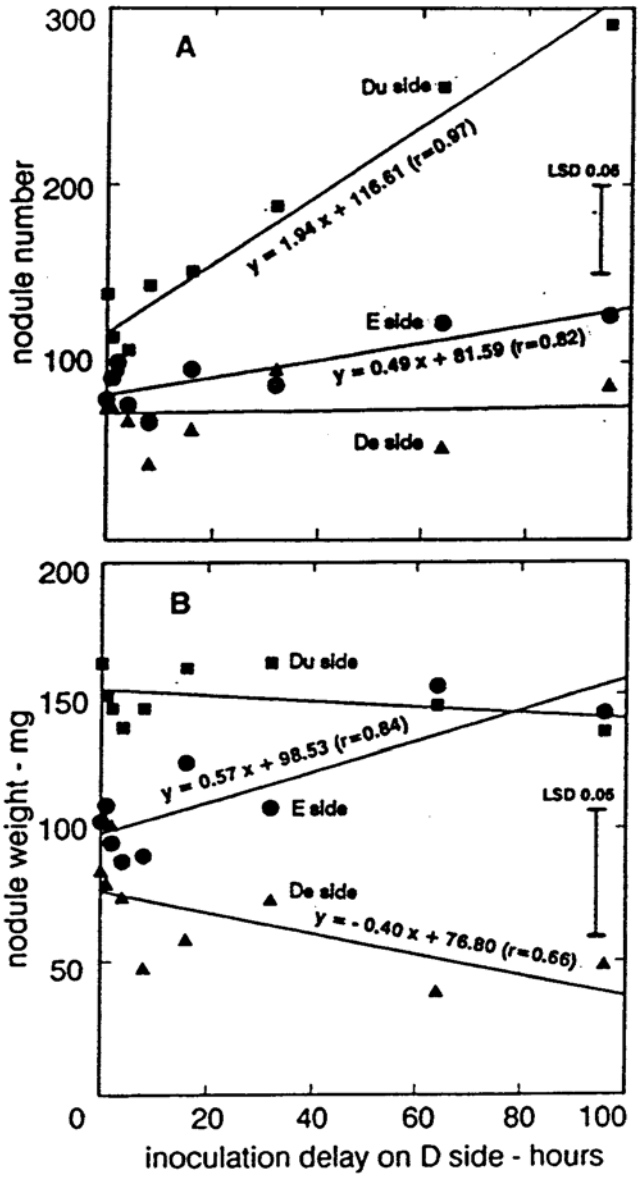


Figure II-4. Nodule numbers - A; and nodule weight - B; on the early/delayed inoculated (E/D treatment) and uninoculated/delayed inoculated (U/D treatment) split-root system of soybean;
- E side = early inoculated side of the E/D treatment;
- De side = delayed inoculated side of the E/D treatment, inoculated O, 1, 2, 4, 8, 16, 32, 64, 96 hours after E side;
- Du side = delayed inoculated side of the U/D treatment, inoculated as De side.

Plants were grown in the green house in a split-root assembly shown in Figure II-1 and harvested 21 days after delayed inoculation.

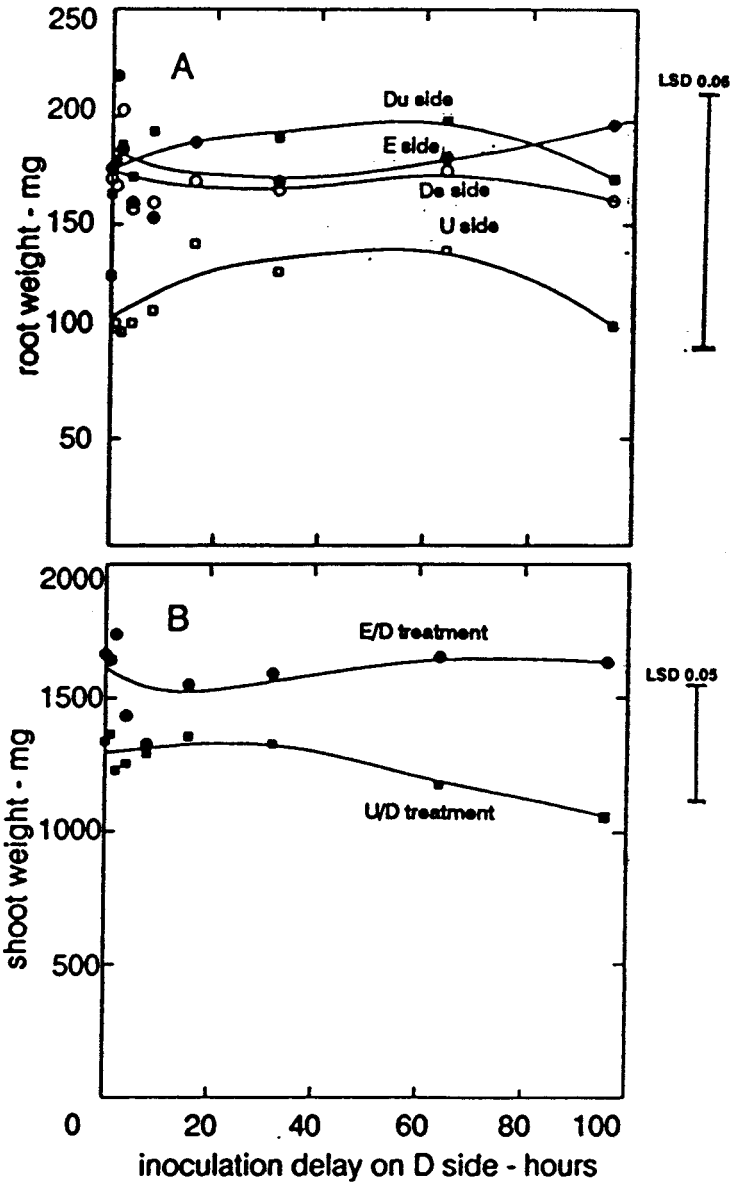


Figure II-5. Root weight - A; and on shoot weight - B; at harvest, 21 days after delayed inoculation of the early/delayed inoculated (E/D treatment) and uninoculated/delayed inoculated (U/D treatment) split-root system of soybean;
- E side = early inoculated side of the E/D treatment;
- De side = delayed inoculated side of the E/D treatment;
- Du side = delayed inoculated side of the U/D treatment
- U side = uninoculated side of the U/D treatment

Plants were grown and inoculated as described in Figure II-4.

Table II-I. Relationships between plant growth parameters and nodule numbers on the inoculated/uninoculated split-root system of soybean. Leaf area and total root length on the inoculated and uninoculated root side were measured daily from 1 to 5 days after inoculation and developing nodules were counted daily from 5 to 14 days after inoculation. Plants were grown in pouches (Figure II-2) and harvested 14 days after inoculation.

parameter	days after inoculation						
	1	2	3	4	5	8	14
leaf area-LA(cm^2)	4.1	12.1	19.0	24.7	30.5	-	-
CV (%)	88.6	47.8	40.5	40.6	38.0	-	-
inoculated side*							
root length-RL(cm)	17.9	33.8	69.2	77.3	95.3	-	-
CV (%)	83.8	71.5	62.9	60.8	52.7	-	-
uninoculated side*							
root length	26.0	48.2	83.2	92.1	109.6	-	-
CV (%)	74.3	64.9	58.9	55.4	50.4	-	-
nodule number-NO	-	-	-	-	6.0	38.6	44.0
CV (%)	-	-	-	-	159.3	54.2	45.8

Pearson's correlation coefficients for leaf area (LA), root length (RL) 1 to 5d after inoculation and nodule numbers (NO) 5d and 14d after inoculation.

	LA1d	LA2d	LA3d	LA4d	LA5d	RL1d	RL2d	RL3d	RL4d	RL5d
NO5d	0.84**	0.80*	0.74*	0.64	0.61	0.88**	0.84**	0.58	0.54	0.49
NO14d	0.98***	0.82**	0.70*	0.57	0.53	0.67	0.70	0.59	0.56	0.55
RL1d	0.61									
RL2d		0.90**								
RL3d			0.79*							
RL4d				0.70*						
RL5d					0.64					

* Inoculated and uninoculated root side did not differ significantly in total root length, according to LSD.

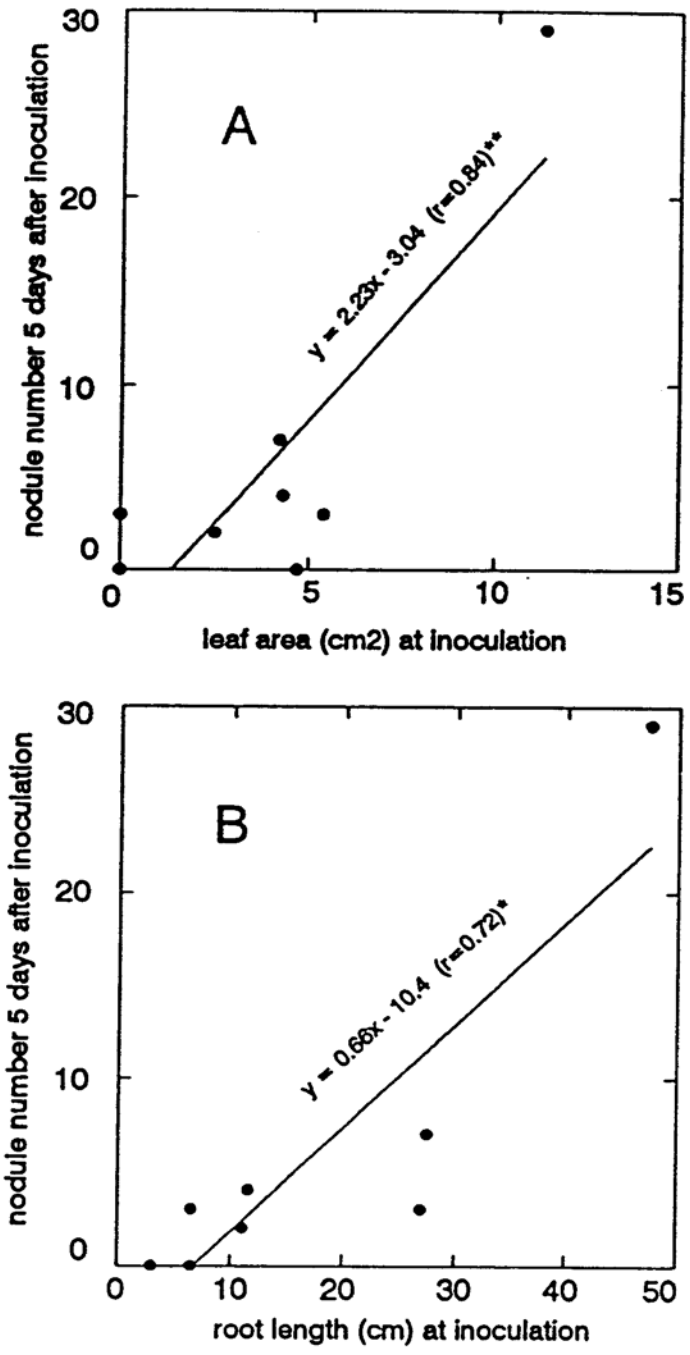


Figure II-6. Relationship between nodule emergence and leaf area - A; and between nodule emergence and root infectible area - B. First emerging nodules were observed 5 days after inoculation. Plants were grown and inoculated as described in Table II-1.

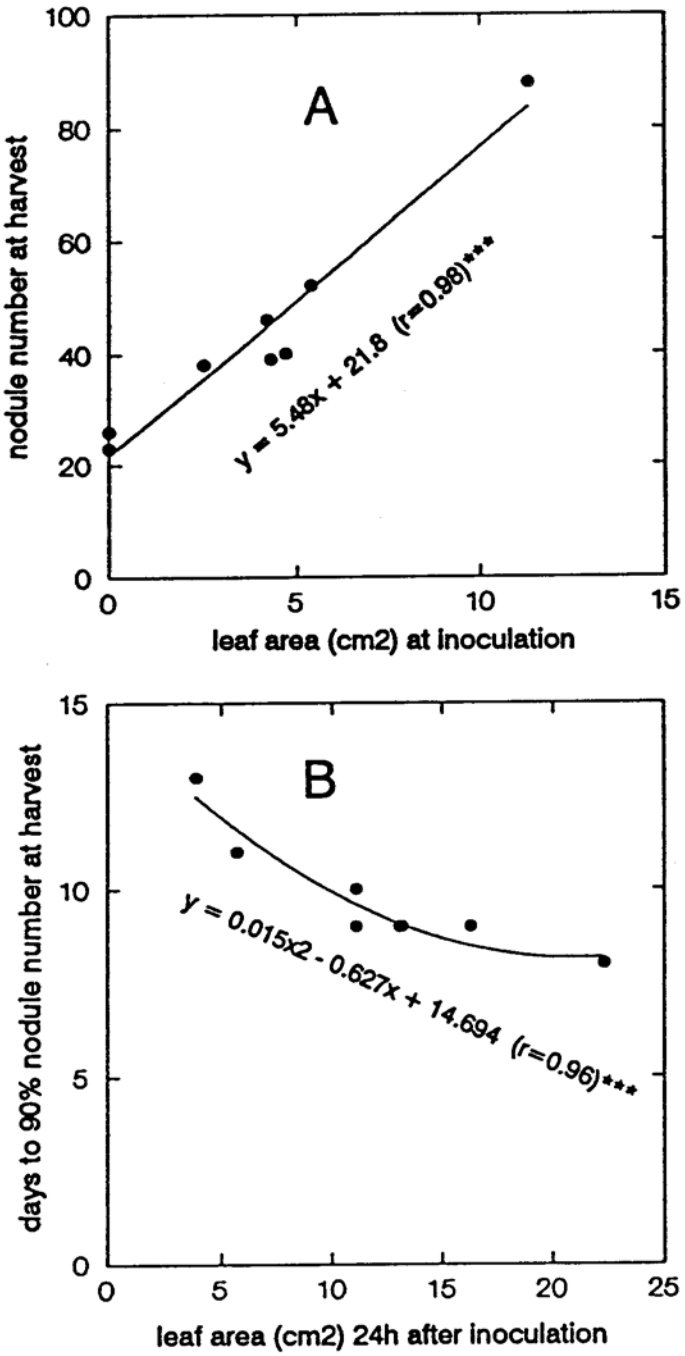


Figure II-7. Relationship between leaf area at the time of inoculation and nodule numbers at harvest - A; and between leaf area at inoculation and the rate of nodule development - B. Soybean plants with inoculated/uninoculated split-root system were grown in pouches and harvested 14 days after inoculation.

Table II-II. Nodule development on the early (E)/delayed (D) inoculated split-root system, when D side was inoculated 4 days after E side. The same plants, grown in pouches, were scored at 8, 12 and 21 days after D inoculation. Percent suppression was calculated as: $[1 - NO_{D\ side} / NO_{E\ side}] \times 100$. Semicircular bumps on the roots less than 1mm in diameter were classified as nodule primordia - NP; round structures with diameter equal to or greater than 1mm were classified as nodules - NO.

days after	E side		D side		
D inocul.	NO	NP	NO	NP	suppression
	number/side				%
8	32.0a[x]	7.1c	0.3c	3.1c	98.9a
12	35.2ab	6.5c	4.9c	3.5c	84.6b
21	45.7b	15.5b	12.0c	15.7b	70.3c

[x] Numbers of NO and NP, followed by different letters within the column or row are significantly different at $p \leq 0.10$, according to Tukey's HSD test.

