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Competition Between *Bradyrhizobium japonicum* Strains for Nodulation: Study of Nodulation Regulation in the Soybean

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Abstract

The study of nodulation regulation in two *Bradyrhizobium japonicum* strains (G2 and GMB1) of very different competitive abilities was carried out by performing double inoculation experiments delayed over time along the primary root of the soybean. Successive inoculation of G2 and GMB1 strains at 15, 24 and 48 hr periods along the root (in zones sensitive to infection at the time of inoculation) induced a phenomenon of nodulation regulation which led to a decrease in the number of nodules formed in the first zone inoculated, regardless of the order in which the strains were inoculated. The serologic analysis of the nodules revealed that the two strains decrease their nodulation (compared to controls inoculated alone) and the G2 strain appears to be dominant. No correlation between strain competitiveness and decrease in nodulation potential during delayed inoculations was observed.

The delayed inoculation experiments in the same root zone (infection sensitive at 0 time) showed that the first hours of infection are determinant in the process of competitiveness between strains. Each strain caused a regulation of nodulation to occur which reduced the number of nodules formed by the other strain. A simple delay of two hours in the inoculation of the G2 strain was sufficient to prevent it from causing regulation of the GMB1 strain and allowed this strain to form 50% of the nodules, where it would have formed only 19% if the two strains had been inoculated simultaneously.

Keywords: *Bradyrhizobium*, competition, regulation of nodulation

1. Introduction

Sensitivity of soybean root cells to infection by *Bradyrhizobium japonicum* is developmentally restricted and transient (Bhuvaneswari et al., 1980). A rapid regulatory phenomenon appears within 15 hr after inoculation which reduces nodulation in developmentally younger regions of the root (Pierce and Bauer, 1983). This phenomenon seems to be dependent on strain x cultivar interactions (Heron and Pueppke, 1987).

When two different strains of *Rhizobium* or *Bradyrhizobium* of identical specificity are present in a host Leguminosae, competition for nodule formation occurs: more nodules are formed by one of the strains, even when both strains were present in equal quantities at the time of inoculation. Strain competitiveness is influenced by many factors, such as soil type (Moawad and Bohlool, 1984), nature of culture medium (Fernandez-Flouret and Cleyet-Marel, 1987) and temperature (Hardarson and Gareth-Jones, 1979).

The two *Bradyrhizobium japonicum* G2 and GMB1 strains are of very different competitive types: the G2 strain appeared to be much more competitive and formed 89% of the nodules although it only represented 50% of the inoculum. In a preceding work (Fernandez-Flouret and Cleyet-Marel, 1988), the study of the nodulation profile of the two strains showed that the high competitiveness of the G2 strain could not be explained by a higher potential for nodule formation when it was inoculated alone; the two strains showed the same individual capacity to initiate nodulation and formed the same number of nodules on the plant. For both strains a phenomenon of nodulation regulation seems to be activated in the plant at high bacterial concentrations, which decreases the number of nodules formed in the root zone sensitive to infection at the time of inoculation. Moreover, we observed that simultaneous inoculation of both strains in the infection sensitive zone led to a decrease in the number of nodules formed by the two strains (Fernandez-Flouret and Cleyet-Marel, 1988).

The present work studies nodulation regulation of the G2 and GMB1 strains by inoculation experiments delayed over time on the root to examine whether the difference in competitiveness between the two strains could be explained by a difference in their nodulation regulation. Prior to the experiments we verified the duration of infection sensitivity of our plant's root cells.

2. Materials and Methods

Bacterial strains and preparation of inocula

Bradyrhizobium japonicum G2 strain (311B 125 USDA, Beltsville) and GMB1 strain (I.R.A. Madagascar) were used in this study. Stock cultures were maintained on yeast extract mannitol (Y.E.M.) agar slants at 5°C (Vincent, 1970).

Liquid cultures for inoculum preparation were grown on Y.E.M. medium. Each strain was shake cultured in 250 ml Erlenmeyer flasks containing 50 ml of Y.E.M. for 3 days at 28°C until the mid-log phase. Rhizobia were harvested by centrifugation (37000 g, 20 min) and resuspended in a sterile saline solution (Bergersen, 1961) to reach the desired concentration of 10^8 cells/ml (0.1 absorbance unit at 620 nm). Viable cells were counted by plating.

