

## Competition Between *Bradyrhizobium japonicum* Strains for Nodulation: Characterization of Nodulation Patterns Using Plastic Growth Pouches

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### Abstract

Competition between two strains of *Bradyrhizobium japonicum*, G2 and GMB1, for nodulation on soybean (*Glycine max* (L.) Merr.) was studied by analysing the nodulation characteristics of each strain, inoculated alone or in a mixture. The competitiveness of the two strains differed considerably, but they showed the same ability to initiate nodulation when tested separately. In the two cases, inoculum concentration similarly affected the percentage of plants nodulated and the average number of nodules formed per plant. The mean percentage of nodules formed above the RT mark (Root Tip at the time of inoculation) was inversely proportional to the number of rhizobia applied to the root. Inoculation of the plants with both strains at once led to a decrease in the average number of nodules formed, without any preferential root localization of nodules initiated by either strain.

Keywords: *Bradyrhizobium*, competition, profile of nodulation frequency

### 1. Introduction

The infectibility of root cells in soybean (*Glycine max* (L.) (Merr.) is a transient property (4-6 hr); epidermal cells susceptible to infection by rhizobia at the time of inoculation are located in the zone of elongation, just above the root tip (RT); as the root grows, these cells mature and lose their susceptibility to nodulation; the newly generated cells then become infectible

(Bhuvanewari et al., 1980). The location of nodules relative to the RT position is a useful quantitative indicator of the initial nodulation rate and efficiency. Bhuvanewari et al. (1983) have used this method to demonstrate that the age of *Bradyrhizobium* cultures affects the efficiency of the infection rather than the rate at which nodulation is initiated, and that bacteria at mid-log phase (third day of culture) are the most effective. Profiles of nodulation frequency, based on the location of the nodules on the primary root, can be used to characterize different properties of the *Rhizobium* strains. For instance, Halverson and Stacey (1984) have shown that *Bradyrhizobium japonicum* strain HS111, a mutant that is slow to nodulate, developed nodules at a lower position on the root than the wild strain, and its ability to initiate infection was thus delayed.

Competition for nodulation among rhizobial strains is an important aspect of rhizobial ecology; the competitiveness of a strain is the second criterion for selection after its capacity to fix nitrogen. Many works report the effect of several factors on this competition phenomenon as the type of soil (Moawad and Bohlool, 1984; Kossiak and Bohlool, 1985) the temperature (Hardarson and Gareth-Jones, 1979), host variety (Diatloff and Brockwell, 1976; Ruiz-Argüeso et al., 1977), bacterial motility (Ames and Bergman, 1981), or the composition of the culture medium (Fernandez-Flouret and Cleyet-Marel, 1987).

The objective of this work was to determine, using nodulation profiles, whether the tendency of two strains of *Bradyrhizobium japonicum* to nodulate in the root zone infectible at the time of inoculation, is an important factor in their respective competitive abilities to nodulate.

## 2. Material and Methods

### *Bacterial strains*

*Bradyrhizobium japonicum* strain G2 (311B125, USDA, Beltsville) and strain GMB1 (I.R.A. Madagascar) were used in this study. Stock cultures were maintained on yeast extract mannitol (YEM) agar slants at 5°C (Vincent, 1970).

### *Strain growth in separate or common cultures*

Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of YEM medium (with only 0.01 g of mannitol to obtain a complete growth curve in a shorter time) on a shaker, at 28°C. One flask was inoculated with 1 ml of a suspension of strain G2, one flask with 1 ml of a suspension of strain

GMB1, and one flask with 0.5 ml of G2 and 0.5 ml of GMB1. Strain growth was studied by monitoring the O.D. at 620 nm of the suspensions and their concentrations: each day, viable cells were counted by plating 0.1 ml of serial dilutions of each culture on YEM agar plates. Since the colony morphologies of strains G2 and GMB1 differ (G2 forms a white circular colony and GMB1 forms a small beige diffuse colony), it was very easy to distinguish between them on the plates.

#### *Preparation of inocula*

Liquid cultures for inoculum preparation were grown on YEM medium. Each strain was shake-cultured in 250 ml Erlenmeyer flasks containing 50 ml of YEM for 3 days at 28°C, until the mid-log phase. Rhizobia were harvested by centrifugation (3000 g, 20 min) and resuspended in a sterile saline solution (Bergersen, 1961) to the desired concentration, assessed by spectrophotometry (O.D. at 620 nm). Viable cells were counted by plating.

