Nodule efficiency of three soybean genotypes inoculated by different methods

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ABSTRACT

The objective of the study was to investigate how the inoculation of soybean seed (variety Afrodita, and lines NS-L-2016 and NS-L-300168) with strains of *Bradyrhizobium japonicum* (1, 1a, 2b), *Azotobacter chroococcum* (3, 13, 14), and GA_3 (gibberellic acid) affected plant dry weight, nitrogen content of nodules and whole plant, the enzymes of nitrogen assimilation (NR, GS) and soluble protein content. The highest dry matter mass and nitrogen content were found in the variety Afrodita, followed by line NS-L-300168. The GS and NR activity was increased significantly by all three inoculation treatments relative to the control. In all three genotypes, the highest values for the enzymatic activity were achieved with treatment mixture of *B. japonicum* and *A. chroococcum* strains. Each measurement was performed with three replications. The results were processed using variance analysis and the values were tested with the *LSD* at 5%.

Keywords: soybean; inoculation; nitrate reductase (NR); glutamine synthetase (GS); soluble protein content

The effect of soybean inoculation is known to be dependent upon the quality of rhizobia from the point of view of their efficiency in N_2 fixation, competition ability, and compatibility to soybean genotypes.

Several authors (Sadowsky et al. 1987, Relić and Sarić 1988, Mrkovački et al. 1992, Hungria and Bihrer 2000) have studied the differences between *Bradyrhizobium japonicum* strains regarding their effectiveness with different soybean genotypes and their results have shown the differences of nodulation, yield, and total N accumulated in grains.

Mixed cultures of *Azotobacter vinelandii* and *Rhizobium* strains when used as inoculants for several legumes caused the formation of increased numbers of root nodules when compared with plants inoculated with rhizobia alone (Burns et al. 1981).

Plant hormones are involved in eliciting early nidulin gene expression. Addition of gibberellin (GA_3) on *Lotus* roots resulted in the formation of nodule. These results show that the pericycle cells of the roots are specifically sensitive to gibberellins (Kawaguchi et al. 1996). Gibberellins elicit changes in Ca^{2+} levels (Bush et al. 1989). When *Rhizobium*-infected roots, the morfogenetic signal is propagated from the root hair to the cortical cells which will undergo an anticlinal cell division. This signal initially could involve changes in membrane potential and Ca^{2+} levels (Hirsch 1992).

In root nodules the N_2 gas is reduced to ammonia, which is assimilated into amino acids. Amino acids are then used to synthesize other nitrogen-containing compounds. Glutamine synthetase (GS) is the key enzyme responsible for the assimilation and reassimilation of ammonia and produce glutamine.

The primary objective of this study was to determine how the inoculation, with three different types of inoculation, of soybean plants effected i) the quality of the symbiotic association, ii) the activity of some enzymes of nitrogen metabolism, such as nitrate reductase (NR, EC 1.6.6.1), glutamine synthetase (GS, EC 6.3.1.2), and iii) soluble protein content in soybean nodules.

The other two objectives were to establish if the inoculation of soybean plants with *B. japonicum* and *A. chroococcum* strains and *B. japonicum* and GA_3, brings any changes in the primary assimilation of nitrogen.

MATERIAL AND METHODS

In the first treatment (1), soybean seeds were inoculated with mixture *B. japonicum* strains (1, 1a, 2b) that are good producer of growth regulators (Milić et al. 1993).

The second inoculant (2) was a mixture of three *B. japonicum* strains and three *A. chroococcum* strains (1v:1v). In the third treatment (3), the plants were inoculated with the mixture of *B. japonicum* strains combined with the GA_3 (0.3 $\mu g.cm^{-3}$ liquid culture) (commercially known as Merck GA_3) that had been determined to be present in the *A. chroococcum* strains (3, 13, 14) (Govedarica et al. 1994).

The *B. japonicum* and the *A. chroococcum* strains used in the study are from the collections of the Microbiology Department of Institute of Field and Vegetable Crops in Novi Sad, registered in the World Data Center on Microorganisms in Japan under the NS.CNFB acronymy.

