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Relation Between the Content of Growth Regulators and Effectiveness of *Bradyrhizobium japonicum*

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Abstract

The objective of the investigation discussed in this paper was to study the effect of two *Bradyrhizobium japonicum* strains, which produce different growth regulators, on the characteristics of the symbiotic association with soybean variety NS-16. The effect of the eluate of IAA spots from TLC plates on the symbiotic association with the strain which is the less effective producer of growth regulators was studied as well. The results obtained showed differences in the mass of dry weight of plants and in nitrogen content, nitrogenase activity and in nitrogen fixation effectiveness.

Keywords: *Bradyrhizobium japonicum*, strain, soybean, symbiotic association, growth regulators, N-fixation, effectiveness, nitrogenase

1. Introduction

Bradyrhizobium japonicum strains differ regarding their morphologic, physiologic and biochemical characteristics and their capacity to fix nitrogen when inoculated to individual soybean varieties (Sarić and Fawzia, 1983; Sarić et al., 1990). As shown previously (Sarić et al., 1978; Milić, 1981; Milić and Mrkovački, 1990) *Bradyrhizobium japonicum* strains synthesize various growth regulators and they also differe in their capacity to synthesize growth regulators in culture (Sarić and Milić, 1981; Milić and Sarić, 1986, 1987). Growth

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regulators affect growth and development, and cause morphologic and physiologic changes in plants as well as in the synthesis of organic matter. Nodule bacteria produce growth regulators of both the stimulatory and inhibitory types (Thimann, 1936, 1939; Philips and Torrey, 1970, 1972; Sekino et al., 1989). However, there are no sufficient data about their effect on the process of nitrogen fixation. Different opinions exist about the function of IAA in the mechanism of infection. It has been considered that IAA stimulates nodule formation (Kefford et al., 1960; Nutman, 1965). Bergersen (1974) and Badenoch-Jones et al. (1982) found that curling of root hair is not caused only by the production of IAA by *Rhizobium* spp. strains. However, they did not negate the role of IAA, either *Rhizobium*- or plant-derived, in other aspects of nodule development and maintenance. IAA stimulates forming and elongation of root hairs (Fahraeus and Ljunggren, 1967), but has no effect without bacterial cells. Sekino et al. (1989) suggested that IAA from trypthopan is synthesized in nodules and that there exists a relationship between the plant and *Bradyrhizobium japonicum* bacteria in IAA biosynthesis. The effect of IAA produced by the cells of *B. japonicum* on the host plant, i.e., the role of these regulators on nitrogen fixation in symbiotic associations remains an open question. The objective of our work was to evaluate the effect of indole-3-acetic acid (IAA) synthesized by *B. japonicum* strains, on nodulation, growth and root mass, plant shoot dry weight, nitrogen content, quantity of nitrogen fixed and the activity of nitrogenase.

2. Material and Methods

Two wild types of *Bradyrhizobium japonicum* strains 2b and 1, isolated from the nodules of soybean grown as monoculture for several years at the experiment field of the Institute of Field and Vegetable Crops, Novi Sad, (Rimski Šančevi, Vojvodina Province) differing in their capacity of producing growth regulators in culture were used. Using the TLC method (Stahl, 1969), 14 indole, phenol and gibberellin spots were separated from the filtrate of liquid culture (Katznelson and Shirley, 1965) of the strain *Bradyrhizobium japonicum* 2b, but only four spots from the strain 1 (Milić et al., 1992, in press). Two trials were established, one in pots and one in tubes. The experiments with the two *B. japonicum* strains and the soybean variety NS-16 bred in the Institute of Field and Vegetable Crops in Novi Sad (Vojvodina Province), were done hydroponically with Jensen nutrient solution 1/4 strength (Vincent, 1970). Soybean seeds were sterilized with ethanol and 0.1% acid solution of $HgCl_2$ and germinated for 5 days on water agar and then transferred to pots (R-11 cm R_2-9 cm). Five days old soybean seedlings were inoculated with

0.5 ml liquid culture of *B. japonicum* strain 2b and strain 1 respectively in the logarithmic phase of growth (28×10^8 cells per ml). Uninoculated plants grown in the identical solution were used as control. The experiments were in three replications with six plants per pot.