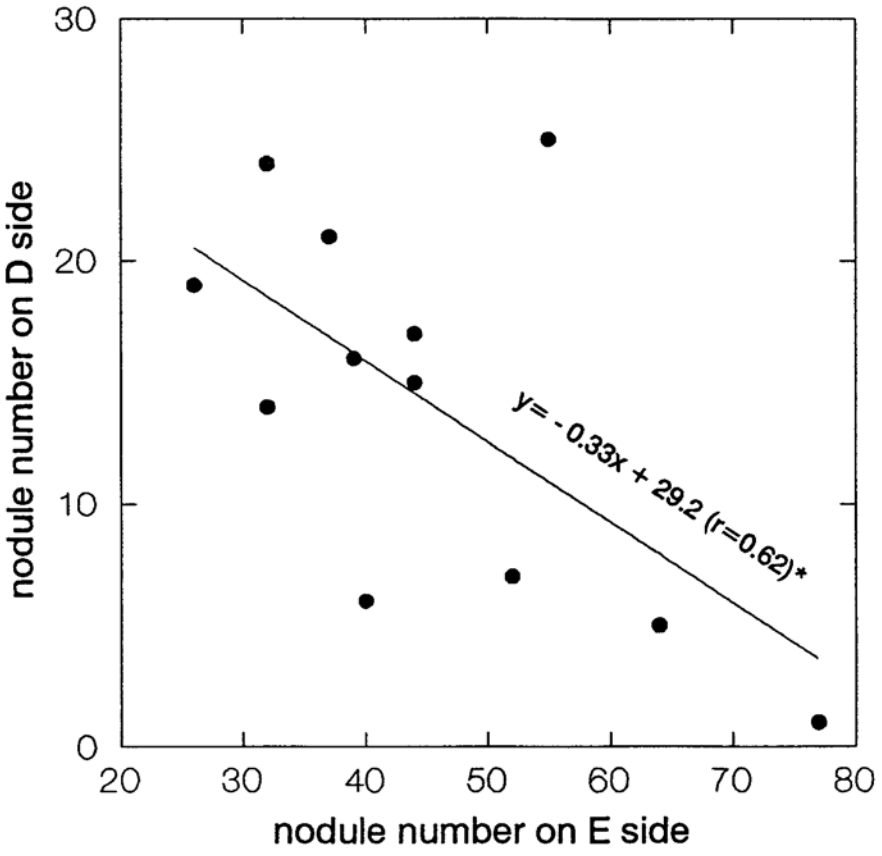


Figure II-8. Relationship between nodule numbers on early (E) and delayed (D) inoculated side of soybean split-root system, 21 days after delayed inoculation. Plants were grown in pouches and D side was inoculated 4 days after E side.

Table II-III. Nodule development on the early (E)/delayed (D) inoculated split-root system of soybean, when D side was inoculated 14 days after E side. Early side was either cut off (E-/D, treatment) or reinoculated (E+/D treatment) at D inoculation. The same plants, grown in pouches, were scored for nodulation at 12 and 21 days after D inoculation. Calculation of suppression and classification of nodule primordia - NP and nodules - NO are described in Table II-II.

days after		E side		D side		E + D side		
D inocul.	treatment	NO	NP	NO	NP	NO	NP	suppression
		number/side				- number/plant -		--- % ---
12	E+/D	25.3a	10.5a	18.0a	10.3a	43.3c	20.8a	23.9a
	E-/D	-	-	10.6a	97.6b	10.6a	97.6b	-
21	E+/D	34.0a	18.0a	26.3a	10.3a	60.3c	28.3a	23.3a
	E-/D	-	-	63.6c	62.1c	63.6c	62.1c	-

Numbers of NO and NP, followed by different letters within the column or row are significantly different at $p \leq 0.10$, according to Tukey's HSD test.

Table II-IV. Plant and nodule parameters in soybean plants with early/delayed inoculated split-root system, grown under different light intensity. Delayed (D) side was inoculated 4 days after early (E) side. Plants were harvested 21 days after delayed inoculation. Percent suppression was calculated as described in Table II-II.

environment	light intensity	nodule number		suppression	dry weight		
					nodules	roots	shoot
	µE/m2/s	E side	D side	%	mg/plant		
green house	1300 - 1700	125.8a	86.5a	32a	191.0a	357.2a	1632.3a
growth room	300 - 400	45.7b	12.0b	74b	72.6b	177.8b	957.3b

Numbers within columns, followed by different letters are significantly different at $p \leq 0.05$, according to Tukey's HSD test.

DISCUSSION

Further to close relationship between light intensity and nodulation observed by Kosslak and Bohlool (49) our results show that nodule numbers per plant and plant autoregulatory response are directly proportional to the light intensity in the growth environment (Table II-IV). Correlation between leaf area at the time of inoculation and nodule numbers at harvest (Fig. II-7 A) suggests that, increased nodulation obtained with delayed inoculation (Fig. II-4 A, Du side) is due to higher photosynthetic potential in older plants, rather than to larger infectible root area, as suggested by Nutman (59) for clover. The conclusion that, plant growth potential at the time of inoculation determines the number of nodule primordia and mature nodules per plant, is further supported by the experiment in which plants with only half root system, inoculated later, initiated more nodule primordia and developed essentially the same number of mature nodules as plants of the same age with complete root system inoculated earlier (Table II-III).

Similar total nodule mass on the E/D and U/D inoculated split-root system (Fig. II-4 B) and inverse relationship between nodule numbers on early and delayed inoculated root half (Fig. II-8) suggest a threshold for nodule numbers and nodule mass, depending on plant developmental stage and its growth potential. Reduced nodule mass on D side (Fig. II-4 B) cannot be accounted for by shorter period for nodule development on that side but, more likely, reflects greater partitioning of current photosynthate and dry matter to early initiated nodules at the expense of late initiated nodules. Approximately 16 hour interval between early and delayed inoculation seems sufficient for early initiated nodules to establish a prevailing sink (Fig. II-4 B).

Pierce and Bauer (66), using different experimental protocol from ours, showed that second inoculation 15 hours after the first produced virtually no nodules when the first inoculum dose was optimized for nodule

yield. By contrast, our results (Fig. II-4) indicate that plant autoregulatory response affected nodule development (nodule mass) much more than nodule initiation (nodule numbers). The rate of nodule development may vary with plant growth potential (Fig.II-7 B) and nodules produced by the second inoculum apparently develop at a slower rate than those produced by the first (Table II-II). Therefore, observed autoregulatory response may vary considerably with experimental conditions affecting plant growth and with the interval after second inoculation, when nodules are scored.

Nitrogenase activity in nodules on the E side was first detected 10 days after (E) inoculation and in nodules on D side 8 days after (D) inoculation. Little suppression of nodule development on D side observed with 14-day delay of the second inoculation (Table II-II), compared to 4-day delay of second inoculation, suggests that, other factors became involved in regulation of nodule development after the onset of nitrogen fixation in first mature nodules. According to Atkins (3) there is a considerable lag period between the start of nitrogen fixation and substantial N export from nodules. In the absence of mineral nitrogen, N deficiency symptoms are regularly observed in plants at the onset of nitrogen fixation, since cotyledonary N reserve is depleted and leaf N is mobilized for nodule development (4). Nitrogen limitation to plant growth generally results in increased photosynthate and dry matter partitioning to roots (85) and presumably allows for development of additional nodules from nodule primordia initially arrested in further development (Table II-III). Singleton and Stockinger (72) have shown differential allocation of plant dry matter to effective and ineffective nodules. It seems therefore, that carbon partitioning related to nitrogen fixing efficiency of early nodules may ultimately determine nodule number and mass on legume roots.

CHAPTER III

PHOTOSYNTHATE PARTITIONING AND AUTOREGULATION OF SOYBEAN (Glycine max L., Merr) NODULE DEVELOPMENT.

ABSTRACT

Control of the number of nodules formed on legume roots is known as autoregulation. Postulated mechanisms of autoregulation involve inhibitory substances produced by either early developing nodules or shoots. Other results, however, have indicated a regulatory role for photosynthate partitioning in autoregulation. In the present study, one side of a split-root system of soybean plants was inoculated at 8 days from planting and the other either inoculated 4 days later (early/delayed) or remained uninoculated (early/uninoculated). Plants were labeled with $^{14}CO_2$ and photosynthate partitioning to developing nodules and roots was evaluated from the time of early inoculation until N_2 fixation (acetylene reduction) was detected. After staining with Eriochrome black T, roots and developing nodules were separated into 4 root categories and 4 nodule categories, based on structure and developmental stage. Differential partitioning of ^{14}C to root and nodule structures was monitored by autoradiography of intact root systems and quantified by scintillation counting of excised root and nodule structures. Specific radioactivity of nodule structures increased with developmental stage and was up to four times greater in early nodules compared to both, nodules on delayed inoculated root half and roots, whose sink intensity decreased progressively as nodules developed. By 7 days after inoculation, early inoculated half root system accounted for over 70% of the radioactivity recovered in the whole root system. These results suggest that, competition between early and late initiated nodules for current photosynthate play an important role in early regulation of nodule development. Nodules initiated

later apparently become deprived of current photosynthate and their development slowed or arrested at an early developmental stage.

INTRODUCTION

Control of the number of nodules formed on legume roots after infection by rhizobia is known as autoregulation. Autoregulation appears to be an intrinsic plant regulatory mechanism (20) and is manifested as a suppression of subsequent nodulation by early developing nodules (35, 49, 58, 59, 66, 69). Studies with different legume species, suggested variable regulatory mechanism(s) in common legumes. Induction of cell divisions in the root cortex by compounds diffused from rhizobia, invading root hairs, is necessary and sufficient to elicit plant regulatory response in soybean (19, 77) and alfalfa (20). However, regulatory response in alfalfa prevents initiation of new nodules (20), while in soybean it suppresses the development of late initiated nodules (24). In common bean (Phaseolus vulgaris) nodule formation and proliferation of rhizobia within them seems to be required for suppressive effect of early nodules on late infections (35).

Nutman (58) proposed that nodule development in red clover was controlled by inhibitors produced by early nodule and root meristems (58, 59) since excision of early nodules and root tips stimulated further nodulation (60). Results of exactly the same type were obtained in alfalfa (20) and soybean (22). Split-root (49) and grafting techniques (27) demonstrated a systemic nature and shoot control (27) of the autoregulatory response in soybean. Caetano-Anolles and Gresshoff (19) postulated a shoot derived inhibitor induced by early infections (first initiated nodules), which then suppresses development of late initiated infections. The shoot derived inhibitor is apparently lacking in supernodulating soybean mutant (61).

Kosslak and Bohlool (49), using different levels of shading, showed that nodule number per plant and intensity of autoregulatory response was directly related to the amount of photosynthetically active radiation available to soybean. These results suggested that competition for current

photosynthate between early and late initiated nodules could provide a mechanism for autoregulation.

Immediate dependence of N_2 fixation on carbohydrate supply from photosynthesis (84), as well as selective partitioning of current photosynthate to effective nodules at the expense of ineffective nodules (71) have been clearly demonstrated. However, differential partitioning of photosynthate to roots and developing nodules before the onset of N_2 fixation has so far not been reported.

To determine the role for photosynthate partitioning in control of nodule development, we evaluated sink intensity of developing nodules and roots from the time of inoculation until the start of nitrogen fixation in first mature nodules.

MATERIALS AND METHODS

Growth system: A variant of a split-root procedure described in material and methods in Chapter II (Figure II-2) was used.

Planting procedure: Seeds of soybean (Glycine max L., Merr.) cv. D-68 (T.E. Carter, Dept. of Crop Science, NC State University at Raleigh) were surface sterilized with 2.5 % NaClO for 5 minutes, rinsed 7 times with sterile H_2O, imbibed for 4 hours and then sown hillum down in moist, sterile horticultural vermiculite. Approximately 48 hours after sowing, uniform seedlings (1.5 to 2.5 cm radicle) were selected, the tips of the radicles cut off and seedlings planted into the center of the trough (Fig. 1-A). Growth assemblies were covered with transparent polyethylene film to provide sufficient humidity for lateral root growth. Strip connectors (Fig. 1-A) between the trough (Fig. 1-B) and the wick (Fig. 1-C) were removed after the roots had grown a few cm down the wick (4 to 5 days after planting). At that point, split roots were selected for uniformity and trimmed to leave only 2 uniform roots per pouch.

At planting, 25 ml N-free plant nutrient solution (PNS) was added per pouch and subsequently maintained at a level 1 to 3 cm from the bottom of the pouch with half strength PNS. Concentrations of nutrients in PNS were as described previously (Chapter II, materials and methods).

Plants were grown in the growth room under average PAR 300 to 400 $\mu E/m^2/sec$, 18 h photoperiod and temperature range 23 to 27°C.

Inoculation treatments: Bradyrhizobium japonicum strain USDA 110 (TAL 102, obtained from the NifTAL Project collection) as peat based inoculant was suspended in N-free PNS so that 2.5×10^7 cells was applied per root half in 4 ml of inoculum. Cell density in PNS was determined by drop plate method (43).

Half root portions were first inoculated (early inoculation) 8 days after planting, when the roots had grown to the bottom of the

pouches and at least one trifoliate leaf had emerged on the shoot. The other root half was either inoculated 4 days later - early(E)/delayed(D) treatment, or remained uninoculated - early(E)/uninoculated(U) treatment.

^{14}C labeling and root processing procedure: Plants of both treatments (E/U, E/D) were placed in a sealed clear plastic chamber at 24 hour intervals from l to 12 days after early and delayed inoculation. Tops of the split-root assemblies were sealed with plastic tape and tape caulk (Mapco, Inc., Cleveland, OH) around the stem to minimize direct 14CO2 incorporation by roots and nodules. The $^{14}CO_2$ (45-80 µCi/plant, progressively increasing with plant development) was generated by injecting 10 to 20 ml 3.6 N sulfuric acid through a serum stopper into a beaker containing 2 ml $NaH^{14}CO_3$ (ICN Biomedicals, Inc.) in 0.1N NaOH. A fan within the chamber was used to circulate $^{14}CO_2$. Plants were allowed to assimilate $^{14}CO_2$ for 70 min and the chamber opened for additional 20 mins for translocation of assimilates. It was shown previously (38) that, root and nodule radioactivity in $^{14}CO_2$ pulsed soybean peaks approx. 90 minutes after the start of $^{14}CO_2$ fixation by the leaf. Plants were then placed on ice, roots separated from the shoot, stained with Eriochrome black T, prepared after Bohlool (13), for 10 to 15 mins, rinsed in half strength PNS, then submerged in 50 ml half strength PNS with 0.1% Thimerosal (Sigma), added as preservative, and stored at 4°C until dissected.

Analysis of labeled tissue: Two plants of each treatment were used for dissection and one for autoradiography. Root halves of one plant/treatment were dissected, immediately one after another, under a dark field microscope (Wild M7 S). Nodule and root meristematic structures (1 to 5 mm root segments) were excised and grouped, based on structure and developmental stage. Classification of root and nodule structures is presented in Figure III-1. The remaining portions of "tap" and lateral roots were grouped

separately. Excised and grouped nodule and root structures were air dried, weighed, placed in the scintillation vials, rehydrated with 0.30 ml H_2O, solubilized in 1 - 2 ml Soluene 350 (Packard) at 45°C in a water bath overnight and then suspended in 15 ml of scintillation cocktail (Hionic-fluor, Packard). Radioactivity was determined on a Packard 22000A Tri-carb scintillation analyzer.

