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Ammonium and nitrate levels of soil inoculated with *Azospirillum brasilense* in maize

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***Azospirillum brasilense* is a nitrogen fixing bacteria used in maize crop production due to its high capacity for plant growth promotion and yield increase. The present study aimed to evaluate ammonium and nitrate levels from soil inoculated with *A. brasilense* in maize crop production. The experiment was carried out in greenhouse conditions, using randomized blocks with split plots for 20 days with four replicates and two treatments where ammonium and nitrate levels were measured daily. The numbers of colony forming units of diazotrophic bacteria at initial and final stages of the experiment were counted in addition to the shoot and root dry mass measurement. The results were analyzed using F, Scott and Knott tests. The treatment which received inoculation did not show any statistically significant difference in the ammonium, nitrate levels either root or shoot dry mass as compared to the control. Also, there was no increase in the colony counts of diazotrophic bacteria. Taken together, the results showed that *A. brasilense* was not able to promote ammonium and nitrate levels after 20 days of its inoculation, suggesting that the plant growth promoting effect are not by fixing nitrogen during the initial plant development period and this period would be not appropriate for the plants to receive the inoculation.**

Key words: *Azospirillum brasilense*, maize, nitrogen fixer.

INTRODUCTION

Maize (*Zea mays* L.) is one of the largest cereal cultivated worldwide, has shown large adaptation capacity at different environmental conditions and being designated for human and animal feed due its high nutritional value (Peter et al., 2000). Nitrogen is the major nutrient, among the nutritional contents present in maize. Nevertheless, due to the complex dynamic of nitrogen in tropical soils, its availability is usually not enough to supply the necessity of the maize crop, so additional nitrogen fertilization is necessary for maize to express its

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Table 1. Soil chemical and physical characteristics.

pH	$CaCl_2$	MO (g dm³)	P resina (mg dm³)	K	Ca	Mg	H+Al	SB	CTC	V (%)
				....	....	....	$mmol_c dm^3$	....	....	....
6.3		7	6	0.4	21	6	15	27,5	42.8	64

high potential yield (Lemaire and Gastal, 1997).

Ferreira et al. (2001) claim that the nitrogen fertilization improves grain quality of maize, nutrients, minerals and proteins levels in addition to yield and yield related parameters such as number of cobs per plant, kernels per cob and kernel biomass. The nitrogen as mineral fertilization is applied in large quantities into soil because it is easily lost by volatilization, leaching, thereby increasing the cost of production up to 20% of total cost of production (Cantarella and Duarte, 2004)

In this context, the nitrogen fixing bacteria can be used as an important strategy to supply nitrogen, since plants are not able to assimilate nitrogen directly from the atmosphere. Other ways such as biological fixing, organic matter and synthetic fertilization must be used to supply plants' need (Amado et al., 2002). The atmospheric nitrogen fixing is fulfilled by a group of bacteria named "diazotrophs". Genus *Azospirillum* has been used as inoculant in several crops, such as cereals, cotton, sugarcane, coffee, grassland and others (Reis, 2007). Among the different genera of bacteria, *A. brasilense* is very useful, because of its ability in nitrogen fixation as well as phytohormones biosynthesis (Fallik et al., 1988).

The major challenge currently in agricultural production is reducing the production cost, by keeping the yield level with minimized environmental impact (Bhardwaj et al., 2014). In this context, utilization of plant growth promoting rhizobacteria can be a great strategy for this purpose. The present study aimed to evaluate the effect of *A. brasilense* on ammonium and nitrate nitrogen assimilation in soil planted with maize crop.

MATERIALS AND METHODS

Study site

The experiment was carried out in greenhouse conditions at Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal (FCAV), Unesp, Sao Paulo State (21° 14' 05" S, 48° 17' 09" W and 615,01 m of altitude).

Maize cultivation in pot culture conditions

Five liter capacity pots were used in this experiment. They were filled with Oxisol Eutrophic soil (USDA, 2016). The chemical and physical characteristics of the soil are described in Table 1. The chemical analysis was done according to Instituto Agronômico de Campinas (IAC, 2011), and the fertilization were carried out according to Raij et al. (1997). No nitrogen formulation was added to the fertilization.

