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SHORT COMMUNICATION

Response of Soybean to Seed Inoculation with *Bradyrhizobium japonicum* and with Mixed Inoculants of *B. japonicum* and *Azotobacter chroococcum*

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Abstract

Effects of pre-sowing soybean seed inoculation with *Bradyrhizobium japonicum* alone or with mixed inoculants containing soybean rhizobia and *Azotobacter chroococcum* were compared. In the pot experiment all the tested strains of soybean rhizobia in pure cultures or in mixtures with *A. chroococcum* significantly improved nodulation of soybean plants and seed yields of this crop. In micro-plot experiments pre-sowing soybean seeds treatment with the inoculant containing the most effective strain 94P of *B. japonicum* alone or with the mixed inoculant of strain 94P and *A. chroococcum* were equally effective in improving nodulation intensity and seed yields of soybean in comparison to the uninoculated soybean.

Key words: *Azotobacter chroococcum*, *Bradyrhizobium japonicum*, inoculants, nodulation, soybean, yield

Rhizobia are ubiquitous soil microorganisms but the diversity and density of soil populations of these bacteria depend on soil properties, crop rotation, agricultural practices and also to the great extent on the presence of wild species of leguminous plants in a given area. In regions or countries, like Poland, where soybean is not an indigenous plant and where this crop is not grown frequently soils are usually void or deficient in rhizobia nodulating soybean (Bushan, 1998; Sadowsky and Graham, 1998; Prevost and Bromfield, 2003, Martyniuk *et al.*, 2005; Cheminingwa and Vessey, 2006). Under such conditions soil inoculation or pre-sowing pelleting of soybean seeds with inoculants containing root-nodule bacteria specific for soybean result in a significant increase of nodulation and seed yields of this crop (Thies *et al.*,1991; Singleton *et al.*, 1992; Bushan, 1998; Graham and Vance, 2003). Moreover, it has been shown under gnotobiotic conditions and in soil-less pot experiments that mixed inoculants containing rhizobia and other beneficial bacteria, like *Azotobacter* spp., were more effective than inoculants consisting of rhizobia alone in stimulation of nodulation, nitrogen fixation and in consequence the yields of legumes were higher (Burns *et al.*, 1981; El-Bahrawy, 1983; *Rodelas et al.*, 1999).

The objective of this study was to determine, in pot and micro-plot experiments with natural soil, whether nodulation and seed yields of soybean are influenced by pre-sowing seed inoculation with *Bradyrhizobium japonicum* alone or with mixed inoculants containing the bradyrhizobia and *Azotobacter chroococcum* and to compare obtained results.

The Culture Collection of N_2-fixing Bacteria belonging to Department of Agricultural Microbiology of the Institute of Soil Sc. and Plant Cultivation in Puławy was the source of all the bacteria used in this work. All *Bradyrhizobium japonicum* strains were isolated from soybean nodules and they originated from: Poland (strains: 78B, L, PO and PR), USA (strains 138, 110 and 94P), Australia (strain CB82), North Korea (strain KR) and from Sweden (strain II). Stock cultures of rhizobia were maintained at 4°C on slants of yeast extract-mannitol agar (YEMA) supplemented with 3 g $CaCO_3$ L^{-1} (Vincent, 1970). These bacteria were used to inoculate soybean seeds in a pot experiment. Viable cell numbers (colony forming units – c.f.u.) of the rhizobia in liquid cultures and on inoculated soybean seeds were counted by standard dilution plate procedures on Congo red-YEMA (Vincent, 1970, Martyniuk *et al.*, 2005). *Azotobacter chroococcum* strain 17/08 was isolated from

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fresh soil collected from an experimental field at the Experimental Station belonging to the Institute of Soil Science and Plant Cultivation (ISSPC) in Puławy, using plates with modified Burk's N-free agar medium inoculated with serial dilutions of this soil in sterile water (Martyniuk and Martyniuk, 2002). This medium was also used to maintain stock cultures of *A. chroococcum* 17/08 and to count numbers of c.f.u. of these bacteria in liquid cultures and on soybean seeds.

