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Early Nodulation in Legumes Inoculated With *Azospirillum* and *Rhizobium*

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Abstract

The effect of *Azospirillum brasilense* combined with *Rhizobium* on nodule formation, was studied during the establishment phase of various forage legumes. The uppermost nodules on the primary roots of pouch-grown burr medic (*Medicago polymorpha*), siratro (*Macroptilium atropurpureum*) and Egyptian clover (*Trifolium alexandrinum*), appeared significantly earlier following combined inoculation with *Rhizobium* and *Azospirillum*, compared with *Rhizobium* alone. Root tip positions were marked on pouches at the time of inoculation and the marks were then used as reference points in following the course of nodule formation. *Azospirillum* increased the total number of nodules by 3.6, 1.8 and 1.3 fold at days 5, 7 and 10 from inoculation, respectively, in pouch-grown medic. The number of nodules located above the root tip mark (RTM) increased by 4, 3 and 2 fold at the same days, respectively, as compared with inoculation with *Rhizobium* alone. Also in pot-grown medic, the number of nodules on the upper part of the main roots increased after inoculation with *Azospirillum*. Significantly higher root dry weight and root surface area were obtained at day 20 and also higher shoot dry weight and N content, 32 days from emergence. The possible mechanism of early nodulation by the combined *Azospirillum*/*Rhizobium* inoculation is discussed.

Keywords: *Azospirillum*, *Rhizobium*, early nodulation, legumes

1. Introduction

Increases in the number of nodules per plant and in yield of legumes inoculated with *Azospirillum* have been reported (Rai, 1983; Singh and Subba Rao, 1979; Iruthayathas et al., 1983; Sarig et al., 1986). Recently we reported on the interaction between *Azospirillum* and *Rhizobium* with regard to the distribution of nodules along the primary root of several forage legumes (Yahalom et al., 1987). As found in growth pouches, the root cells of several legume species appear to remain susceptible to nodulation by rhizobia for only short periods of time (Bhuvaneswari et al., 1981). This time limit for the initiation of nodule can be measured by marking the positions of developing root tips of seedlings on the plastic pouches in which they grow. The most susceptible region is between the root tip and the smallest emerging root hair (RT-SERH). The marking technique enables one to monitor the rate and time of infection, by the position of the uppermost nodule relative to RTM made at the time of inoculation (Bhuvaneswari et al., 1980, 1981; Halverson and Stacey, 1984; Pueppke, 1986). Using this method we investigated the influence of combined *Azospirillum*/*Rhizobium* inoculation on early nodulation in several forage legumes.

2. Materials and Methods

Organisms and growth conditions: *Rhizobium meliloti* RT-1 was obtained from the South Africa *Rhizobium* Collection, Rietondale Research Farm, Pretoria. *Rhizobium* sp. 280-A for inoculating siratro, and *R. trifolii* were obtained from the Dept. of Field Crops, Volcani Center, Bet Dagan, Israel. Cultures used for inoculation were grown 3 days in yeast mannitol broth (YMB) (Vincent, 1970) at 30°C in 250 ml Erlenmeyer flasks on a rotary shaker (110 rpm). The cells were harvested by centrifugation at 12,000 g and washed twice with one-tenth strength of 30 mM phosphate-buffered saline pH 7.0 (Bhuvaneswari et al., 1980). *Azospirillum brasilense* Cd (ATCC-29729) was grown on malate liquid medium (Okon et al., 1977) supplemented with 0.25% NH_4Cl and 0.01% yeast extract for 20 hr at 30°C. Cells were harvested at the late logarithmic growth phase by centrifugation (1,000 g) and were washed twice in 30 mM potassium phosphate-buffered saline pH 7 (Yahalom et al., 1987). The bacterial suspensions that were used for inoculation were diluted to the appropriate cfu/ml by spectrophotometer measurements of culture samples at 540 nm (A_{540}) in 1 cm light-path cuvettes. Bacteria also were counted by dilution plate counts.

Plant assays

Seeds of burr medic (*Medicago polymorpha*), siratro (*Macroptilium atropurpureum*) and Egyptian clover (*Trifolium alexandrinum*) were obtained from the Dept. of Field Crops, Volcani Center, Bet Dagan. The seeds were disinfected, germinated, and grown in polyester pouches as described by Yahalom et al. (1987).

