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Maize initial growth with the inoculation of plant growth-promoting bacteria (PGPB) under different soil acidity levels

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Abstract

The effects of plant growth promoting bacteria (PGPB) on the initial growth and leaf gas exchange parameters of maize plants (*Zea mays* L.), and bacterial population of the root and non-rhizosphere soil were investigated under different soil acidity levels. Maize plants were grown in 13-dm³ pots filled with clayey Rhodic Hapludox in a greenhouse. Treatments were arranged in a randomized block design in a 3 × 4 factorial: three soil acidity levels [pH in 0.01 mol L^{-1} $CaCl_2$ solution of 4.5; 5.0 and 5.5] and four seed inoculation treatments [control (non-inoculated); inoculation with *Azospirillum brasilense* strain AbV5; inoculation with *Herbaspirillum seropedicae* strain SMR1; and inoculation with two bacteria strains (*A. brasiliense* + *H. seropedicae*)]. Seeds inoculation with two PGPB strains (AbV5 and SMR1) improved the leaf area (14%), stem diameter (7%), relative chlorophyll content (14%), but had no effect on dry matter yield of maize plants, even with the changes of soil acidity levels. At 15 days after sowing, the inoculation of *A. brasilense* resulted in increased of diazotrophic bacteria density in the soil (15%). At 41 days after sowing, the inoculation of PGPB strains (AbV5 and SMR1) resulted in increased of diazotrophic bacteria density in the maize roots (13%). Seed inoculation with *H. seropedicae* enhances the nitrogen concentration in the leaf tissue of maize (12%) under soil acidity conditions and without the nitrogen supply, indicating increase in the biological nitrogen fixation. Inoculation of PGPB in acidic sandy soil (4.5 to 5.0 pH) resulted in higher phosphorus concentration in the leaf tissue of maize, indicating increase of phosphorus solubilization promoted by the diazotrophic bacteria. Leaf CO_2 assimilation rate was not affected by the maize seed inoculation with PGPB. Soil acidity resulted in the reduction of stomatal conductance (–25%), leaf CO_2 assimilation rate (–14%), leaf transpiration rate (–21%) and water use efficiency (–6%) of maize plants. The NFb Lactate (selective for *A. brasilense*) and NFb Malate (selective for *H. seropedicae*) culture media were not effective in differentiating of the two diazotrophic species studied and *Herbaspirillum seropedicae*, for both the root and soil samples.

Keywords: *Azospirillum brasilense*, *Herbaspirillum seropedicae*, soil pH, gas exchange, *Zea mays*.
Abbreviations: *A*_leaf CO_2 assimilation rate, AbV5_*Azospirillum brasilense*, BNF_biological nitrogen fixation, *E*_transpiration rate, g_S_stomatal conductance, NFb_semi-solid culture media, PGPB_plant growth-promoting bacteria, SMR1_*Herbaspirillum seropedicae*, WUE_water-use efficiency.