The plants were grown for 5 weeks (35 days), to the stage of flowering. Sterilized Jensen solution was added to the pots according to the following scheme: for the first 7 days, the solution remained unchanged; after 7 days, the solution was added to the pots with glass funnel to maintain the required quantity of the liquid; after 14 days, the solution was discarded and chanted with a new one every week, up to the period of flowering, when the plants were manually harvested. The plant material was evaluated for the length of the plant shoot, root, the number of nodules and the dry weight of the plant expressed in mg per plant. Nitrogen fixation effectiveness was determined according to the amount of fixed nitrogen evaluated as difference of nitrogen content between inoculated treatments and the non-inoculated control. Total nitrogen was determined by Kjeldahl, and nitrogenase activity by the method Acetylene Reduction Assay (ARA) of Hardy et al. (1968) on a Hewlett Packard A Gas Chromatograph 5840.

The second experiment was established in tubes (25×2.5 cm) with one plant per tube, and performed in ten replications with sterilized Jensen solution. Five days old seedlings were inoculated separately with *B. japonicum* strains 2b, 1 and strain 1 which contained an additional amount of IAA spot extract from TLC sample on silica gel HF_{254} of strain 2b. In the filtrate of *B. japonicum* culture, the growth materials of indole and phenole type were determined by the method of Durkee and Poapat (1962) with TLC on sylica gel HF_{254} (Stahl, 1969). Strain 2b synthesized violet fluoroscence spots with Rf (0.23–0.31). According to its Rf value, these spots corresponded to the standard IAA. The spots were extracted with 80% water solution of methanol. The extract was evaporated under a stream of dry nitrogen and its sediment was dissolved in distillated water. This amount was added to inoculated plants with strain 1 proportional to the IAA concentration observed in strain 2b (app. 0.036 μg in 0.5 ml liquid culture). The results obtained in our study were statistically analyzed by Duncan's Multiple Range analysis.

3. Results

Effect of B. japonicum *strains on the morphology of soybean plants*

The morphology of roots and nodules differed depending on the strains used. After harvesting, the roots of uninoculated plants were less developed than the inoculated ones. The nodules on the plant roots inoculated with strain 2b were larger, reddish in section, and located at the central part of the root, while strain 1 formed smaller nodules, which were located on lateral roots (Fig. 1).

Effect of B. japonicum *strains on the length of soybean plants*

The results obtained indicated that the length of individual plant parts (above-ground part, root and the entire plant) depended on the strain of *B. japonicum* used. When compared with the uninoculated plants, the plants inoculated with *B. japonicum* 2b and *B. japonicum* 1 increased the length of the plants by 14.25 cm and 2.89 cm, respectively. Strain 2b increased the length of the above-ground part, root, and the whole plant by 4.91 cm, 6.12 cm and 11.36 cm, respectively. The number of nodules varied as well. *B. japonicum* strain 1 produced a significantly higher number of nodules than strain 2b which produced less but large nodules (Table 1).

Effect of B. japonicum *strains on the dry weight of soybean plants*

The inoculated strains affected the production of dry weight of the root, the nodules and the whole plants. *B. japonicum* strain 2b significantly increased the dry weight of the roots and the whole plant, when compared with the control plants. *B. japonicum* 1 significantly decreased the dry weight of roots. Five weeks after growing, there occurred significant differences in the mass of nodules. However, strain 2b formed less nodules per plant but higher mass of nodules than strain 1 (Table 2).

Effect of B. japonicum *strains on total nitrogen, fixed nitrogen and nitrogenase activity in soybean plants*

Compared with the uninoculated plants (control), both strains affected the total nitrogen content fixed nitrogen and nitrogenase activity, so that they significantly increased these parameters in the shoot, root and the whole plant (Tables 3, 4 5). Regarding the significant differences achieved between the two strains, we suggest that growth regulators produced by the *B. japonicum* strains, have a direct or indirect role in the enzyme activity of the soybean plant.