Marking and inoculation

The seedlings were inoculated with *Rhizobium* 2 days after transfer to the pouches, when the roots were approximately 3 cm long. In most experiments *Azospirillum* was either applied 24 hr before inoculation with *Rhizobium*, or together with *Rhizobium*. A waterproof marking pen was used to indicate the position of the root tip (RTM), and the position of the smallest emerging root hair (SERH), at the time of inoculation, by viewing through a stereo microscope. The region between the RTM and the SERH is called the non root hair zone (NRH) (Bhuvaneswari et al., 1980). Seedlings were inoculated by applying 0.5 ml bacterial suspension of *Rhizobium* or *Azospirillum* to the roots of the seedlings in each pouch. The controls received phosphate-buffered saline without *Azospirillum* inoculum.

Scoring of nodulation

After inoculation, the pouches with seedlings were kept at temperature of 24/18°(day/night), for 6–8 days and scored when most of the formed nodules on the primary root were at least 0.75 mm in diameter. The position of the nodules on the primary root was marked with different colors at days 5, 7 and 10 after inoculation, with the aid of a stereo microscope. Dead or injured seedlings were discarded. The effect of *Azospirillum* on nodulation of legumes was tested in half liter pots, filled with quartz sand, in which imbibed seeds were placed at 1 cm depth previous to inoculation with 0.5 ml suspension (10^7 cfu/ml) of *Azospirillum*. The *Rhizobium* inoculation was carried out 24 hr later by applying 5 ml bacterial suspensions followed by 10 ml of water. Shoot and washed root dry weights were obtained after drying at 70°C during 48 hr in a forced-air oven. Total N was determined by a semimicro Kjeldahl procedure (Bremner, 1965). The root surface area was determined by the titration method (Carley and Watson, 1966).

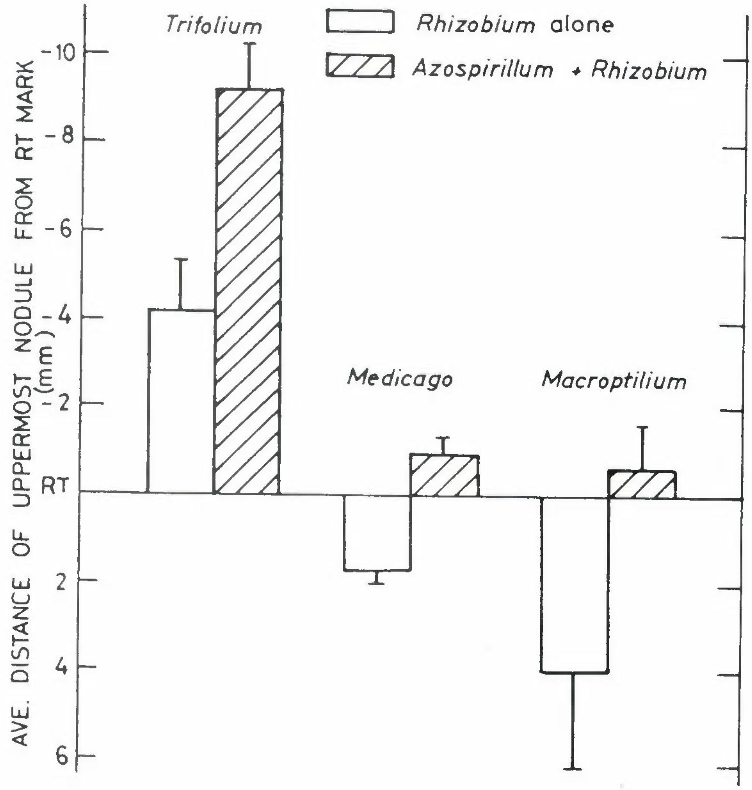

Figure 1. Effect of preinoculation with *Azospirillum* (10^6 cfu/ml) prior to inoculation with *Rhizobium* (10^6 cfu/ml), compared with inoculation with *Rhizobium* (10^6 cfu/ml) alone, on the distance between the uppermost nodule and the root tip mark (RTM) in seedlings of pouch-grown clover (*Trifolium alexandrinum*), medic (*Medicago polymorpha*) and siratro (*Macroptilium atropurpureum*). The position of the root tip was marked, at the time of inoculation and the distance of the uppermost nodule from the mark was recorded 8 days later. Negative and positive numbers on the vertical axis indicate nodule formation above or below the mark, respectively. Means of 24 seedlings. Vertical bars indicate SE.