Seed germination and plant culture

Surface disinfected soybean seeds (*Glycine max* (L.) Merr. cv. Kingsoy) were germinated in a growth chamber for 2 days and then transferred to sterilized plastic pouches moistened with 10 ml of N-free hydroponic growth nutrient solution (Kalia and Drevon, 1985). Plants were maintained in a controlled environment growth chamber (20°C at night, 25°C by day; light intensity: 200 μmol photon $m^{-2}s^{-1}$; photoperiod: 14 hr; 80% RH).

Inoculation and determination of nodulation frequency profiles

Three day old seedlings were inoculated under sterile conditions with 5 μl of a bacterial suspension (with a Hamilton syringe dispenser) in the root zone where infection sensitive cells were located (Bhuvaneswari et al., 1980). Plastic growth pouches were kept moist and nodules appeared within 8 days. The position of each nodule was measured relative to the RT mark and expressed relative to the SERH-RT distance in the nodulation frequency profile.

Duration of infection sensitivity of root cells

After noting the position of the infection sensitive zone under a magnifying glass, soybean seedlings were inoculated at variable intervals (30 plants per treatment). Strains were inoculated at a concentration of $6 \cdot 10^6$ bacteria.plant^{-1} to obtain 100% nodulation of plants and the maximum amount of nodules (Fernandez-Flouret and Cleyet-Marel, 1988). Nodulation was scored one week later.

Delayed inoculations

Two delayed inoculations were tested:

- Delayed inoculation in the same root zone (the zone sensitive to infection at the time of the first inoculation). Plants were first inoculated with the GMB1 strain and then with the G2 strain at intervals ranging from 2 to 72 hr. G2 and GMB1 strains were inoculated in each experiment at a concentration of $1 \cdot 10^6$ bacteria.plant^{-1}. The percentage of nodules formed for each strain was obtained after serologic analysis of nodules harvested 15 days after inoculation.

  In the nodulation frequency profile, the root was expressed relative to the SERH-RT1 distance (RT1: root tip at the time of the first inoculation). Thirty plants were inoculated for each treatment.

- Delayed inoculation in the zone sensitive to infection at the time of each inoculation. Inoculation intervals were 15, 24 and 48 hr. The root was expressed relative to the RT1-RT2 distance (RT2: root-tip at the time of the second inoculation). Thirty plants were tested for each treatment and nodulation controls were performed at each inoculation time for the strain added. Strains were inoculated at concentrations of approximately 10^6 bacteria.plant^{-1}.

  At 0 time, the first strain was inoculated in the zone sensitive to infection at that moment. After a 15, 24 or 48 hour interval, the second strain was inoculated in the new root zone which did not yet have any root hair. Root tips were marked on the pouch at each inoculation time, either RT for the root tip at 0 time or RT15, RT24 or RT48 for the root tip at the second inoculation time.

  The distance measured between RT and RT15 (or 24 or 48) serves as the reference value (1 RDU) to determine nodulation profiles rather than the SERH-RT distance. This technique to establish nodulation profiles makes it possible to compare profiles obtained after double inoculation of the root for all the time intervals tested between the two inoculations.

Strain identification

Nodules were harvested two weeks after the inoculation and tested for strain occupancy by immunofluorescence as described by Schmidt (1974).

3. Results

Measure of duration of sensitivity to infection in root cells

The percentage of plants with nodules and the number of nodules formed above the RT decreased when an interval of more than 2 hr was left between the time when the infection sensitive zone was marked and inoculation (Table 1). After a 4-hour interval, the number of nodules formed above the RT decreased nearly by half and after 6 hr, only a quarter of the number of nodules formed in the infection sensitive zone initially marked. However, after 9 hr, nodules once again formed above the RT. The same results were obtained with the GMB1 strain (data not shown). The duration of sensitivity to infection of root cells is thus higher here than that observed by Bhuvaneswari and co-workers (1980), who did not obtain nodule above the RT after a 6-hour delay between marking and inoculation of the infection

Table 1. Effects of delayed inoculation on nodulation above the root tip mark. Sets of 30 plants were marked at the root tip at various time intervals prior to inoculation with *Bradyrhizobium japonicum* strain G2. After one week, the number and position of primary root nodules above the RT mark were determined for each plant.

