The Initial Development of Soybean Subjected to Co-Inoculation with *Azospirillum brasilense* and *Bradyrhizobium japonicum*

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Abstract— *The aim of the current study is to assess the initial development of soybean (Glycine max (L.) Merrill) subjected to inoculation and co-inoculation with Bradyrhizobium japonicum and Azospirillum brasilense using phenological parameters such as leaf area and dry matter partitioning of leaves, stems and roots, as well as leaf nitrogen content. The experiment was conducted in a greenhouse at Lagoa da Cruz Farm, a research base belonging to Dom Bosco Catholic University. The soybean variety CD 2728 IPRO, which has a 120-day cycle, was sown in 5,000-ml plastic pots. The experimental design followed a completely randomized methodology, with four treatments and four repetitions, using the inoculation with nitrogen fixing bacteria such as Bradyrhizobium japonicum, Azospirillum brasilense, Bradyrhizobium japonicum + Azospirillum brasilense (co-inoculation), and the witness. The leaf nitrogen content analysis, as well as the phenological measurements of the leaf area and the dry weight of the shoot and root system, were performed 30 days after sowing. According to the herein obtained results, it was possible concluding that the initial soybean development was benefited by the co-inoculation with Azospirillium brasilense + Bradyrhizobium japonicum in the root dry matter partitioning.*

Keywords— ***symbiosis, rhizosphere, nitrogen, partitioning.***

I. INTRODUCTION

Nitrogen (N) is classified as an essential element for plants, since it is found in the composition of the most important biomolecules, such as ATP, NADPH, NADH, chlorophyll, proteins and several enzymes (MIFLIN; LEA, 1976; HARPER, 1994; BREDEMEIER; MUNDSTOCK, 2000).

The main N sources required for plant growth are the soil nitrogen derived from the decomposition of organic matter and rocks, the nitrogen derived from the application of fertilizers, and the nitrogen resulting from the atmospheric nitrogen fixation process (HUNGRIA et al., 1994). There is also the reaction between electrical discharges and N. Such reaction results in nitrate, which is added to the soil (COSTA, 2011).

As soon as the nitrogen is fixed in the soil, in the form of ammonia or nitrate, a biogeochemical cycle takes place and it makes the nitrogen go through different organic and inorganic forms before it goes back to molecular N (TAIZ; ZEIGER, 2013). The amino acids, ammonia and ammonium resulting from the nitrogen, or released due to organic matter decomposition in the soil, are disputed by plants and microorganisms. Consequently, the plants develop several nitrogen fixation mechanisms such as the symbiosis with fixing bacteria (SIQUEIRA; FRANCO, 1988; FISHER; NEWTON, 2002).

On the other hand, the organisms living near the soil surface are able to fix nitrogen through the decomposition of dead animals and plants. The saprophytic bacteria and several fungal species are the main responsible for dead organic matter decomposition (SIQUEIRA; FRANCO, 1988). These microorganisms use proteins and amino acids, and release the nitrogen excess in the form of ammonium.

The nitrogen assimilation is one of the several stages in the nitrogen cycle, which encompasses different biosphere N forms and their interconversions. The main sources of nitrogen available for plants are nitrate (NO^{-3}), ammonium (NH^4) and atmospheric N_2 biological fixation (PRADO, 2008).

The biological nitrogen fixation is the most important way to fix the atmospheric nitrogen (N_2) into ammonium (TAIZ & ZEIIGER, 2013). Thus, the atmospheric gases also diffuse into the porous space of the soil, and the N is used by the microorganisms living in it. It happens due to nitrogenase enzyme activity, which is able to break the triple bond in N_2 and reduce it to ammonia (HUNGRIA et al., 2001).

According to Gitti (2015), the symbiotic association between the soybean roots and the bacteria belonging to genus Bradyrhizobium provides all the nitrogen required to obtain approximately 3,600 kg ha^{-1} soybean (mean yield). It also provides 20-30 kg ha^{-1} nitrogen to the successive culture.

According to Moreira et al. (2010), the biological fixation of atmospheric nitrogen is done by diazotrophic microorganisms - which can be free-living, associated with plant species or may establish symbiosis with leguminous plants. This integrated association between leguminous plants and bacteria belonging to the genus Bradyrhizobium occurs through the development of new structures in the root system, called nodes, where the BNF takes place.

Therefore, the symbiosis process benefits both the host plant and the microorganism. The bacterium benefits from products supplied by the host plant, such as photosynthates or organic carbon, whereas the rhizobium provides the plant with the nitrogen compound that is readily available to it (CASSINI et al., 2006).

The nodulation process starts right after germination since the rhizobium is already in the soil or adhered to the seed due to the inoculation process. Thus, the process is divided in three stages: infection, nodular development, and node activation and functioning (CASSINI et al., 2006).

The phenomenon known as chemotaxis occurs in the pre-infection process. The root system produces several substances such as carbohydrates, amino acids, as well as phenolic compounds (flavonoids), which comprise a chemical gradient in the rhizosphere and, thus, attract the bacteria to the root surface (STRALIOTTO et al., 2002).