Roots for autoradiography were stained as described above, stored in half strength PNS overnight at 4°C, then freeze-dried, pressed in a vice as described by Turgeon and Wimmers (80) and autoradiographed with Kodak X-OMAT AR film (Eastman Kodak). Exposure time was 2 to 3 days at room temperature. Identical autoradiographs were obtained from stained and unstained (control) roots, that were freeze-dried immediately after labeling.

Scintillation counts of root and nodule structures on stained and unstained test roots, labeled 7 or 14 days after inoculation and stored over 6 weeks showed that neither staining nor prolonged storage significantly affected distribution of radioactivity in roots.

Determination of nitrogenase activity: Half root systems of extra plants (2 to 4 replicates) for each treatment were placed into 100 ml test tubes at 24 h intervals from 6 - 12 days after E or D inoculation. Tubes were injected through a serum stopper with 5 ml acetylene and ethylene production was determined by gas chromatography (Varian 940 GC).

Statistical analysis: Data were analyzed by Tukey's HSD test using SYSTAT statistical package (87).

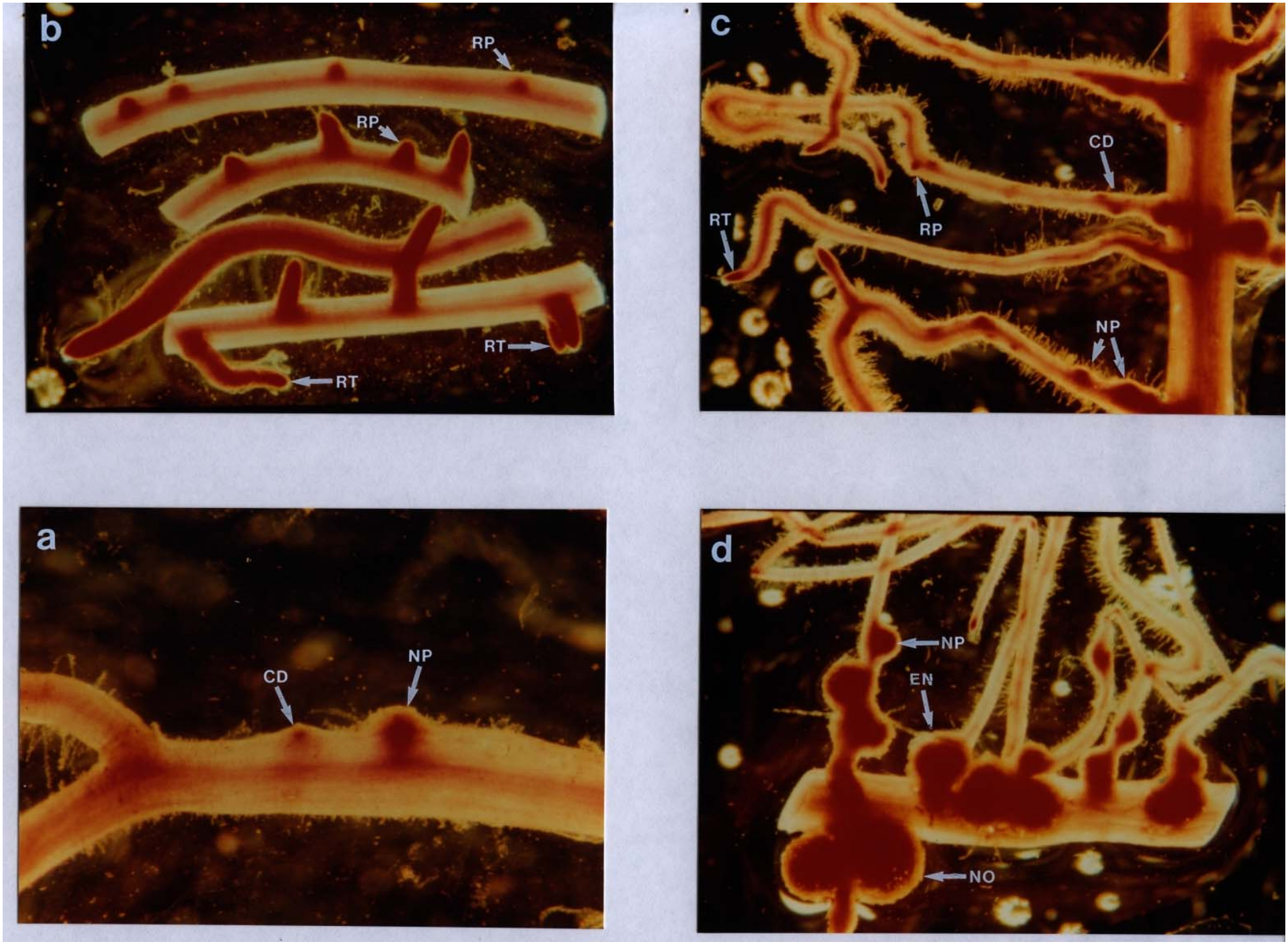
Figure III-1. Classification of nodule and root structures:

a - subepidermal cortical cell division centres (CD) which may represent the initial nodule meristems. Nodule meristems with vascular connection to the root steele and diameter up to 0.5 mm were classified as nodule primordia (NP).

b - root primordia (RP) were distinguished from NP by the site of their initiation and their shape; RP higher than 0.5 mm (measured from the root surface) were classified as root tips (RT).

c - distribution of root and nodule meristems on the root system.

d - round structures with diameter between 0.5 and 1 mm were classified as emerging nodule (EN); round structures with diameter equal to or greater than 1 mm were classified as nodules (NO).


b
RP
RP
RT
RT
c
CD
RP
RT
NP
a
CD
NP
d
NP
EN
NO

RESULTS

Nodule initiation and development on early and delayed inoculated root half.

Individual nodule developmental stages, defined in Figure III-1, occurred at the same time from inoculation on early (E) and on delayed (D) inoculated root side. First cortical cell division centers (CDs) could be identified at 3 days from inoculation, first nodule primordia at day 5, emerging nodules at day 6 and nodules at day 7 after inoculation. Numbers of symbiotic and root meristematic structures, on the E/U and E/D inoculated split-root systems are presented in appendices III-1 and III-2. There were essentially the same number of CDs on E and D half root systems but significantly less CDs advanced to successive developmental stages on D side compared to E side (Table III-I). Nitrogenase activity (acetylene reduction) was first detected at 10 days after inoculation on E side and 8 days after inoculation on D side. The onset of nitrogenase activity coincided with the occurrence of nitrogen deficiency symptoms in plants and was not related to nodule size or number. No further increase of nodule mass on D side was observed after the onset of nitrogen fixation on E side, where nodule mass increased well into the nitrogen fixation stage (Figure III-7).

Sink intensity of developing nodules and roots

Respiratory loss of ^{14}C from root and nodule structures was not measured. Based on findings of Gordon et al. (38) that, $^{14}CO_2$ respiration parallels ^{14}C-sugar content in the sink tissue, it was assumed relative differences in radioactivity of separated (excised) root and nodule structures reflect differences in the import of labeled photosynthate.

Distribution of radioactivity within the root system, presented in Figure III-2, indicates the flow of labeled photosynthate to developing nodules and root tips which appear as major sink for current photosynthate. Based on scintillation counts, sink intensity

of individual root and nodule structures was characterized by two parameters: 1) specific radioactivity (SA = dpm / mg dry weight), which indicates the flux of labeled photosynthate to or through the sink tissue; 2) relative specific radioactivity (RSA = % dpm / % dry weight), which indicates partitioning of labeled photosynthate to the sink tissue relative to its size. Specific and relative specific radioactivity of individual nodule and root structures is presented in appendices III-3 to III-8.

Pooled data for nodule and root structures are presented in Figures III-3 and III-4. Both, specific and relative specific radioactivity indicate sink intensity of developing nodules on the E side was 3 - 4 fold that of the roots. By contrast, specific radioactivity of nodules on D side indicates reduced flux of photosynthate to those nodules (Figure III-3) compared to nodules on E side. However, relative to the amount of infected tissue (sink size), early (E) and late (D) nodules show similar sink intensity characterized by relative specific radioactivity (Figure III-4). Following the appearance of first mature nodules, their associated roots became increasingly deprived of labeled photosynthate (Figure III-2 E, Figure III-4).

Current photosynthate and dry matter partitioning within and between the opposite sides of the split roots.

Relative to roots, partitioning of labeled photosynthate to nodules increased exponentially with nodule development. The rate of increase was similar on the E side in both treatments (E/U, E/D), but significantly slower on D side. At the onset of nitrogen fixation on the respective sides, nodules accounted for over 60 of the radioactivity within the E side and for less than 10% of the radioactivity within D side (Figure III-5). In both treatments (E/U, E/D), increased photosynthate partitioning to E side coincided with the development of first mature nodules on that side. Even

before first mature nodules developed on D side that side was already deprived of current photosynthate (Figure III-6).

Differences in photosynthate partitioning to roots and nodules on the opposite sides of the split-roots were clearly reflected in dry matter partitioning, which represents an integrated value of photosynthate partitioning over time (Figure III-7). Increased photosynthate partitioning to early nodules (Figure 6) reduced root growth on early and delayed inoculated side as well as nodule development on D side (Figure III-7).

Table III-I. Number and propotion of cortical cell division centers (CD) that developed into nodule primordia (NP), emerging nodules (EN) and mature nodules (NO) on the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root system of soybean. D side was inoculated 4 days after E side. Numbers of nodule structures (CD, NP, EN, NO), classified in Figure III-1, represent mean values for observations between 9 and 12 days after E or D inoculation when all numbers reached a constant average.

inoculation treatment	root side	CD		NP		EN		NO	
		number per side	%CD	number per side	%CD	number per side	%CD	number per side	%CD
E/U	E	202a	100a	91a	45a	57a	29a	31a	16a
E/D	E	209a	100a	75a	36a	54a	26a	41a	20a
E/D	D	197a	100a	34b	17b	16b	8b	9b	5b

Numbers within columns, followed by different letters, are significantly different at $p \leq 0.01$, according to Tukey's HSD test.

Figure III-2. Autoradiographs of the early (E) and delayed (D) inoculated side of the split-root system of the same plant, harvested 11 days after E inoculation (7 days after D inoculation). Framed areas (E1 to D4) show the corresponding details on the stained intact roots. Note that eriochrome black dye accumulates in the same structures as radioactivity and that nodule primordia (NP) in the vicinity of nodules (NO), as well as roots, distal from nodules are deprived of current photosynthate. Abbreviations: NO = nodule, EN = emerging nodule, NP = nodule primordium, CD = cortical cell division center, RT = root tip, RP = root primordium.

X 1.4
E
1
2
X 27
E1
NO
NP
X 21.6
E2
NO
X 1.4
D
1
2
3
4
X 21.6
D1
NP
X 28.8
D3
RT
RP
X 21.6
D2
NP
CD
EN
NP
X 21.6
D4
RP

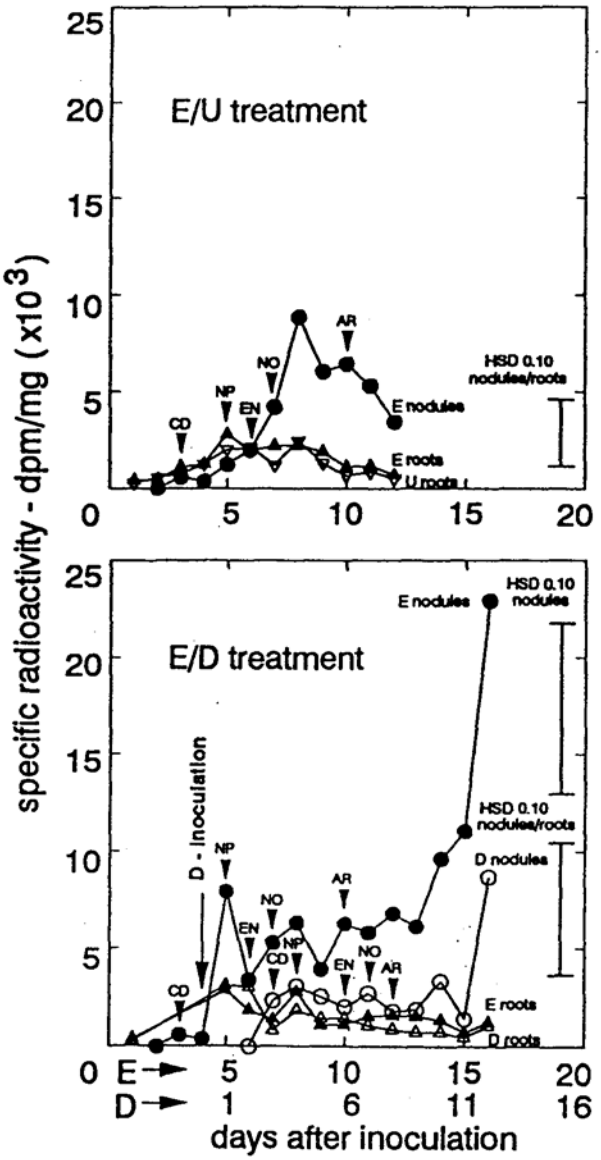


Figure III-3. Specific radioactivity of developing nodules and roots on the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root systems of soybean. Arrowheads indicate the appearance of nodule developmental stages (classified in Fig.III-1) and the onset of nitrogen fixation (acetylene reduction): CD = cortical cell division centers, NP = nodule primordia, EN = emerging nodules, NO = nodules, AR = acetylene reduction.

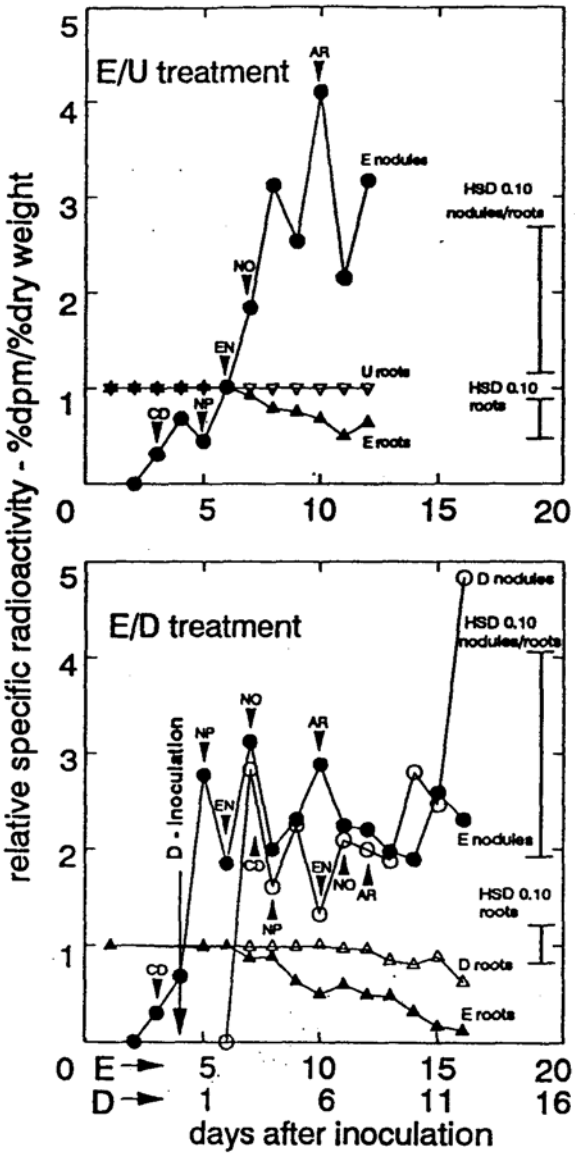


Figure III-4. Relative specific radioactivity (ratio of proportion of radioactivity recovered in below ground tissue to proportion of below ground tissue dry weight) of developing nodules and roots on the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root systems of soybean. Arrowheads indicate the appearance of nodule developmental stages (classified in Fig.III-1) and the onset of nitrogen fixation (acetylene reduction): CD = cortical cell division centers, NP = nodule primordia, EN = emerging nodules, NO = nodules, AR = acetylene reduction.