Each vase was seeded with four maize seeds. After seed germination, the less vigorous seedling were thinned out and left with three uniform seedlings in each pot in order to effectively achieve this study. The plants were irrigated to 70% water holding capacity of soil. In order to achieve that, all vases were weighted, replenished evapo-transpiration volume every day.

Azospirillum brasilense inoculum

The *A. brasilense* bacterium used in this study was obtained from a commercial liquid formulation, which belongs to Ab-V5 and Ab-V6 lineage. The inoculum was prepared by adding an aliquot of *A. brasilense* culture with the aid of inoculation handle into Erlenmeyer flask with 100 ml of *A. brasilense* specific culture medium. The medium formulation is glucose, 5 g ($C_6H_{12}O_6$); potassium phosphate monobasic, 0.4 g (KH_2PO_4); potassium phosphate dibasic, 0.4 g (K_2HPO_4); ammonium phosphate monobasic, 0.5 g ($NH_4H_2PO_4$); 0.1 g magnesium sulfate heptahidrate ($MgSO_4.7H_2O$); 0.2 g sodium chloride (NaCl); 0.017 g ferric chloride heptahidrate ($FeCl_3.6H_2O$), 0.002 g magnesse sulfate Hexahydrate ($MnSO_4.6H_2O$); 1 g yeast extract to 1000 ml of deionized water pH 7.4 and sterilized by autoclaving. The inoculum was inoculated as previously described then incubated at 28°C for 48 h. Then, the inoculum concentrations were adjusted to 1 x 10^8 colony forming unit (CFU) per ml. The study had a treatment which received the inoculum and control with no bacterial treatment. 3 ml of inoculum per vase were added with aid of pipette at the concentration of 3 x 10^8 CFU/vase, weekly starting after the seed germination. The control treatment did not receive the inoculum.

Analysis of ammonium and nitrate level from soil

Daily, for 20 days, four vases from each treatment were dismounted. Then, the soil samples were taken to the laboratory and dried at environmental temperature for 20 h. After that, the soil samples were sieved using 2 mm sieve and estimated ammonium (NH_4) and nitrate (NO_3) nitrogen levels according to Keeney and Nelson (1982).

Analysis of shoot and root dry mass

The shoot and root mass of the maize plants were separated and kept in kiln at 65°C up to constant weight. After complete drying at 65°C, all the plant materials were weighted and the values of shoot and root dry matter were recorded.

Counting of diazotrophic bacteria

The diazotrophic bacterial colony forming units were counted twice. First counting was done at zero time and second counting at 20th

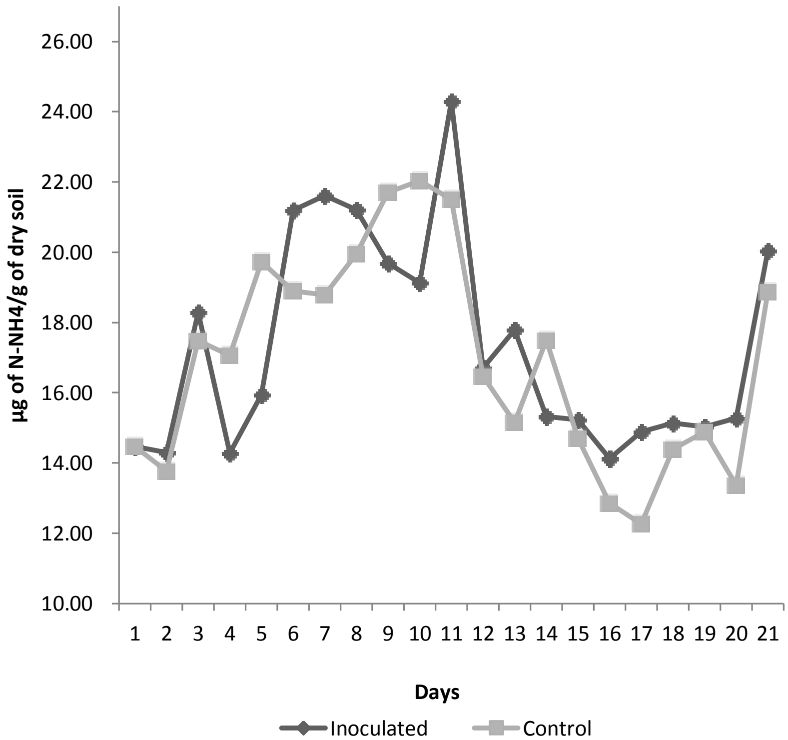

Figure 1. Means of ammonical nitrogen levels in the soil from treatment inoculated and control pots during 20 days under maize crop in greenhouse conditions.

day after germination in both treatment and control plants using the selective medium NFb (Döbereiner et al., 1995).