Introduction

Maize (*Zea mays L.*) is an important cereal crops from Brazil. In 2013/2014 season, the maize production area was of 15.8 million hectares, producing 80 million tons of grains (Conab, 2014). Paraná is the largest maize producing state with approximately 16 million tons, followed by Mato Grosso and Goiás (IBGE, 2013). An interesting alternative to avoid or reduce the use of N-fertilizers could be the exploitation of plant growth-promoting bacteria (PGPB), capable of enhancing growth and yield of many crop species, several of agronomic and ecological significance (Pedraza, 2008). When PGPB are associated with non-leguminous plants, they are divided into two groups: facultative endophytic bacteria (*Azospirillum* spp.) and obligate endophytic bacteria (*Herbaspirillum* spp.). Facultative endophytic bacteria can survive in the soil and on the plant surface as well as in the interior of the plant, *Azospirillum* strains capable of colonizing the interior of the plant could be considered facultative endophytes because they are also commonly found in the rhizosphere (Baldani et al., 1997). Bacteria such as *Herbaspirillum* spp. and *Burkholderia* spp., however, seem to be found only inside plant tissues and could be considered to be obligate endophytes. The PGPB or diazotrophic bacteria can be affected by various environmental factors such as temperature, oxygen concentration, humidity, soil fertility and pH of the colonization media (Baldani and Baldani, 2005). Therefore, the count of colony forming units (CFU) of diazotrophic bacteria has become practice carried out with the purpose of prove its benefits to plants (Oliveira et al., 2004). The PGPB are capable of promoting plant growth through different mechanisms, such as biological nitrogen fixation (BNF), phytohormone production (Moreira and Siqueira, 2006), phosphate solubilization (Stamford et al., 2004; Walpola and Yoon, 2013) and antagonism to plant pathogens (Moreira et al., 2010). The BNF occurs through the enzymatic reduction of the atmospheric dinitrogen (N_2) to ammonia (NH_3), catalyzed by nitrogenase enzyme (Pedraza, 2008). The facultative endophytic characteristic contributes to the variability of reactions by association with the rhizosphere region, because in this region there is intense extrusion of protons that interfere in hydrogenic potential (pH) of the medium. Thus, in addition to genotypic variation of plants and growth-promoting endophytic groups, soil pH can also influence the BNF. Bacteria of the genus *Azospirillum* require near neutral pH at a temperature between 32 and 37 °C for their growth (Baldani et al., 1986), and the genus *Herbaspirillum* are microaerophilic and develop extreme changes in pH between 5.3 and 8.0 (Döbereiner et al., 1995). Therefore, the development of obligate endophytic bacteria may be more responsive that to facultative endophytic bacteria in acid soils. Soil pH directly influences the availability of nutrients to the plant. Tropical soils are in general acidic (pH 4.0 to 5.5) causing toxicity of iron (Fe) and aluminum (Al) to the plants, and result in the phosphorus (P) fixation, making it unavailable by plants (Malavolta, 1979). Soil acidity can be identified in agricultural soils when the pH in $CaCl_2$ range from 4.0 to 7.0. Low cation exchange capacity (CEC), low base saturation and high aluminum (Al^{3+}) levels, sufficient to alter the normal growth of many plant species, characterize these soils. The greater nitrogen (N) availability in the soil is found at pH above 5.5 (Malavolta, 1979) and the uptake of nutrients by plants can be reduced from 79.5% to 26.7% when soil pH is reduced from 6.0 to 4.5 (Embrapa, 1980).

The toxicity caused by the Al uptake can be reversed by physiological mechanisms for organic acids such as citrate and malate, which act as chelating agents of Al (Hartwig et al., 2007). Therefore, is important to mention that the physiological processes are directly stimulated by the photosynthetic rate of plants, which is affected by inoculation of diazotrophic bacteria (Bashan et al., 2004). Measurements of gas exchange of plants may be important observation to quantify the influence of diazotrophic bacteria on plant growth under different soil conditions. The pH range considered optimal for maximum microbial activity in the soil ranges from 6.0 to 6.5 (Moreira and Siqueira, 2006); however, studies with lower pH values showed promising results with the use of PGPB in grasses (Rodrigues et al., 2014). Dartora et al. (2013) reported increased maize yield by inoculation of *Azospirillum brasilense* and *Herbaspirillum seropedicae* under acidic pH. These authors attributed these results to the behavior obligate endophytic of the *H. seropedicae* specie; however, the count of diazotrophic bacteria was not performed yet. The PGPB activity in the rhizosphere depend on various environmental factors, as soil pH that can interfere in the colonization affecting both obligate endophytes as the facultative endophytes. Therefore, the objective of this study was to investigate the effect of PGPB on the initial plant growth and leaf gas exchange parameters of maize, and bacterial population of the root and non-rhizosphere soil under different soil acidity levels.

Results and Discussion

Soil acidity levels significantly affected the plant height (Table 1), root dry matter (Table 2), stem diameter (Table 3), relative chlorophyll content (Table 4), leaf P concentration (Table 5), and leaf gas exchange parameters of maize (Table 6). Seed inoculation with diazotrophic bacteria strains significantly affected the leaf area (Table 3), stem diameter (Table 3), relative chlorophyll content (Table 4), leaf N concentration (Table 5), diazotrophic bacteria density in the non-rhizosphere soil at 15 days after sowing of maize using the NFb lactate media (Table 7) and diazotrophic bacteria density in the roots at 41 days after sowing of maize using the NFb malate media (Table 8). There was significant interaction between the factors of soil pH and PGPB inoculation only for the variable of diazotrophic bacteria density in the non-rhizosphere soil at 15 days after sowing of maize using the NFb malate media (Table 7).

