Co-inoculation with rhizobacteria in association with humic acid and nitrogen on common bean development

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Studies on co-inoculation of strains with high symbiotic potential, as well as the use of humic substances are of great importance for obtaining increases in nodulation, biological nitrogen fixation and yield of the bean crop. Thus, this study aimed to evaluate the effects of the co-inoculation of Rhizobium tropici and Azospirillum brasilense in association with humic acids and nitrogen (N) on the behavior of different bean genotypes, cultivated in a protected environment. The experiment was carried out at the Federal Institute of Education, Science and Technology of Rondônia, Campus of Colorado do Oeste-RO, Brazil, from February to April 2015. The experimental design was completely randomized, with four replicates. Common bean seeds of the cultivars ‘Pérola’ and ‘BRS Esplendor’ were previously co-inoculated with Azospirillum brasilense and Rhizobium tropici. Shoot dry matter production in the cultivar ‘Pérola’ increased by 76.12% when co-inoculated with rhizobacteria. N use efficiency in the bean cultivar ‘Pérola’ is higher when co-inoculated with Azospirillum brasilense and Rhizobium tropici, confirming that co-inoculation alone is sufficient to provide the N necessary for plant development.

Key words: Phaseolus vulgaris L., Rhizobium tropici, Azospirillum brasilense, biological N fixation (BNF).

INTRODUCTION

The common bean (Phaseolus vulgaris L.) constitutes the staple food of many developing countries and is considered as one of the most important constituents in the diet of the Brazilian population, for being recognizably an excellent source of protein, carbohydrates and iron. Global bean production has progressively increased since the 1960s. Brazil is the world’s largest producer and consumer of this leguminous plant, with production of about 3.39 million tons. However, the mean yield of bean in the country is relatively low, only 1071 kg ha\(^{-1}\) in the 2014/2015 season, considering 1\(^{st}\), 2\(^{nd}\) and 3\(^{rd}\) crops (Conab, 2015). These low crop yields reflect the low technological level employed by farmers, as well as the cultivation of bean in soils with low fertility, especially poor in nitrogen (N) (Pelegrin et al., 2009). Among the factors that most contribute to the increase in production costs of bean crops, the use of mineral fertilizers, especially with N, stands out, because they are required in larger amounts by plants. However, although N fertilizers are the fastest form of absorption by plants,
they have high costs, high expenditure of energy sources in their manufacturing, low efficiency of use by plants, rarely exceeding 50%, besides being closely related to environmental pollution (Hungria et al., 2013). Thus, alternatives aiming to reduce input application in agricultural production areas, capable of promoting high yields and maintaining environmental sustainability, with focus on food safety, are of great interest. One alternative for the reduction in the necessity of N fertilizers is the biological N fixation (BNF), which is performed by a restrict group of bacteria, referred to as diazotrophic (Reis, 2007).

Currently, the commercial inoculant for bean in Brazil is produced with a species of rhizobium adapted to tropical soils, *Rhizobium tropici*, which is able to fix 20 to 30% of the N required by the plant through BNF and can provide 20 to 40 kg ha\(^{-1}\) of N (Fancelli and Dourado Neto, 2007). Besides rhizobia that are specific for leguminous plants, other microorganisms can bring great benefits to crops. One of the most promising groups is represented by associative bacteria, which are capable of promoting plant growth through various processes, such as the production of growth hormones, capacity to perform BNF, etc. Among these bacteria, those belonging to the genus *Azospirillum* stand out.

In this context, an alternative co-inoculation technique, also referred to as mixed inoculation, with symbiotic and non-symbiotic bacteria, has been studied in leguminous plants. This technique consists in the use of combinations of different microorganisms, which produce a synergetic effect, surpassing the production results obtained when they are used in separate (Bárbaro et al., 2011). In the cases in which *Azospirillum brasilense* has been used in leguminous plants, the beneficial effect of the association with rhizobium is mostly due to the capacity of the bacteria to produce phytohormones, which results in higher root system development and, therefore, the possibility to explore a greater soil volume (Bárbaro et al., 2008). In the bean crop, studies have shown that the combined inoculation of *Rhizobium* and *Azospirillum* can increase the amount of N fixed and grain yield (Yadegari, 2010).