Statistic analysis

This study was designed in randomized blocks with split plot in the time with four repetitions with two treatments with or not inoculation with *A. brasilense* and the second factor were the days. The data were submitted to variance analysis by F test 5% and the mean values were compared by Scott and Knott test using Agroestat version 1.0 (Barbosa and Maldonado, 2010).

RESULTS AND DISCUSSION

After 20 days of evaluation, there was no observed statistical difference as to ammonium levels in the soils of bacterial inoculated plants, as compared to the control treatment. The measured range of ammonium level were from 12.26 to 22.03 and 14.13 to 24.30 µg NH_4-N g^{-1} of dry soil, for the control and inoculated treatment, respectively (Figure 1).

The ammonium levels measured daily from each treatment did not differ each other. The ammonium levels increased from fourth day up to 10^{th} day and decreased between the days 11 and 19 (Figure 2). *A. brasilense* is able to grow using atmospheric nitrogen as only nitrogen source (Eckert et al., 2001). According to the author, depending on situation, bacteria *Azospirillum* may use different nitrogen sources such as ammonia, nitrate, nitrite, molecular nitrogen and amino acid for its own metabolism without making it available for plants (Eckert et al., 2001).

The nitrate levels estimated in the soil from control treatment and the treatment inoculated with bacterium, did not differ statistically, the values varied from 6.10 to 13.83 and 6.52 to 15.47 µg NO_3-N g^{-1} of dry soil, respectively (Figure 3). There was a significant increase ($p<0.05$) in the soil nitrate levels between each day during the 20 days of experimentation (Figure 4). Probably, the *A. brasilense* who is a nitrogen fixer does not use this characteristic as mechanism of plant growth promotion at initial development period of maize. The *A. brasilense* is a phytohormone producing bacterium, and in certain conditions, this characteristic may promote a greater plant growth and development (Didonet et al., 2000).

Martínez-Morales et al. (2003) showed a phytohormone biosynthesis mainly by auxin by bacterial isolates belong

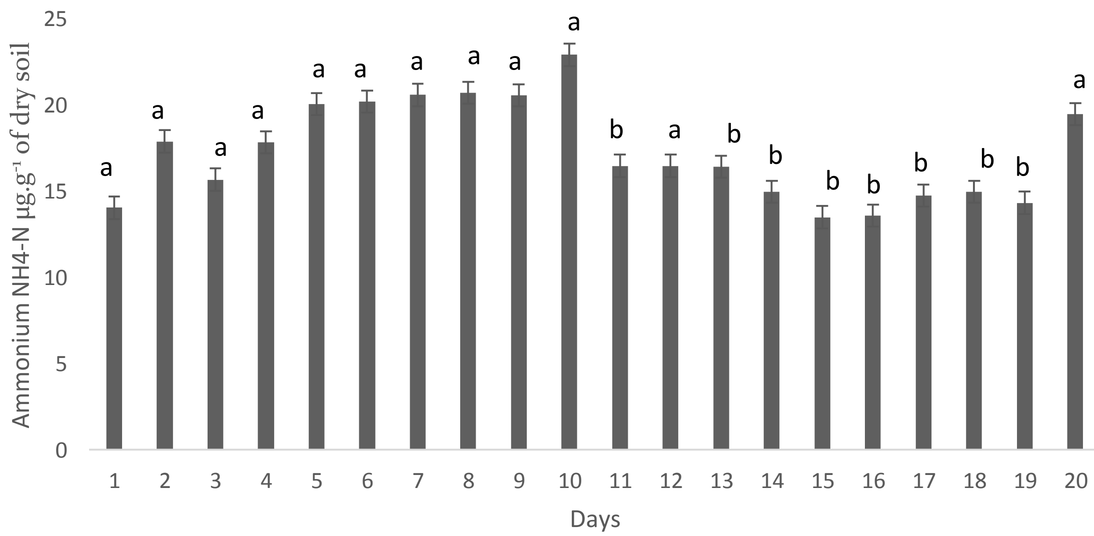

Figure 2. Means and standard deviation of ammonium levels during 20 days in the soil planted with maize crop under greenhouse conditions. Means followed by the same letter indicate no statistical difference between each other by Scott and Knott 5%.