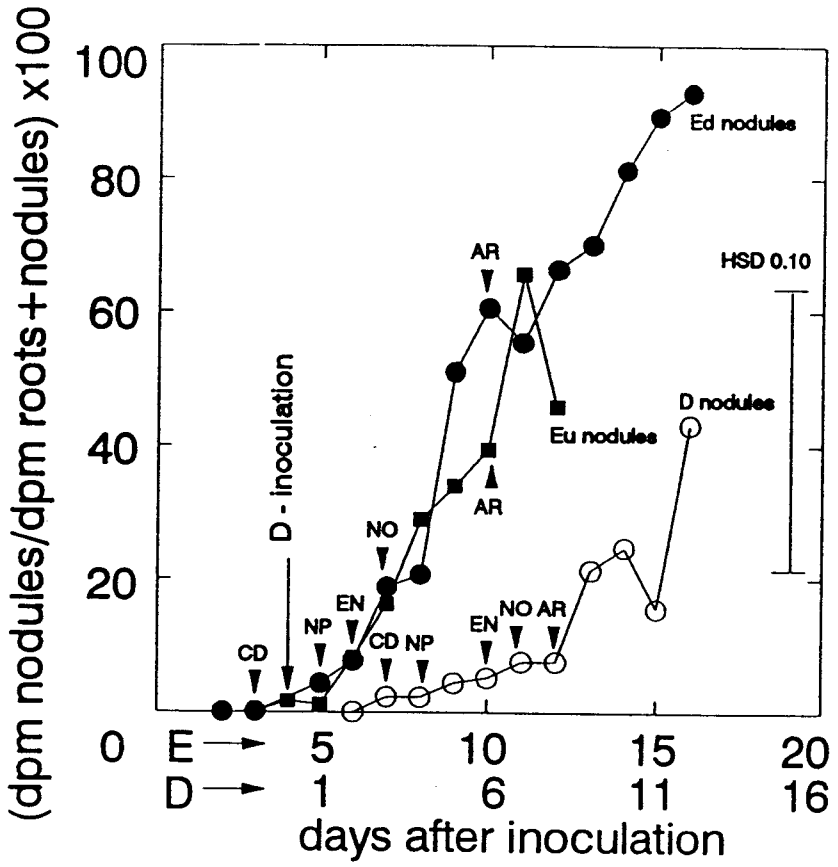


Figure III-5. Relative amount of total radioactivity recovered in soybean half root systems partitioned to nodules.
Eu = early inoculated side of ealy/uninoculated split-root system;
Ed = early inoculated side of early/delayed inoculated split-root system;
D = delayed inoculated side;
Arrowheads indicate the appearance of nodule developmental stages (classified in Fig.III-1) and the onset of nitrogen fixation (acetylene reduction): CD = cortical cell division centers, NP = nodule primordia, EN = emerging nodules, NO = nodules, AR = acetylene reduction.

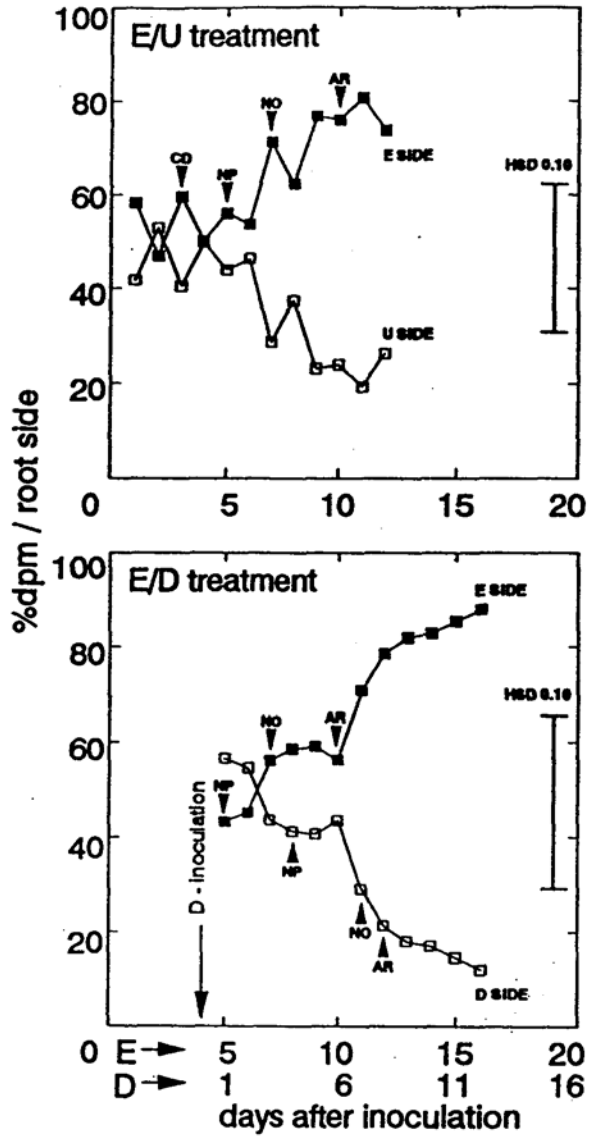


Figure III-6. Relative amounts of ^{14}C radioactivity recovered from the opposite sides (roots+nodules) of the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root systems of soybean. Arrowheads indicate the appearance of nodule developmental stages (classified in Fig.III-1) and the onset of nitrogen fixation (acetylene reduction): CD = cortical cell division centers, NP = nodule primordia, EN = emerging nodules, NO = nodules, AR = acetylene reduction.

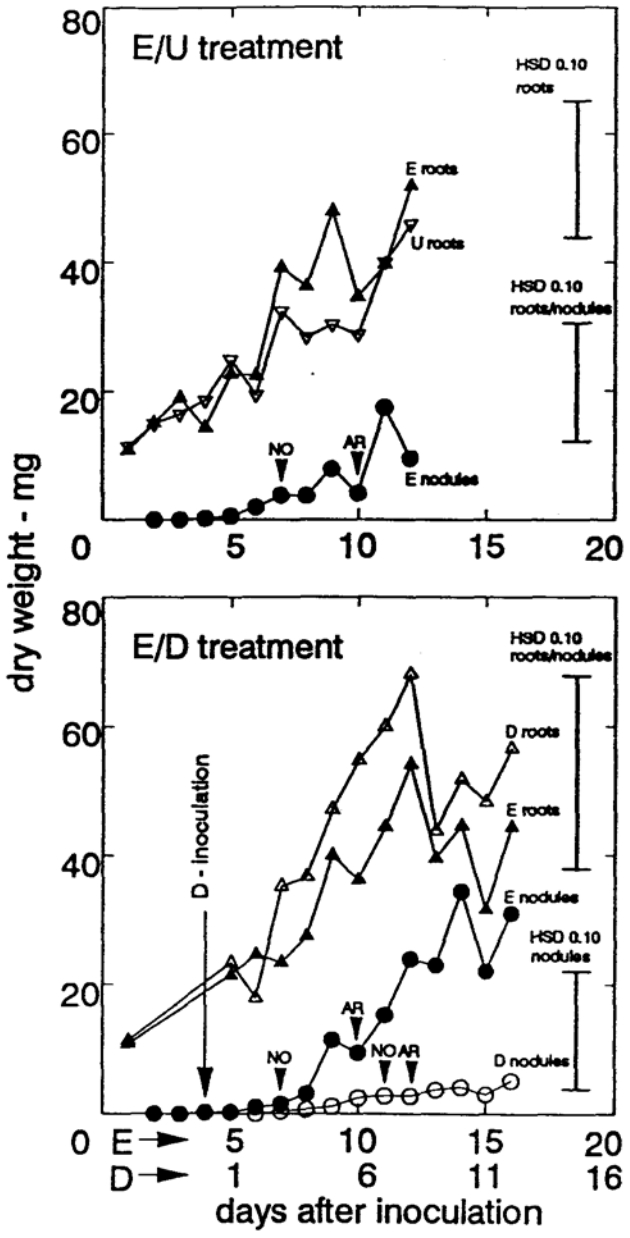


Figure III-7. Dry weight of roots and nodules on the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root system of soybean. Arrowheads indicate the appearance of first nodules (NO - classified in Fig.III-1) and the onset of nitrogen fixation (AR = acetylene reduction).

DISCUSSION

Further to the findings of Calvert at al. (24), that autoregulation operates via the arrest of development of cortical cell division centers, the pattern of nodule development on the early and delayed inoculated roots in our experiment (Table III-I) indicates that, autoregulation is not a single step but, rather a continuous process where nodule development can be arrested at any stage of development of cortical cell division centers (CDs) into functional nodules. From equal numbers of CDs on early (E) and delayed (D) inoculated half root system, progressively less CDs advanced into each successive developmental stage on D side than on E side.

Much higher specific and relative specific radioactivity of developing nodules, compared to roots (Fig. III-3, Fig. III-4), indicate that even early nodule structures are much stronger sinks for current photosynthate than roots. Root tips among root structures and mature nodules among root structures were the most intense sinks (Appendices III-3 to III-8). Nodule sink intensity, as measured by specific radioactivity (Fig. III-3), increased with nodule developmental stage and, with the development of advanced nodule structures, sink intensity of early nodule structures, as well as, sink intensity of root structures decreased (App. III-5, App. III-8, Fig. III-3, Fig. III-4).

Current photosynthate and dry matter partitioning to early and late initiated nodules and their associated roots (Figures III-5 to III-8) shows the tremendous cost in terms of carbon for early nodule development and clearly indicates that early nodules develop at the expense of late initiated nodules, as well as, at the expense of root growth. Immediate dependence of nodule numbers per plant and of the intensity of autoregulatory response on the amount of photo synthetically active light, available to soybean plant (49, chapter II - Table II-IV) strongly suggest early nodule development is limited by

carbon from photosynthesis. Thus, competition between early and late initiated nodules for a limited amount of photosynthate becomes more vigorous as nodules develop.

Regulation of organ growth and development by photosynthate partitioning, controlled by source limitation and sink demand, is a common process in higher plants (85). Once a potential sink is established, competitive success of that organ depends on the development of an adequate vascular link for the supply of carbon and nutrients, apart from the growth characteristics of that organ, imposed by growth regulators (85, 86). A model postulating translocatable signals acting as growth regulators in the earliest stages of soybean nodule development has been proposed by Caetano-Anolles and Gresshoff (19, 39). Their study (19) also shows that at least some meristematic activity in the early inoculated root is necessary to induce feedback suppression of nodulation in delayed inoculated root.

According to Calvert et al. (24), in soybean, only CDs closely associated with the infection threads develop into nodule primordia, which are characterized by vascular connection between the nodule meristem and root steele (Fig. III-1). In our study, the beginning of selective photosynthate partitioning to early inoculated root side coincided with the onset of nodule development - i.e. with the development of first nodule primordia on that side (Fig. III-6). Thus, vascular connection of potential nodule meristems to the root vascular system - a transition of a cortical cell division center into a nodule primordium, may clearly represent the early determinant for successful development of infection into a nodule. Consequently, due to their developmental and thus competitive advantage as sinks for current photosynthate, first established nodule primordia are also the first to develop into functional nodules, while nodule primordia

initiated later are deprived of current photosynthate and their development slowed or completely arrested. Caetano-Anolles et al. (22) showed that excision of first formed nodules allows for development of nodule primordia, that are clustered around early nodules (Fig. III-1 d), and were initially suppressed.

High proportion of CDs that develop into nodules in alfalfa (20) and in supernodulating soybean mutant (55) suggest that, autoregulatory response in different species or genotypes of the same species may be related to the overall pattern of nodule development (e.g. indeterminate type nodules in alfalfa compared to determinate type in soybean) or to some underlying mechanism controlling the rate of transition of CDs into nodule primordia (55). Nodule primordia formed at high rate (55) are likely to be equally competitive as individual sinks and most of them may therefore develop into functional nodules.

CHAPTER IV

RELATIONSHIP BETWEEN COMPETITION PATTERN AND THE RATES OF NODULE FORMATION BY THE TWO STRAINS OF *Bradyrhizobium japonicum* ON A SPLIT-ROOT SYSTEM OF SOYBEAN (*Glycine max*, L., Merr.)

ABSTRACT

Due to auto regulatory control of nodule numbers on legume roots rhizobial strains compete for nodule occupancy on the common host. Interstrain differences in the rate of root colonization and in the rate of nodule initiation have been proposed as determinants of the outcome of interstrain competition. We compared competition pattern and nodule initiation rates by a highly competitive (USDA 110) and poorly competitive (USDA 38) strain of *B. japonicum* on a split-root system of soybean using 3 inoculation treatments: 1) two strains inoculated on half root, the other half remained uninoculated (direct competitive system); 2) two strains inoculated on the opposite root halves (indirect competitive system); 3) single strain inoculated on half root, the other half remained uninoculated (noncompetitive system). The same experiment was done in vermiculite and in growth pouches. Root staining and a serological procedure for blocking the fluorescence of surface attached rhizobia were used to identify the occupants in early nodule primordia 5 days after inoculation, in early nodules 10 days after inoculation and in mature nodules 21 days after inoculation. In the indirect competitive system (on the opposite root halves), USDA 110 formed 85% and 63% of nodule primordia and 75% and 74% of mature nodules per plant in vermiculite and in growth pouches. When the two strains were in direct competition (on the same root half), USDA 110 formed 70% of nodule primordia and 94% of mature nodules in vermiculite but only 25% of nodule primordia and 48% of mature nodules in growth pouches. Even

though USDA 110 still dominated among singly occupied nodules, since 75% of nodule primordia and 31% of mature nodules was occupied by both strains. In the noncompetitive system the two strains formed similar numbers of infections (cortical cell division centers and nodule primordia) and similar numbers of mature nodules, whereas in the indirect competitive system USDA 110 initiated 3 to 5 times as many infections and formed 3 times as many nodules as USDA 38.

These results suggest that interstrain competition pattern is determined before the formation of nodule primordia and well before the release of rhizobia into plant cells. Environmental factors (growth medium, light intensity) that affected nodulation and competition, apparently affect the early interactions between the symbiotic partners, which determine the number of infections initiated by competing strains.

INTRODUCTION

Response of leguminous plants to inoculation with superior nitrogen fixing strains of rhizobia in the field depends to a large extent on the size and characteristics of soil indigenous populations of homologous rhizobia (78). Indigenous strains compete with the introduced strain for nodule occupancy on the common host. Knowledge of mechanisms involved in interstrain competition provides criteria for selection and genetic engineering of superior symbiotic partners.