Biometric characteristics

Soil acidity levels only significant effected the plant height at 41 days after sowing (DAS) (Table 1). Maize plants grown at pH 4.5 had lower plant height (56.9 cm) compared to the plants grown at pH 5.0 (59.7 cm). Diazotrophic bacteria inoculation did not affect the plant height up to 41 DAS, regardless of soil acidity levels (Table 1). In field experiments, Dartora et al. (2013) testing the maize Pioneer 30F53H hybrid and seed inoculation with *A. brasilense* and *H. seropedicae*, also found no differences in plant height, in soil of pH 5.5. However, Inagaki et al. (2014) verified that associated inoculation of *A. brasilense* and *H. seropedicae* provided maize plants of smaller stature compared to the uninoculated control. Root dry matter of maize increased from 3.96 g $plant^{-1}$ in the treatment with pH 4.5 to 4.87 and 4.59 g $plant^{-1}$ in the treatment with pH 5.0 and 5.5, indicating mean reduction of 18% (Table 2). Root, stem, leaf and total dry matter of maize plants did not affected by the inoculation of PGPB under different soil acidity levels (Table 2). Rodrigues et al. (2014) also found no effect of inoculation of AbV5 strain and humic acids in the stem plus leaf sheath dry matter of wheat plants in soil with pH 4.8. Similarly, Sala et al. (2008) verified that the inoculation of *A. brasilense*. *H. seropedicae* and *Achromobacter insolitus* isolated did not affect the production of shoot dry matter of maize. Testing an *A. amazonense* strain (resistant to acidity of the medium) in nutrient solution with pH 5.1, Reis Júnior et al. (2008) found an increase in shoot dry matter yield of maize compared to the uninoculated control. Diazotrophic bacteria are capable of plant growth promoting through different mechanisms, including the BNF, phytohormone production and phosphate solubilization (Stamford et al., 2004; Moreira and Siqueira, 2006; Walpola and Yoon, 2013). In common bean, the increase of soil pH improved the symbiosis of BNF bacteria, but these effects varied according with to strains (Rufini, et al., 2011). These results are beneficial for the common bean because the correct soil acidity improves the growth and development of plants (Campanharo et al., 2010). The inoculation of two PGPB strains (AbV5 and SMR1) resulted higher leaf area (31.7 dm^2 $plant^{-1}$) compared to the maize plants inoculated only with SMR1 strain (24.7 dm^2 $plant^{-1}$), indicating mean increase of 22% (Table 3). The increase in leaf area can intensify leaf transpiration rate; therefore, its reduction may be a strategy to reduce the water loss from plants. In this study, however, it is not possible to confirm this behavior because the PGPB inoculation showed leaf transpiration rate (E) similar to the uninoculated control (Table 6). Stem diameter was significantly affected by the inoculation of diazotrophic bacteria and soil pH levels (Table 3). Maize grown at pH 5.5 resulted in the greatest stem

Table 1. Plant height (cm) at different times during the maize initial growth as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	14 DAS[(1)]			21 DAS			41 DAS		
	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5
Control[(2)]	22.8	23.4	21.7	34.2	33.9	30.5	56.8	59.9	56.8
AZ[(3)]	22.7	22.8	22.2	32.2	32.5	30.2	56.4	60.1	61.4
HE[(4)]	21.6	22.7	22.1	30.8	30.4	32.9	56.8	58.6	55.6
AZ+HE[(5)]	22.1	22.1	22.5	31.3	32.0	30.6	57.8	60.1	59.1
Mean	22.3	22.7	22.1	32.1	32.2	31.1	56.9^{B}	59.7^{A}	58.2^{AB}
F test				F value					
Inoculation (I)	0.43^{ns}			1.44^{ns}			1.91^{ns}		
Soil pH (pH)	1.05^{ns}			1.58^{ns}			4.32*		
I × pH	0.77^{ns}			2.04^{ns}			1.12^{ns}		
CV (%)	5.5			6.4			4.6		
$LSD_{(I)}$	1.36			2.26			2.96		
$LSD_{(pH)}$	1.06			1.77			2.32		

(1) DAS: Days after sowing. (2) Non-inoculated. (3) AZ: Seeds inoculation with *Azospirillum brasilense*. (4) HE: Seeds inoculation with *Herbaspirillum seropedicae*. (5) AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.* Values represented by the different letters, for the soil pH levels show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. *: statistical significance at 5% by F test. CV: coefficient of variation. LSD: least significant difference.