Effect of IAA extracted from B. japonicum *2b on the effectiveness of* B. japonicum *1*

The eluate of IAA spots extracted from TLC of *B. japonicum* strain 2b cultures was added to the inoculum of *B. japonicum* 1 and the effect of the

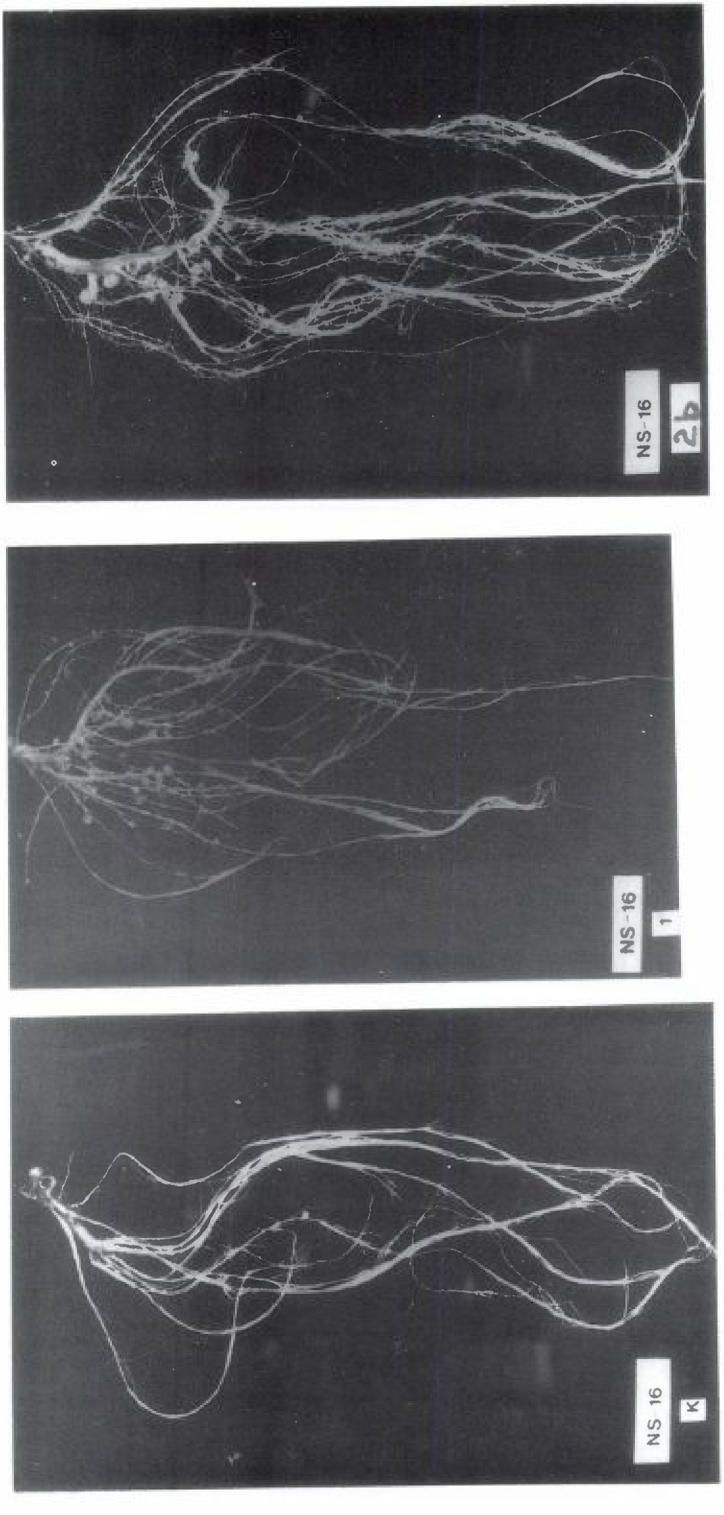

Figure 1. Root of the uninoculated plant (K) and plant inoculated with *B. japonicum* 1 and 2b, 35 days after sowing

Table 1. Effect of *B. japonicum* strains 2b and 1 on the length of the above-ground part, root, whole plant and the number of nodules, 35 days after sowing

Strain	Length in cm			Number of nodules/plant
	above-ground	root	whole plant	
Control	23.21^{C}	28.32^{B}	51.79^{B}	0.01^{C}
2b	35.61^{A}	30.43^{A}	66.04^{A}	29.33^{B}
1	30.70^{B}	24.31^{C}	54.68^{B}	36.39^{A}
LSD at P 0.05	1.818	1.574	3.063	3.110

There are no significant differences between the values marked with the same letter at the level of 0.05. Each sample represents three replicates.