Finely milled brown coal, mixed with 1% of $CaCO_3$ to adjust the pH to 6.6–6.8, was used as the bacterial carrier in all inoculants. The carrier material was obtained from a commercial producer of rhizobial inoculants in Wałcz, Poland. Portions of moistened (5%) carrier weighing 200g were placed in polypropylene bags and sterilized by autoclaving in 121°C for 60 min. Batches of 50 ml of YEMB medium and N-free Burk's medium were used to culture the rhizobia and *A. chroococcum* 17/08, respectively, in 250-ml Erlenmeyer flasks on a shaker platform rotating at 100 rpm. Cultures of the bacteria in the late log-phase of growth with approximately $2–5 \times 10^9$ c.f.u. ml^{-1} were used to inoculate bags containing sterile carrier. To prepare mixed inoculants bags with sterile carrier were inoculated with 40 ml of broth cultures of particular rhizobial strains and 40 ml broth culture of *A. chroococcum*. In the case of inoculants containing the rhizobia alone 40 ml of Burk's liquid medium was added instead of *A. chroococcum* culture. The inoculants were incubated for 24 hours at room temperature and then used to inoculate soybean seeds at the rate of 2 g per 100 g of seeds.

Polish cultivar "Aldana" of soybean [*Glycine max* (L) Merrill] was grown in a pot experiment (2010) and in two micro-plot experiments under field conditions conducted in 2011 and 2012. In the pot experiment, Mitscherlich pots filled with 7 kg of the soil collected from the experimental field at the ISSPC Experimental Station in Puławy were used. This soil (sandy loam) had the following basic characteristics: pH 6.2, 1% org. C, 60% sand, 31% silt and 9% clay. The soil contained low populations of soybean rhizobia (about 12 cells g^{-1}), as assessed by plant infection test and the most probable number estimation (Vincent, 1970; Martyniuk *et al.*, 2005), and about 10 c.f.u. g^{-1} of bacteria belonging to the genus *Azotobacter* spp., as determined by plate dilution method on Burk's agar medium (Martyniuk and Martyniuk, 2002). Soybean seeds used in this experiment were surface disinfected with 95% ethanol for 3 min and washed five times with sterile water. Disinfected seeds were than treated with inoculants containing only different strains of soybean rhizobia or with mixed inoculants consisting of the same strains of the genus *Bradyrhizobium* and *A. chroococcum* 17/08 (Table I). There were, all-together 21 treatments (including uninoculated seeds), each consisting of 5 replicated pots completely randomized in a greenhouse. The pots were sown in April 28 of 2010 with 8 soybean seeds and after germination the seedlings were thinned to 5 uniform plants per pot. Each pot filled with soil was fertilized with: KH_2PO_4 – 1,92g, K_2SO_4 – 1,11g and $MgSO_4$ – 1,0 g and soil moisture adjusted to 60% of WHC (water holding capacity). This soil moisture level was maintained throughout the entire experimental period.

At the flowering stage of soybean plants, one pot from each treatment was removed to examine root systems for nodulation intensity according to the following scale: 0 – no nodules on the roots, 1 – single nodules on lateral roots only, 2 – small clusters of nodules on the tap root and single nodules on the laterals, 3 – large clusters of nodules on the tap root and numerous nodules on the laterals. At physiological maturity of the soybean plants the experiment was terminated to determine seed yields and some yield components.

Micro-plot experiments, arranged in a randomized split-plot design, were carried out during the 2011 and 2012 growing seasons on a field at the ISSPC Experi-

Table I
Effect of soybean seeds inoculation with different strains of root-nodule bacteria (*Bradyrhizobium japonicum*) and with the same strains in mixture with *Azotobacter chroococcum* on nodulation rating, number of pods and seed yields of soybean.