Delayed inoculation (hours)	0	2	4	6	9
% plants nodulated	93	93	58	36	28
% plants nodulated above RT mark	86	64	58	14	14
Nodules* above RT mark per plant	2.57±0.86	2.23±1.25	1.57±0.67	0.40±0.67	0.50±0.90

*Mean ±SE

Table 2. Percentages of nodules formed by strain G2 in delayed inoculation experiments. Sets of 30 plants were first inoculated with strain GMB1 in the infectible root zone (time 0) and after a 2 to 10 hour interval, strain G2 was added in the same root zone. Nodulation was scored two weeks later and nodules were tested for strain occupancy.

First experiment		Second experiment	
Delay (hours)	% of nodule occupancy by strain G2	Delay (hours)	% of nodule occupancy by strain G2
0	81	0	93
2	49.1±18.6	6	22.6±11.6
4	56.2±18.3	8	26.7±13.4
6	24.9±18.1	10	17.5±13.8
8	24.1±19.5		

sensitive zone. The type of plant material used (we used a Kingsoy cultivar, while Bhuvaneswari and co-workers used Williams and Beeson cultivars) as well as the bacterial strains could account for the differences observed in sensitivity to infection.

Delayed inoculation of G2 strain in the infection sensitive zone at 0 time

In order to determine the critical time period necessary for occupancy by the least competitive strain, the GMB1 strain was inoculated first in the root followed by the G2 strain at different time intervals in the same zone. The experiment was carried out in duplicate.

The results shown in Table 2 demonstrate that a two hour delay in inoculation of the G2 strain is sufficient to favor nodulation of the GMB1 strain, inoculated first. However, the G2 strain formed most of the nodules after a 4 hour delay in inoculation and the analysis of variance did not show any difference between the two and four hour treatments ($P<0.05$). On the contrary, 6 and 8 hour treatments (undifferentiated) were significantly different from the 2 and 4 hour treatments ($P<0.05$): the GMB1 strain formed most of the nodules after a 6 hour delay in inoculation of the G2 strain, although it would have formed only 19% if the two strains had been inoculated simultaneously.

Beyond a 24 hour delay, no nodule was formed by the G2 strain. However, the G2 strain formed nodules up to 10 hr in the zone sensitive to infection at 0 time. These results concur with those for the duration of sensitivity to infection in root cells that we observed (Table 1).

In vermiculite culture, Kosslak et al. (1983) showed that adding a less competitive *Bradyrhizobium* strain 6 hr before another more competitive strain allowed the first strain to form more nodules than it would have if the two strains had been added simultaneously. However, a 48 hour delay was necessary before the first strain formed most of the nodules.

An interesting point which should be noted is the fact that the nodulation intensity for the GMB1 strain does not appear to be affected by delayed inoculation of the G2 strain: the nodulation profiles for different delayed inoculation treatments are identical to controls for GMB1 strain nodulation, although we previously observed that simultaneous inoculation of the two strains caused their nodulation to decrease (Fernandez-Flouret and Cleyet-Marel, 1988).

Delayed inoculation in the zones sensitive to infection at each inoculation time

The results obtained by Pierce and Bauer (1983) on the development of a regulation of nodulation less than 15 hr after inoculation led us to test delayed inoculation of the G2 strain 15 hr after inoculation of the GMB1 strain.

Figure 1A shows the nodulation profiles obtained for GMB1 control (inoculated alone) at 0 time and G2 control (inoculated alone) at 15 hr, as well as the nodulation profile (GMB1 and G2 nodules) obtained for a double inoculation at a 15 hr interval. Surprisingly, the first nodulation peak is much lower than its control, while the second peak is not. However, a serologic analysis of nodules revealed the presence of numerous GMB1 nodules at the level of the second nodulation peak and showed a sharp decrease in the number of G2 nodules formed (Fig. 1B). Successive inoculation of GMB1 and G2 strains caused a decrease in nodulation for both strains without changing the respective localization of the nodules. Moreover, we did not observe any dominance of the G2 strain in the number of nodules formed when the two strains were inoculated at the same concentration.