The experiment was carried out using pots (R-11 cm, R_2-9 cm) filled with soil, and two soybean lines (NS-L-2016, NS-L-300168) and one soybean variety (Afrodita). All three genotypes were developed at the Institute of Field and Vegetable Crops.

The soil was brought in from the field of the Institute in Rimski Šančevi (Vojvodina Province) and then mixed with sand in the proportion of 4:1. The sand was sterilized in an autoclave for two hours at 1.5 atm., the soil was not sterilized.

Agrochemical analyses of the soil type for the experiment identified that was slightly calcareus chernozem soil, medium provided with humus, optimum phosphorous content and with neutral reaction (4.65% of $CaCO_3$, 2.99% of humus, 0.15% of nitrogen, 7.05 pH in KCl, 7.93 pH in H_2O, P_2O_5 12.00 mg.100 g^{-1} soil and K_2O 25.5 mg.100 g^{-1} soil).

The pots were first sterilized using a 10% HCl solution and then washed with distilled water. The soybean seeds were sterilized with ethanol and then with a 0.1% acid solution of $HgCl_2$, and washed with distilled water several times.

Immediately after sowing, the seeds were inoculated with the mixtures described above, each seed receiving 1 cm^3 of inoculum (10^9 cells per cm^3). Non-inoculated plants were used as control.

The experiments included four replications and six plants per pot. The plants were watered with tap water and grown until flowering (45 days) in a greenhouse under semi-sterile conditions. The plants were harvested by hand and evaluated for plant dry weight and nodule dry weight (expressed in mg per plant). Total nitrogen was determined according to Kjeldahl.

The activity of NR and GS was determined *in vitro* in an extract from nodules of 45-day old plants, according to Coombs and Hall (1982).

NR activity was expressed in nM NO_2^-.g^{-1} fresh nodules.h^{-1}.

GS activity was calculated from the hydroxamate content produced and expressed in µM γGH.g^{-1} fresh weight of nodules.h^{-1}. The activity of the enzymes was monitored by measuring absorbency at 540 nm on a UNICAM SP 600 spectrophotometer.

Soluble proteins (mg proteins.g^{-1} fresh nodules) were determined according to Lowry (1951).

Each measurement was performed with three replications. The results were processed using variance analysis and the values were tested with the *LSD* at 5%.

RESULTS

The results are shown separately for each parameter and all three soybean genotypes in figures (Figures 1–7) with averaged values. All results have the same legend: control – non-inoculated plants, 1. inoculated with mixture of *B. japonicum*, 2. inoculated with *B. japonicum* + *A. chroococcum* strains, and 3. inoculated with *B. japonicum* + GA_3 (gibberellic acid).

Effect of inoculation on dry weight of soybean plants and root nodules. As shown in Figure 1, all the soybean genotypes achieved nodule dry weight in the non-inoculated treatment as well. However, these nodule dry weights (4.20–21.9 mg.$plant^{-1}$) were much lower than in the inoculated treatment (30.29–69.30 mg.$plant^{-1}$). With all the genotypes, significant increases were recorded in treatment 3. As regards the dry weight of whole plant, a significant increase of this parameter was observed in treatment 3 with NS-L-2016 and NS-L-300168 genotypes (Figure 2).

Effect of inoculation on nitrogen content of soybean plants and root nodules. As far as plant nitrogen content is concerned, all of the genotypes exhibited statistically significant differences with treatment relative to the control. In the variety, Afrodita and line NS-L-2016 there were a statistically significant increase with treatment 1 as well (Figure 3).

Both soybean lines as well as the variety Afrodita exhibited a statistically significant increase in nodule nitrogen content with treatment 3. In Afrodita, a statistically significant increase was also recorded with treatment 1 (Figure 4).

As shown root nodules were formed on the non-inoculated, control plants as well, i.e. a symbiotic association was established between the soybeans and the root nodule bacteria that had already been present in the soil on which the plants were sown before five years ago. These associations, however, were less effective than those found in the inoculated treatments.