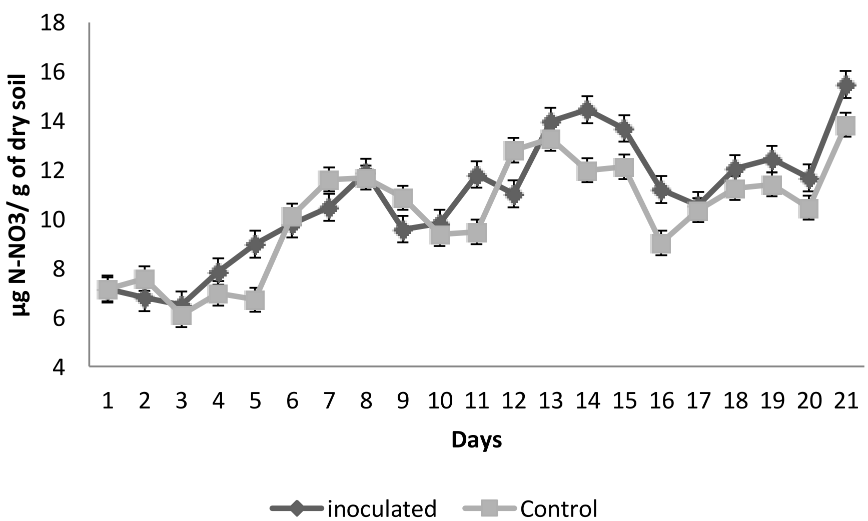

Figure 3. Means and standard deviation of nitrate levels in the soil from inoculated and control treatment during the 20 days of experimental period.

to the *Azospirillum* genus. This auxin may promote the plant growth and development by mainly affecting root morphology and increasing radicular hair strength and number (Barbieri et al., 1986). In addition to auxin, *Azospirillum* can also produce other phytohormones such as cytokine, gibberellin and indole acetic acid (Moreira et al., 2010) and has proven bio-control effect (Raaijmakers et al., 2009).

The survival and establishment of diazotrohic bacteria in soil was measured two times during the experiment one at zero time before planting another at the 20th day (while the termination of experiment) by plating the soil sample in diazotrohic bacterial specific nutrient media. Comparing the cfu of diazotrophic bacteria between two different times, the later (20 days after planting) was higher than the initial time point (Figure 5). It suggests

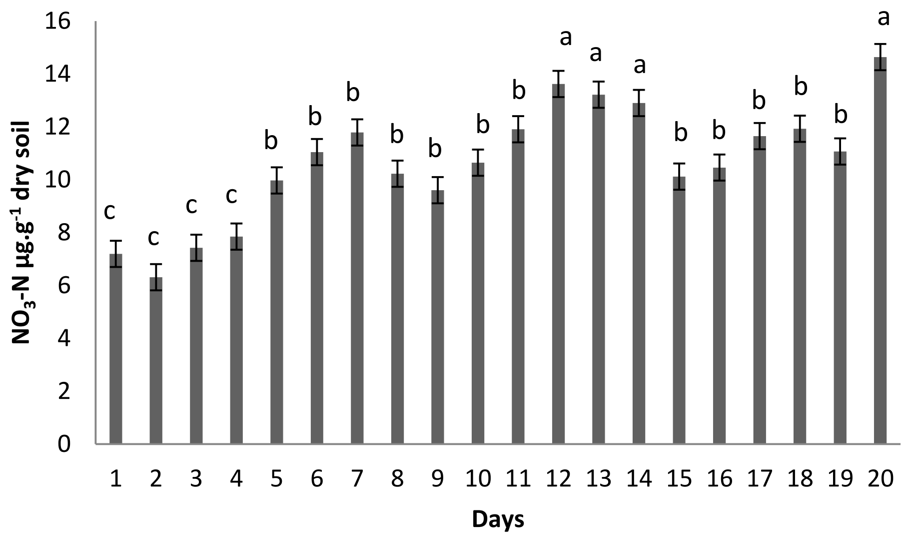

Figure 4. Means and standard deviation of ammonium levels during 20 days. Means followed by the same letter indicate no statistical difference between each other by Scott and Knott 5%.