Table 2. Root dry matter (RDM), stem dry matter (SDM), leaves dry matter (LDM) and total dry matter (TDM) of maize plants at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	RDM (g plant^{-1})			SDM (g plant^{-1})			LDM(g plant^{-1})			TDM (g plant^{-1})		
	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5	pH 4.5	pH 5.0	pH 5.5
Control[(1)]	3.81	4.80	4.64	4.41	4.18	4.50	4.21	3.65	4.00	12.4	12.6	13.1
AZ[(2)]	3.65	4.96	4.73	3.88	4.72	4.50	3.83	4.11	4.05	11.4	13.8	13.3
HE[(3)]	3.85	5.09	4.09	4.49	4.38	4.13	3.61	4.38	3.56	12.0	13.8	11.8
AZ+HE[(4)]	4.52	4.64	4.89	4.32	4.73	4.64	4.19	4.53	4.29	13.0	13.4	13.8
Mean	3.96^{B}	4.87^{A}	4.59^{A}	4.28	4.38	4.44	3.96	4.17	3.97	12.2	13.4	13.0
F test							F value					
Inoculation (I)	0.68^{ns}			0.03^{ns}			1.26^{ns}			0.91^{ns}		
Soil pH (pH)	9.22**			0.38^{ns}			0.51^{ns}			3.20^{ns}		
I × pH	1.26^{ns}			1.24^{ns}			0.92^{ns}			1.16^{ns}		
CV (%)	13.8			12.5			16.1			10.8		
$LSD_{(I)}$	0.68			0.60			0.72			1.54		
$LSD_{(pH)}$	0.53			0.47			0.56			1.21		

(1) Non-inoculated. (2) AZ: Seeds inoculation with *Azospirillum brasilense*. (3) HE: Seeds inoculation with *Herbaspirillum seropedicae*. (4) AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.* Values represented by the different letters, for the soil pH levels show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. **: statistical significance at 1% by F test. CV: coefficient of variation. LSD: least significant difference.

Table 3. Leaf area and stem diameter of maize plants at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	Leaf area (dm^{2} plant^{-1})				Stem diameter (mm)			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[(1)]	30.5	25.0	25.7	27.0^{ab}	22.2	21.9	22.9	22.4^{b}
AZ[(2)]	27.0	27.1	26.7	26.9^{ab}	20.7	22.5	24.8	22.7^{ab}
HE[(3)]	22.4	27.1	24.7	24.7^{b}	23.3	23.6	25.1	24.0^{ab}
AZ+HE[(4)]	32.1	34.2	28.8	31.7^{a}	23.8	22.8	25.9	24.2^{a}
Mean	28.0	28.3	26.5		22.5^{B}	22.7^{B}	24.7^{A}	
F test					F value			
Inoculation (I)	3.02*				3.81*			
Soil pH (pH)	0.45^{ns}				8.91**			
I × pH	0.70^{ns}				1.04^{ns}			
CV (%)	21.2				7.0			
$LSD_{(I)}$	5.08				1.81			
$LSD_{(pH)}$	6.46				1.42			

(1) Non-inoculated. (2) AZ: Seeds inoculation with *Azospirillum brasilense*. (3) HE: Seeds inoculation with *Herbaspirillum seropedicae*. (4) AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.* Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

diameter compared to other pH levels. The inoculation of two PGPB strains (AbV5 and SMR1) resulted higher stem diameter (24.2 mm) compared to the uninoculated control (22.3 mm), indicating mean increase of 7% (Table 3). These results indicate that the pH 5.5 and inoculation of *A. brasilense* + *H. seropedicae* can increase the tolerance of maize plants to lodging, as well as improve the accumulation of soluble solids in the stem (Fancelli and Dourado Neto, 2000). In a field experiment with soil pH 5.5, Dartora et al. (2013) also reported that the inoculation of the two strains (AbV5 and SMR1) increased the stem diameter of the maize compared to the uninoculated control. This study, soil acidity conditions and the absence of N supply may have provided stress conditions for the maize plants, which can be improved colonization of plants by diazotrophic bacteria, thus causing an increase in stem diameter and leaf area (Table 3) and relative chlorophyll content (Table 4).