The highest nitrogen content was found in the variety Afrodita, followed by NS-L-2016 and NS-L-300168.

Effect of inoculation on nodule enzymatic activity. All three inoculation treatments had a positive effect on NR,

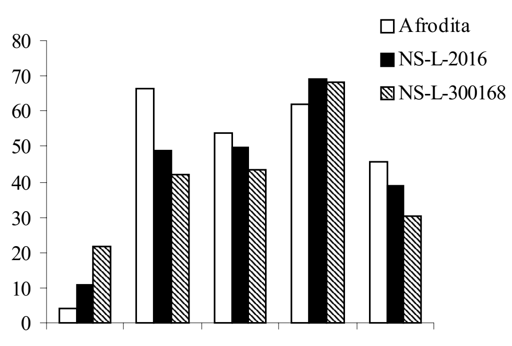

Figure 1. Effect of inoculation on dry weight in root nodules of soybean genotypes (mg.$plant^{-1}$)

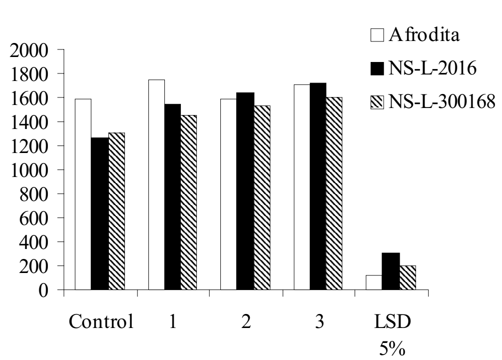

Figure 2. Effect of inoculation on dry weight in whole plant of soybean genotypes (mg.$plant^{-1}$)

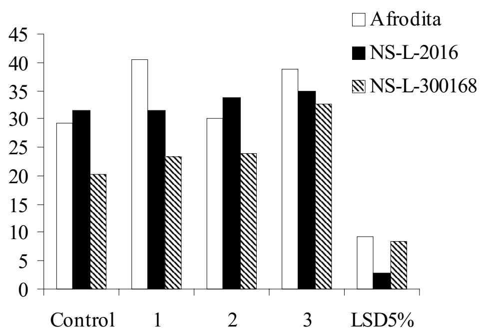

Figure 3. Effect of inoculation on nitrogen content in whole plant in soybean genotypes ($mg.plant^{-1}$)

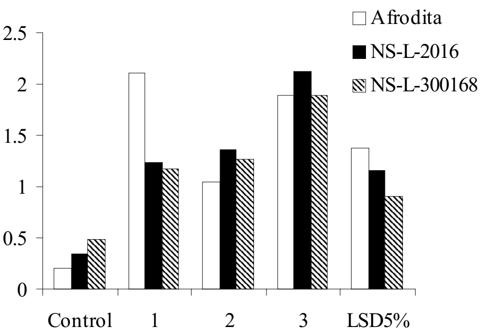

Figure 4. Effect of inoculation on nitrogen content in root nodules of soybean genotypes ($mg.plant^{-1}$)

GS, activities, and soluble protein content in the nodules of all the genotypes. In all three genotypes, the highest values for enzymatic activity and soluble protein content were achieved with treatment 2. In NS-L-2016 and NS-L-300168 NR activity ranged between 32.0 and 501.6 and between 49.6 and 463.3 nM $NO_2^-.g^{-1}$ fresh $nodules.h^{-1}$, respectively. In Afrodita, the activity of the enzyme ranged from 64.0 to 531 nM $NO_2^-.g^{-1}$ fresh $nodules.h^{-1}$. The highest statistically significant increase in NR activity was achieved with treatment 2, namely 531.0 in Afrodita, 501.64 in NS-L-2016, and 463.30 nM $NO_2^-.g^{-1}$ fresh $nodules.h^{-1}$ in NS-L-300168 (Figure 5).