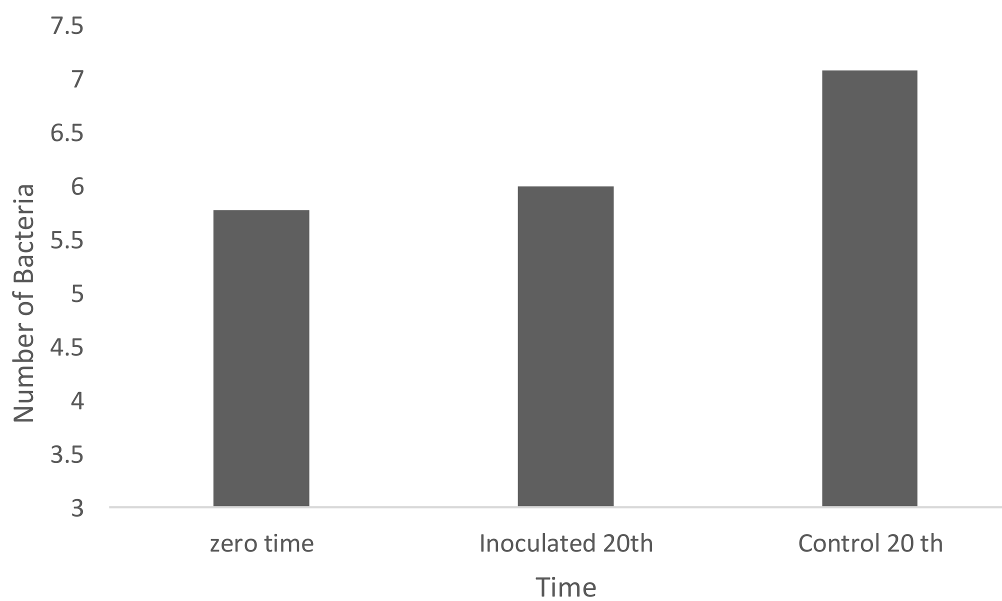

Figure 5. Number of diazotrophic bacteria in the soil before maize planting (zero time) and at 20th day of both treatment inoculated and control under greenhouse conditions. Means followed by the same letter indicate no statistical difference between each other by Scott and Knott 5%.

that, probably the plant has induced the nitrogen fixing bacterial growth under nitrogen deficiency conditions. Moreover, the number of diazotrophic bacteria from inoculated treatment was higher than control treatment, even though they were not statistically significant.

This indicates that *A. brasilense* inoculum did not increase the number of diazotrophic bacteria when compared with the control treatment as unexpected. This result suggests that *A. brasilense* inoculum may have promoted a competition with other soil microbiota, then led to a population rearrangement, that might have caused the delay in diazotrophic bacterial inoculum