To elucidate this mechanisms, competition has been studied extensively in soil and in artificial growth systems. Amarger and Lobreau (1) proposed that the numerical ratio between the strains (introduced vs. indigenous) in soil or in the inoculum mixture, determined nodule occupancy by each strain. They introduced competitive index (1) to account for differences in strain competitiveness when various pairs of strains are inoculated in equal numbers.

Kosslak et al. (48) have shown that, preexposure of soybean roots to a less competitive strain for as little as 6 hours, before the introduction of a more competitive strain, substantially increases nodule occupancy by a less competitive strain. Fernandez-Flouret and Cleyet-Marel (33), using different pairs of strains, obtained similar results with even shorter preexposure periods. Both groups of authors concluded that early events in the infection process determine the outcome of interstrain competition.

Bohlool (14) postulated that competition is a post infection phenomenon, related to the speed of nodule development by competing strains. McDermot and Graham (56) correlated competitiveness of B. japonicum strains, determined in paired tests with a standard strain in vermiculite, to their individual "nodule forming efficiency", determined by the number of nodules

formed above the root tip mark at the time of inoculation in growth pouches (cf.56). Correlation between nodule initiation rate and competitiveness did not, account for all the strain combinations and did not hold for inoculum doses higher than 10^6 cells per plant.

Infection and nodulation process includes several morphologically and physiologically distinct steps. Strain attributes responsible for root infection (7, 21, 68) and host attributes controlling nodule development (21, 24, 68, 77) may determine the outcome of interstrain competition.

In studies published so far, interstrain competition patterns were determined in functional nodules. Available evidence, however, indicates that host/strain interactions in the earliest stages of the infection process determine the outcome of interstrain competition.

We developed two procedures to identify early nodule primordia and rhizobial cells within. To evaluate the contribution of early (during infection) and late (during nodule development) interactions between the symbiotic partners to the outcome of competition we compared competition patterns between a highly competitive (USDA 110) and poorly competitive (USDA 38) strain of B. japonicum in nodule primordia and in functional nodules. Competition patterns were then related to nodule initiation rates by the two strains, inoculated simultaneously on the opposite halves of the split-roots (competitive system) or singly on half root system (noncompetitive system). To include the effects of environmental factors, the same experimental protocol was carried out under greenhouse and growth room conditions.

MATERIALS AND METHODS

Growth systems: Two variants of a split-root system described by Singleton (70) were used:

1) The growth pouch assembly described in Chapter II (Figure II-2) was used in the growth room experiment.

2) For the greenhouse study, two square pots (0.7 L) were taped together on a tongue depressor, serving as a base. Polyethylene bags were placed inside the pots and filled with dry horticultural vermiculite. Plastic elbows (90 deg. angle, 1/2" diameter) with a 13mm hole, drilled in the center were used to direct split roots into pots.

Planting procedure: Seeds of soybean (Glycine max L., Merr.) cv. Lee were surface sterilized, germinated and planted as described in Chapter II. The top of the pot split-root assembly was covered with aluminum foil. At planting, 30 ml of N-free plant nutrient solution (PNS) was added per pouch and subsequently maintained at a level 1 to 3 cm from the bottom of the pouch with half strength PNS. Plants in pots received 200 ml PNS per pot at planting and additional 200 ml per pot 10 days after inoculation. Concentrations of nutrients in PNS were as described in materials and methods in Chapter II. Plants in pouches were grown in the growth room under average PAR 350 $\mu E/m^2/sec$, 18 h photoperiod and temperature range 23 to 27°C. Plants in vermiculite were grown in the greenhouse under PAR 1350 - 1600 $\mu E/m^2/sec$, approximately 13 h photoperiod and temperature range between 13 and 37°C.

Inoculation treatments: Bradyrhizobium japonicum, strains USDA 110 and USDA 38 were obtained from the Niftal Project collection. Six day old YEM broth cultures were diluted with N-free PNS adequately, so that 10^8 cells was applied per root side - in 1 ml of inoculum per pouch and in 50 ml of inoculum per pot. This presumably ensured similar distribution of bacteria along the roots in the two growth media. Cell density in broth cultures was determined by counts on black polycarbonate filters (Poretics Corp.,

Livermore, CA) using specific FAs for the two strains and later verified by drop plate counts (43). For mixed inoculations, strains were mixed in the ratio 1:1, according to filter counts. Plate counts indicated ratio of USDA 110 : USDA 38 of 1.0 : 1.1 in vermiculite and 1.0 : 1.3 in pouches.

Plants were inoculated 8 days after planting, when the roots had grown to the bottom of the pouches and pots and at least one trifoliate leaf had emerged on the shoot. Three inoculation treatments were imposed on split roots in each growth medium: 1) one side inoculated with either USDA 110 or USDA 38, other side uninoculated; 2) one side inoculated with USDA 110, other side with USDA 38; 3) one side inoculated with a mixture of USDA 110 and USDA 38, other side uninoculated.

Identification of early nodule structures and rhizobial strains: Sets of plants were harvested at 5, 10 and 21 days after inoculation. For the identification of cortical cell division centers and nodule primordia (for classification see Chapter III, Figure III-1), roots were separated from the shoot, stained with Eriochrome Black T, prepared after Bohlool (13) for 10 to 15 min, quickly rinsed in PBS, then submerged in 50 ml PBS with 0.01 Thimerosal (Sigma) and stored at 4°C until dissected. Double inoculated roots were shaken in 250 ml 0.5 N NaOH with 4g glass beads (75 - 150 µm) for 1 hour and then washed 3 X 15 mins in phosphate buffer (pH 7.1) on a wrist action shaker prior to staining. Prior to dissection these roots were incubated in 1:1: 50 mixture of unconjugated (to FITC) antisera specific for each strain and phosphate buffer saline (PBS) for 1 hour at 37°C with minimum rotary shaking; then rinsed in PBS (pH 7) incubated 1/2 hour in PBS at room temperature and stored in H_2O for dissection.

Nodule primordia and early nodules (10 days after inoculation) were excised under the dissecting microscope, air dried and stored for strain identification. Rhizobia within nodule primordia and nodules were identified with FAs specific for each strain, according to Bohlool (13).

Rehydrated nodule primordia were crushed on microscopic slides and nodules in microtiter plates (Immulon2, Dynatech Laboratories, Inc.) and smeared in duplicates. Examples of FA reactions in nodule primordia and in nodules are presented in Figure IV-1. Treatment with NaOH removed most of the rhizobia attached to the root surface (Fig. IV-1) and antiserum treatment efficiently blocked the fluorescence of the remaining cells. Test observations of the intact root segments and of crushed nodule primordia proved that these two treatments virtually eliminated interference of surface attached bacteria with identification of rhizobia within nodule primordia and nodules. Rhizobia within nodule primordia could be first detected 4 to 5 days after inoculation.

Determination of nitrogenase activity: Extra plants for each treatment were tested for nitrogenase activity at 24 h intervals from 7 to 10 days after inoculation. Half root systems were placed into 100 ml test tubes. Tubes were injected through a serum stopper with 5 ml acetylene and ethylene production was determined by gas chromatography (Varian 940 GC).

Statistical analysis: Four to six plants per inoculation treatment on each sampling date were scored for nodulation and nodule occupancy and 2 plants per inoculation treatment were used in acetylene reduction assays. Data were analyzed by Tukey's HSD test, using SYSTAT statistical package (87).

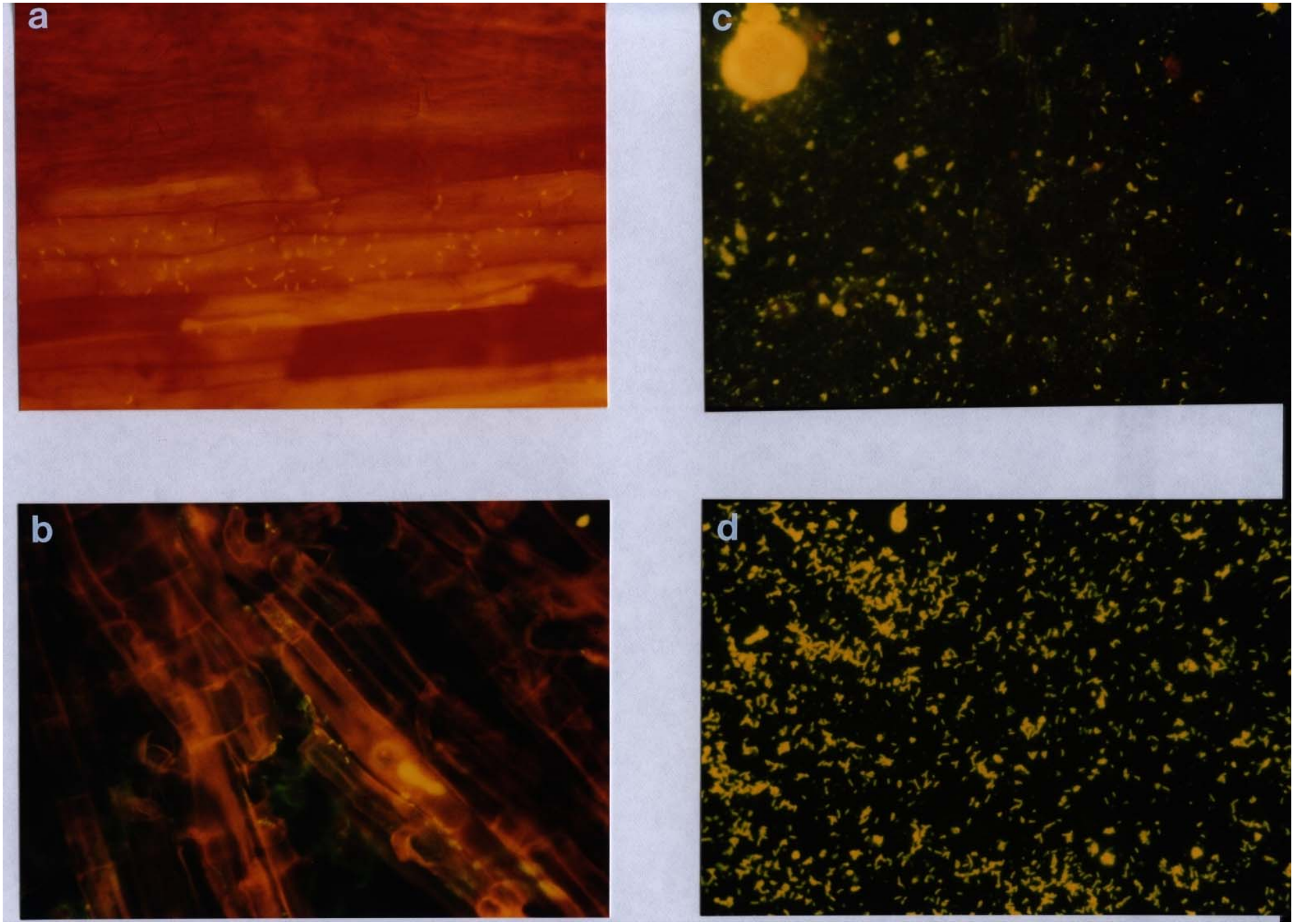
Figure IV-1.

a - cells of B. japonicum, strain USDA 110, colonizing soybean root surface, stained with fluorescent antibodies (FA);

b - an example of FA reaction observed in crushed nodule primordia, 5 days after inoculation

c - an example of FA reaction observed in nodule smears, 10 days after inoculation

d - an example of FA reaction observed in nodule smears, 21 days after inoculation


a
c
b
d

RESULTS

Growth conditions affected plant growth and nodulation, as well as interstrain competition. Plants grown in vermiculite, in the greenhouse, produced more shoot and root mass and greater total nodule number and mass, than plants grown in pouches, in the growth room (Table IV-I).

Nodule occupancy by USDA 110 and USDA 38 in nodule primordia 5d after inoculation and in functional nodules, 10d and 21d after inoculation, is presented in Table IV-II. Strain USDA 110 was a superior competitor to USDA 38, regardless whether the two strains were competing for nodule sites on the same side or, on the opposite sides of the split-root system. When the two strains were inoculated together on the same root half, competition pattern was significantly affected by the growth medium. Although USDA 110 dominated among singly occupied nodules in vermiculite and in pouches, significantly more nodule primordia and nodules were occupied by both strains in growth pouches than in vermiculite. When the two strains were inoculated on the opposite root halves, a similar competition pattern was observed in vermiculite and in pouches.

Table IV-III shows the extent of infection and nodule initiation by the two strains, as measured by the number of cortical cell divisions centers (CCD) and nodule primordia (NP) 5 days after inoculation and subsequent nodule development up to 21 days after inoculation. When inoculated alone on a half root, both strains developed similar numbers of CCD and NP, as well as, similar nodule numbers and nodule mass in either growth medium. Conversely, when the two strains were inoculated on the opposite root halves in vermiculite, USDA 110 initiated significantly more nodule primordia and developed significantly greater nodule number and mass than USDA 38, whereas in pouches, nodulation characteristics of the two strains did not differ significantly.

Acetylene reduction assay (ARA) data are presented in table IV-IV. Nitrogenase activity (ARA) was first detected 8 days after inoculation in nodules formed by USDA 110 and 10 days after inoculation in nodules formed by USDA 38. At 21 days after inoculation the two strains exhibited similar nitrogenase activity when inoculated alone on half roots, while USDA 110 showed higher (nonsignificant) total and specific nitrogenase activity than USDA 38 when the two strains were inoculated on the opposite root halves.

Table IV-I. Plant and nodule parameters, 21 days after inoculation of soybean split-root system with a highly competitive (USDA 110) and poorly competitive (USDA 38) strain of B. japonicum; (un=uninoculated).

	plants grown in vermiculite				plants grown in pouches			
inoculum	plant dry weight			nodule	plant dry weight			nodule
side1/side2	shoot	roots	nodules	number	shoot	roots	nodules	number
	---- mg/plant ----			no/plant	---- mg/plant ----			no/plant
110+38/un	730ab	206ab	102ab	105a	566b	107c	51c	65a
110 / 38	896a	260a	128a	110a	651b	111c	54c	71a
110 / un	664ab	198ab	92b	81a	548b	105c	47c	60a
38 / un	565b	173b	87b	67a	535b	108bc	43c	54a

Numbers for individual parameters (within column or row) not followed by the same letters are significantly different at $p \leq 0.05$, according to Tukey's HSD test.

Table IV-II. Competition pattern between highly competitive (USDA 110) and poorly competitive (USDA 38) strain of B. japonicum, inoculated on the same or, on the opposite sides of the split-root system of soybean (un = uninoculated). Competition pattern was determined in nodule primordia - NP (plants harvested 5 days after inoculation), in nodules at the start of N_2 fixation - NO1 (plants harvested 10d after inoculation) and in mature, functional nodules - NO2 (plants harvested 21 d after inoculation).

			plants grown in vermiculite			plants grown in pouches		
			relative nodule occupancy (%)*					
inoculum side1/side2	days after inoculation	nod.dev. stage	USDA110	USDA38	both	USDA110	USDA38	both
			%					
110+38/un	5	NP	70a	3bc	27b	25b	0c	75a
"	10	NO1	90a	0c	10bc	64d	14bc	22bc
"	21	NO2	94a	4c	2c	48bd	21bc	31b
110 / 38	5	NP	85a	15b	-	63a	37b	-
"	10	NO1	62ab	38b	-	61ab	39b	-
"	21	NO2	75a	25b	-	74a	26b	-

* Total nodule number/plant = 100%; numbers represent the average of four plants; Numbers within the inoculation treatment (column or row) not followed by the same letters are significantly different at $p \leq 0.05$, according to Tukey's HSD test.