Table 2. The effect of *B. japonicum* strains 2b and 1 on plant dry weight of the above-ground part, root, nodules and the whole plant, 35 days after sowing

Strain	Dry weight in mg			
	above-ground part	root	nodules	whole plant
Control	358.0^{A}	223.7^{B}	0.01^{C}	581.0^{C}
2b	532.8^{A}	256.1^{A}	49.90^{A}	838.8^{A}
1	469.0^{A}	178.0^{C}	39.01^{B}	686.0^{B}
LSD at P 0.05	75.87	7.219	10.08	73.05

There are no significant differences between the values marked with the same letter at the level of 0.05. Each sample represents three replicates.

Table 3. Effect of inoculation with *B. japonicum* strains 2b and 1 on the nitrogen content in NS-16 soybean variety, 35 days after sowing

Strain	Nitrogen content mg/plant			
	above-ground part	root	nodules	whole plant
Control	5.977^{C}	2.717^{C}	0.010^{C}	8.397^{C}
2b	17.740^{A}	3.867^{A}	2.603^{A}	24.21^{A}
1	13.41^{B}	2.770^{B}	1.813^{B}	18.00^{B}
LSD at P 0.05	0.7853	0.07169	0.1242	0.6839

There are no significant differences between the values marked with the same letter at the level of 0.05.

Table 4. Amount of nitrogen fixed (mg/plant) expresses as difference between nitrogen content in inoculated treatments and the non-inoculated control in NS-16 soybean variety, 35 days after sowing

Strain	Nitrogen content mg/plant			
	above-ground part	root	nodules	whole plant
Control	0.01^{C}	0.01^{C}	0.01^{C}	0.01^{C}
2b	11.76^{A}	1.750^{A}	2.603^{A}	15.81^{A}
1	7.439^{B}	0.353^{B}	1.813^{B}	9.604^{B}
LSD at P 0.05	0.7886	0.07169	0.124	0.6801

There are no significant differences between the values marked with the same letter at the level of 0.05.

Table 5. Nitrogenase activity in soybean variety NS-16 inoculated with *B. japonicum* strains 2b and 1, 35 days after sowing

Strain	Nitrogenase activity μmol/plant
Control	0.010^{C}
2b	127.4^{A}
1	70.73^{B}
LSD at P 0.050	1.715

There are no significant differences between the values marked with the same letter at the level of 0.05.

Table 6. Effect of IAA spots eluated of the effective *B. japonicum* strain 2b on the activity of *B. japonicum* 1, 35 days after sowing

Strain	Whole plant dry weight mg/plant	Nitrogen content mg/plant	Nitrogen fixed mg/plant	Nitrogenase activity μmol C_2H_4/plant hr	Dry weight %	Nitrogen content %
Control	544.0^{D}	5.570^{D}	0.01^{D}	0.01^{C}	100.0^{D}	100.0^{D}
2b	833.3^{B}	26.94^{A}	21.37^{A}	128.5^{A}	162.3^{D}	483.7^{A}
1	654.7^{C}	19.55^{C}	13.98^{C}	72.18^{B}	120.0^{C}	351.0^{C}
1 + "IAA"	1225.0^{A}	23.4^{B}	18.49^{B}	86.07^{B}	207.0^{A}	430.0^{B}
LSD at P 0.05	74.32	0.7259	0.7967	17.52	1.290	12.68

There are no significant differences between the values marked with the same letter at the level of 0.05.

inoculum on soybean plants was observed. The results obtained confirmed that strain 2b is more effective than strain 1. Dry weight of the plants inoculated with strain 2b increased by 62% while the mass of the plants inoculated with strain 1 increased by 20% only. IAA extract from strain 2b added to strain 1 affected, first of all, the dry weight of the whole plant, which increased 107% in comparison to uninoculated plants. It is interesting that a significantly higher increase of dry weight was achieved with strain 1 and IAA spots than with strain 2b. This would confirm the role of IAA in the stimulation of plant growth and consequently dry weight of soybean plants.