#### *Seed germination and plant culture*

Soybean seeds (*Glycine max* (L.) Merr. cv. Kingsoy) were surface-sterilized for 20 min in 3% calcium hypochlorite, washed five times with sterile water, and soaked in water for 2 hr prior to germination on water agar plates (7 g l<sup>-1</sup>). Seeds were germinated in a growth chamber for 2 days at 28°C. Seedlings without visible microbial contamination were transferred to plastic growth pouches (containing a paper towel and sterilized with gamma radiation), moistened with 10 ml of hydroponic growth nutrient solution (Kalia and Drevon, 1985), and maintained in a controlled environment growth chamber (20°C night, 25°C day; light intensity: 200  $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ ; photoperiod: 14 hr; 80% RH).

#### *Inoculation and determination of nodulation frequency profiles*

Three-day-old seedlings were inoculated under sterile conditions with 25  $\mu\text{l}$  of a bacterial suspension in the root zone where infectible cells were located, between the zone of the smallest emerging root hairs (SERH) and the root tip (RT), which were marked at the same time (Bhuvanewari et al., 1980). Plastic growth pouches were kept moistened, and nodules appeared within 8 days. The position of each nodule from the RT mark was measured to the nearest 0.2 mm, and expressed relative to the SERH-RT length (the distance from SERH to RT) in the nodulation frequency profile. Plants that died or were injured were not scored, and the nodulation frequency was expressed as the number of nodules per plant and per 20% of the SERH-RT distance, taking into account all the plants inoculated.

### *Competition between the two strains of Bradyrhizobium japonicum G2 and GMB1*

Inocula were prepared by mixing suspensions of strains G2 and GMB1 to obtain frequencies ranging from 0.01% to 100%. Thirty plants were inoculated with each suspension. Concentrations of the mixed inocula varied from  $4 \times 10^6$  to  $6 \times 10^7$  bacteria per plant. After 15 days of growth, all nodules were scored and tested for strain occupancy by immunofluorescence, as described by Schmidt (1974).

#### *Effect of bacterial concentration on nodulation*

Plants were inoculated with 25  $\mu$ l of bacterial suspensions of each strain ranging from  $10^3$  to  $10^8$  cells per plant. Thirty plants were tested with each concentration. After 10 days of growth, each plant was scored to determine nodulation frequency profiles.

## 3. Results and Discussion

### *Competition for nodule sites*

The competition pattern of the two strains, G2 and GMB1, for nodule sites is given in Table 2. When the proportion of G2 in the mixed inoculum was 0.8%, this strain formed 17% of the nodules. With less than 10% of this strain in the mixed inoculum, it was able to form half of the nodules. Our results show that the competitive ability of the two strains is different, G2 being the more competitive.

### *Strain growth when cultured together*

The strains were cultured until they reached the stationary phase, which took 130 hr. Growth rates calculated in the mid-log phase showed little difference: GMB1:  $\mu=0.0349$ ; G2:  $\mu=0.0358$ ; G2+GMB1:  $\mu=0.0316$ . Figure 1 shows the variation of the concentrations of strain GMB1 versus that of strain G2 when they were cultured together. The logarithm of the concentration of strain GMB1 varies almost linearly with the logarithm of the concentration of strain G2. This shows that there was only competition between the two strains for substrate uptake and that there was no antagonism between them. Interference between various *Rhizobium* strains in culture has previously been detected (De Antoni et al., 1981), but no correlation with the competitive ability of the strains has been shown (Martensson and Gustafsson, 1985). In the present study, the greater competitiveness of strain G2 does not appear to be correlated with superior growth in the culture medium when the two strains are mixed.



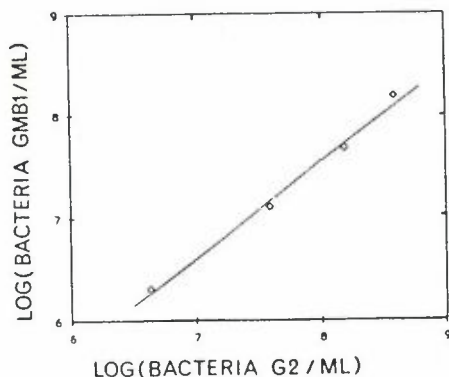


Figure 1. Growth of strain GMB1 of *Bradyrhizobium japonicum* relative to the growth of strain G2 when cultured together. The two strains were cultured on YEM medium (0.01 g of mannitol) until they reached the stationary phase. Viable cells were counted daily by plating on YEM agar. The curve was fitted to the data points by the least-squared method.