Relative chlorophyll content

The relative chlorophyll content in the lower leaves (SPAD 1), central leaves (SPAD 2) and top leaves (SPAD 3) measured through SPAD readings at 41 DAS was affected by the three soil acidity levels (Table 4). The lowest relative chlorophyll content was obtained in lower soil pH values, regardless of leaf position on the plant. These results show that the soil acidity has high influence on the relative chlorophyll content of maize plants, by reduce the growth of the roots (Table 2). In turn, the reduction of the relative chlorophyll content of new leaves (up leaves) to the older leaves (lower leaves) was due to chlorophyll degradation when the leaves begin to senescence.

The inoculation with the combination of the two bacteria strains (AbV5 and SMR1) showed higher relative chlorophyll content in the central leaves (SPAD index of 16.6) compared to the uninoculated control (SPAD index of 15.0) (Table 4). The new leaves are considered as metabolic drain organs, which are not capable of totally supplying their own demands for carbon. Thus, in order to development, they depend on other assimilated parts of the plant with source tissues until they become self-sufficient. When leaves begin senescence, they resort on a nutrient remobilization (Taiz and Zeiger, 2013). Therefore, the inoculation of *A. brasilense* and *H. seropedicae,* predominantly in the rhizosphere and internal region of the roots, respectively, provided higher relative chlorophyll content in the central leaves (photosynthetically active). The measurements of SPAD index are directly related to the N assimilation by plants. The increase in the relative chlorophyll content in the tomato leaves was also verified under increasing rates of nitrogen fertilizer (Guimarães et al., 1999).

Nitrogen, phosphorus and potassium concentration in leaf tissue

The highest N concentration in the maize leaves was obtained in the treatment with inoculation of SMR1 strain (7.77 g kg^{-1}) and is higher to the uninoculated control (6.78 g kg^{-1}). These results report the importance of diazotrpphic bacteria on BNF, when there is no supply of N fertilizer as in the present study. The inoculation of two PGPB strains (AbV5 and SMR1) and inoculation of AbV5 strain also increased the N concentration in the maize leaves compared to the uninoculated, but no significant difference ($p > 0.05$). The *Azospirillum* species has an important role in plant growth promoting, excreting indole acetic acid (IAA) (Bashan et al., 2004), which can improve nutrient uptake and increase nutrient use efficiency (Hungria, et al., 2010). The presence of diazotrophic bacteria associated to the plants does not necessarily mean that high N amounts are provided by BNF (Boddey et al., 1995).

Nitrogen concentration in the maize leaves was not significantly affected by soil acidity levels (Table 5). It has been reported differentiated responses between maize hybrids with regard to the N supply by diazotrophic bacteria (Hungria et al., 2010; Dartora et al., 2013). Araujo et al. (2013) evaluated the *H. seropedicae* inoculation in 35 maize genotypes in an experiment without N supply and soil pH of 5.3 found that only nine hybrids had a significant increase in the N concentration. Sala et al. (2008) found no effect of N rates, but noted an increase in N concentration in maize plants when there was inoculation diazotrophic bacteria associated with N fertilization. The higher P concentration in maize leaves were obtained on soil pH conditions of 4.5 and 5.0 (Table 5). Stamford et al. (2004) found a reduction in soil pH and increase the availability of P after the cultivation of cowpea (*Vigna unguiculata* L. Walp) in the treatments with the application of phosphate biofertilizers with *Acidithiobacillus*. According to the authors, the use of *Acidithiobacillus* resulted in the release of sulfuric acid that was sufficient to solubilize phosphorus from the phosphate rocks. Walpola and Yoon (2013) evaluating the inoculation of phosphate solubilizing bacteria in mung beans (*Vigna radiata* L.) found significant correlation between the soil acidity and populations of *Pantoea agglomerans* and *Burkholderia anthina,* resulting in increased P concentration in the plant roots and shoots. Therefore, there is necessity of more research in relation to the PGPB inoculation with the purpose of solubilize phosphate in acidic soils which can be taken up by plant roots.

The K concentration in the maize leaves was not significantly affected by the inoculation of PGPB and soil acidity levels (Table 5). Campanharo et al. (2010) found that increased soil pH in association with the BNF in common bean, increased the availability of K in the soil. This increased soil pH improved the K uptake due to improvements in soil conditions for the growth and development of bean roots.