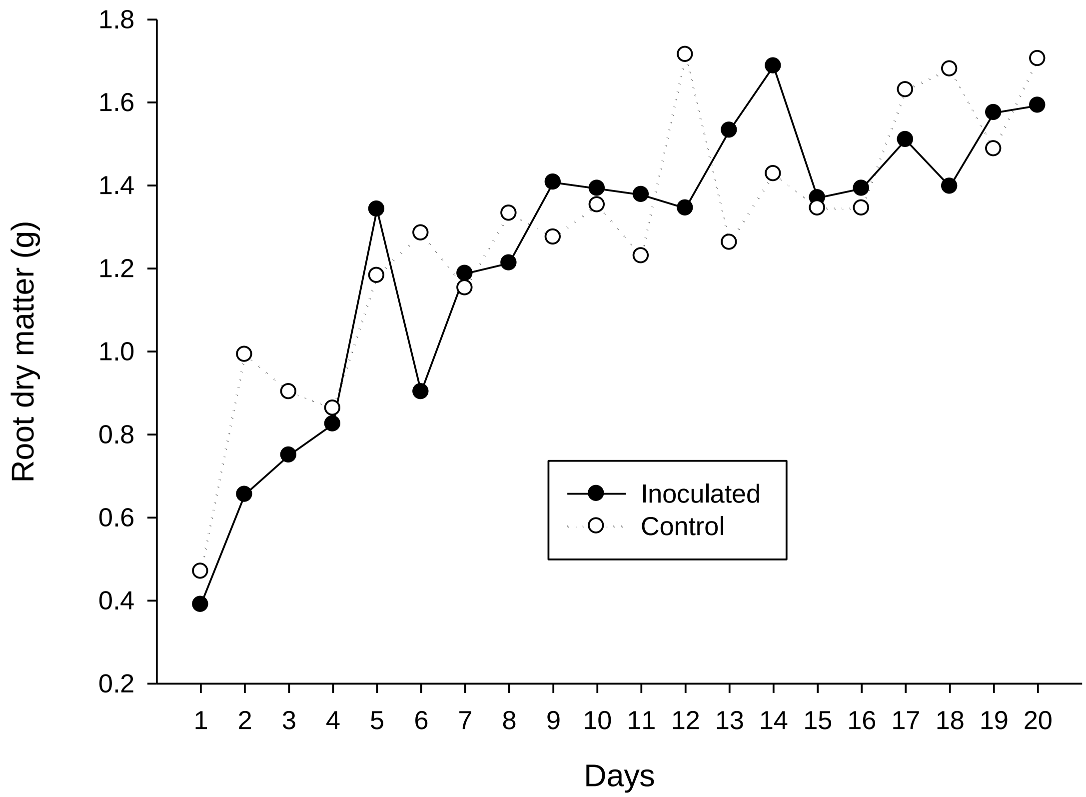

Figure 6. Root dry matter biomass (means) of maize plants which received *A. brasilense* treatment in comparison with control treatment, which did not receive any treatment during the 20 days trial period at greenhouse conditions.

establishment. Didonet et al. (2000) also reported that the inoculation of *A. brasilense* may promote a competition between normal soil microbiota.

The root dry mass varied from 0.47 to 1.72 g and from 0.39 to 1.69 of treatments control and inoculated with *A. brasilense*, respectively (Figure 6). There was no significant difference in both root and shoot dry mass of maize plants between the treatments. The values of shoot dry mass varied from 0.18 g to 1.16 g in control and from 0.26 g to 1.70 in inoculated plants (Figure 7). Probably, the duration of evaluation period was not appropriate to measure the plant development enhanced by the plant growth promoting bacteria *A. brasilense*. Quadros et al. (2014) reported an increase of root dry matter inoculated with *A. brasilense* when the evaluation was done at V_{12} stage of maize and this result was attributed to auxin produced by *A. brasilense*.

Dartora et al. (2013) verified a growth promoting effect in maize inoculated with *A. brasilense* and *Herbaspirillum* at reproductive stage, this effect probability was not found because maize was evaluated in initial stage of development. Certainly, the evaluation period of maize growth promotion at initial stages, to study the growth promoting effect of *A. brasilense*, is not appropriate.

Conclusion

The experimental findings indicate that *A. brasilense* did not promote an increase of nitrogen in the soil either nitrate or ammonical form during the initial 20 days of planting, suggesting that the growth promotion effects are not via nitrogen fixing at the initial period of plant development. Also, this early period would not be appropriate for the plants to receive *A. brasilense* inoculum, also in this period, the mode of interaction of this microorganism with plants may not be predominately nitrogen fixing. Probably, it will occur at later growth stages.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