Table IV-III. Nodule initiation and development by a highly competitive (USDA 110) and poorly competitive (USDA 38) strain of B. japonicum on soybean half root systems. Plants were inoculated and harvested as described in Table IV-II; (un = uninoculated).

A - number of cortical cell division centers (CCD) and nodule primordia (NP);

days after inocul.	inoculum side1/side2	plants grown in vermiculite CCD side1	CCD side2	NP side1	NP side2	plants grown in pouches CCD side1	CCD side2	NP side1	NP side2
		number/half root				number/half root			
5	110 / 38	781a	206b	164a	28b	313ab	298ab	37b	22b
	110 / un	1001a	-	238a	-	355b	-	50b	-
	38 / un	892a	-	201a	-	223b	-	32b	-
	110+38/ un	461a	-	195a	-	134ab	-	26b	-

B - nodule numbers and weight

days a.inoc.	inoculum side1/side2	number side1	number side2	weight side1	weight side2	number side1	number side2	weight side1	weight side2
		no/half root		mg/half root		no/half root		mg/half root	
10	110 / 38	53a	33a	11.3a	6.8a	36a	23a	8.3a	4.5a
	10 / un	56a	-	12.1a	-	38a	-	7.4a	-
	38 / un	57a	-	9.9a	-	37a	-	6.3ab	-
	110+38/ un	60a	-	9.1a	-	43a	-	4.5a	-
21	110 / 38	82a	28b	97.8a	30.3b	53ab	19b	39.0b	15.5b
	110 / un	81a	-	91.6a	-	60a	-	47.2b	-
	38 / un	67a	-	86.7a	-	54a	-	43.5b	-
	110+38/ un	105a	-	102.0a	-	65a	-	51.0b	-

Numbers for individual nodule parameters within a sampling date (column or row), not followed by the same letters are significantly different at $p \leq 0.10$, according to Tukey's HSD test.

Table IV-IV. Nitrogenase activity (ARA) in nodules formed by a highly competitive (USDA 110) and poorly competitive (USDA 38) strain of B. japonicum, inoculated singly on half root or, together on the opposite sides of the split-root system of soybean; (un = uninoculated).

		plants grown in vermiculite				plants grown in pouches			
		ARA				ARA			
days after inoculation	inoculum side1/side2	per side side1	per side side2	per g nodule side1	per g nodule side2	per side side1	per side side2	per g nodule side1	per g nodule side2
		u moles C_2H_4 h^{-1}				u moles C_2H_4 h^{-1}			
8	110 / 38	1.6a	0.0a	0.9a	0.0a	0.4a	0.0a	0.2a	0.0a
	110 / un	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
	38 / un	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
10	110 / 38	10.4a	5.1a	1.2a	0.4a	6.1a	2.1a	1.2a	0.5a
	110 / un	18.5a	0.0a	1.0a	0.0a	8.0a	0.0a	0.8a	0.0a
	38 / un	0.1a	0.0a	0.1a	0.0a	3.8a	0.0a	0.6a	0.0a

Numbers within column or row not followed by the same letter are significantly different at $p \leq 0.10$, according to Tukey's HSD test.

DISCUSSION

The relationship between nodule initiation rates of the two strains and their competition pattern observed in this study supports the correlation between these two processes proposed by McDermot and Graham (56). Comparison of infection and nodule initiation rates of strains USDA 110 and USDA 38 in the indirect competitive system (Table IV-III, inoculation treatment 110/38) with their nodule occupancy in a direct competitive system (Table IV-II, inoculation treatment 110+38/un) indicates that, superior competitiveness of USDA 110 could be attributed to its superior rate of infection and nodule initiation in a competitive system. In vermiculite, USDA 110 initiated significantly more cortical cell division centers and significantly more nodule primordia, compared to USDA 38 (Table IV-III) and at the same time occupied more nodule primordia than USDA 38 when strains were in direct competition (Table IV-II). In growth pouches, USDA 110 showed only moderate advantage over USDA 38 in the infection and nodule initiation rate (Table IV-III), appeared far less dominant in forming nodule primordia and, consequently, occupied less nodules when strains were in direct competition (Table IV-II). The rates of infection and nodule development by USDA 110 and USDA 38 did not differ significantly in a noncompetitive system (Table IV-III; inoculation treatments 110/un, 38/un).

Occupancy by the two strains, of nodule primordia 5d after inoculation and of functional nodules 21d after inoculation (Table IV-II) indicates that, in both, direct and indirect competitive system, the outcome of competition between the two strains was determined during the earliest stages of infection, where more competitive strain (USDA 110) initiated more potential nodule sites (cortical cell division centers) than USDA 38 (Table IV-III) and also initiated nodules faster, as indicated by greater total number and higher proportion of advanced nodule primordia (data not shown) formed by USDA 110 compared to USDA 38.

Earlier nitrogenase activity in nodules formed by USDA 110 (Table IV-IV) also indicates earlier penetration of the infection thread into plant cells and earlier differentiation into N_2 fixing bacteroids, as a result of faster infection by USDA 110.

When double inoculations, using the same strain but delayed second inoculation, are performed on a single (24) or on a split-root (Chapter III, Table III-I) system, similar numbers of infections (cortical cell division centers) are observed on early and delayed inoculated root region but, further development of infections on the delayed inoculated root region is suppressed. This phenomenon, collectively known as autoregulation is a plant controlled response, which is morphologically detectable within 3 to 7 days after inoculation (24, 77). By contrast, our present results indicate that, the outcome of interstrain competition is determined, to a large extent already at the cortical cell division stage or, at the latest, at nodule primordia stage. Autoregulation may simply favor the development of early initiated nodule primordia regardless to their occupancy. Consequently, initial competition pattern observed in nodule primordia is clearly reflected in nodule numbers and nodule mass produced by the two strains (Table IV-III). Thus, the rate of infection rather than rate of nodule development appears to be determinant of strain's competitiveness. Halverson and Stacey (40) demonstrated that short term preincubation of rhizobia in soybean root exudate increases nodule initiation rate of a slow to nodulate strain. Inoculation with one strain 4 - 6 hours before a second strain is introduced (33, 48), seems sufficient for a less competitive strain to colonize the roots and initiate infections to a stage where infection by a more competitive strain is suppressed.

At optimal inoculum doses for nodule yield, the attachment of rhizobia to soybean roots is virtually completed within 1 hour after inoculation (83)

and motility and chemotaxis appear important determinants of root colonization and nodule initiation (7).

In our study, environmental factors, such as growth medium and light intensity, clearly affected early events in the infection process. Therefore, it seems likely that in soil, which is by far more complex environment, strain response to plant symbiotic signals in the rhizosphere, their motility and chemotaxis, determine the proportion of root colonization and nodule initiation by competing strains, which is then manifested in numbers of functional nodules formed by each strain.

REGULACIJA NODULACIJE IN KOMPETICIJE MED SEVI BAKTERIJE Bradyrhizobium japonicum PRI FORMIRANJU SIMBIOZE S SOJO (Glycine max [L], Merrill).

POVZETEK

Formiranje simbioze med strocnicami in bakterijami rodu Rhizobium poteka preko kompleksnega redosleda genetskih in fizioloskih interakcij med partnerjema, ki kulminirajo v zrelih nodulah, v katerih poteka fiksacija atmosferskega dusika. Gostiteljska rastlina regulira stevilo nodul na koreninah s procesom imenovanim autoregulacija. Doslej je bila autoregulacija nodulacije dokazana pri stevilnih zelnatih strocnicah. Ko je gostiteljska rastlina izpostavljena vecim sevom homolognih Rhizobijev hkrati, ti tekmujejo za omejeno stevilo nodul na skupnem gostitelju. Cimbolj kompetitiven je sev, temvecji delez v skupnem stevilu nodul okupira oz. formira. Kompeticija med indigenimi in introduciranimi sevi Rhizobiuma v praksi predstavlja glavno oviro za uspesno inokulacijo s sevi, ki so sposobni fiksirati vec atmosferskega dusika.

Mehanizmi autoregulacije in kompeticije med sevi so se vedno nepojasnjeni. V nasih studijah proucujemo stadije infekcijskega procesa, v katerih poteka autoregulacija nodulacije in kompeticija med sevi Bradyrhizobium japonicum za nodulacijo na soji, ter mehanizme preko katerih gostiteljska rastlina regulira infekcijo in razvoj nodul.

V literaturi predlagani mehanizem avtoregulacije vkljucuje 2 tipa regulatornih substanc: 1) zgodaj zasnovani nodulni in koreninski meristemi producirajo inhibitor(je), ki neposredno zavira(jo) razvoj kasneje zasnovanih nodul; 2) zgodnje infekcije preko se neidentificiranega molekularnega signala izzovejo produkcijo sistemskega inhibitorja v poganjku, to pa nato deluje na pozne infekcije. Med stevilnimi objavljenimi studijami, ki obravnavajo autoregulacijo, ena sama ugotavlja vlogo fotosintatov pri regulaciji stevila nodul na rastlini. Zato v prvi seriji

eksperimentov ugotavljamo vpliv rastnega potenciala gostiteljske rastline na stevilo in maso nodul pri optimalni dozi inokuluma ter opredelimo casovni interval po inokulaciji, v katerem nastopa autoregulacija. Uporabljamo razdeljen koreninski sistem (Figures II-1 in II-2), kjer eno polovico korenin inokuliramo zgodaj (cas 0) ali pustimo neinokulirano, drugo polovico korenin pa inokuliramo z dolocenim casovnim zamikom. Razvoj nodul na obeh polovicah korenin koreliramo z kolicino fotosintetsko aktivne svetlobe (PAR - $\mu E/m^2/sec$) ter z listno povrsino in dolzino korenin v obdobju od inokulacije do pojava prvih nodul.

V primeru ko je bila polovica korenin inokulirana 1, 2, 4, 8, 16, 32, 64 ali 96 ur za drugo polovico (Figure 11-4), je bilo stevilo nodul na pozno inokulirani polovici korenin znacilno nizje kot na zgodaj inokulirani polovici korenin pri zamiku druge inokulacije za 64 ur ali vec. Masa nodul na pozno inokulirani polovici korenin pa znacilno nizja kot na zgodaj inokulirani polovici korenin ze pri zamiku druge inokulacije za 16 ur ali vec. Stevilo nodul na neinokuliranem/zakasnjeno inokuliranem koreninskem sistemu je narascalo linearno (r=0.97; Figure II-4) s casovnim zamikom inokulacije (1 - 96 ur). Izracunana korelacija med stevilom nodul na rastlino in listno povrsino v casu inokulacije (r=0.98; Figure II-7A, Table II-I) je znacilno visja kot korelacija med stevilom nodul in dolzino korenin v casu inokulacije (r=0.67; Table II-1). Zabelezen je bil hitrejsi razvoj nodul na rastlinah z vecjo listno povrsino v casu inokulacije (Figure II-7B). Stevilo in masa nodul na rastlino sta bila premosorazmerna z intenziteto svetlobe v rastnem okolju (Table II-IV), Stevilo nodul na pozno inokulirani polovici korenin pa obratno sorazmerno s stevilom nodul na zgodaj inokulirani polovici korenin (Figure II-8). Intenziteta svetlobe je tudi znacilno vplivala na razmerje med stevilom nodul na zgodaj inokulirani polovici korenin in stevilom nodul na pozno inokulirani polovici korenin (Table II-IV). Pri 4 - dnevnem intervalu med zgodnjo in

pozno inokulacijo razdeljenega koreninskega sistema je bilo stevilo nodul na pozno inokulirani polovici znacilno nizje, kot pri 14 - dnevnem intervalu med inokulacijama (prim. Table II-II in II-III). Nitrogenazno aktivnost v nodulah na zgodaj inpkulirani polovici korenin smo ugotovili (acetilenski test) deseti dan po (zgodnji) inokulaciji. V poskusu, kjer smo zgodaj inokulirano polovico korenin odrezali v casu inokulacije druge polovice korenin (14 dni za zgodnjo inokulacijo) se je na preostali polovici korenin razvilo skoraj 5- krat toliko nodulnih primordijev kot na celotnem zgodaj in pozno inokuliranem razdeljenem koreninsken sistemu kontrolnih rastlin (Table II-III); Po stevilu zrelih (funkcionalnih) nodul, 3 tedne po pozni inokulaciji, pa se rastline s polovico korenin in kontrolne rastline niso razlikovale (Table II-III) .

Navedeni rezultati ka2yejo, da je stevilo zasnovanih nodul (nodulnih primordijev) modno odvisno od rastnega potenciala (e.g. listne povrsine) rastline v casu inokulacije, medtem ko na stevilo zrelih nodul na rastlini odlocilno vpliva intenziteta fotosintetsko aktivne svetlobe. Regulatorni mehanizem (avtoregulacijo), ki uravnava stevilo in maso in nodul na rastlino je mogoce opaziti ze 16 ur po inokulaciji. Nizja stopnja autoregulacije pri 14 dnevni zakasnitvi druge inokulacije (po zacetku fiksacije dusika v zgodnjih nodulah) kot pri 4 dnevni zakasnitvi (pred zacetkom fiksacije dusika v zgodnjih nodulah) kaze na fizioloske spremembe v nodulirani rastlini, glede na omejujoce dejavnike rasti rastline in razvoja nodul. Simptomi pomankanja dusika na rastlinah, ki se pojavijo ob zacetku nitrogenazne aktivnosti v nodulah (10 dni po inokulaciji), kazejo na pomankanje N za rast poganjka na kar rastlina reagira s povecanim dotokom asimilatov v korenine. Ti asimilati skupaj s fiksiranim dusikom omogocajo razvoj vecjega stevila nodul.

V naslednji studiji proucujemo vlogo selektivne porazdelitve asimilatov pri autoregulaciji nodulacije. Spet uporabimo razdeljen koreninski sistem (Figure 11-2) z dvema obravnavanjema:
1) zgodaj inokuliran/neinokuliran koreninski sistem (E/U treatment) kot kontrola;
2) zgodaj/pozno inokuliran koreninski sistem (E/D treatment), kjer drugo polovico korenin inokuliramo 4 dni za prvo.

Po 2 rastlini vsakega obravnanja izpostavimo $^{14}CO_2$ vsakih 24 ur od inokulacije do zacetka fiksacije dusika v prvih nodulah. Porazdelitev radioaktivnih asimilatov v koreninah najprej zasledujemo z autoradiografijo (Figure III-2). Po barvanju korenin z Eriochrome Black T, pod svetlobnim mikroskopom (binokularno lupo) na temnem polju lahko opredelimo razvojne stadije nodul (Figure III-1). Celotni koreninski sistem nato (pod mikroskopom) seciramo in grupiramo nodulno in koreninsko tkivo glede na razvojni stadij in strukturo. Po raztapljanju tkiva in suspenziji v scintilacijskem koktajlu merimo radioaktivnost v razvijajocih se nodulah in koreninah posameznih rastlin na scintilacijskem stevcu.

