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Diazotrophic bacteria inoculation associates with acids and nitrogen in corn

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A successful application of humic acids and diazotrophic bacteria in corn represents a potential that collaborates to break the current energetic consumption paradigm, which is based on unsustainable fossil sources. Thus, this study aimed to quantify the contribution of diazotrophic bacteria in association with humic acids and nitrogen (N) in corn, in an experiment conducted under controlled conditions in a greenhouse. The experiment was carried out at the Federal Institute of Rondônia, Campus of Colorado do Oeste-RO, Brazil. The experimental design was completely randomized with four replicates and the treatments consisted of: control; inoculation of *Azospirillum brasilense*; 80 kg ha^{-1} of N; inoculation of *A. brasilense* + humic acid; inoculation of *A. brasilense* + 80 kg ha^{-1} of N; and inoculation of *A. brasilense* + 80 kg ha^{-1} of N + humic acid. At 40 days after emergence, plants were collected, divided into shoots and roots, and the variables were analyzed. According to the results, the joint use of plant growth-promoting bacteria and humic acids increased in plant height, stem diameter and root length and volume. Inoculation of *A. brasilense* combined with 80 kg ha^{-1} of N and humic acid increased N use efficiency in corn plants by 60%, while inoculation of *A. brasilense* combined with 80 kg ha^{-1} of N increased shoot N contents in corn plants.

Key words: *Zea mays* L., *Azospirillum brasilense,* humic substances, biological nitrogen fixation (BNF).

INTRODUCTION

Brazil occupies the third position in the ranking of corn grain production, after UAE and China. Corn planted area in the 2014/2015 season is estimated at 15,769 million hectares with production of 78,554 million tons of corn (Conab, 2015). Despite its high photosynthetic rate, corn is influenced by problems of environmental stress, such as those related to low fertility of soils, which mostly have nitrogen (N) deficiency (Araujo et al., 2014).

Identifying, selecting and using corn genotypes more tolerant to N deficiency and more efficient to absorb this nutrient constitute an important strategy (Reis Junior et al., 2008). Thus, the search for genotypes that form more-efficient associations with diazotrophic bacteria must be considered. Besides the capacity of biological N

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fixation, diazotrophic bacteria associated with grasses are known to act directly on the production of phytohormones (Radwan et al., 2004; Creus et al., 2004); solubilization of phosphates (Rodriguez et al., 2004); increase in nitrate reductase activity, when occur endophytically (Cassan et al., 2008); and indirectly on the biological control of pathogens and synthesis of siderophores (Correa et al., 2008; Vessey, 2003).

Currently, endophytic diazotrophic bacteria, from the most different genera and species, have been reported in association with a large number of grasses, from both tropical and temperate climates (Reis Júnior et al., 2008). In addition, the possibility of occurrence of significant increases in yield and N availability through biological nitrogen fixation (BNF) in corn has been described by many authors. Among the diazotrophic microorganisms found in association with cereals and grasses, the species of *Azospirillum* constitute, currently, one of the most studied groups. Although the number of researches involving endophytic bacteria has increased in the last years in Brazil, little is known about the effects of using endophytic diazotrophic bacteria combined with humic substances. Humic substances, the main component of soil organic matter, can promote increase in the population of endophytic diazotrophic bacteria, acting as a physical-chemical conditioner, as well as stimulating the increase in the establishment of the bacterial inoculum inside the plant. This can be hypothetically explained as part of the effects of humic substances to 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).

However, a successful application of humic acids and diazotrophic bacteria in corn represents a potential that collaborates to break the current energetic consumption paradigm, which is based on unsustainable fossil sources. The use and the knowledge on the potentialities of these bacteria, which supply N through biological fixation and increase fertilizer use efficiency, as an alternative for N nutrition, as well as the application of humic substances, represent an economically viable, ecologically sustainable strategy. As given earlier, this study aimed to quantify the contribution of diazotrophic bacteria in association with humic acids and N in corn, in an experiment conducted under controlled conditions in a greenhouse.