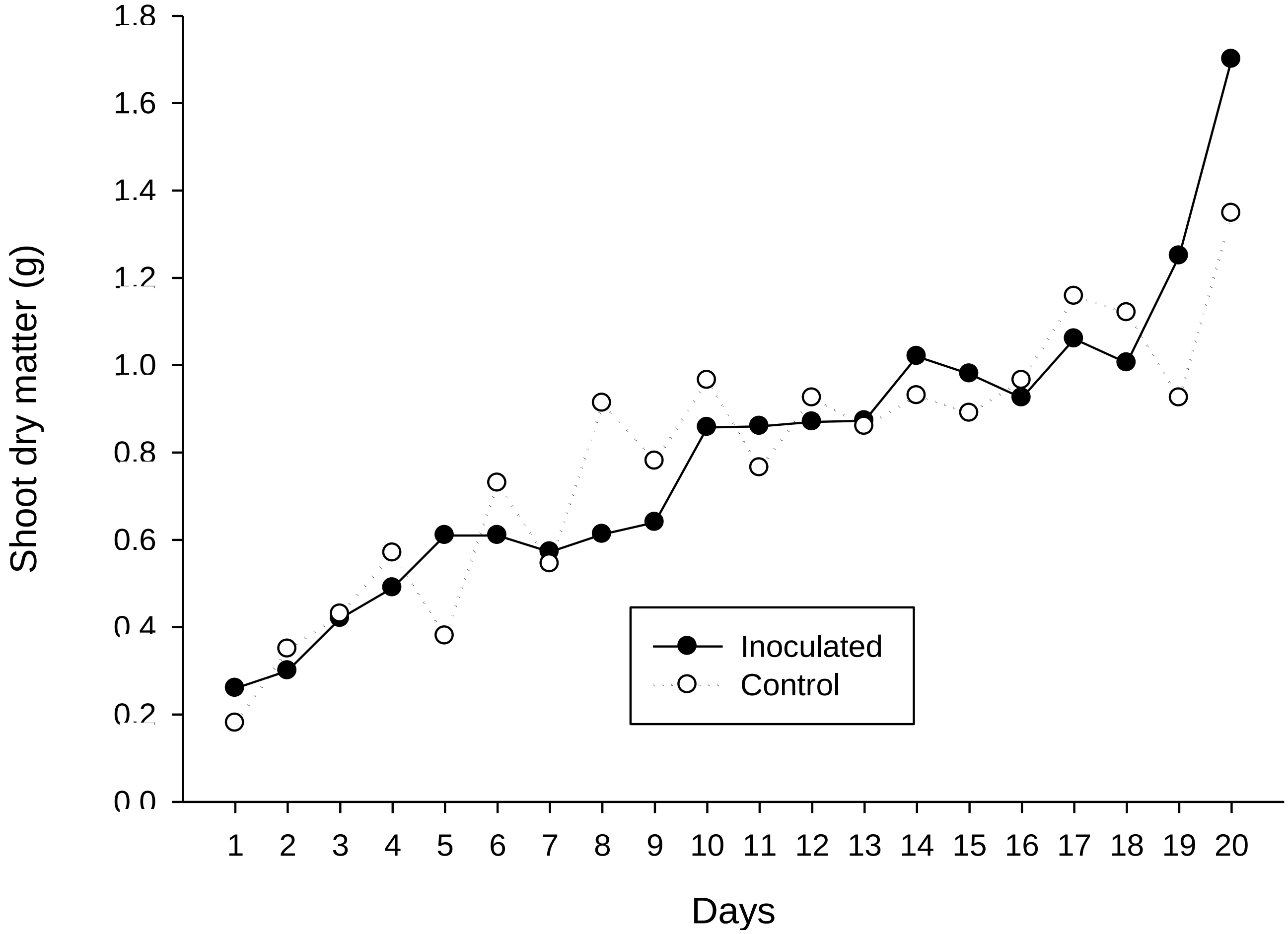

Figure 7. Shoot dry matter biomass (means) of maize plants which received *A. brasilense* treatment in comparison with control treatment, which did not receive any treatment during the 20 days trial period at greenhouse conditions.

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REFERENCES

Amado TJC, Mielniczuk J, Aita C (2002). Recomendação de adubação nitrogenada para o milho no RS e SC adaptada ao uso de culturas de cobertura do solo, sob sistema plantio direto. Rev. Bras. Ciênc. Solo 26:241-248.