#### *Profile of nodule distribution*

The nodulation profiles of the two strains (G2 and GMB1 of *Bradyrhizobium japonicum*) are shown in figs. 2A and 2B. They show that the two strains have a maximum frequency of nodulation in the area between RT and SERH. This confirms the work of Bhuvaneswari et al. (1980, 1981) and Halverson and Stacey (1984). The nodulation profiles obtained with the two strains change slightly with the rhizobial concentration in the inoculum. More nodules were produced below the RT and in the younger areas of the root when the inoculum concentration was increased.

Correspondence analysis was applied to the nodulation profiles to compare the nodule numbers initiated in each 20% fraction of the SERH-RT distance. This showed a clear distinction between the nodulation profiles corresponding to different inoculum concentrations. The different treatments, i.e. the different numbers of rhizobia inoculated per plant, are situated along an axis with a high level of inertia (59%) showing a clear-cut distinction between high and low inoculum concentrations. In contrast, the analysis was unable to distinguish between the nodulation profiles of the two strains. These data do not suggest that the poor competitiveness of strain GMB1 is due to a defective initiation of infection leading to nodulation. When the two strains were inoculated separately, they showed the same ability to initiate infection

Table 1. Effect of the inoculum concentration on the number of nodules formed on the primary root of soybean. Plants were grown in growth pouches and separate sets of 30 plants were inoculated with different concentrations of the strain G2 or the strain GMB1. Nodulation was scored 10 days later.

Strain	log (bacteria per plant)	nodules* per plant	nodules* above RT per plant
G2	3.60	2.00 ± 1.61	2.00 ± 1.61
	4.60	3.90 ± 1.14	3.55 ± 1.12
	5.40	7.82 ± 1.24	5.32 ± 1.25
	6.20	8.50 ± 2.07	5.83 ± 1.90
	6.60	8.80 ± 2.62	5.14 ± 2.29
	8.15	8.52 ± 1.58	1.92 ± 0.78
GMB1	2.48	1.30 ± 1.44	1.30 ± 1.44
	3.48	1.20 ± 1.55	1.00 ± 0.86
	4.48	2.62 ± 0.77	2.45 ± 0.70
	5.68	5.20 ± 1.14	4.35 ± 1.06
	6.34	13.10 ± 1.74	9.82 ± 1.62
	7.48	10.10 ± 1.45	3.63 ± 0.97
	7.94	9.00 ± 2.13	5.25 ± 1.71

\*Mean ±SE

and to promote nodulation, but a difference in competitiveness for nodule sites appeared when the two bacteria were both present on the root system.

#### *Effect of the inoculum dose on the nodulation pattern of the two strains*

Several effects of the inoculum dose were observed. First, the proportion of plants that were nodulated increased with the inoculum dose, reaching 95–100% with  $3 \times 10^5$  G2 rhizobia per plant or  $10^8$  GMB1 rhizobia per plant (Fig. 3A). Moreover, more than 70% of the plants were nodulated when a minimal concentration of  $3 \times 10^4$  rhizobia of either strain was administered. Second, the mean number of nodules formed per plant increased with the inoculum concentration and stabilized to  $8.50 \pm 2.07$  with strain G2. The stabilization with strain GMB1 was less evident, but when 90% of the plants were nodulated, the number of nodules formed per plant no longer increased. The mean data are given in Table 1.