MATERIALS AND METHODS

The experiment was carried out under controlled conditions, in a greenhouse, from February 2015 to March 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 region is Awa, hot and humid tropical, with two well-defined seasons. The soil used in the study was classified as Red Yellow Argisol of very clayey texture (Embrapa, 2013) and 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 cmmolc dm^{-3}; H+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) inoculation of *Azospirillum brasilense*; 3) 80 kg ha^{-1} of N; 4) inoculation of *A. brasilense* + humic acid; 5) inoculation of *A. brasilense* + 80 kg ha^{-1} of N; and 6) inoculation of *A. brasilense* + 80 kg ha^{-1} of N + humic acid, totaling 24 experimental units.

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_2O_5 and 60 kg ha^{-1} of K_2O, as single superphosphate and potassium chloride, 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 with the dose 80 kg ha^{-1} of N, as urea (45%), by applying 40 kg ha^{-1} of N at sowing and 40 kg ha^{-1} of N as top-dressing, 15 days after plant emergence (DAE).

The experimental units consisted of plastic pots with capacity for 6 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.

The experiment used seeds of 'BRS Caatingueiro' corn, previously inoculated with a product containing a combination of two strains of *A. brasilense* (Ab-V5 and Ab-V6), in inoculant with peat formulation, produced by the Total Biotecnologia company. The dose used was 100 g of the peat inoculant and 50 ml of the sugar solution (10% of sugar concentration) for 50 g of seeds, mixed with the seeds, in order to cover them completely. After that, seeding was performed. Seeds germinated directly in the pots and at 8 DAE, thinning was performed, leaving only one plant in each experimental unit.

Humic acids were extracted and provided by the Biotechnology Laboratory of the Norte Fluminense State University (UENF), established in the Campus of Goytacazes-RJ, Brazil. Humic acids were isolated from vermicompost, according to Canellas et al. (2010). The material was previously dissolved in water, in the proportion of 13.5 mg L^{-1}. Then, plants were sprayed using 20 ml per pot, at the beginning of the stem elongation stage, at 15 DAE.

At 40 DAE, plant height and stem diameter were determined. Plant height was obtained through the measurement from the basis to the apical meristem of the plants, using a ruler. Stem diameter was determined using a digital caliper, at height of 2 cm from the soil surface. Then, plants were collected and divided into roots and shoots. 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 root volume is determined by the difference between the initial and final volumes in the cylinder. After that, samples were placed in paper bags and dried in a forced-air oven at temperature of 65°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 acid 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).

Table 1. Plant height (PH), stem diameter (SD), root length (RL), root volume (RV), shoot dry matter (SDM), root dry matter (RDM) and dry mass of corn plants in response to the inoculation of *Azospirillum brasilense* in association with humic substances and nitrogen (Colorado do Oeste-RO, Brazil, 2015).

Treatment	PH (cm)	SD (mm)	RL (cm)	RV (cm^3/planta)	SDM (g)	RDM (g)	TDM (g)
Control	40.50^{b}	6.19^{b}	44.62^{b}	8.50^{b}	0.20	5.12	5.32
Inoculation	52.25^{ab}	6.62^{b}	48.75^{ab}	12.00^{ab}	0.60	5.29	5.90
80 kg ha^{-1} N	46.75^{ab}	6.03^{b}	57.00^{ab}	10.50^{ab}	0.41	5.20	5.61
Inoculation + Humic acids	57.00^{a}	7.22^{a}	66.25^{a}	16.00^{a}	1.10	5.59	6.69
Inoculation + 80 kg ha^{-1} N	55.62^{ab}	6.59^{b}	62.75^{ab}	11.25^{ab}	0.68	5.28	5.97
Inoculation + 80 kg ha^{-1} N + Humic acids	51.75^{ab}	6.94^{b}	55.12^{ab}	9.75^{ab}	0.71	5.29	6.01
Medium	50.64	6.60	55,75	11.33	0.62	5.29	5.91
Test F	0.03*	0.04*	0,01*	0.05*	0.15^{NS}	0.11^{NS}	0.07^{NS}
CV (%)	13.43	15.73	14,24	26.88	71.30	4.04	9.57

* 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.