The same type of results were obtained when G2 was inoculated 15 hr before GMB1 (Fig. 2A and 2B). Nodulation of both strains sharply decreased, particularly in the first infection sensitive zone of the root.

Table 3 shows the percentage of nodulation reduction for each strain when inoculated successively. These percentages were obtained by comparing the average number of nodules formed per plant in controls with that obtained in double inoculation. There does not appear to be any relationship between the differences in competitiveness of the G2 and GMB1 strains and the percentages of nodulation reduction after 15 hr.

We therefore tested other inoculation delays to see if the same type of nodulation regulation could be observed for longer time periods.

The nodulation profiles obtained for 24 hour inoculation intervals with the delayed inoculation of G2 or GMB1 always demonstrated the same type of regulation (profiles not shown). As for 15 hr, a reduction in nodulation was observed in the first inoculated zone, but not in the second. The serologic analysis showed that the nodules in the second infection sensitive zone were formed by both strains. For a 48 hour inoculation interval, highly differentiated nodulation peaks were obtained, though they were always lower than controls (Fig. 3).

For a high bacterial concentration at the first inoculation ($8 \cdot 10^6$ bacteria per plant), Pierce and Bauer (1983) observed a reduction in the nodulation

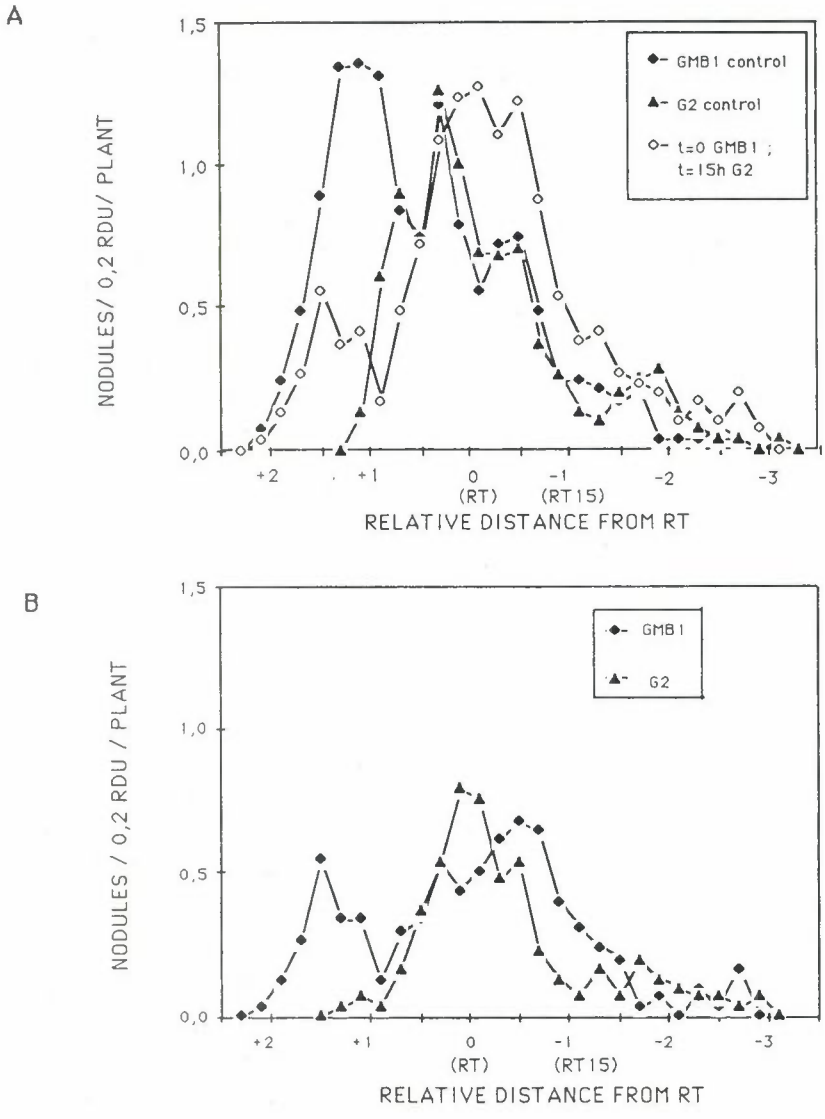

Figure 1. Distribution of primary root nodules on soybean seedlings inoculated with strain GMB1 at time 0 and with strain G2 at time 15 hr. (A) Nodulation profile obtained in double inoculation and nodulation profiles of controls obtained in single inoculation (GMB) at time 0 or G2 at time 15 hr). (B) Nodulation profiles of strains GMB1 and G2 obtained after serological analysis of nodules formed on doubly inoculated seedlings. One RDU is equal to the length of the root between the root tip at time 0 (RT) and the root tip at time 15 hr (RT 15).

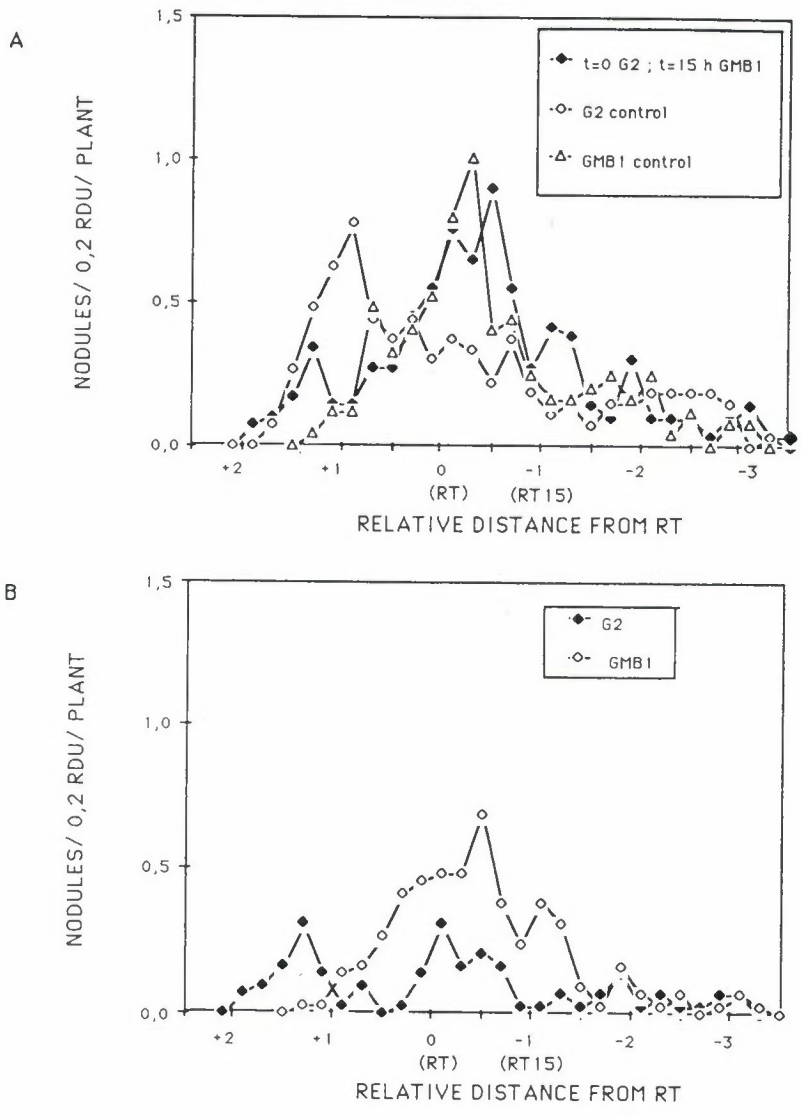

Figure 2. Distribution of primary root nodules on soybean seedlings inoculated with strain G2 at time 0 and with strain GMB1 at time 15 hr. (A) Nodulation profile obtained in double inoculation and nodulation profiles of controls obtained in single inoculation (G2 at time 0 or GMB1 at time 15 hr). (B) Nodulation profiles of strains G2 and GMB1 obtained after serological analysis of nodules formed on doubly inoculated seedlings. One RDU is equal to the length of the root between the root tip at time 0 (RT) and the root tip at time 15 hr (RT 15).