The interaction between the bean crop and atmospheric N-fixing bacteria has shown the capacity to substitute N fertilization, at least partially, for obtaining high yields (Pelegrin et al., 2009). However, although the number of researches involving these bacteria has increased in the last years in Brazil, little is known about the effects of using these microorganisms together with humic substances (HS).

Substance humic, the main component of soil organic matter (85 to 90%), can promote effects on plants that are related to the increase in nutrient absorption, due to the influence in cell membrane permeability and to the chelating power, as well as to photosynthesis and the formation of ATP, amino acids and proteins. In addition, HS directly alter plant biochemical metabolism and, consequently, can influence growth and development, as well as promote increase in the population of endophytic bacteria, stimulating the increase in the establishment of the bacterial inoculum inside the plant. This can be hypothetically explained as part of the effects of HS on the increase in the number of lateral roots, which constitute the major site of infection of the host plant by endophytic bacteria (Marques Júnior et al., 2008).

Therefore, studies on the association of rhizobium strains with high symbiotic potential and rhizobacteria, as well as the use of HS, become of great importance for obtaining increases in nodulation, biological N fixation and bean yield, under tropical conditions. Given the above, this study aimed to evaluate the effects of co-inoculation of *R. tropici* and *A. brasilense* in association with humic acids and N on the behavior of different bean genotypes, cultivated in a protected environment.

**MATERIALS AND METHODS**

The experiment was carried out from February to April 2015, at the Plant Production Sector of the Federal Institute of Education, Science and Technology of Rondônia, Campus of Colorado do Oeste-RO, Brazil (13° 06' S; 60° 29' W; 407 m). According to Köppen's classification, the climate in the area is Awa, hot and humid tropical, with two well-defined seasons. The soil used in the study, classified as Red Yellow Argisols of very clayey texture (Embrapa, 2013), was collected in the layer of 0 to 20 cm. The soil chemical analysis before the experiment showed the following results: O.M. - 10.00 g dm\(^{-3}\); pH (CaCl\(_2\)) - 5.30; P - 1.10 mg dm\(^{-3}\); K - 0.14 cmolc dm\(^{-3}\); Ca - 5.56 cmolc dm\(^{-3}\); Mg - 1.15 cmolc dm\(^{-3}\); Al - 0.0 cmolc dm\(^{-3}\); H\(_2\)Al - 2.25 cmolc dm\(^{-3}\); SB - 6.90 cmolc dm\(^{-3}\); CEC - 9.10 cmolc dm\(^{-3}\) and base saturation - 75.30%. Granulometric analysis showed 199 g kg\(^{-1}\) of sand, 166 g kg\(^{-1}\) of silt and 635 g kg\(^{-1}\) of clay.

The experiment was set in a completely randomized design, with four replicates, and the treatments were: 1) Control; 2) Co-inoculation with *R. tropici* and *A. brasilense*; 3) 30 kg ha\(^{-1}\) of N; 4) Co-inoculation with *R. tropici* and *A. brasilense* + Humic acid; 5) Co-inoculation with *R. tropici* and *A. brasilense* + 30 kg ha\(^{-1}\) of N and 6) Co-inoculation with *R. tropici* and *A. brasilense* + 30 kg ha\(^{-1}\) of N + Humic acid, totaling 24 experimental units for each bean genotype evaluated.

Based on the results of soil chemical analysis, basal fertilization was performed in order to guarantee the establishment of the crop, by mixing the soil with 110 kg ha\(^{-1}\) of P\(_2\)O\(_5\) and 60 kg ha\(^{-1}\) of K\(_2\)O, as single superphosphate (18% P\(_2\)O\(_5\)) and potassium chloride (60% K\(_2\)O), respectively. Micronutrients were applied based on crop requirements, in the form of a solution, using deionized water and salts (A.R.), according to Epstein and Bloom (2006). N fertilization was performed at sowing, using the dose of 30 kg ha\(^{-1}\), as urea (45%).