Gas exchange

Although the PGPB inoculation have significantly affected leaf area, relative chlorophyll content and N concentration in the corn leaves, this factor did not affect any of the gas exchange parameters (Table 6). These results may be due to the absence of nitrogen fertilization limiting the growth of photosynthetically active leaves, that is, the absence of nitrogen fertilizer directly affected gas exchange measures. Thus, the absence of effect of PGPB inoculation on the gas exchange measurements may explain the non-significant result in the dry matter yield of maize plants (Table 2). According to Bashan et al. (2004), the highest dry matter yield obtained by plants inoculated compared to the non-inoculated plants come from the higher photosynthetic activity influenced by PGPB inoculation. Soil acidity levels significantly affected the gas exchange parameters of maize plants (Table 6). The lowest values of leaf CO_2 assimilation rate (A), stomatal conductance (g_S), transpiration rate (E) and water use efficiency – WUE (A/E) of maize plants were obtained in treatment with soil pH of 4.5 compared to pH 5.5. These data show that increasing soil acidity can reduce the transpiration rate and photosynthesis rate, and thus reducing the WUE. The soil acidity (i.e., pH 4.5) and the absence of

Table 4. Relative chlorophyll content in different maize leaves measured through SPAD readings at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	SPAD 1[1]				SPAD 2				SPAD 3			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[2]	14.4	15.4	16.4	15.4^{a}	14.2	15.4	15.4	15.0^{b}	15.0	15.1	15.7	15.3^{a}
AZ[3]	14.4	15.7	16.6	15.6^{a}	15.3	16.4	16.2	16.0^{ab}	13.2	14.6	17.6	15.1^{a}
HE[4]	14.8	15.1	17.0	15.6^{a}	16.3	15.9	17.8	16.7^{ab}	15.7	15.9	17.8	16.5^{a}
AZ+HE[5]	13.8	16.4	18.7	16.3^{a}	16.4	16.5	19.9	17.6^{a}	15.8	16.2	19.8	17.3^{a}
Mean	14.3^{C}	15.9^{B}	17.2^{A}		15.5^{B}	16.0^{AB}	17.3^{A}		15.0^{B}	15.5^{B}	17.7^{A}	
F test					F test							
Inoculation (I)	1.04^{ns}				4.13*				2.99*			
Soil pH (pH)	18.59**				4.02*				8.15**			
I × pH	1.65^{ns}				0.93^{ns}				0.79^{ns}			
CV (%)	8.4				11.4				12.7			
$LSD_{(I)}$	1.46				2.06				2.25			
$LSD_{(pH)}$	1.15				1.62				1.77			

[1] SPAD 1: SPAD index of the lower leaves, SPAD 2: SPAD index of the central leaves, and SPAD 3: SPAD index of the top leaves. [2] Non-inoculated. [3] AZ: Seeds inoculation with *Azospirillum brasilense*. [4] HE: Seeds inoculation with *Herbaspirillum seropedicae*. [5] AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae*. Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

the use of nitrogenous fertilizers resulted in stomatal closure (g_S) of maize plants. This effect is a plant defense mechanism to soil acidity stress and N deficiency, directly influencing the leaf CO_2 assimilation rate and finally, reducing the transpiration rate. The leaf CO_2 assimilation rate can be explained by stomatal conductance, because when the stomatal closure occurs, the transpiration and CO_2 diffusion are blocked. The main producing regions of grains in Brazil are located in acid soils, characterized by low base saturation and high Al^{3+} levels, sufficient to alter the normal growth of many species of cultivated plants (Hartwig et al., 2007). Research results have shown that Al negatively affects the uptake of essential nutrients such as phosphorus (P), calcium (Ca) and magnesium (Mg) (Steiner et al., 2012).