Table 3. Percentages of nodulation reduction of strains G2 and GMB1 in double inoculation experiments. Sets of 30 plants were first inoculated with one strain and after a 15, 24 or 48 hour interval, the second strain was inoculated in the new root zone which did not yet have any root hair. Controls were performed with single inoculation at each time. Nodulation was scored two weeks later and nodules were tested for strain occupancy. The mean number of nodules formed by each strain in double inoculation experiment was compared with that of control singly inoculated.

Experiment	Delayed inoculation (hours)		
	15	24	48
first inoculum : strain GMB1	35%	61%	63%
second inoculum : strain G2	45%	8%	41%
first inoculum : strain G2	67%	42%	nd
second inoculum : strain GMB1	26%	41%	nd

nd : not determined

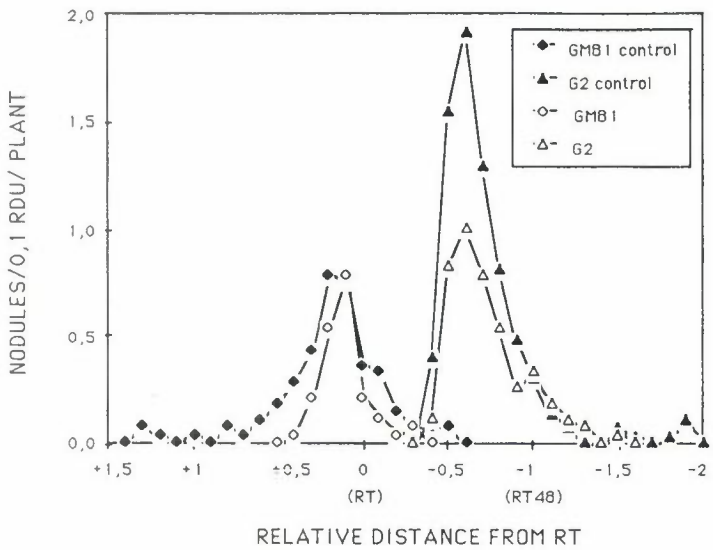

Figure 3. Double inoculation of soybean seedlings with strain GMB1 at time 0 and strain G2 at time 48 hr : nodulation profiles of controls obtained with single inoculation (strain GMB1 at time 0 or strain G2 at time 48 hr), and nodulation profiles of the two strains obtained after serological analysis of nodules formed on doubly inoculating seedlings. One RDU is equal to the length of the root between the root tip at time 0 (RT) and the root tip at time 48 hr (RT 48).