3. Results

On the average less than one nodule formed on the main root of medic seedlings, 5 days after inoculation with *Rhizobium*, as compared with 3 nodules in seedlings inoculated with *Azospirillum*, 24 hr before *Rhizobium* (Table 1). At day 10 the number of nodules on the roots of seedlings inoculated with *Azospirillum* was 30% higher than with *Rhizobium* alone. The number of nodules above RTM following *Azospirillum* inoculation increased

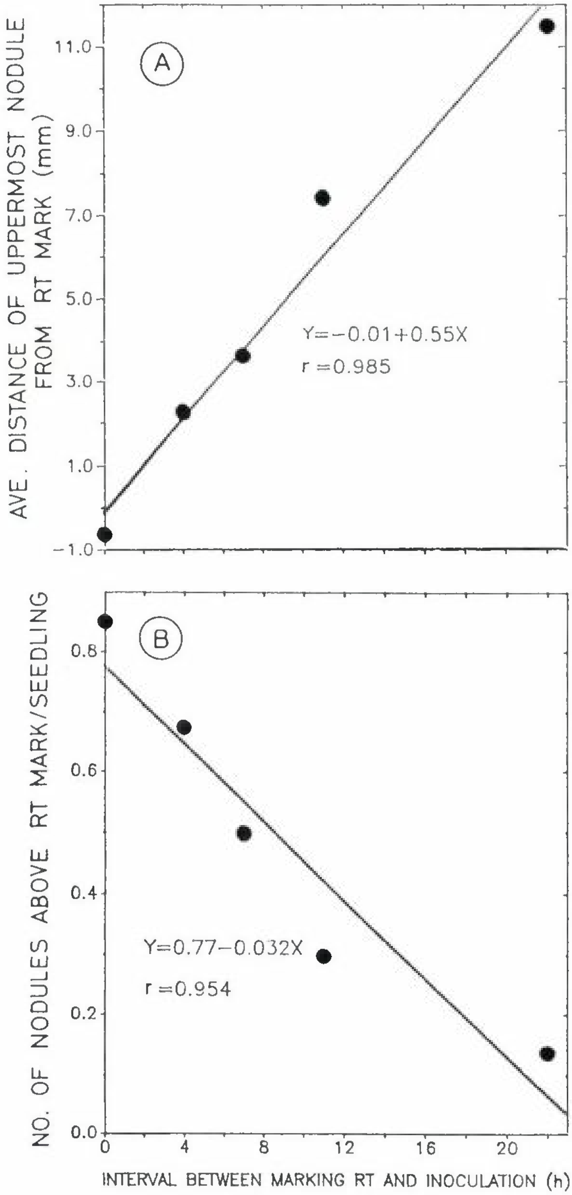

Figure 2. The relationship between the time of *Rhizobium* (10^6 cfu/ml) application and the distance between the uppermost nodule and the root tip mark (A) and the number of nodules above the root tip mark (B), in *Medicago* seedlings 8 days after inoculation. Means of 24 seedlings for each time of inoculation.

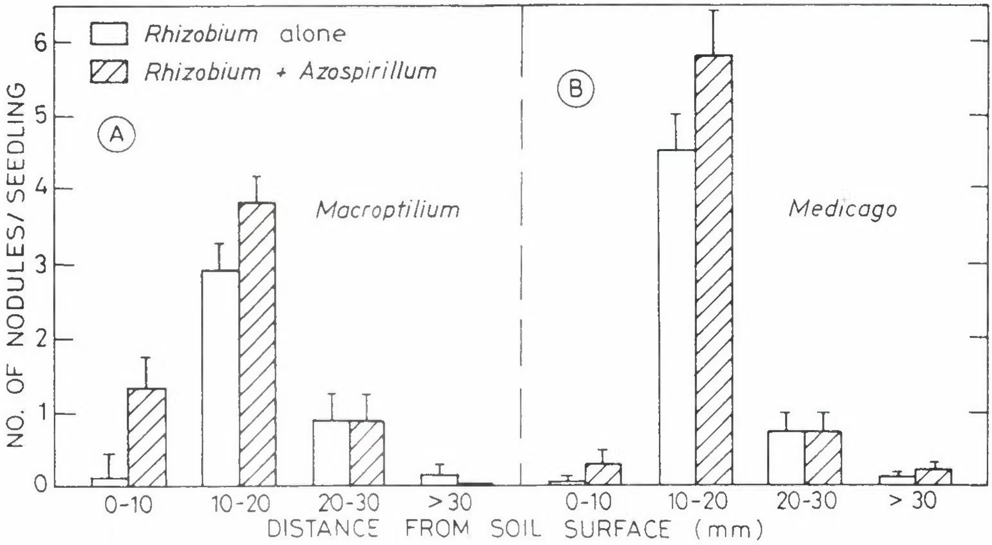

Figure 3. Effect of preinoculation with *Azospirillum* (10^6 cfu/ml) prior to inoculation with *Rhizobium* (10^6 cfu/ml), compared with inoculation with *Rhizobium* (10^6 cfu/ml) alone, on the distribution of nodules on the primary roots of 20 day pot-grown plants of: (A) *Macroptilium* at 25/18°C (day/night), (B) *Medicago* at 28/20°C (day/night). Means of 72 and 49 plants, respectively. Vertical bars indicate SE.