Seeds inoculated with:	Nodulation rating[1]	Number of pods $plant^{-1}$	Seed weight g $plant^{-1}$
Control, no inoculation	0.8 a[2]	6.3 a	3.0 a
B. japonicum II	2.5 c	11.4 b	4.9 b
B.j. II+*Azotobacter*	2.6 c	11.7 b	4.8 b
B. japonicum 78B	3.0 c	10.9 b	5.0 b
B.j. 78B+ *Azotobacter*	2.8 c	11.1 b	4.8 b
B. japonicum 138	3.0 c	11.6 b	4.8 b
B.j. 138+ *Azotobacter*	3.0 c	11.4 b	5.0 b
B. japonicum 110	2.2 b	9.3 b	4.4 b
B.j. 110+ *Azotobacter*	2.0 b	10.0 b	4.5 b
B. japonicum KR	2.8 c	10.9 b	4.9 b
KR+ *Azotobacter*	2.8 c	12.8 bc	5.3 b
B. japonicum L	2.8 c	10.6 b	4.9 b
B.j. L+ *Azotobacter*	2.6 c	11.6 b	4.6 b
B. japonicum PO	2.0 b	9.6 b	4.4 b
B.j. PO+ *Azotobacter*	2.8 c	11.4 b	4.6 b
B. japonicum PR	3.0 c	13.5 c	5.3 b
B.j. PR+ *Azotobacter*	3.0 c	11.7 b	5.0 b
B. japonicum CB82	2.6 c	10.0 b	4.8 b
B.j. CB82+ *Azotobacter*	2.8 c	12.4 bc	5.0 b
B. japonicum 94P	3.0 c	11.6 b	5.1 b
B.j. 94P+ *Azotobacter*	3.0 c	13.2 c	5.3 b

[1] Nodulation rating – 0–3 scale, where 0 = no nodules on roots and 3 – numerous nodules on lateral and the tap root; [2] Numbers in columns followed by the same letter are not significantly different ($\alpha = 0.05$

Table II
Effect of soybean seeds inoculation with *Bradyrhizobium japonicum* 94P or with the same strain in mixture with *Azotobacter chroococcum* on rhizosphere counts of *Azotobacter chroococcum*, nodulation rating, pods number and seed yields of soybean in microplot experiments (means for 2011 and 2012)

Seeds inoculated with:	Counts of *A. chroococcum* c.f.u g soil^{-1}	Nodulation rating	Number of pods plant^{-1}	Seed weight g square m^{-1}
Control, no inoculation	35 a*	0.4 a	8.7 a	172 a
B. japonicum 94P	28 a	2.1 b	12.2 b	286 b
B.j. 94P + *Azotobacter*	61 b	2.1 b	13.1 b	292 b

* Values in columns followed by the same letter are not significantly different ($\alpha = 0.05$)

mental Station in Puławy. The experiments included the following treatments, each consisting of four replicated plots: I – soybean seeds (cv. Aldana) treated with inoculant containing *Bradyrhizobium japonicum* strain 94P, II – soybean seeds treated with mixed inoculant of strain 94P and *A. chroococcum* 17/08 and III – uninoculated seeds. About 24 hours before inoculation soybean seeds were treated with commercial chemical seed dressing "Sarfun". Plants were grown on 1m^2 plots consisting of 3 rows spaced 33 cm apart. Soil fertilization and other agro-technical practices followed general recommendations for cultivation of soybean.

At the flowering stage of soybean development, five plants from each replicated plots were dug out to assess root systems for nodulation intensity according to the scale used in the pot experiment and to count the total number of bacteria from the genus *Azotobacter* in non-rhizosphere soil samples and in the rhizosphere soil closely adhering to soybean roots. At the physiological maturity stage all soybean plants from the central rows were collected to determine seed yields and some yield components. All data were subjected to the analysis of variance using Anova test.