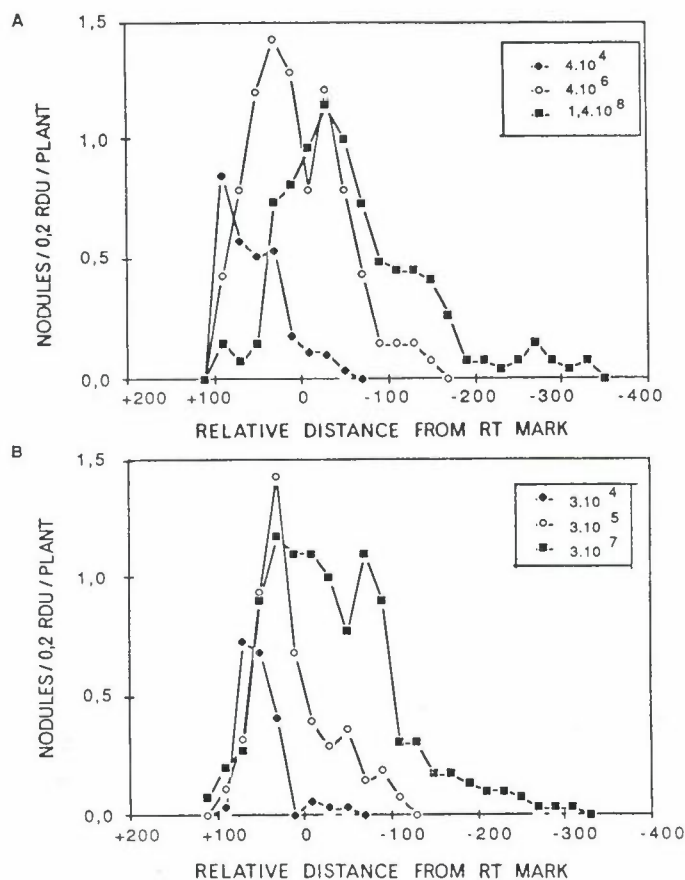


Figure 2. Nodulation frequency profile of *Bradyrhizobium japonicum*: (A) strain G2; (b) strain GMB1. Sets of 30 plants were inoculated with 25  $\mu$ l of a bacterial suspension between the SERH and the RT positions. Three different concentrations were tested and are expressed in number of bacteria per plant. Nodulation on the primary root was scored at about 10 days. One RDU (Relative Distance Unit) is equal to the length of the root between SERH (taken as +100) and RT (taken as 0). The direction of root growth is from positive to negative values.

Another point of interest is the relationship between the inoculum dose and the proportion of plants with nodules above the RT mark or the mean number of nodules per plant above the RT mark (Fig. 3B). The variation of the proportion of plants nodulated above the RT mark was roughly the same as that of the proportion of plants nodulated on the whole root system. Nevertheless, slight differences appeared at high inoculum concentra-