The experimental units consisted of plastic pots with capacity for 8 dm\(^{3}\), filled with air-dried soil, sieved through a 4-mm mesh. The moisture in the pots was daily controlled through weighing, in order to maintain the soil at 60% of field capacity. Irrigation was performed using distilled water.

Seedling was performed using common bean seeds, cultivars “Pérola” (“Canica” group) and “BRS Esplendor” (“Pretó” group), which were previously inoculated with a product containing a combination of two strains of *A. brasilense* (Ab-V5 and Ab-V6), in...
inoculant with liquid formulation, and an inoculant containing the strain R. tropici, in peat-based formulation, produced by the company Total Biotecnologia. The applied dose was 150 mL for each 50 kg of bean seeds, for the inoculant with liquid formulation, and 250 g for each 10 kg of bean seeds, for the inoculant with peat-based formulation. For the inoculation with R. tropici, 60 mL of a sugar solution at 10% (m/v) were added to each 10 kg of seeds, in order to increase the adhesion of the inoculant to the seeds. Thus, co-inoculation corresponded to the mixed inoculation of the two inoculants, according to the recommendations for the crop.

Humic acids were extracted and provided by the Biotechnology Laboratory of the Norte Fluminense State University – UENF, Campus of Goytacazes-RJ, and were isolated from vermicompost, according to Canellas et al. (2005). The material was previously dissolved in water, in the proportion of 50 mg L\(^{-1}\). HS was directly applied on the seeds, inside plastic bags, with a volumetric pipette. After application, the plastic bags were closed and vigorously agitated for two minutes, for a homogeneous distribution of the product on the seeds. The seeds were placed to germinate directly in the pots and, 8 days after emergence (DAE), thinning was performed, leaving only one plant in each experimental unit.

At 35 DAE, plant height and stem diameter were determined. Plant height was measured from the basis to the apical meristem of the plants, using a ruler. Stem diameter was determined using a digital caliper, at the height of 2 cm from the soil surface. Then, plants were collected and divided into roots and shoots. After that, all the collected plant material was washed in running water, HCl solution at 0.1 mol L\(^{-1}\) and deionized water, respectively. Root length was determined using a ruler and root volume through the graduated cylinder method, in which roots are submerged in a graduated cylinder containing a known volume of water and their volume is determined by the difference between the initial and final volumes of the container. Then, the samples were placed in paper bags and dried in a forced-air oven at temperature of 65\(^\circ\)C for 72 h. After drying the plant material, its dry matter was weighed and ground in a Wiley-type mill and the samples were subjected to sulfuric digestion, for the determination of N contents in the different plant parts (roots and shoots), according to the methodology described in Embrapa (2009).

N absorption efficiency, ratio between total N content in the plant and root dry matter, was calculated according to Swiader et al. (1994). N transport efficiency, ratio between shoot N content and total N content in the plant, and N use efficiency, ratio between the total dry matter production and total N accumulation in the plant, were calculated according to Siddiqi and Glass (1981).

Nitrogen absorption efficiency, ratio between the total N content in the plant and root dry matter, was calculated according to Swiader et al. (1994), while N use efficiency, ratio between total dry matter production and total N accumulation in the plant, was calculated according to Siddiqi and Glass (1981).

The results were subjected to analysis of variance and the means were compared by Tukey test at 0.05 probability level, using the statistical program Sisvar.

RESULTS AND DISCUSSION

There was significant difference (p≤0.05) for plant height, stem diameter, root length, root volume, shoot dry matter, root dry matter and N use efficiency in response to the co-inoculation of A. brasilense and R. tropici and application of humic substances and N in bean plants, cv. 'Pérola', from the 'Carioca' group (Tables 1 and 2).

On the other hand, the cultivar 'BRS Esplendor', from the 'Preto' group, showed significant response (p≤0.05) only for plant height, and no significant effects of treatments for the other studied variables (Tables 3 and 4).

The lack of response to the application of A. brasilense and R. tropici can be related to the period of evaluation of the experiment (V4 stage), when BNF activity is still low. Brito et al. (2009), evaluating the uptake rate of N derived from BNF, N fertilizers and from the soil for bean growth, observed that the highest N fixation rates occur from the pre-flowering (R5 stage) on. These results corroborate those of Souza (2014), who observed no effect of R. tropici, isolated or combined with A. brasilense, on shoot, root and total dry matter production of bean plants, cv. ‘Pérola’.