In the pot experiment, all inoculants containing either *Bradyrhizobium japonicum* strains alone or mixtures of the rhizobial strains with *Azotobacter chroococcum* 17/08 significantly increased nodulation rate, pod numbers and seed yields per plant of soybean as compared to the untreated control plants (Table I). Of the tested *B. japonicum* strains only two of them (110 and PO) had significantly lower ability to stimulate nodulation of soybean than other strains of *B. japonicum*, but with respect to pod numbers and soybean seed yields these differences were less pronounced. The inoculants containing mixed cultures of *B. japonicum* strains and *A. chroococcum* were generally similar in their effectiveness in increasing nodulation rate of soybean plants with the exception of the strain PO which was less effective when used as pure culture (Table I). Strain 94P of *B. japonicum* both applied as pure culture or in mixture with *A. chroococcum* 17/08 gave relatively the highest soybean seed yields in the pot experiment and this strain has been chosen for further studies under field conditions (microplot experiments).

In the micro-plot experiments conducted in 2011 and 2012 pre-sowing inoculation of soybean seeds with the inoculant containing *B. japonicum* strain 94P alone and with the mixed inoculant containing the strain 94P and *A. chroococcum* 17/08 resulted in a significant increase of nodulation intensity, pod numbers and seed yields of soybean, as compared to the uninoculated control (Table II).

Previous studies with different legumes have clearly shown that seed inoculation with symbiotic root-nodule bacteria is very effective in improving nodulation and yields of these crops when they are grown on soils deficient in infective strains of rhizobia or on soils containing low numbers of these bacteria (Thies *et al.*, 1991; Singleton *et al.*, 1992; Bushan, 1998; Graham and Vance, 2003). This was the case with the soil used in our experiments. This soil contained only about 12 cells g^{-1} of rhizobia nodulating soybean and thus pre-sowing seed inoculation with the tested inoculants resulted in markedly higher nodulation rates and soybean seed yields as compared to those obtained in the control treatment without seed inoculation (Table II).

Results shown in Table II, which are means for two growing seasons (2011 and 2012), indicate that the mixed inoculants containing the rizobial strain 94P and *A. chroococcum* strain 17/08 were not superior in comparison to the inoculant containing *B. japonium* 94P alone.

In pot experiments conducted under gnotobiotic conditions or with the use of sterile soils (Burns *et al.*, 1981; El-Bahrawy, 1983; Rodelas *et al.*, 1999) it has been shown that nodulation intensity, efficiency of nitrogen fixation and yields of some legumes inoculated with mixed cultures of rhizobia and other beneficial microorganisms, like *Azotobacter* spp. and *Azospirillum* spp., were higher as compared to those inoculated with the rhizobia alone. Results of our pot and micro-plot experiments with unsterile soil naturally

colonized with various soil microorganisms indicate that addition *Azotobacter chroorcoccum* strain 17/08 to the inoculant did not improve symbiotic interaction between *Bradyrhizobium japonicum* 94P and soybean plants (Table II).

The prerequisite to obtain positive effects of seed or soil inoculation with beneficial microorganisms on the plant growth is proliferation of microorganisms on roots or in the rhizosphere soil (Bashan, 1998; Rodelas *et al.*, 1999). To find out if *A. chroococcum* introduced onto soybean seeds proliferated in the rhizosphere soil of this crop we counted total numbers of *Azotobacter* spp. cells in non-rhizosphere soil and in soil closely adhering to the roots of soybean inoculated with the tested inoculants. The results presented in Table II show that the number of *Azotobacter* spp. in the rhizosphere soils of soybean treated with the inoculant containing *A. chroococcum* 17/08 remained low (61 c.f.u. g soil^{-1}), even though it was significantly higher than that in the rhizosphere soil of soybean treated with *B. japonicum* 94P alone and in the non-rhizosphere soil. Competitive interactions with other soil microorganisms and at slightly acid pH 6.2 of the soil used in our studies were probably the main reasons for the limited proliferation of *A. chroococcum* in the rhizosphere soil of soybean. Bacteria of the genus *Azotobacter* prefer neutral soils in which their numbers range from several hundreds to 10^4 c.f.u. in g of soil (Bashan, 1998; Rodelas *et al.*, 1999; Martyniuk and Martyniuk, 2002).

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