Different inoculation treatments had different effects on GS activity in the soybean genotypes studied. GS activity ranged from 697 to 1083 µM $\gamma GH.g^{-1}$ fresh $nodules.h^{-1}$ in line NS-L-2016, from 653 to 1213 in NS-L-300168, and from 712 to 1366 in Afrodita. The highest GS activity value was recorded in Afrodita with treatment 2 (1366). In NS-L-2016, statistically significant increases in GS activity were achieved with all three treatments, while in NS-L-300168 this was the case only in treatments 2 and 3. In Afrodita, the increase was significant only in treatment 2 (Figure 6).

Soluble protein content. Nodule soluble protein content in the soybean plants is shown in Figure 7. Significant increases were recorded in all three treatments relative to the control plants. The values ranged as follows: from 16.0 to 45.6 in NS-L-2016, from 13.3 to 47.6 in NS-L-300168, and from 17.6 to 42 mg $proteins.g^{-1}$ fresh nodules in Afrodita. The highest soluble protein content was found in treatment 2. As far as the genotypes are concerned, line NS-L-300168 had the highest nodule soluble protein content in all three treatments.

DISCUSSION

Co-inoculation in the field with *Rhizobium* and *Azospirillum* promoted root development, nodulation, nitrogen fixation, and crop yield in a wide variety of legumes. Many investigations suggested that there are good possibilities for increasing nodulation, nitrogen fixation and crop yield of legumes in the field by inoculation with *Rhizobium* and *Azospirillum* or *Azotobacter* (Singh and Subba 1979, Iruthayathas et al. 1983, Plazinski et al. 1984).

Our results have shown that the effectiveness of the symbiotic associations in the soybean genotypes under investigation varied according to the inoculation treatments.

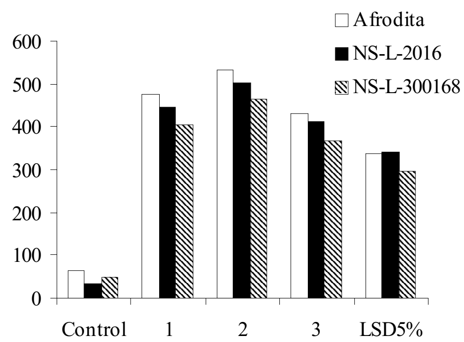

Figure 5. Effect of inoculation on nitrate reductase activity (NR) in root nodules of soybean genotypes (nM $NO_2^-.g^{-1}$ fresh $nodules.h^{-1}$)

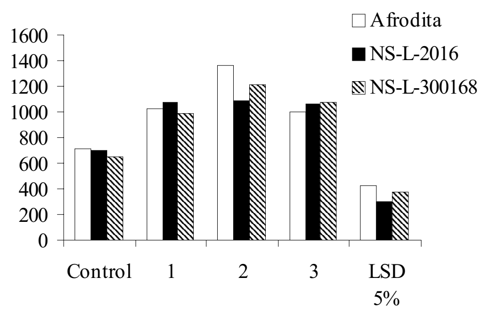

Figure 6. Effect of inoculation on glutamine synthetase activity (GS) in root nodules of soybean genotypes (µM $\gamma GH.g^{-1}$ fresh $nodules.h^{-1}$)

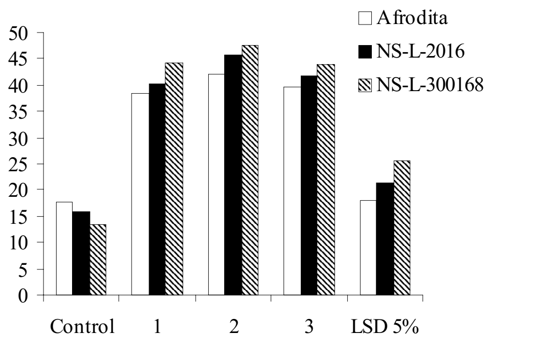

Figure 7. Effect of inoculation on soluble protein content in root nodules of soybean genotypes (mg proteins.g^{-1} fresh nodules)

The dry weight of nodules on non-inoculated treatments were many times lower than in the inoculated treatments and varied according the soybean variety.