Table 1. Effect of early inoculation with *Azospirillum* (10^7 cfu/ml), compared with inoculation with *Rhizobium* (10^7 cfu/ml) alone, on nodule formation on the primary roots of medic seedlings, means of 24 seedlings

	Days from inoculation			
	5		10	
	Number of nodules per pouch			
	Total	above RT	Total	above RT
Rhizobium alone	0.9±0.4b	0.3±0.2b	10.0±1.2a	1.4±0.4b
Rhizobium + *Azospirillum*	3.2±0.6a	1.3±0.5a	13.3±1.1a	3.3±0.6a

* Values followed by the same letter were not significantly different (P=0.05) according to the Kruskal-Wallis test (Conover, 1980).

more than 4 fold at day 5, and almost 3 fold at day 10, compared with the inoculation with *Rhizobium* alone (Table 1). The average position of the uppermost nodule on the main root was significantly higher not only in seedlings of medic, but also of siratro and clover inoculated with *Azospirillum*

Table 2. Effect of inoculation with *Azospirillum* (10^7 cfu/ml) and/or *Rhizobium* (10^7 cfu/ml) on various parameters of pot-grown *Medicago polymorpha* at 25/18°C (day/night). Means of 9 pots, 4 seedlings/pot. Numbers followed by the same letter of each column do not differ significantly at p=0.05.

Treatment	Shoot dry weight	Shoot N content	Root dry weight	Root surface area
	——— mg/pot ———			ml NaOH 0.01 M
	20 days from emergence			
Rhizobium alone	79a	2.4a	35b	10.5b
Rhizobium + *Azospirillum*	85a	2.5a	43a	12.5a
	32 days from emergence			
Rhizobium alone	162b	5.2b	96b	27.0b
Rhizobium + *Azospirillum*	244a	7.5a	126a	32.0a

and *Rhizobium*, compared with *Rhizobium* alone (Fig. 1). The average distance of the uppermost nodule from RTM increased proportionally with the lengthening of the interval between marking the medic root tip (RT) and inoculation (Fig. 2A). The number of nodules above the RTM decreased proportionally (Fig. 2B). Inoculation with *Azospirillum* increased nodule numbers on the upper part of the primary roots (0–20 mm from soil surface) as compared to *Rhizobium* alone (Fig. 3AB). At day 20 from emergence, the root weight and surface had increased by almost 20% and the number of nodules on the primary roots by 26% in pot-grown medic inoculated with *Azospirillum* compared with *Rhizobium* alone (Table 2). After 32 days of growth, shoot weight and N content were significantly greater (Table 2). On the other hand the number of nodules on the lateral roots was not affected (data not shown). When *Azospirillum* was accompanied by low concentrations of *Rhizobium* (10–100 cfu/ml), the number of lateral root nodules was 2–3 times greater compared with *Rhizobium* alone (Table 3). At these low *Rhizobium* concentrations the number of nodules on the primary roots was relatively low. However, when rhizobia concentration was 10^6 cfu/ml, no nodules were observed on the lateral roots. The inoculation with *Azospirillum* and *Rhizobium* increased by 2.5 times the number of nodules on the upper part of primary medic roots (5–15 mm from soil surface) as compared to plants inoculated with *Rhizobium* alone (Table 3).

Table 3. Effect of inoculation with *Azospirillum* (10^7 cfu/ml), 24 hr before inoculation with *Rhizobium*, on nodule distribution on the roots of 21 day pot-grown *Medicago polymorpha* seedlings at 25/18°C (day/night). Average of 4 replications, 8 seedlings per pot. Numbers followed by the same letter do not differ significantly at p=0.05.