The added IAA spots also significantly increased the symbiotic effectiveness so that the tested plants had a higher amount of fixed nitrogen. It also increase the nitrogenase activity, but not significantly, because it remained at the level of strain *B. japonicum* 1 (Table 6).

4. Discussion

According to the results, it can be concluded that the two strains (2b,1) of *B. japonicum* affect the symbiotic association with soybean differently. The symbiotic association of soybean with *B. japonicum* 2b, which is a better producer of growth regulators, showed that the plants grew better, had a higher mass produced nitrogenase activity and a larger nitrogen content than the symbiotic association of soybean with *B. japonicum* 1. These differences are statistically significant.

Fixed nitrogen was two times higher in the association with *B. japonicum* 2b than in the association with *B. japonicum* 1. This can be explained by the effect of growth regulators on plant growth, and consequently increased dry weight and increased nitrogen fixation per plant. Growth regulators have been found in bacteria which inhabit the rhizosphere. *Azotobacter paspali* and *A. chroococcum*, which live in association with plants, produce growth regulators and improve the growth and yield of plants (Barea and Brown, 1974; Brown, 1976). Tien et al. (1979) reported that *Azospirillum brasilence*, which produces auxin (IAA), gibberellin (GA_3) and cytokinin (Kinetin), provides nitrogen to the associated plants and increases their productivity.

We assume that the production of growth regulators in the tested strains of *B. japonicum* affected the pattern of nodulation. Strain *B. japonicum* 2b which produced several types and a large number of growth regulators in culture (Milić et al., 1992, in press) formed large, reddish nodules mainly on the central root. On the other hand, *B. japonicum* 1 formed small nodules on lateral roots. In one of our investigations (Millić et al., 1992, in press) we used the HPLC method for determination of growth regulators of idole, phenole and gibberelline type, and determined a larger peak area from RT in pure culture of *B. japonicum* 2b which corresponds to gibberellic acid (GA_3), than from *B. japonicum* 1. However, it formed less nodules per plant than strain 1.

According to Fletcher et al. (1958), Thurber et al. (1958) and Mes (1959), exogenous adding of small concentrations of GA_3 did not affect the growth of root and shoot of various legumes, including soybean, but inhibited nodulation. According to this, we may assume that GA_3, as well as other gibberellin-like substances act as growth regulators in nodule formation. The effective strain 2b synthesized about eight times more IAA than the poorly effective strain *B. japonicum* 1, i.e. strain 2b synthesized 72.28 μg IAA/l culture and strain 1, 9.28 μg IAA/l culture (Milić et al., 1992, in press). It should be emphasized that the extract of IAA spots from strain 2b added to strain 1 produced a better symbiotic association than with strain 1.

According to Fahraeus and Ljunggren (1968), nodules were not formed on plants when only synthetic IAA without bacterial cells was added. However, our unpublished results proved that synthetic IAA added to the plant in a similar quantity as produced by *B. japonicum* 2b, did not form nodules, only stimulating and elongating root hairs, unlike in plants which had no synthetic IAA added.

The differences between strains 2b and 1 of *B. japonicum* are related to nitrogenase activity in the soybean plants. We suggest that the growth regulators affected nitrogenase activity, which is in agreement with the results obtained by Hess and Fauereissen (1980). IAA and other growth regulators are seen to have a significant role in the effectiveness of *B. japonicum* strains. It appears that IAA affects not only nodulation but the process of nitrogen fixation as well. It is obvious from our results that the plants which had added extract of IAA spots significantly increased dry weight, total N, total fixed nitrogen of strain 1.

Kaneshiro and Kwolek (1985) found increased effectiveness of nitrogen fixation in soybean plants with *R. japonicum* mutant B-14075 which synthesized more IAA in culture. Hunter (1987) obtained similar results. The results presented here confirm our previous results and the existence of a relationship between nitrogenase activity and the production of growth regulators (Sarić et al., 1990). It remains unclear how *B. japonicum* affects the processes in nodules and plants via growth regulators.

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