Plant height for the cultivar ‘Pérola’ showed the highest values in the treatment corresponding to co-inoculation of A. brasilense and R. tropici, co-inoculation + 30 kg ha\(^{-1}\) of N and co-inoculation + 30 kg ha\(^{-1}\) of N + humic acid, being superior to the control (without co-inoculation and without N) and similar to the other treatments. The values...
Table 2. Shoot nitrogen content (SNC), root nitrogen content (RNC), total nitrogen content (TNC), nitrogen absorption efficiency (NAE), nitrogen transport efficiency (NTE) and nitrogen use efficiency (NUE) of bean plants, cv. ‘Pérola’, in response to co-inoculation of *Azospirillum brasilense* and *Rizhobium tropici* and application of humic substances and nitrogen. Colorado do Oeste-RO, Brazil (2015).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SNC (g kg(^{-1}))</th>
<th>RNC (g kg(^{-1}))</th>
<th>TNC (g kg(^{-1}))</th>
<th>NAE (mg g(^{-1}))</th>
<th>NTE (%)</th>
<th>NUE (mg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>41.60</td>
<td>24.22</td>
<td>65.82</td>
<td>309.17</td>
<td>73.89</td>
<td>0.02</td>
</tr>
<tr>
<td>2. Co-inoculation</td>
<td>45.25</td>
<td>24.62</td>
<td>79.70</td>
<td>627.27</td>
<td>85.58</td>
<td>0.07</td>
</tr>
<tr>
<td>3. 30 kg ha(^{-1}) N</td>
<td>44.20</td>
<td>26.80</td>
<td>69.87</td>
<td>621.92</td>
<td>86.60</td>
<td>0.05</td>
</tr>
<tr>
<td>4. Co-inoculation + Humic acid</td>
<td>48.46</td>
<td>26.63</td>
<td>75.10</td>
<td>346.49</td>
<td>91.07</td>
<td>0.03</td>
</tr>
<tr>
<td>5. Co-inoculation + 30 kg ha(^{-1}) N</td>
<td>45.73</td>
<td>27.35</td>
<td>73.08</td>
<td>484.54</td>
<td>91.08</td>
<td>0.03</td>
</tr>
<tr>
<td>6. Co-inoculation + 30 kg ha(^{-1}) N + Humic acid</td>
<td>43.56</td>
<td>26.16</td>
<td>69.72</td>
<td>514.14</td>
<td>94.31</td>
<td>0.03</td>
</tr>
<tr>
<td>Medium</td>
<td>44.80</td>
<td>25.96</td>
<td>72.21</td>
<td>483.92</td>
<td>87.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Test F</td>
<td>0.11(^{NS})</td>
<td>0.21(^{NS})</td>
<td>0.08(^{NS})</td>
<td>0.57(^{NS})</td>
<td>0.48(^{NS})</td>
<td>0.04(^{*})</td>
</tr>
<tr>
<td>CV (%)</td>
<td>7.05</td>
<td>7.63</td>
<td>5.79</td>
<td>61.80</td>
<td>17.09</td>
<td>20.01</td>
</tr>
</tbody>
</table>

* and \(^{NS}\) - significant 5% probability and not significant, respectively. Medium followed by the same letter in the columns, do not differ statistically between themselves by Tukey test, the 5% probability. CV: coefficient of variation.