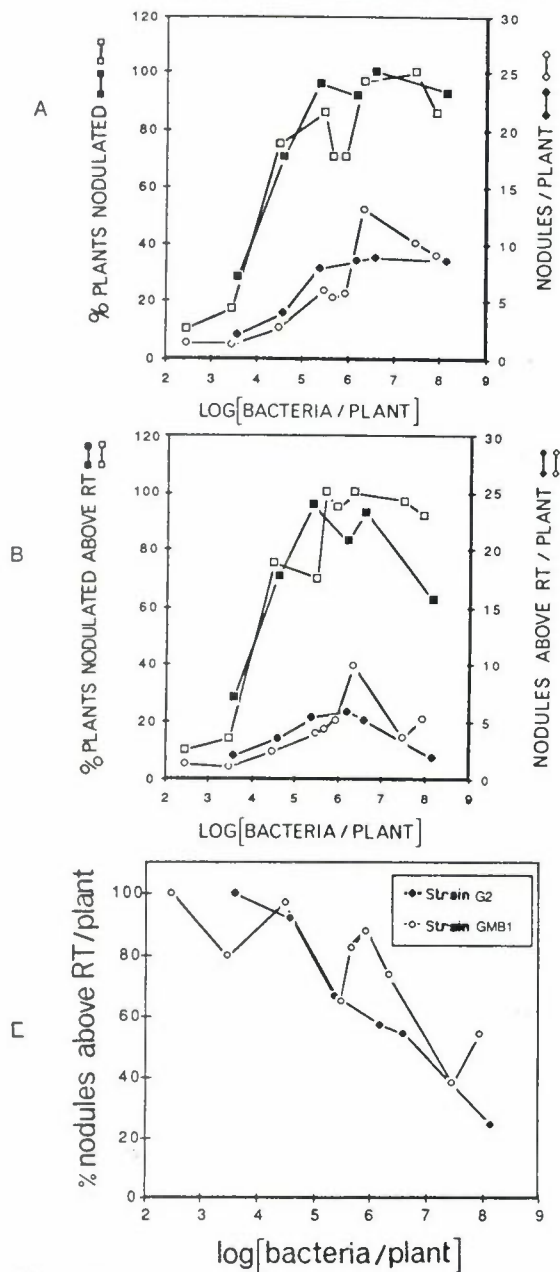


Figure 3. Effect of inoculum concentration: (A) on nodulation of soybean cv. Kingsoy, (B) on nodulation above the root tip (RT) marked at the time of inoculation, (C) on the percentage of nodules formed above the RT mark. Sets of 30 plants were inoculated with strain G2 or GMB1 at different concentrations ranging from  $10^2$  to  $10^8$  bacteria per plant. Data on the number of nodules formed are average values for all the plants nodulated, but SE are not shown. Solid symbols represent strain G2 and open symbols strain GMB1.



tions. The mean number of nodules above the RT mark reached a maximum with  $10^6$  rhizobia per plant and then decreased at higher doses (Fig. 3B and Table 1). A similar result was reported by Pierce and Bauer (1983), but at lower concentrations, since the nodule maximum above the RT mark was observed with  $10^4$  rhizobia per plant. These authors considered that this result suggests a regulatory phenomenon affecting nodulation in soybean inoculated with high concentrations.

We also observed that the mean number of nodules formed per plant has a tendency to stabilize at around 9 nodules with high concentrations of inoculum (Fig. 3A). The maximum of nodulation frequency in fact shifts below the RT mark as the dose increases (Fig. 2A and B), and the proportion of nodules above the RT mark appears to vary inversely with the inoculum dose (Fig. 3C). This observation is particularly evident with strain G2. An increase in the mean number of nodules per plant above the RT mark (Fig. 3B) in the range  $10^2$  to  $10^6$  rhizobia per plant was observed because the inoculum dose was not optimal and the regulatory phenomenon affecting nodulation in this area did not occur.

The decrease in the proportion of nodules per plant above the RT mark occurred even at low inoculum concentrations, which is consistent with the study by Turgeon and Bauer (1983). With our inoculum technique, rhizobia are applied directly and mainly on the more infectible cells on the primary root, but with an inoculum volume of 25  $\mu$ l, some is spread around the infectible cells and small droplets adhere to the root tip, which moves as the root grows. According to Turgeon and Bauer (1983) the number of bacteria required to achieve a given probability of nodule formation appears to be linked with a precise application of the inoculum on the infectible cells. Thus, it appears understandable that small droplets dispersed below the RT or adhering to it increase their infection potential with the inoculum concentration and give rise to more nodules below the RT mark.

#### *Mixed inoculation*

Nodulation frequency profiles obtained after inoculation of the plants with a mixture of strains G2 and GMB1 confirm the results obtained with single-strain inocula: nodules were distributed uniformly along the root, in the zone infectible at the time of inoculation. No particular localization of the nodules was observed. On the other hand, at a given dose, the average number of nodules per plant obtained with the mixed inoculum (Table 2) was lower than with the single-strain inocula (Table 1, Fig. 3A). In treatment where

Table 2. Nodule occupancy by the strain G2 in competition with the strain GMB1 and effect of mixed inoculation on nodulation of *Glycine max* cv. Kingsoy. Separate sets of 30 seedlings were inoculated with a mixture of strains G2 and GMB1 of *Bradyrhizobium japonicum* in various proportions. Nodulation on the primary root was scored 15 days later and strain occupancy was assessed by immunofluorescence. RT is the mark made at the root tip at the time of inoculation.

log bacteria added per plant	% of strain G2 in the mixed inoculum	% of nodule occupancy by strain G2*	% plants nodulated	% plants nodulated above Rt mark	nodules* per plant	nodules* above RT mark per plant	% nodules* above RT mark per plant
7.81	97	100.00 ± 0.00	90	50	4.64 ± 1.06	1.75 ± 0.81	29.92 ± 14.21
7.72	80	95.70 ± 3.49	100	66	5.41 ± 0.99	2.17 ± 0.78	37.48 ± 11.89
7.35	31	83.89 ± 7.82	87	81	4.19 ± 1.13	2.42 ± 0.93	49.52 ± 13.29
6.63	8.6	62.18 ± 9.20	96	74	4.48 ± 0.71	1.74 ± 0.61	37.89 ± 12.48
6.63	0.8	17.30 ± 6.68	97	93	5.79 ± 0.96	4.27 ± 0.89	75.01 ± 10.24
6.64	0.08	1.71 ± 2.39	96	96	5.14 ± 0.98	3.48 ± 0.89	67.50 ± 12.33