Diazotrophic bacteria density of the roots and non-rhizosphere soil

Density of diazotrophic bacteria (log cell number) using the NFb lactate media (selective for *A. brasilense*) and NFb malate media (selective for *H. seropedicae*) in the root samples at 15 DAS was not affected by the PGPB inoculation and soil acidity levels (Table 7). In non-rhizosphere soil samples the highest diazotrophic bacteria density using the NFb lactate media were obtained with inoculation of *A. brasilense* AbV5 strain, differing from the other treatments. This result confirms the methodology of Döbereiner et al. (1995) that determines the NFb lactate media as selective genus *Azospirillum*. Using the NFb malate media was observed significant interaction between the factors inoculation and soil acidity for the diazotrophic bacteria density in the non-rhizosphere soil samples at 15 DAS (Table 7). Inoculation of the *H. seropedicae* SMR1 strain in soil pH of 5.0 resulted in lower diazotrophic bacteria density, differing from the other treatments (Table 7). The uninoculated control in soil pH 4.5 showed lower densities of diazotrophic bacteria in the soil compared to soil with pH 5.0, but not different from pH 5.5. Chanway et al. (1988) reported that the diazotrophic bacteria population in the soil is affected by the amount and composition of organic materials secreted by plant roots, which vary during growth and development of plants. Density of diazotrophic bacteria using the NFb malate media in the root samples at 41 DAS was affected by the PGPB inoculation (Table 8). The highest diazotrophic bacteria density was obtained with the inoculation of *A. brasilense* and *H. seropedicae* isolated or associated compared to the uninoculated control. These results show that PGPB inoculation increases the diazotrophic bacteria population in the roots of maize plants in acidic pH levels, especially when the seeds are subjected to inoculation (Table 8). The PGPB inoculation and soil acidity levels at 41 DAS did not affect bacterial density in the root samples using the NFb lactate and non-rhizosphere soil samples using the NFb lactate and NFb malate media (Table 8). In general, the results show that even the NFb lactate media being considered selective for *A. brasilense* strains and NFb malate media considered selective for H. seropedicae strains, this preferred bacterial growth was not observed in this study. The acidic soil pH levels may have influenced these results. The same can be reported concerning to the type of association; *A. brasilense* is considered a facultative endophytes whereas *H. seropedicae* is considered an obligate endophytes. In general, the results indicate that the PGPB inoculation in 30F53H hybrid maize seed resulting in high population density of diazotrophic bacteria in acidic soil conditions and without nitrogen fertilizer application (Tables 7 and 8). Some important variables coincided in the results, such as leaf area and stem diameter (Table 3), relative chlorophyll content in the leaves (Table 4) and leaf N concentration (Table 5). Despite the high population density of diazotrophic bacteria not presenting efficiency enough to promote a significant increase in the amount of dry matter, N became more available to the roots, that is, the PGPB, mainly *H. seropedicae,* were able to facilitate the assimilation of N, thus increasing their leaf content, which may be a benefit until the end of the crop cycle. The density of diazotrophic bacteria was not influenced by the soil pH levels at 41 DAS; however, the inoculation with diazotrophic bacteria in maize seeds did not promote the P solubilization. In this case, it is necessary to investigate the possible phosphorus solubilization promoted by microorganisms in the soil in non-legumes, especially the influence of the rhizosphere and non-rhizosphere pH of maize plants.

Materials and Methods

Experimental conditions and Treatments

The experiment was carried out under greenhouse conditions, localized in the Western Paraná State University (Unioeste), in Marechal Cândido Rondon, Paraná, Brazil (24º46' S, 54º22' W, and altitude of 420 m), in 13dm³ plastic pots.

Table 5. Nitrogen (N), phosphorus (P) and potassium (K) concentrations in the leaf tissue of maize at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	N ($g\ kg^{-1}$)				P ($g\ kg^{-1}$)				K ($g\ kg^{-1}$)			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[(1)]	6.56	7.00	6.78	6.78^{b}	2.04	2.10	1.98	2.04	24.0	23.2	24.5	23.9
AZ[(2)]	7.00	6.86	7.29	7.05^{ab}	2.24	1.90	1.41	1.85	25.4	23.4	23.5	24.1
HE[(3)]	7.87	7.84	7.58	7.77^{a}	2.18	2.09	1.66	1.98	25.1	24.0	25.1	24.7
AZ+HE[(4)]	8.53	7.29	6.41	7.41^{ab}	2.23	1.93	1.88	2.01	23.6	24.6	26.8	25.0
Mean	7.49	7.25	7.01		2.17^{A}	2.00^{A}	1.73^{B}		24.5	23.8	25.0	
F test	F value											
Inoculation (I)	3.53*				0.98^{ns}				1.64^{ns}			
Soil pH (pH)	1.41^{ns}				9.34**				2.67^{ns}			
I × pH	2.15^{ns}				1.46^{ns}				2.16^{ns}			
CV (%)	11.0				14.9				5.9			
$LSD_{(I)}$	0.69				0.25				1.25			
$LSD_{(pH)}$	0.88				0.32				1.59			

(1) Non-inoculated. (2) AZ: Seeds inoculation with *Azospirillum brasilense*. (3) HE: Seeds inoculation with *Herbaspirillum seropedicae*. (4) AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.* Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, p ≤ 0.05). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