Table 3. Plant height (PH), stem diameter (SD), root length (RL), root volume (RV), shoot dry matter (SDM) and root dry matter (RDM) of bean plants, cv. ‘BRS Esplendor’, in response to co-inoculation of *Azospirillum brasilense* and *Rizhobium tropici* and application of humic substances and nitrogen. Colorado do Oeste-RO, Brazil (2015).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PH (cm)</th>
<th>SD (mm)</th>
<th>RL (cm)</th>
<th>RV (cm(^{3})/planta)</th>
<th>SDM (g)</th>
<th>RDM (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>35.05(^{ab})</td>
<td>3.56</td>
<td>31.00</td>
<td>12.00</td>
<td>1.01</td>
<td>0.28</td>
</tr>
<tr>
<td>2. Co-inoculation</td>
<td>50.75(^{a})</td>
<td>3.61</td>
<td>32.33</td>
<td>12.25</td>
<td>1.28</td>
<td>0.43</td>
</tr>
<tr>
<td>3. 30 kg ha(^{-1}) N</td>
<td>37.80(^{b})</td>
<td>4.15</td>
<td>29.95</td>
<td>15.50</td>
<td>2.50</td>
<td>0.68</td>
</tr>
<tr>
<td>4. Co-inoculation + Humic acid</td>
<td>45.40(^{a})</td>
<td>3.82</td>
<td>27.62</td>
<td>11.75</td>
<td>1.85</td>
<td>0.42</td>
</tr>
<tr>
<td>5. Co-inoculation + 30 kg ha(^{-1}) N</td>
<td>50.17(^{a})</td>
<td>3.90</td>
<td>26.55</td>
<td>14.50</td>
<td>2.58</td>
<td>0.52</td>
</tr>
<tr>
<td>6. Co-inoculation + 30 kg ha(^{-1}) N + Humic acid</td>
<td>44.20(^{ab})</td>
<td>3.66</td>
<td>28.72</td>
<td>12.25</td>
<td>1.82</td>
<td>0.35</td>
</tr>
<tr>
<td>Medium</td>
<td>43.89</td>
<td>3.78</td>
<td>29.36</td>
<td>13.04</td>
<td>1.67</td>
<td>0.45</td>
</tr>
<tr>
<td>Test F</td>
<td>0.01(^{*})</td>
<td>0.19(^{NS})</td>
<td>0.61(^{NS})</td>
<td>0.53(^{NS})</td>
<td>0.08(^{NS})</td>
<td>0.15(^{NS})</td>
</tr>
<tr>
<td>CV (%)</td>
<td>19.51</td>
<td>9.02</td>
<td>17.14</td>
<td>26.05</td>
<td>57.56</td>
<td>45.37</td>
</tr>
</tbody>
</table>

* and \(^{NS}\) - significant 5% probability and not significant, respectively. Medium followed by the same letter in the columns, do not differ statistically between themselves by Tukey test, the 5% probability. CV: coefficient of variation.

ranged from 22 cm (control) to a mean of 41.16 cm (co-inoculation, co-inoculation + 30 kg ha\(^{-1}\) of N and co-inoculation + 30 kg ha\(^{-1}\) of N + humic acid), with increment of 87.09% in relation to the control (Table 1). On the other hand, the cultivar ‘BRS Esplendor’ showed greater height in the treatment with co-inoculation of *A. brasilense* and *R. tropici* and co-inoculation + 30 kg ha\(^{-1}\) of N, statistically differing from the control. A mean increment of 43.96% was observed in plant height, in comparison to the control (Table 3).

This increase in plant height is associated with the stem elongation promoted by N associated with co-inoculation. According to Marschener (1995), the application of N doses in the initial development stage of plants causes increments in the production of growth-promoting phytohormones (auxins, gibberellins and cytokines), which are responsible for processes of cell division and expansion. It is important to point out that, even with rhizobium inoculation, many studies (Pelegrin et al., 2009; Brito et al., 2011) suggest the need for the application of an initial N dose in the bean crop.

As to the stem diameter of the cultivar ‘Pérola’, the treatment with co-inoculation of *A. brasilense* and *R. tropici* was superior, statistically differing (p<0.05) only from the control (Table 1). Higher stem diameter is directly related to the increase in production, since it acts in the storage of soluble solids that will be used later for grain formation (Fancelli and Dourado Neto, 2008).

Co-inoculation of *A. brasilense* and *R. tropici* influenced root length and volume of bean plants cv. ‘Pérola’ (Table 1). Co-inoculated plants showed increment of about 55.26% in root length and 35.13% in root volume, compared with the control (not inoculated), but did not differ statistically from the treatment with co-inoculation + 30 kg ha\(^{-1}\) of N and co-inoculation + 30 kg ha\(^{-1}\) of N + humic acid. This effect of increase in root length and volume is due to the production of auxins by the bacteria, which stimulates the growth of secondary roots, thus...
increasing the specific area of absorption of water and nutrients by plants (Radwan et al., 2004).