Rhizobium	*Azospirillum*	Primary root nodules Distance from soil surface (mm) 5–15	15–25	25–35	35–45	Lateral nodules No./plant
10	–	0.3	0.5	0.9	0.5	4.9b
10	+	0.3	0.6	0.5	0.0	9.3a
10^2	–	1.0	2.2	0.9	0.6	1.7b
10^2	+	1.1	2.4	1.0	0.5	5.4a
10^6	–	1.1	3.0	1.2	0.1	0.0
10^6	+	2.8	2.4	0.7	0.4	0.0

4. Discussion

Increases in final nodule numbers in legumes inoculated with *Azospirillum* have been reported (Rai, 1983; Plazinski and Rolfe, 1985; Singh and Subba Rao, 1979). These studies did not follow the dynamics of nodule formation during plant development. In our work, the growth of legume seedlings in pouches offered a non-destructive method for monitoring the process of nodule formation as affected by *Azospirillum* (Yahalom et al., 1987). The infection of *Rhizobium* leading to nodulation of primary roots was mostly restricted to the area between the RT and SERH of soybean seedlings. During root elongation of soybeans, there was a transient susceptibility of root hair cells to infection by *Rhizobium*. The position of the uppermost nodule from RT indicated the rate of infection (Bhuvaneswari et al., 1981). In medic seedlings inoculated at the time of marking RT, the uppermost nodule formed above the RTM. Lengthening the interval between marking RT and inoculation caused: (1) The uppermost nodule to form at increasing distance below RTM, in linear relation with time; (2) A decrease of nodule number that formed above RTM in linear relation with time (Fig. 2AB). These results are comparable to those found by Bhuvaneswari et al. (1981) in soybean. Inoculation with *Azospirillum* caused earlier infection by *Rhizobium* in medic, siratro and clover. This was concluded from the observations that *Azospirillum* caused the uppermost nodule to appear at a significantly higher position of the root (Fig. 1) and accelerated nodule development above RTM compared with *Rhizobium* alone (Table 1). *Azospirillum* inoculation changed nodulation patterns in pouch-grown medic and siratro (Yahalom et al., 1987). Similar changes in nodule profile were observed in pots (Fig. 3AB). It has been suggested that *Azospirillum* may stimulate epidermal cells to differentiate into infectable root hair cells, thus increasing their susceptibility to *Rhizobium* infection (Yahalom et al., 1987). In pouch-grown medic, inoculated with *Rhizobium* alone at 10 cfu/ml, no nodulation was observed. In seedlings preinoculated with *Azospirillum* (10^6 cfu/ml), 25% were nodulated (Yahalom et al., 1987). In pots, the effect of *Azospirillum* inoculation at low $10^1 - 10^2$ cfu/ml *Rhizobium* concentration was significant. Increase in nodule numbers as affected by *Azospirillum* was found only in lateral roots but not on the primary roots (Table 3). The increase in the number of lateral root nodules may be due to increased lateral root formation in plants inoculated with *Azospirillum* (unpublished results), thus increasing the percentage of successful encounters between roots and *Rhizobium* in the soil. This result indicates that in presence of *Azospirillum*, a lower number of *Rhizobium* is already sufficient for nodule formation as compared to *Rhizobium* alone and could explain previous results of promotion of nodulation in legumes in the field, inoculated with *Azospirillum* and nodulated by indigenous *Rhizobium* (Singh and Subba Rao, 1979; Sarig et al., 1986). In the absence of *Azospirillum* the total number of nodules per pot was not affected by *Rhizobium* concentration. Increase in rhizobial numbers (10^6 cfu/ml) favoured nodulation of primary roots with reduced nodulation of lateral roots. Under high rhizobial numbers, *Azospirillum* favoured nodulation only in the upper part of the primary root (Table 2) thus supporting early nodulation. The differences between nodulation in pouches and pots can be due to the fact that soil environment affected nodule initiation and distribution (Pueppke, 1986). Acetylene reduction activity in pouch-grown medic and siratro was markedly increased by *Azospirillum/Rhizobium* inoculation (Yahalom et al., 1987). The results obtained in this work in pot experiments indicate that *Azospirillum* by causing earlier nodulation, significantly increased N content of 32 day plants (Table 2). In addition, as previously shown in cereals (Kapulnik et al., 1985; Okon, 1985), the total root surface area of medic was favorably affected by *Azospirillum* 20 day after inoculation. These favorable effects resulted in an increased dry matter content in roots and shoots in 32 day plants. It is possible that plant growth promoting substances (IAA, cytokinins) produced by *Azospirillum* (Tien et al., 1979) may be involved in root development and in root nodule initiation. Demonstration of this hypothesis is under investigation.

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