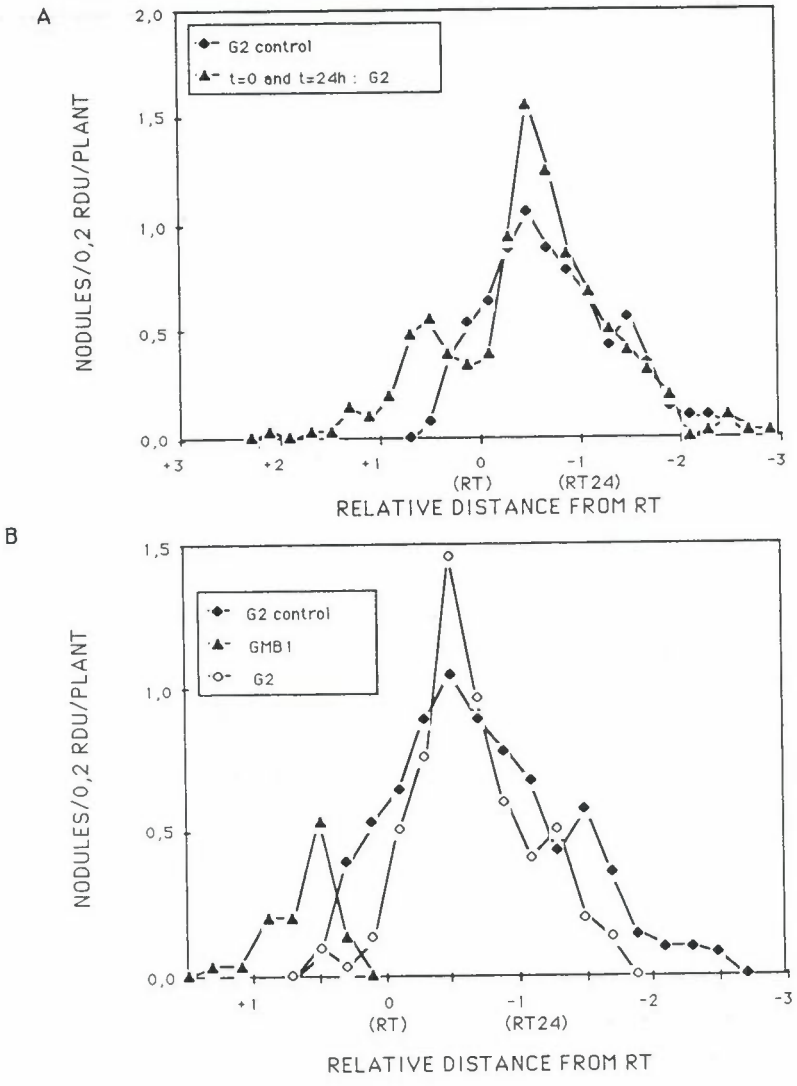

Figure 4. Distribution of primary root nodules on soybean seedlings doubly inoculated. (A) Nodulation profile obtained with double inoculation of strain G2 at times 0 and 24 hr, and nodulation profile of the control obtained with single inoculation (G2 at time 24 hr). (B) Nodulation profiles of strains GMB1 and G2 obtained after serological analysis of nodules formed on doubly inoculated seedlings (GMB1 at time 0 and G2 at time 24 hr) and nodulation profile of the control (single inoculation of G2 at time 24 hr). One RDU is equal to the length of the root tip at time 0 (RT) and the root tip at time 24 hr (RT 24).

peak obtained in the first inoculation zone. In our experiments, we inoculated plants with concentrations ranging from $9 \cdot 10^5$ to $3,4 \cdot 10^6$ bacteria per plant (which were optimal nodulation concentrations in our study); the reduction in nodulation obtained could be due to an overly high concentration of our inoculum.

We therefore inoculated a series of plants with the GMB1 strain (5.3×10^5 bacteria per plant), and another series of plants with the G2 strain (2.4×10^5 bacteria per plant). The plants were then reinoculated 24 hours later with the G2 strain (2.5×10^5 bacteria per plant). A control series was inoculated at 24 hours with the G2 strain.

Figure 4 shows the nodulation profiles obtained: for both types of experiments, nodulation in the first inoculation zone was low, while a nodulation peak for the G2 strain can be observed in the second inoculation zone which is only slightly lower than controls. The nodulation reduction observed in the first inoculation zone thus appears to be independent of the inoculum concentration and of which strain was inoculated first.

4. Discussion

Pierce and Bauer (1983) demonstrated the phenomenon of nodulation regulation in the soybean by performing delayed inoculation and using a single *Bradyrhizobium* strain. These authors notably showed that nodulation on the primary root due to a first inoculation significantly reduces the formation of nodules due to a second inoculation delayed by 15 hr. However, Heron and Pueppke (1987) showed that the intensity of this regulation depended on the strain x cultivar couple used.

Inhibition of nodulation of a strain by pre-inoculation with another strain was also demonstrated during the studies of competition between *Bradyrhizobium* strains in nodule formation. In field cultivated soybeans, Skrdleta (1970) observed that for two *Bradyrhizobium japonicum* strains, the longer the inoculation interval between strains, the more nodulation of the second strain added was inhibited. After a 7 day interval, all the nodules formed on the primary root came from the strain added to the crop. Using the split-root technique, other authors have shown that nodulation of the second half of the system was inhibited by pre-inoculating the first half after a 96 hour inoculation interval for the soybean (Kosslak and Bohlool, 1984) and a 24 hour interval for clover (Sargent et al., 1987).