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 (Ferreira, 2000).

RESULTS AND DISCUSSION

There was significant difference (p≤0.05) for plant height, stem diameter and root length and volume in response to *A. brasilense* inoculation associated with humic substances and N (Table 1).

Plant height and stem diameter of corn showed the highest values in the treatment with *A. brasilense* inoculation associated with humic acids, statistically differing from the control (without inoculation and without N) (Table 1). There were increases of 40.74 and 16.63% in plant height and stem diameter, respectively, in relation to the control. It should be pointed out that 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).

The inoculation of *A. brasilense* in association with humic acids influenced the root length and volume in corn plants (Table 1). Plants inoculated and under the application of humic acids showed increases of 48.47% in root length and 88.23% in root volume, compared with the control (not inoculated), but did not differ from the other treatments. 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 reported by Canellas et al. (2013), who observed increase in root area of corn plants when inoculated with *Herbaspirillum seropedicae* combined with humic substances.

The positive responses of the association of Ab-V5 and Ab-V6 + humic acid may have been due to what was found by Marques Júnior et al. (2008), under controlled conditions. These authors observed that inoculation of bacteria from the genus *Herbaspirillum* + humic substances in heat-treated seed pieces of sugarcane (variety 'RB72454') showed effects of inoculation, combined or not with humic substances, on the increase in the population of the inoculated bacteria, as well as on the increment in root growth, induced by both, inoculation of the selected bacteria and humic acids, which suggests new models of utilization of diazotrophic bacteria in plants. However, based on the results, it can be inferred that inoculation of *A. brasilense* associated with humic acids is capable of providing the N necessary for corn growth and development, allowing a reduction in the use of synthetic N fertilizers and consequently, a reduction in production costs.

N contents in the shoots, roots and in the plant were higher in the treatment with inoculation + 80 kg ha^{-1} of N, not differing statistically from the control and from those treatments with inoculation and inoculation + humic acids (Table 2). It should be pointed out that, commonly, in grasses, there is greater contribution of inoculation associated with N fertilization. According to Baldani et al. (1996), the inoculation of diazotrophic bacteria in the presence of small N doses proves to be more efficient for the plant-bacteria system, in comparison to the isolated use of bacteria. This is due to the fact that the amount of organic compounds excreted, deposited and/or exuded in the rhizosphere by the plant in the presence of small N doses produces intense microbial activity and interactions, which allow these bacteria to colonize, that is, it allows the emission of signals to the microorganisms.

As observed in the present study, Dobbelaere et al. (2002) reported that the effect of inoculation of *A. brasilense,* strain Sp 245, and *Azospirillum irakense*, strain KBC1, was higher when associated with N doses. Dalla Santa et al. (2004), in tests with corn, using the *Azospirillum* species strains RAM-7 and RAM-5, observed that the use of these strains was able to reduce

Table 2. Shoot nitrogen content (SNC), root nitrogen content (RNC), total nitrogen content (TNC), shoot nitrogen accumulation (SNA), root nitrogen accumulation (RNA) and total nitrogen accumulation (TNA) in corn in response to the inoculation of *Azospirillum brasilense* associated with humic substances and nitrogen. Colorado do Oeste-RO, Brazil (2015).