The highest nitrogen content was found in the variety Afrodita, followed NS-L-2016 and NS-L-300168. Nitrogen content in soybeans and their nodules depends upon a specific relationship between the soybean cultivar and the *B. japonicum* strain (Mrkovački et al. 1989). The previous results of our studies confirm that nodule dry weights, nitrogen content, and nitrogen fixation are correlated with growth regulators produced by the tested strains of *B. japonicum* (Milić et al. 1993).

A. chroococcum strains are capable to produce growth substances (Barea and Brown 1974) and they too can affect the host plant via plant metabolic processes.

This study's results showed that the activity of the GS enzyme increased significantly in all three treatments and in all of the soybean genotypes relative to the control. In all three genotypes, the highest values for the enzymatic activity and protein content were achieved with treatment 2 (a mixture of *B. japonicum* and *A. chroococcum* strains). This results is in agreement with our previously results – the highest values for the enzymes activity GS and GOGAT in root nodules of different soybean genotypes were with treatment mixture of *B. japonicum* and *A. chroococcum* strains (Milić and Mrkovački 1998). The dual inoculation brought significant increases in the dry weight of nodules and the nitrogen content in the whole plant in field trial experiment with different soybean genotypes (Milić et al. 2001).

Other studies with *Azospirillum* or *Azotobacter* and *Rhizobium* inoculum combinations (Singh and Subba 1979, Iruthayathas et al. 1983, Plazinski et al. 1984) have resulted in increased nodulation.

Treatment 3 (a mixture of *B. japonicum* strains and GA_3) also significantly increased the activity of the enzymes and nitrogen content in relation to the control. However, the increases of enzymatic activity were generally smaller than those achieved with treatment 2.

In the non-inoculated plants, which were used as control, the NR, GS, and soluble protein content were lower, compared to the inoculated treatments (1, 2 and 3). The low level of enzymatic activity was probably due to the low nitrate and ammonium levels as well as the low effectiveness of *B. japonicum* strains from the soil, since only the sand (and not the soil) was sterilized.

GS activity in root nodules is well correlated with the level of nitrogen fixation during plant development (Stripf and Werner 1978, Sengupta-Gopalon and Pitas 1986).

In our experiment, GS activity was increased significantly by inoculation in all three treatments (1, 2 and 3), and the highest increase was recorded in treatment 2 (a mixture of *B. japonicum* and *A. chroococcum* strains).

The amount of GA_3 that was added to the *B. japonicum* increased nitrogen content in NS-L-2016 and NS-L-300168. In Afrodita, however, there was no statistically significant increase in the parameters studied relative to treatment 1. How this variety would respond if larger amounts of gibberellic acid were added is something to be investigated in a future study.

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ABSTRAKT

Účinnost hlízek u tří genotypů sóje při různých typech inokulace

Cílem práce bylo zjistit, do jaké míry inokulace osiva sóje (odrůdy Afrodita a linie NS-L-2016 a NS-L-300168) kmeny *Bradyrhizobium japonicum* (1, 1a, 2b), *Azotobacter chroococcum* (1, 13, 14) a GA_3 (kyselinou giberelinovou) ovlivňuje hmotnost sušiny rostlin, obsah dusíku v hlízkách a v celé rostlině, aktivitu enzymů asimilace dusíku (NR,GS) a obsah rozpustných bílkovin. Nejvyšší hmotnost sušiny a obsah dusíku jsme zjistili u odrůdy Afrodita, a dále u linie NS-L-300168. Ve srovnání s kontrolou se aktivita GS a NR po inokulaci u všech tří variant významně zvýšila. U všech tří genotypů bylo dosaženo nejvyšších hodnot enzymatické aktivity u varianty, u níž byla použita směs *B. japonicum* a *A. chroococcum*. Každé měření mělo tři opakování. Výsledky byly zpracovány pomocí analýzy variance a hodnoty byly vyhodnoceny pomocí *LSD* na hladině 5 %.

Klíčová slova: sója; inokulace; nitrátreduktáza (NR); glutaminsyntetáza (GS); obsah rozpustných bílkovin

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