\* Mean ± SE

strain G2 formed most of the nodules (100, 96 and 80%), the average number of nodules per plant varied between 4 and 5 at a dose of the order of  $10^7$  bacteria, whereas 8 or 9 nodules per plant would be obtained with the same concentration of strain G2 (Fig. 3A). Likewise, in treatments where strain GMB1 formed most of the nodules (18 and 1.2% G2 nodules), we obtained an average number of 5 to 6 nodules per plant at a dose of  $4 \times 10^6$  bacteria per plant, whereas strain GMB1 alone at the same concentration would have resulted in 12 nodules per plant (Fig. 3A).

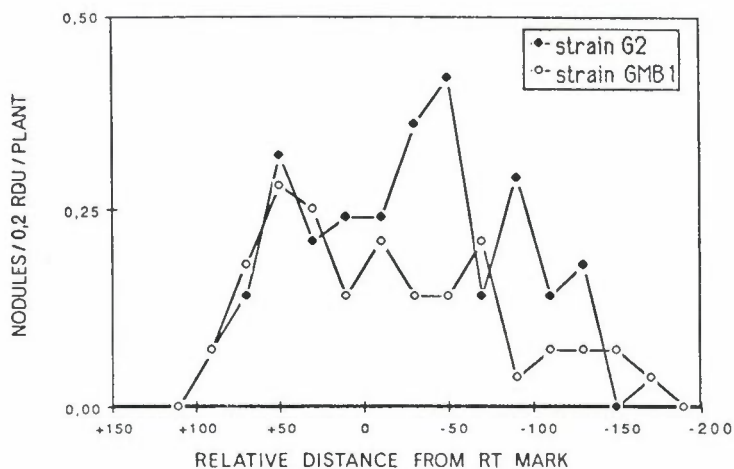


Figure 4. Nodulation frequency profile obtained with an inoculum consisting of a mixture of the two strains G2 and GMB1. Sets of 30 seedlings were inoculated with an inoculum consisting of 8.6% strain G2 and 91.4% strain GMB1. Nodules were scored at about 15 days and tested for strain occupancy. Strain G2 formed 59% of the nodules and strain GMB1 formed 41%. One RDU is equal to the length between SERH (taken as +100) and RT (taken as 0). The direction of root growth is from positive to negative values.

On the other hand, the average percentages of nodules above the RT mark were of the same order as those obtained with single-strain inocula (Table 2 and Fig. 3C). When strain G2 formed most of the nodules, we obtained an average percentage of the order of 39% (average of 3 treatments), and when strain GMB1 formed most of the nodules, the percentage was of the order of 70% (average of two treatments). The corresponding percentages with single-strain inocula were of the order of 34% for strain G2 and 65% for strain GMB1 (Fig. 3C). This kind of reduction of nodulation with mixed

inocula has been observed with soybean (Diatloff and Brockwell, 1976) and with the pea (Winarno and Lie, 1979), but in the latter case no antagonism between the two strains was observed either in liquid culture or in the rhizosphere of the plant (Broughton et al., 1982). The existence of many infections preventing the formation of nodules on inoculated roots (single strain) has been demonstrated by Calvert et al. (1984). As Broughton et al. (1982) have proposed, this phenomenon suggests that the regulation of nodulation occurs at a stage following bacterial penetration into the root. This hypothesis may also explain the reduction in nodulation when two strains are present, as we observed no change in the location of the nodules on the root (and consequently in the rate of nodule initiation).

#### 4. Conclusion

The growth-pouch technique (Bhuvanewari et al., 1980) was used to study competition for nodule formation on soybean between two strains of *Bradyrhizobium japonicum*. This technique has allowed us to demonstrate that the competitiveness of strains G2 and GMB1 could not be distinguished on the basis of differences in the rates of the nodule initiation in the root zone infectible at the time of inoculation, in the percentage of plants nodulated, or in the average number of nodules formed. The average number of nodules formed was reduced when the two strains were administered together, without any change in the location of the nodules on the root, suggests that a nodulation regulation must occur during the stages following bacterial infection.

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