The activation of the nodulation genes occurs through substances produced by the plant (TAIZ & ZEIIGER, 2013). The rhizobium starts producing compounds known as nod factors after the activation, and it changes the structure of the root system cells.

After the initial adhesion, the rhizobium dissolves the cell wall of the modified absorbing hair, penetrates the cortical cells, forms a special structure known as infection thread, and multiplies the rhizobial cells within such structure (CASSINI et al., 2006).

Once the rhizobium reaches the cortical region, it enters the cortical cells, adapts to the new nitrogen fixation function, and forms bacteroids (CASSINI et al., 2006). The first soybean root nodules can be seen 10 to 15 days after the emergence of the plants, according to favorable environmental and management conditions (CÂMARA, 2000).

The plant growth-promoting bacteria (PGPB) are a group of microorganisms that can be beneficial to plant development due to their ability to colonize the root surface, the rhizosphere, the phyllosphere and the internal tissues of plants (DAVISON, 1988; KLOEPPER et al., 1989).

These bacteria belong to free-living gram-negative bacterial groups and their versatile carbon and N metabolism makes them competitive during the colonization process (QUADROS, 2009). According to Trentini (2010), these bacteria use N sources such as ammonia, nitrate, nitrite, amino acids and molecular nitrogen in their metabolism.

According to Barassi et al. (2008), the inoculation with *Azospirillum brasilense* improves the photosynthetic parameters of the leaves such as chlorophyll content and stomatal conductance, increases the proline content in the shoots and roots, improves the water potential, and increases the water content in the apoplast, the cell wall elasticity, the biomass production, and the plant height. According to Bashan et al. (2006), there is increase in several photosynthetic pigments such as chlorophyll a and b, as well as in *auxiliary photoprotective pigments* such as *violaxanthin, zeaxanthin, antheraxanthin, lutein, neoxanthin* and *beta-carotene*, and it results in greener plants and in no water stress.

According to Bárbaro et al. (2008), when *Azospirillum brasilense* was applied to leguminous plants, the beneficial effect from the association with rhizobium have mostly resulted from the bacteria's ability to produce phytohormone. Thus, it resulted in better root system development and allowed better exploring the soil volume.

Molla et al. (2001) have found that the genus Azospirillum has the potential to significantly stimulate root growth, even in plants whose roots were subjected to mechanical damage. Thus, this genus may have positive influence on root growth and development, since it not only promotes root growth, but it may also favor the emergence and development of nodes in soybean plants.

The alternative co-inoculation technique is seen as a mixed inoculation that consists of applying different combinations of microorganisms able to produce a synergistic effect, wherein the productive results obtained from such combination overcome those obtained when microorganisms are individually used (FERLINI, 2006; BÁRBARO et al., 2008).

According to Cassán et al. (2009), the number of nodes and the rate of nodulated plants were higher in soybean plants subjected to co-inoculation with *B. japonicum* and *A. brasilense*. It happened due to the excretion of metabolic products, such as root growth regulating compounds (IAA), by *A. brasilense*.

The use of co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* in soybean has promoted high yield in leguminous plants subjected to water stress [10].

II. MATERIALS AND METHODS

The CD 2728 IPRO soybean cultivar used in the current study has a 120-day cycle, is resistant to lodging, moderately susceptible to root-knot nematodes (*Meloidogyne incognita*) and susceptible to *Meloidogyne javanica*. It was seeded in 5,000 ml plastic pots.

The experiment consisted of four inoculation treatments comprising nitrogen fixing bacteria such as *Bradyrhizobium japonicum*, *Azospirillum brasilense*, *Bradyrhizobium japonicum* + *Azospirillum brasilense* (co-inoculation), and the witness. The experimental design followed a completely randomized block methodology, with four repetitions.

The analysis of nitrogen content in the leaf was performed 30 days after sowing, according to the methodology by Malavolta, Vitti and Oliveira (1997). The leaf area, the dry weight of the shoot and the root system were measured.

The data were subjected to statistical analysis according to the PROC GLM procedure of the SAS statistical package version 9.1. (2004). The variables were assessed through the Dunnett's (P <0.10) and the T tests (P <0.05) in order to compare the means.

III. RESULTS AND DISCUSSION

Table 1 shows the results of the analysis of the soybean leaf area means. It is possible seeing that the results were very close to the global mean, at 90% confidence interval.

TABLE 1
ANALYSIS OF THE MEAN SOYBEAN (*GLYCINE MAX* (L.) MERRILL) LEAF AREA, 30 DAYS AFTER SOWING, USING DIFFERENT INOCULANTS.

	Witness	***A. brasilense***	***B. japonicum***	***A. brasilense + B. japonicum***
Mean (cm^2)	46.31± 8.9	43.42 ± 10.5	48.89 ± 11.0	48.47 ± 11.5

Global mean 46.52cm²; Standard error – 1.313 at 90% confidence interval

According to the results, the leaf area did not significantly differ between treatments. However, the treatment with *B. japonicum* and the co-inoculation have shown the highest mean leaf area. Kolchinski et al. (2006) have used phenological traits to assess soybean seed vigor in the early development period and concluded that more vigorous plants present greater leaf area and dry matter weight in the first 30 days after emergence.