Kot je pokazala mikroskopska analiza, se na zgodaj in pozno inokulirani polovici korenin zasnuje priblizno enako stevilo zacetnih nodulnih meristemov (cortical cell division centers, Figure III-1), od katerih pa napreduje v nadaljne razvojne stadije na pozno inokulirani polovici korenin znacilno nizji delez kot na zgodaj inokulirani polovici korenin (Table III-1). Distribucija radioaktivnosti v koreninskem sistemu prikazana na autoradiografih (Figure III-2) kaze, da so razvijajoce se nodule in koreninski vrsicki glavni ponori asimilatov. Na osnovi scintilacijskih podatkov, smo intenziteto ponorov opredelili z dvema parametroma: 1) specificna radioaktivnost ((dpm (disintegrations per minute) / mg suhe teze], ki kaze dotok radioaktivnih asimilatov v ponorno tkivo (sink tissue);

2) relativna specificna radioaktivnost (dpm / o suhe teze tkiva), ki kale porazdelitev radioaktivnih asimilatov glede na delez ponornega tkiva v skupni masi noduliranih korenin.

Specificna in relativna specificna radioaktivnost sta bili 3 do 4 - krat visji v nodulah kot v koreninah (Figure III-3, Figure III4). Specificna radioaktivnost v nodulah je narascala sorazmerno z razvojnim stadijem in je bila znacilno visja v nodulah na zgodaj inokulirani polovici korenin kot v nodulah na pozno inokulirani polovici korenin (Figure III-3). Glede na delez nodulnega tkiva v masi zgodaj in pozno inokuliranih korenin pa kazejo zgodaj in pozno zasnovane nodule podobno intenziteto ponora oz. relativno specificno radioaktivnost (Figure III-4, E/D treatment). Z razvojem prvih zrelih nodul postanejo korenine prikrajsane za radioaktivne asimilate (Figure III-2, Figure III-4) . Ob zacetku fiksacije dusika na zgodnji oz. pozno inokulirani polovici korenin je odpadlo na zgodnje nodule preko 60%, na pozne nodule pa manj kot lot skupne izmerjene radioaktivnosti v zgodaj oziroma pozno inokulirani polovici korenin (Figure III-5). Znacilno vecji dotok radioaktivnih asimilatov v zgodaj inokulirano polovico korenin na racun preostale polovice korenin (neinokulirane ali pozno inokulirane) je bil opazen socasno z pojavom prvih zrelih nodul na zgodaj inokulirani polovici korenin (Figure III-6). Razlike v porazdelitvi radioaktivnih asimilatov v nodulah in koreninah na obeh polovicah razdeljenega koreninskega sistema so se jasno odrazale v tezi suhe snovi korenin in nodul (Figure III-7). Teza suhe snovi namrec predstavlja integralno vrednost porazdelitve asimilatov v nekem casovnem obdobju. Posledica vecjega dotoka asimilatov v zgodnje nodule je bila zmanjsana rast korenin na obeh polovicah koreninskega sistema in znacilno nizja masa nodul na pozno inokulirani polovici korenin kot na zgodaj inokulirani polovici korenin (Figure III-7).

Po razvoju nodul na zgodaj in pozno inokulirani polovici korenin (Table III-1) lahko sklepamo, da je autoregulacija kontinuiran proces, ki poteka v celotnem obdobju razvoja nodul od prvih centrov celicnih delitev v korteksu do funkcionalnih nodul. Porazdelitev radioaktivnih asimilatov v razdeljenem koreninskem sistemu (Figures III-2, III-4, III-6) jasno kaze na kompeticijo med nodulnimi in koreninskimi meristemi za razpolozljivo kolicino asimilatov. Da se zgodnje nodule razvijajo na racun pozno zasnovanih nodul in na racun rasti korenin dokazujeta tako intenziteta ponorov (specificna in relativna specificna radioaktivnost) kot teia nodul in korenin pri obeh obravnavanjih razdeljenega koreninskega sistema. Neposredna odvisnost skupnega stevila nodul na rastlino in stopnje autoregulacije od razvojnega stadija rastline v casu inokulacije in intenzitete fotosintetsko aktivne svetlobe kaze, da so asimilati zelo pomemben omejitveni dejavnik razvoja nodul. Na osnovi nasih rezultatov predlagamo mehanizem autoregulacije, ki predpostavlja da so, zaradi narascajocega dotoka asimilatov v zgodnje nodule, pozno zasnovane nodule prikrajsane za asimilate, zato se njihov nadaljni razvoj znatno upocasni ali popolnoma ustavi.

V zadnjem eksperimentu ugotavljamo vlogo autoregulacije pri kompeticiji med sevi Bradyrhizobium japonicum za stevilo nodul na skupnem gostitelju - soji. v dosedaj publiciranih studijah avtorji ugotavljajo kompetitvnost sevov po stevilu funkcionalnih nodul, ki jih formirajo posamezni sevi, ce so v inokulacijski mesanici sevi (stevilo celic) v enakem stevilcnem razmerju. Cela vrsta studij pa kaze, da so za izid kompeticije odlocilni najzgodnejsi stadiji infekcijskega procesa. Z naso tehniko barvanja (eriochrome black) in blokiranjem fluorescence povrsinsko pritrjenih celic B. japonicum lahko identificiramo seve v zgodnjih nodulnih primordijih z za posamezni sev specificnimi fluorescencnimi protitelesci

(Figure IV-1). S primerjavo razmerja sevov v nodulnih primordijih in v funkcionalnih nodulah, ugotavljamo prispevek zgodnjih (infekcija) in poznih (razvoj nodul) stadijev infekcijskega procesa h koncnemu izidu kompeticije med sevoma, v funkcionalnih nodulah.

Iz predhodnih raziskav znano kompetitivni sev (USDA 110) in slabo kompetitivni sev (USDA 38) B. japonicum inokuliramo istocasno, v enakem stevilcnem razmerju celic, na razdeljen koreninski sistem soje v treh variantah: 1) oba seva na eni polovici korenin, druga polovica ostane neinokulirana - neposredni kompetitivni sistem; 2) oba seva vsak na svoji polovici korenin - posredni kompetitivni sistem, kjer rastlina igra vlogo posrednika; 3) en sev na polovici korenin, druga polovica neinokulirana - nekompetitivni sistem. Enak eksperimentalni protokol uporabimo pri rastlinah gojenih v rastlinjaku, v vermikulitu - Figure II-1 in rastlinah gojenih v rastni komori v PE vreckah (growth pouches) - Figure II-2.

Stevilo nodul, ki jih formirata seva (posamezno ali skupaj) ugotavljamo 5 dni po inokulaciji - nodule v primordialnem stadiju, 10 dni po inokulaciji - zacetek fiksacije N_2 v nodulah, in 21 dni po inokulaciji - zrele funkcionalne nodule.

Rastni pogoji (medij in intenziteta svetlobe) so znacilno vplivali na kolicino rastlinske biomase (Table IV-I) in na kompeticijo med sevoma (Table IV-II). V posrednem kompetitivnem sistemu v vermikulitu je sev USDA 110 formiral 85 % vseh nodulnih primordijev in 75 o zrelih nodul, v PE vreckah pa 63 % vseh nodulnih primordijev in 74 % zrelih nodul. V direktnem kompetitivnem sistemu v vermikulitu je sev USDA 110 formiral 70 vseh nodulnih primordijev in 94 % zrelih nodul, v PE vreckah pa le 25 % vseh nodulnih primordijev in 48 % zrelih nodul (Table IV-II). Kljub temu se je sev USDA 110 tudi v PE vreckah izkazal kot bolj kompetitiven, saj sta 75 % vseh nodulnih primordijev in 31 % vseh zrelih nodul okupirala oba seva

skupaj. V nekompetitivnem sistemu sta seva zasnovala podobno stevilo infekcij (centrov celicnih delitev v korteksu in nodulnih primordijev) in formirala podobno stevilo nodul (Table IV-III). V posrednem kompetitivnem sistemu v vermikulitu pa je sev USDA 110 zasnoval 3 do 5 - krat toliko infekcij kot sev USDA 38 in formiral 3 - krat toliko nodul kot USDA 38 (Table IV-III). Sev USDA 110 je pokazal tudi zgodnejso in vecjo zacetno aktivnost nitrogenaze v nodulah (Table IV-IV).

Priblizno enako razmerje med sevoma po stevilu nodulnih primordijev in po stevilu nodul, ki jih formirata seva v neposredni kompeticiji, kaze, da je koncni izid kompeticije dolocen ze v najzgodnejsih stadijih infekcijskega procesa. Znacilno vecje stevilo infekcij, ki jih zasnuje bolj kompetitivni sev pa kaze, da na kompetitivnost seva oziroma na izid kompeticije med sevi lahko odlocilno vpliva zacetna interakcija med mikro - in makro - simbiontom. Pri zaporedni inokulaciji koreninskega sistema z istim sevom, zgodnji in pozni inokulum zasnujeta priblizno enako stevilo infekcij - zacetnih nodulnih meristemov, autoregulacija pa nato zavira nadaljni razvoj poznih infekcij. Nasa zadnja studija pa kaze, da je pri socasni inokulaciji s sevi, ki se razlikujejo po kompetitivnosti, izid kompeticije (razmerje med sevi v funkcionalnih nodulah) dolocen ze v zacetnih nodulnih meristemih in autoregulacija nato pac favorizira razvoj zgodaj zasnovanih infekcij (nodul), ne glede na to kateri sev jih zasnuje.

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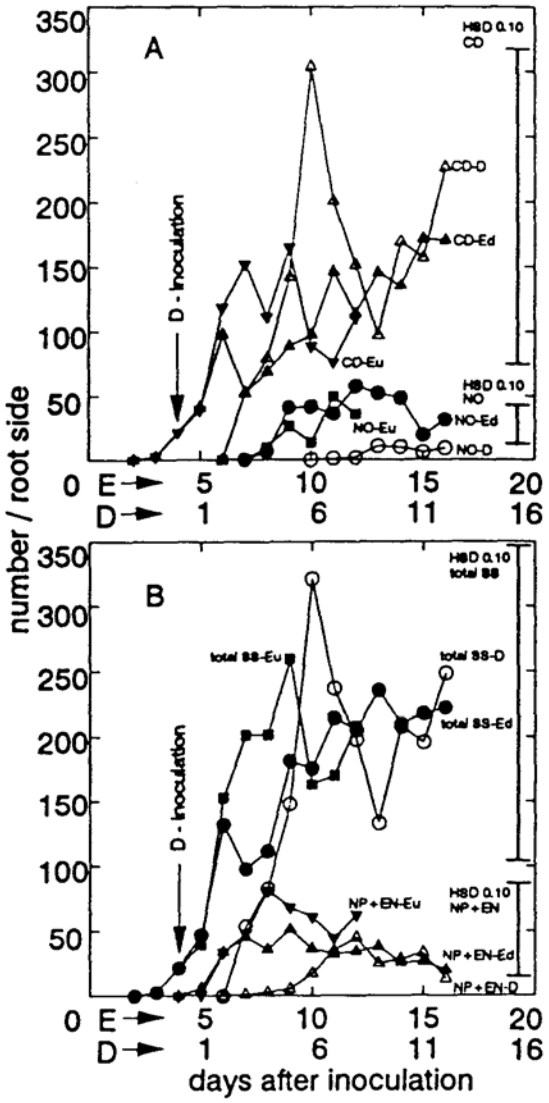
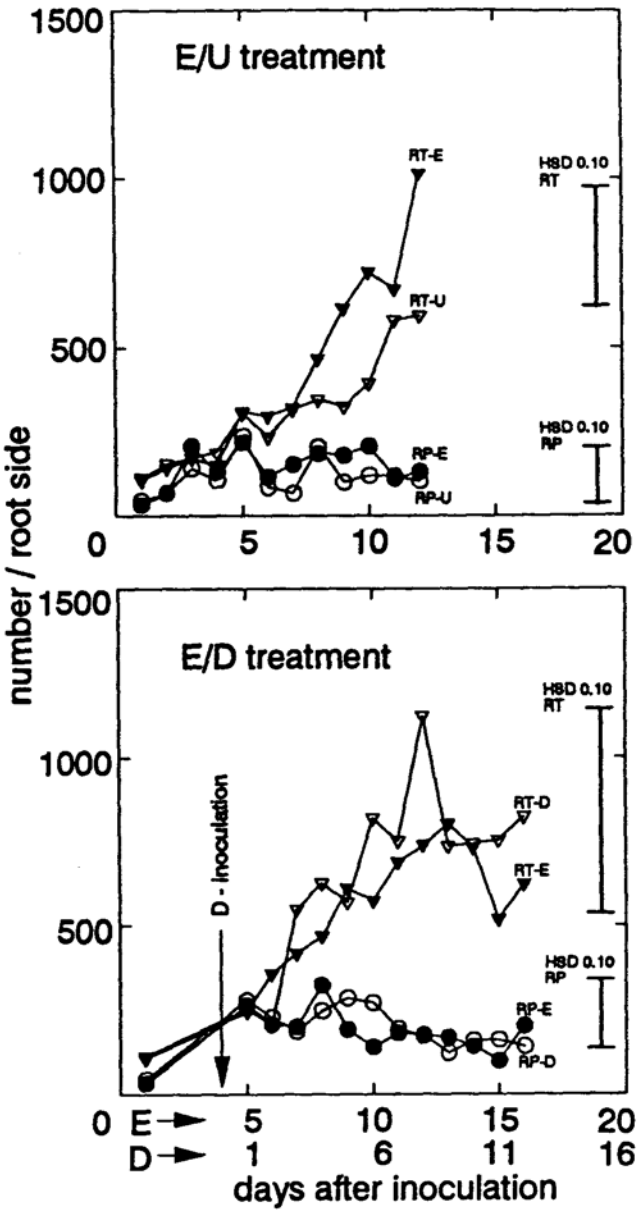
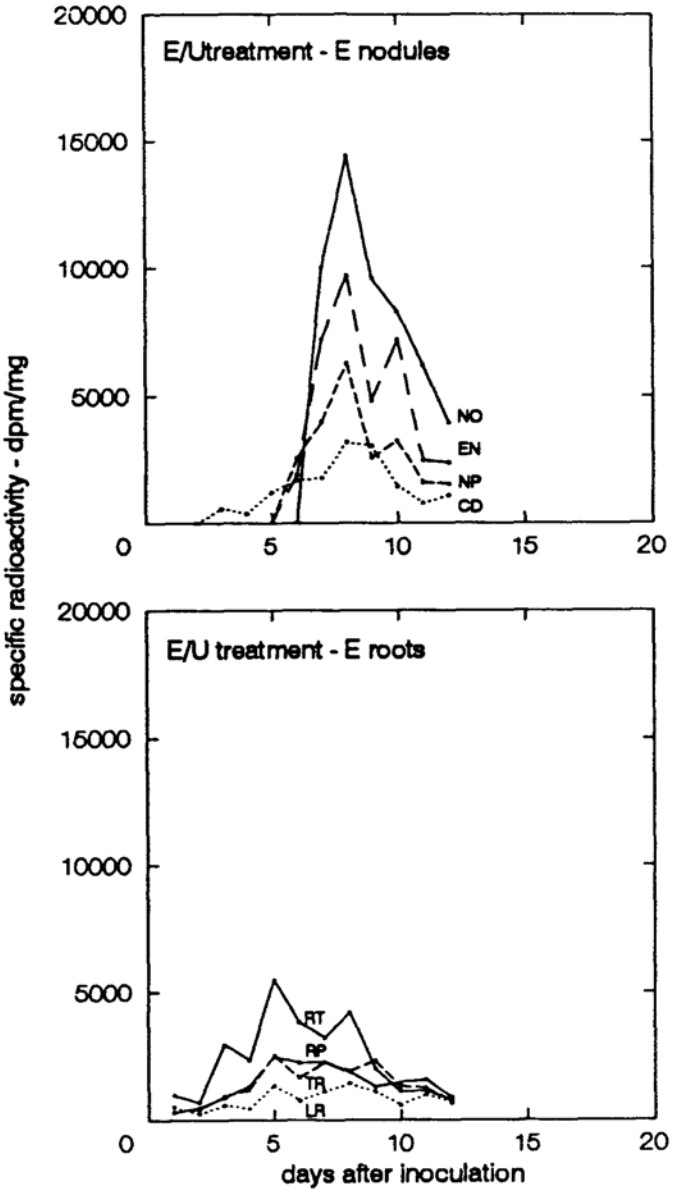