Our experiments in delayed inoculation in the root zones sensitive to infection at the time of inoculation showed a different type of nodulation regulation than that observed by Pierce and Bauer (1983) for any order of strain

inoculation. When added 48 hr after the GMB1 strain, nodulation by the G2 strain was only reduced by 40% while Pierce and Bauer (1983) observed very few nodules formed in the second inoculation zone for the same time period. In fact, Heron and Pueppke (1987) repeated Pierce and Bauer's experiment using the same plant material and obtained a nodulation reduction in the two inoculated zones but they accounted for this by the high variability in nodulation observed for single inoculations. Takats (1986), who used the same *Bradyrhizobium* strain as Pierce and Bauer on a different soybean cultivar, did not obtain a true reduction in the second nodulation peak and only obtained a reduction in the first nodulation peak when the second inoculum applied 10 hr later was superoptimal. However, using the same strain for two successive inoculations not make it possible to differentiate between the nodules formed at each inoculation as the total nodulation profiles obtained cannot account for the real phenomenon of regulation. The results obtained by Takats (1986) and Heron and Pueppke (1987) could be due to a reduction in nodulation in the two inoculated zones, as the time interval of 10 and 15 hr does not make it possible to clearly separate the two nodulation peaks.

Considering the G2 and GMB1 strains, no significant difference was observed in the respective percentages of nodulation reduction (Table 3). Apparently, the phenomenon of nodulation regulation observed during delayed inoculations along the primary root acts in the same way for the two strains. The decrease observed in the number of nodules formed by the first strain inoculated does not appear to be related to competitiveness of this same strain.

However, the phenomenon of nodulation reduction of the first inoculated strain could explain the decrease observed in the number of nodules formed above the RT when the concentration of the inoculum increases (Fernandez-Flouret and Cleyet-Marel, 1988). The greater the number of inoculated bacteria, the more these bacteria are distributed towards the root base. The newly formed root cells are thus infected, causing a reduction in nodulation of the first cells infested.

The phenomenon of nodulation regulation was not observed with preinoculation of bacteria killed by U.V. irradiation (Pierce and Bauer, 1983) or of non-infectious bacteria (Heron and Pueppke, 1987). Along the same lines, Sargent et al. (1987) observed no regulation of nodulation in a *Rhizobium trifolii* strain by its mutant lacking plasmid nodulation genes. Regulation of nodulation seems thus to be induced only by bacteria capable of infecting the plant.

Using the material of Pierce and Bauer (1983) for anatomical observation, Calvert et al. (1984) showed the presence of numerous infections which did

not yield nodule formation in the second inoculation zone. Thus, regulation of nodulation seems to be induced at a stage following meristem induction.

Sargent et al. (1987) observed that simultaneous inoculation of *Rhizobium trifolii* strain and one of its mutants (affected in its nodulation genes) in different parts of the root system led to a reduction in nodules formed by the mutant. The strains, however, presented the same nodulation profiles. According to the authors, a delay of several hours in initiation of nodules by mutant make it possible for the wild type to induce regulation of nodulation by the mutants. The same hypothesis does not seem to be applicable to G2 and GMB1 strains as these two strains appear to exert the same regulation of nodulation. However, the hypothesis of Sargent et al. (1987) could explain the fact that we did not observe a reduction in nodulation in the GMB1 strain after the delayed addition of the G2 strain in the same root zone (while simultaneous inoculation of the two strains led to a sharp reduction in their nodulation (Fernandez-Flouret and Cleyet-Marel, 1988). In our experiments, when the GMB1 strain was added a few hours before the G2 strain, it should induce a regulation of nodulation which decreases the number of nodules formed by the G2 strain; the longer the inoculation interval, the lower the amount of nodules formed by the G2 strain as the sensitivity to infection of the root cells also plays a role in this regulation phenomenon which inhibits the formation of nodules by the other strain and leads to a reduction in the number of nodules formed by both strains. In extreme cases of competition there can thus be a total absence of nodulation (Broughton et al., 1982).

The first hours of infection thus appear to be determinant in the occupancy of a *Bradyrhizobium* strain on the roots of a host plant, but the results obtained from nodulation profiles for the soybean did not show any significant difference between the G1 and GMB1 strains, neither for initiation of nodulation, nor for regulation of nodulation during delayed inoculations.

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