Table 2 presents the analysis of phenological parameters such as the dry matter weight of stem, leaf and root, in the different treatments.

TABLE 2
ASSESSMENT OF DRY MATTER WEIGHT IN SEVERAL ORGANS OF SOYBEAN PLANTS (*GLYCINE MAX (L.) MERRILL*) - 30 DAYS AFTER SOWING - SUBJECTED TO DIFFERENT INOCULATION TREATMENTS

Plant organ	**Witness**	***A. brasilense***	***B. japonicum***	***A. brasilense + B. japonicum***	***C.V.***	***P***
Steam $_{(mg)}$	412bB	419bB	421bB	469aB	**19.79**	**0.077**
Leaf $_{(mg)}$	565bA	612bA	570bA	657aA	**23.90**	**0.088**
Root $_{(mg)}$	522bAB	605bA	523bA	624aA	**25.18**	**0.060**
Standard error	**23.01**	**22.14**	**15.71**	**22.54**		
P	**0.017**	**0.001**	**0.001**	**0.001**		

The means with different lower case letters in the same row show significant treatment effect, according to the Dunnett's test (P <0.10). The means with different capital letters in the same column show significant treatment effect on the plant's organ, according to the T test (P <0.05); C.V: Coefficient of Variation.

Table 2 shows that the dry matter of the stem, leaf and root significantly differed in the treatment using co-inoculation (*A. brasilense* + *B. japonicum*). Such treatment led to dry matter amount greater than that found in the other treatments and resulted in increased stem (13.83%), leaf (19.54%) and root (16.28%) dry matter, in comparison to the witness treatment.

By analyzing the dry matter partitioning between plant organs, it was noticed that the witness treatment showed the largest dry matter accumulation in the leaves and the smallest partitioning in the stem. Thus, it was possible seeing the differentiation in such pattern when the witness treatment was compared to the inoculation treatments, i.e., there was increased partitioning in the roots and the dry matter weight in the leaves remained high. Therefore, the co-inoculation treatment showed the best results.

Consistent data were found by Madhaiyan et al. (2010), who studied the physiological effects of inoculation with *Azospirillum brasilense* and found indole acetic acid (IAA) production by the bacterium, as well as increased root length in tomato and red pepper. The authors also found increased shoot development in these plants. The same result was found by German et al. (2000), who have studied beans subjected to water deficit and inoculated with *Azospirillum*.

Okon and Itzigsohn (1995) have studied the co-inoculation of leguminous plants with *Rhizobium* and *Azospirillum* and found the best plant growth by using this treatment. According to the authors, a possible explanation for such result may be associated with the strong microaerophilic attraction of the *Azospirillum* to the rhizosphere niche, as well as with the fact that it is faster than *Rhizobium*. Thus, it may lead to the conclusion that *Azospirillum* firstly occupies the roots of leguminous plants to allow the preconditioning of the roots prior to *Rhizobium* colonization.

The co-inoculation, among all treatments, has shown dry matter accumulation in the root, stem and leaves higher than that of the witness treatment. This result is consistent with the study by Gitti (2015), who has assessed soybean co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* and found the highest dry matter weight in co-inoculated plants.

The nitrogen content in the leaf was not significant at 90% confidence interval, according to the Dunnett's test, as shown in Table 3.

TABLE 3
MEAN NITROGEN EXTRACTION IN SOYBEAN LEAVES (*GLYCINE MAX (L.) MERRILL*), 30 DAYS AFTER SOWING, UNDER DIFFERENT INOCULATIONS

	Witness	*A. brasilense*	*B. japonicum*	*A. brasilense + B. japonicum*
Mean g kg^{-1}	38.32 ± 4.8	38.51 ± 4.1	40.42 ± 3.9	41.82 ± 2.2

Global mean 39.76 g kg-1; Standard error- 1.041 at 90% confidence interval

According to the results shown in Table 3, there was tendency of nitrogen content increase in the leaf in the *A. brasilense* + *B. japonicum* treatment in comparison to the other treatments. Thus, the co-inoculation treatment has shown 9.13% leaf nitrogen increase when it was compared to the witness treatment. Such result may be associated with the appropriate level of organic matter found in the soil.

Similarly, Peres (2014) has found no significant differences in the leaf nitrogen content between bean plants subjected to nitrogen fertilization and co-inoculated with *Azospirillium brasilense* + *Rhizobium tropici*. According to the author, this result has shown the co-inoculation efficiency in the nitrogen metabolism. Thus, Zuffo et al. (2015) have reported that the inoculation of leguminous plants with nitrogen fixing bacteria was effective in soils with low nitrogen content.

IV. CONCLUSION

The initial development of the soybean crop was benefited by the co-inoculation with *A. brasilense* + *B. japonicum,* with respect to the dry matter partitioning in the roots.

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