Barbieri P, Zanelli T, Galli E, Zanetti G (1986). Wheat inoculation with *azospirillum-brasilense* sp6 and some mutants altered in nitrogen-fixation and indole-3-acetic-acid production. Fems Microbiol. Lett. 36:87-90.

Barbosa JC, Maldonado JW (2010). AgroEstat: sistema para análises estatísticas de ensaios agronômicos. versão 1.1.0.626. Jaboticabal: FCAV, Departamento de Ciências Exatas.

Bhardwaj U, Bhardwaj A, Kumar R, Leelavathi S, Reddy VS, Mazumdar-Leighton S (2014). Revisiting rubisco as a protein substrate for insect midgut proteases. Archiv. Insect Biochem. Physiol. 85:13-35.

Cantarella H, Duarte AP (2004). Manejo de fertilidade de solo para a cultura do milho. In J. C. C. Galvão and G. V. Miranda (ed) Tecnologia de produção de milho. Editora UFV, Vicoça, Minas Gerais, Brasil. pp. 139-182.

Dartora J, Guimaraes VF, Marini D, Sander G (2013). Nitrogen fertilization associated to inoculation with Azospirillum brasilense and Herbaspirillum seropedicae in the maize. Rev. Bras. Eng. Agric. Ambient. 17:1023-1029.

Didonet AD, Lima OD, Candaten AA, Rodrigues O (2000). Reallocation of nitrogen and biomass to the seeds in wheat inoculated with *Azospirillum* bacteria. Pesqui. Agropecu. Bras. 35:401-411.

Döbereiner J, Baldani VL, Baldani JI (1995). Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. Embrapa, Brasilia, Distrito Federal, Brasil.

Fallik E, Okon Y, Fischer M (1988). The effect of *Azospirillum-brasilense* inoculation on metabolic enzyme-activity in maize root seedlings. Symbiosis 6:17-27.

Ferreira ACB, Araújo GAA, Pereira PRG, Cardoso AA (2001). Características agronômicas e nutricionais do milho adubado com nitrogênio, molibdênio e zinco. Sci. Agric. 58:131-138.

IAC - Instituto Agronomico de Campinas (2011). Análise química para avaliação da fertilidade de solos tropicais. Instituto Agronômico, Campinas, São Paulo, Brasil.

Keeney DR, Nelson DW (1982). Nitrogen- Inorganic Forms. In Page, A.L. (ed) Methods of soil analyses. Springer, Berlin, Germany pp. 643-698.

Lemaire G, Gastal FN (1997). N uptake and distribution in plant canopies. In Lamaire G. (ed) Diagnosis of the nitrogen status is crops. Springer, Berlin, Germany pp. 3-43.

Martínez-Morales LJ, Soto-Urzua L, Baca BE, Sanchez-Ahedo JA (2003). Indole-3-butyric acid (IBA) production in culture medium by wild strain Azospirillum brasilense. Fems Microbiol. Letters 228:167-173.

Moreira FMS, Silva K, Nóbrega RSA, Carvalho F (2010). Bactérias diazotróficas associativas: diversidade, ecologia e potencial de aplicações. Comun. Sci. 2:74-99.

Peter CM, Faulkner DB, Merchen NR, Parrett DF, Nash TG, Dahlquist JM (2000). The effects of corn milling coproducts on growth performance and diet digestibility by beef cattle. J. Anim. Sci. 78:1-6.

Quadros PD, Roesch LFW, Silva PRF, Vieira VM, Roehrs DD, Camargo FAO (2014). Desempenho agronômico a campo de híbridos de milho inoculados com *Azospirillum* 61:209-218.

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moenne-Loccoz Y (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341-361.

Raij BV, Cantarella H, Quaggio JA, Furlani ÂMC (1997). Boletim técnico n.° 100. Recomendações de adubação e calagem para o estado de São Paulo. 2 ed. Instituto Agronômico, Campinas, São Paulo, Brasil.

Reis VM (2007). Uso de bactérias fixadoras de nitrogênio como inoculante para aplicação em gramíneas. Embrapa, Seropédica, Rio de Janeiro. Brasil.

USDA - United States Department of Agriculture (2016). Soil Classification. Available at: https://www.nrcs.usda.gov/wps/portal/nrcs/main/soils/survey/class/ (Accessed September 2016).