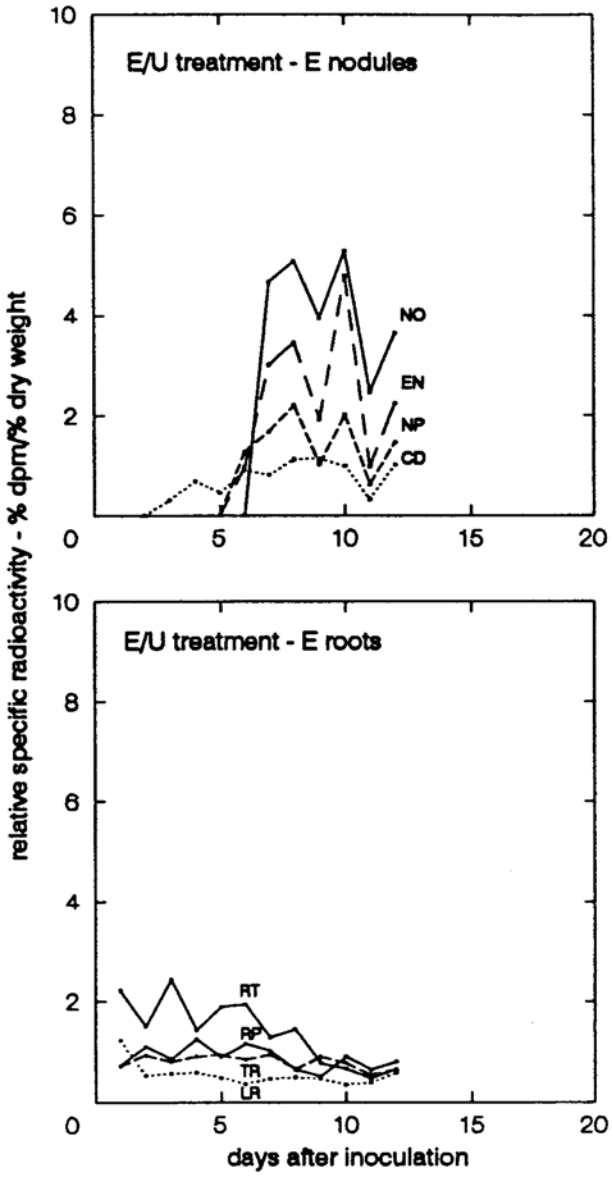
Appendix III-1. Development of symbiotic structures (classified in Fig. III-1) on the early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root system of soybean. Delayed side was inoculated 4 days after E side. A - numbers of cortical cell division centres (CD) and nodules (NO); B - sums of nodule primordia and emerging nodules (NP+EN) and total numbers of symbiotic structures (total SS) per root side; Eu = early inoculated side of early/uninoculated treatment; Ed = early inoculated side of early/delayed inoculation treatment; D = delayed inoculated side.



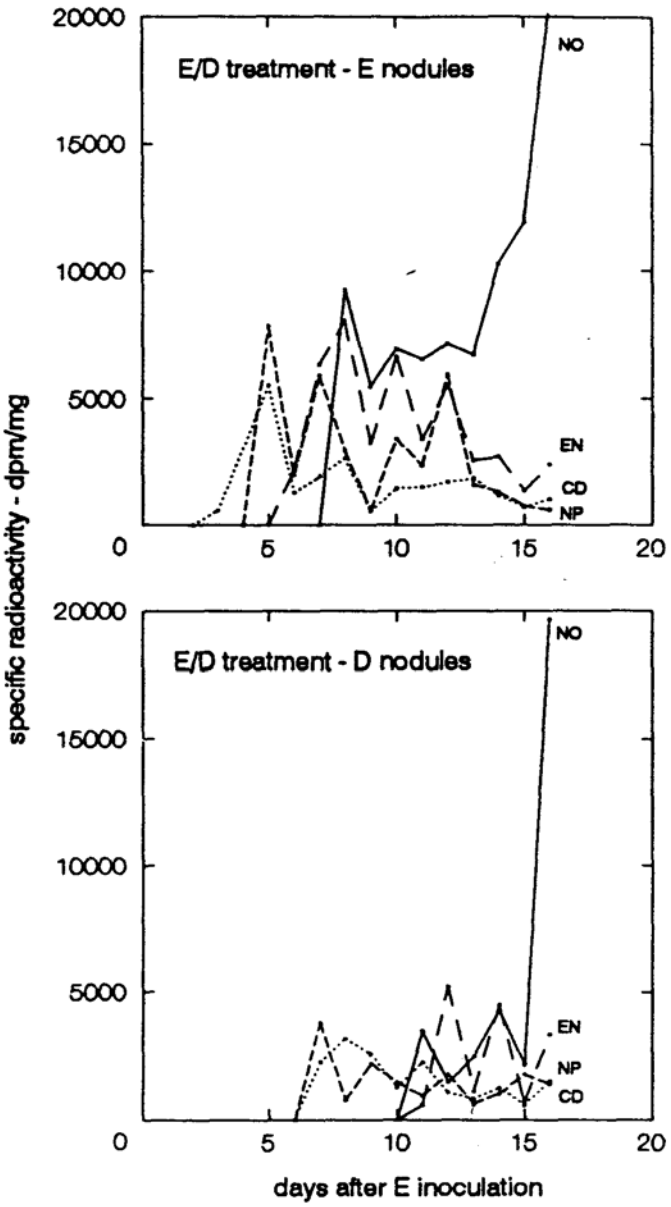
Appendix III-2. Numbers of root primordia (RP) and root tips (RT) (classified in Fig. III-1) on early(E)/uninoculated(U) and early(E)/delayed(D) inoculated split-root system of soybean. Delayed side was inoculated 4 days after E side.



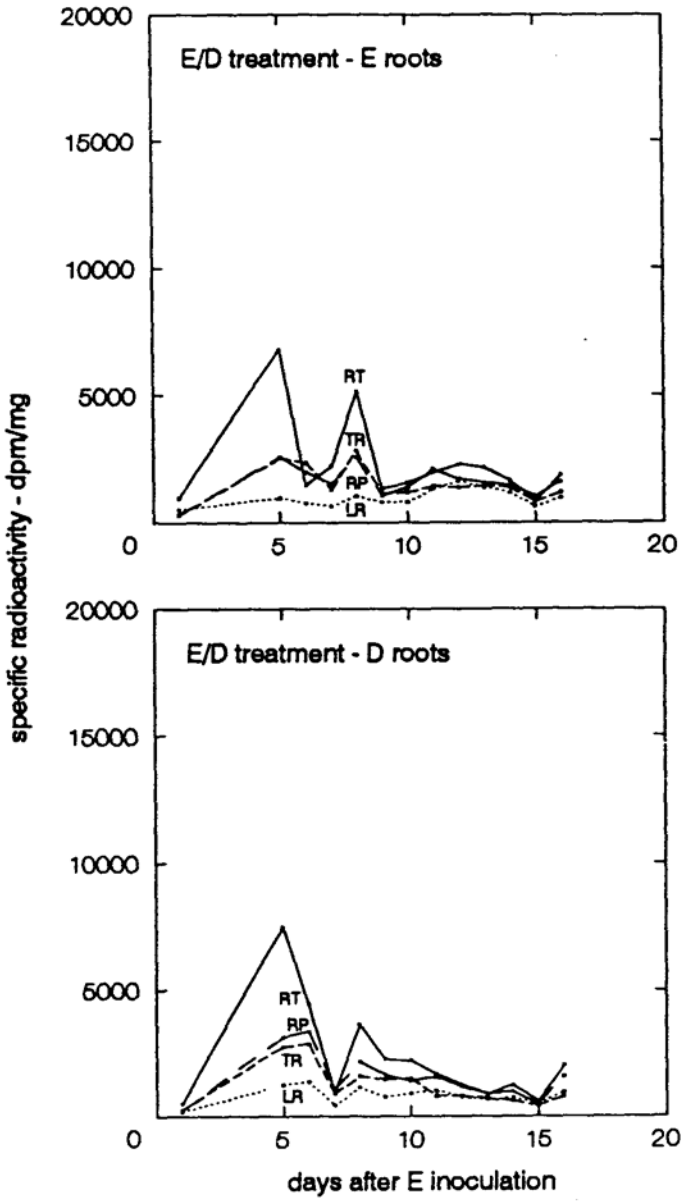
Appendix III-3. Specific radioactivity of symbiotic and root structures (classified in Fig. III-1) on early(E)/uninoculated(U) split-root system of soybean; symbiotic structures: CD=cortical cell division centers, NP=nodule primordia, EN=emerging nodules, NO=mature nodules; root structures: RT=root tips, RP=root primordia, TR="tap" root, LR=lateral roots.



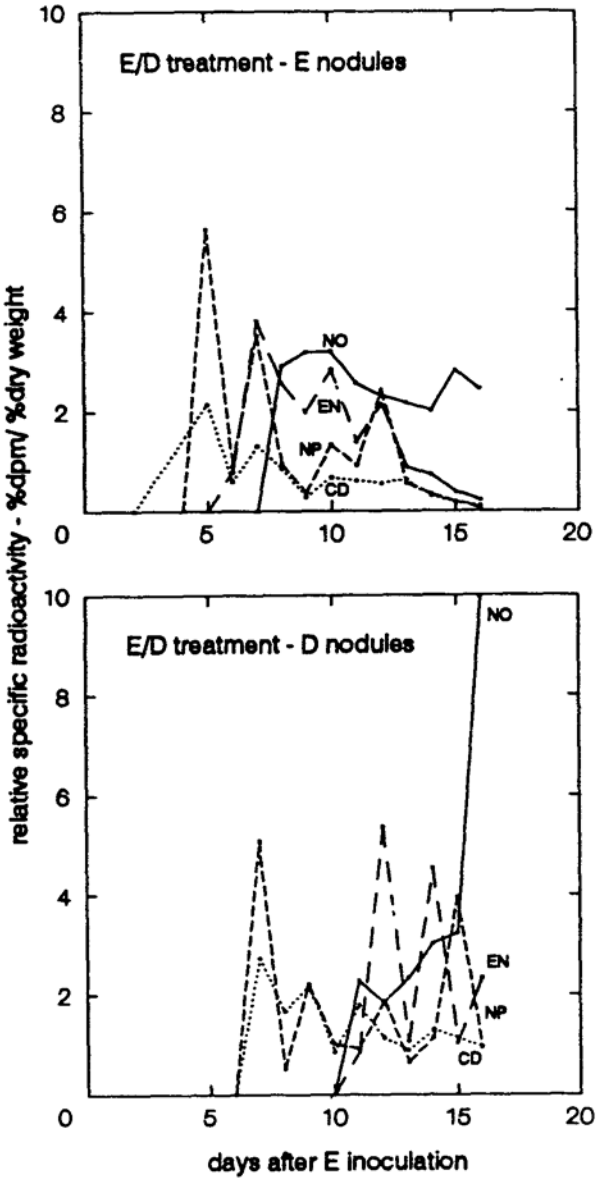
Appendix III-4. Relative specific radioactivity of symbiotic and root structures (classified in Fig. III-1) on early(E)/uninoculated(U) split-root system of soybean; symbiotic structures: CD=cortical cell division centers, NP=nodule primordia, EN=emerging nodules, NO=mature nodules; root structures: RT=root tips, RP=root primordia, TR="tap" root, LR=lateral roots.



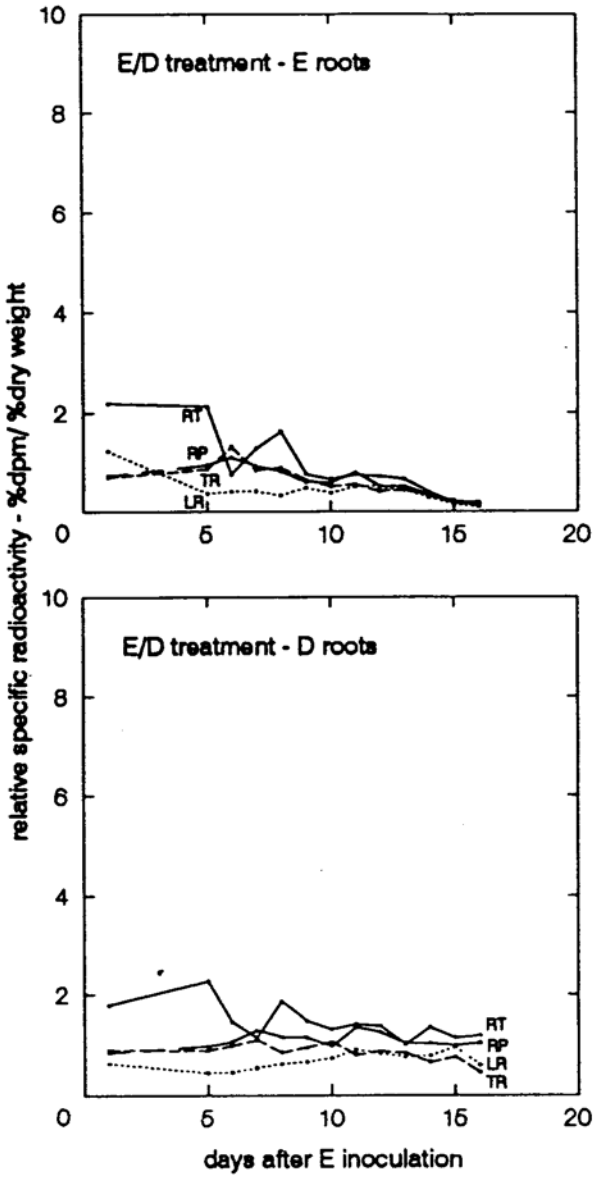
Appendix III-5. Specific radioactivity of nodule structures (classified in Fig. III-1) on the early (E) and delayed (D) inoculated side of the split-root system of soybean; D side was inoculated 4 days after E side; CD=cortical cell division centers, NP=nodule primordia, EN=emerging nodules, NO=mature nodules.



Appendix III-6. Specific radioactivity of root structures (classified in Fig. III-1) on the early(E)/delayed(D) inoculated split-root system of soybean; RT=root tips, RP=root primordia, TR="tap" root, LR=lateral roots.



Appendix III-7. Relative specific radioactivity of nodule structures (classified in Fig. III-1) on the early (E) and delayed (D) inoculated side of the split-root system of soybean; D side was inoculated 4 days after E side; CD=cortical cell division centers, NP=nodule primordia, EN=emerging nodules, NO=mature nodules.



Appendix III-8. Relative specific radioactivity of root structures (classified in Fig. III-1) on the early(E)/delayed(D) inoculated split-root system of soybean; RT=root tips, RP=root primordia, TR="tap" root, LR=lateral roots.