Similar results were obtained by Burdman et al. (1997), who claim that the inoculation with *Azospirillum* ssp. increases the number of root hairs and, since there is an increment in the root system, the combined inoculation with *Rhizobium* contributes to colonizing a greater number of roots, increasing the number of atmospheric N-fixing nodules. Canellas et al. (2013) observed increase in root area of corn plants when inoculated with *Herbaspirillum seropedicae* in combination with humic substances. Gitti et al. (2012) observed increase in root system and higher number atmospheric N-fixing nodules in common bean when co-inoculated with *A. brasilense* and a strain of *Rhizobium*.

Shoot dry matter production increased by approximately 76.12% in the treatment with co-inoculation of *A. brasilense* and *Rhizobium* in relation to the control (without co-inoculation and without N), evidencing the beneficial effects of co-inoculation with symbiotic and associative bacteria in N assimilation by bean plants. In agreement with the obtained results, Oliveira (2011) observed increase in shoot dry matter production of common bean with co-inoculation of CIAT 899 and UFLA 04-155. In addition, Peres (2014) reported that co-inoculation allowed higher production of shoot dry matter and that did not differ from *A. brasilense*. On the other hand, Veronezi et al. (2012) observed no differences in shoot dry matter between the treatments with inoculation of bean seeds with *R. tropici*, co-inoculation with *R. tropici* and *A. brasilense* and without inoculation added or not to mineral N.

It is important to point out that, based on the previously discussed results, most treatments with co-inoculation of *A. brasilense* and *R. tropici* showed results similar to those of treatments with co-inoculation + humic acids and co-inoculation + 30 kg ha⁻¹ of N for plant height, stem diameter and root volume (Tables 1 and 2). This allows suggesting that co-inoculation of bean plants alone is able to supply the N necessary for crop growth and development, which can lead to a reduction in the use of synthetic N fertilizers and, consequently, reduce production costs.

There was no significant effect (p>0.05) of the treatments for shoot N content, root N content, total N content and N absorption and transport efficiencies in bean plants, cv. ‘Pérola’ (Table 3). Only N use efficiency showed significant response (p<0.05) to the treatments. Co-inoculation with *R. tropici* and *A. brasilense* promoted significant increase in N use efficiency, in relation to the control, indicating synergism of the bacteria inoculated in the seeds, which increases the efficiency of initial BNF in the plants (Table 3). This shows that the N use efficiency obtained through the interaction of these microorganisms is equivalent to or higher than that observed with only mineral fertilization (30 kg ha⁻¹ of N). Hungria et al. (2013a) observed positive effects on plant total mass, leaf N and nodulation characteristics of bean with co-inoculation. However, there are still doubts about the origin of the benefits of *Azospirillum* in this interaction, whether from the hormonal effects that they cause on plants or from the improvements in nodulation caused by the rhizobia. Darnadelli et al. (2008) associated the positive effect of co-inoculation to root expansion and reduction in acetylene activity. However, Cásan et al. (2009) related the benefits of this interaction to the release of growth-regulating compounds, which besides promoting increase in root development rate, favors the capacity of plants to absorb water and nutrients, making them more tolerant to possible environmental stresses.

### Conclusions

1. Co-inoculation of *A. brasilense* and *R. tropici* allows obtaining higher initial growth in bean plants, cv. ‘Pérola’.
(2) Shoot dry matter production of bean plants, cv. 'Pérola', increased by approximately 76.12% when co-inoculated with rhizobacteria.

(3) N use efficiency of bean plants, cv. 'Pérola', is superior when co-inoculated with *A. brasilense* and *R. tropici*, confirming that co-inoculation alone is sufficient to provide the N necessary for plant development.

(4) Co-inoculation of *A. brasilense* and *R. tropici* did not interfere with growth and initial development of bean plants, cv. 'BRS Espendor', confirming the different responses of bean genotypes regarding the co-inoculation with rhizobacteria.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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