Treatment	SNC (g kg^{-1})	RNC (g kg^{-1})	TNC (g kg^{-1})	SNA (mg)	RNA	TNA
Control	39.40^{ab}	14.80	53.97^{ab}	16.52	77.89^{b}	298.94
Inoculation	37.35^{ab}	15.40	53.77^{ab}	24.81	78.35^{b}	319.90
80 kg ha^{-1} N	30.65^{abc}	19.22	48.85^{ab}	20.69	101.45^{a}	319.42
Inoculation + Humic acids	29.30^{ab}	17.42	47.42^{ab}	32.29	97.37^{ab}	318.66
Inoculation + 80 kg ha^{-1} N	39.65^{a}	20.27	59.92^{a}	18.16	105.19^{a}	291.59
Inoculation + 80 kg ha^{-1} N + Humic acids	24.67^{c}	18.95	39.00^{b}	14.74	99.96^{ab}	234.33
Medium	33.48	17.67	50.35	21.20	93.37^{ab}	297.14
Test F	0.09*	0.12	0.01*	0.23	0.04*	0.19
CV (%)	13.33	17.18	13.83	69.15	14.95	17.05

* 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. Nitrogen absorption efficiency (NAE), nitrogen transport efficiency (NTE) and nitrogen use efficiency (NUE) by corn plants in response to the inoculation of *Azospirillum brasilense* associated with humic substances and nitrogen (Colorado do Oeste-RO Brazil, (2015).

Treatment	NAE (mg g^{-1})	NTE (%)	NUE (mg g^{-1})
Control	58.39	5.49	0.10^{b}
Inoculation	60.40	7.09	0.11^{ab}
80 kg ha^{-1} N	61.39	8.42	0.09^{b}
Inoculation + Humic acids	56.82	9.60	0.14^{ab}
Inoculation + 80 kg ha^{-1} N	55.11	6.96	0.12^{ab}
Inoculation + 80 kg ha^{-1} N + Humic acids	44.26	6.00	0.16^{a}
Medium	56.06	7.26	0.12
Test F	0.17	0.12	0.00*
CV (%)	16.51	52.16	17.57

* 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.

by 40% the amount of N fertilization recommended. Araújo et al. (2014) observed that the inoculation of the strain Z-94 of *H. seropedicae* combined with 80 kg ha^{-1} of N promoted an increase of about 25.74% in shoot N contents of corn plants, in comparison to the control, fertilized with 80 kg ha^{-1}. These authors reported that the higher N content in inoculated plants is the result of both BNF and the mechanisms of root growth promotion, which can increase the capacity of plants to absorb this nutrient.

N use efficiency increased by approximately 60% with the inoculation of *A. brasilense* associated with 80 kg ha^{-1} of N and humic acids, in relation to the control (Table 3). This shows the beneficial effects of bacteria on N assimilation by corn plants when associated with humic substances and small N doses, since the dose recommended for corn under field conditions and clayey soils is 120 kg ha^{-1} of N to supply its requirement. Araujo et al. (2015) observed that plants fertilized with 30 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae* showed the highest percentage of N use efficiency. On average, in these treatments, nitrogen use efficiency was equal to 84.65%, against 64.63% in treatments fertilized with 120 kg ha^{-1} of N and inoculated with *A. brasilense* and *H. seropedicae.* Studies on N use efficiency in production systems are essential, because as the applied amount exceeds the capacity of plants to absorb the nutrient for production, N can be leached or accumulated in the tissues, reducing its use efficiency (Araujo et al., 2015).

However, despite not causing increments in dry matter production, the combined use of plant growth-promoting bacteria and stabilized organic matter (humic acid) should be more studied because, based on the other analyzed variables, it was promising for the corn crop.

Conclusions

The joint use of plant growth-promoting bacteria and humic acids promoted increase in plant height, stem diameter and root length and volume.

Inoculation of *A. brasilense* combined with 80 kg ha^{-1} of N and humic acids increased N use efficiency of corn plants by 60%, while the inoculation of *A. brasilense* combined with 80 kg ha^{-1} of N increased N content in the shoots of corn plants.

Conflict of Interests

The authors have not declared any conflict of interests.

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