Table 6. Leaf CO_2 assimilation rate (*A*), stomatal conductance (g_S), transpiration rate (*E*) and water use efficiency – WUE (*A*/*E*) of maize plants at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	*A* ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)				g_S ($mol\ m^{-2}\ s^{-1}$)				*E* ($mmol\ H_2O\ m^{-2}\ s^{-1}$)				*WUE*			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[(1)]	15.2	18.9	18.4	17.5	0.07	0.11	0.11	0.10	2.84	4.21	4.17	3.74	4.56	4.73	4.86	4.72
AZ[(2)]	16.0	18.8	16.4	17.1	0.08	0.10	0.09	0.09	3.22	3.94	3.65	3.60	4.52	4.67	4.96	4.72
HE[(3)]	17.2	18.1	19.6	18.3	0.10	0.10	0.11	0.10	3.85	3.98	4.14	3.99	4.74	4.69	4.92	4.78
AZ+HE[(4)]	16.0	19.4	19.1	18.1	0.09	0.11	0.11	0.10	3.31	4.26	4.07	3.88	4.69	4.62	4.98	4.76
Mean	16.1^{B}	18.79^{A}	18.4^{A}		0.08^{B}	0.10^{A}	0.10^{A}		3.31^{B}	4.10^{A}	4.01^{A}		4.63^{B}	4.68^{AB}	4.93^{A}	
F test	F value															
Inoculation (I)	1.27^{ns}				1.95^{ns}				1.73^{ns}				0.136^{ns}			
Soil pH (pH)	11.48**				9.29**				14.88**				4.735*			
I × pH	1.32^{ns}				1.58^{ns}				1.71^{ns}				0.277^{ns}			
CV (%)	9.77				14.89				11.79				6.30			
$LSD_{(I)}$	1.91				0.02				0.50				0.35			
$LSD_{(pH)}$	1.50				0.01				0.39				0.27			

(1) Non-inoculated. (2) AZ: Seeds inoculation with *Azospirillum brasilense*. (3) HE: Seeds inoculation with *Herbaspirillum seropedicae*. (4) AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.* Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, p ≤ 0.05). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

Table 7. Density of diazotrophic bacteria (log cell number)[1] in the maize roots and non-rhizosphere soil at 15 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels, using the NFb lactate media (*Azospirillum brasilense*) and NFb malate media (*Herbaspirillum seropedicae*)

Treatments	NFb lactate media (roots)				NFb malate media(roots)				NFb lactate media (soil)				NFb malate media (soil)			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[2]	6.11	5.54	6.13	5.93	5.54	5.18	5.50	5.41	4.04	5.10	5.26	4.80^{b}	4.25aB	5.83aA	5.39aAB	5.16
AZ[3]	6.25	5.89	5.40	5.85	5.49	5.21	4.71	5.14	6.07	5.80	5.13	5.67^{a}	5.25aA	5.85aA	4.84aA	5.31
HE[4]	6.26	6.07	6.12	6.15	6.72	5.12	6.03	5.96	4.79	4.45	5.08	4.77^{b}	4.90aA	4.13bA	5.41aA	4.81
AZ+HE[5]	6.62	5.74	6.19	6.18	4.79	5.27	4.01	4.69	4.42	4.62	5.26	4.76^{b}	5.38aA	4.90aA	4.77aA	5.02
Mean	6.30	5.81	5.96		5.64	5.19	5.06		4.83	4.99	5.18		4.95	5.17	5.10	
F test	F value															
Inoculation (I)	0.32^{ns}				1.83^{ns}				4.86**				0.74^{ns}			
Soil pH (pH)	0.01^{ns}				0.78^{ns}				1.01^{ns}				0.31^{ns}			
I × pH	0.31^{ns}				0.63^{ns}				2.30^{ns}				2.54*			
CV (%)	16.8				25.7				13.9				16.8			
$LSD_{(I)}$	1.12				1.50				0.77				1.48			
$LSD_{(pH)}$	0.88				1.18				0.60				1.63			

[1] Logarithm of the Most Probable Number (MPN) of colony forming units (CFU) of diazotrophic bacteria. [2] Non-inoculated. [3] AZ: Seeds inoculation with *Azospirillum brasilense*. [4] HE: Seeds inoculation with *Herbaspirillum seropedicae*. [5] AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.*Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

Table 8. Density of diazotrophic bacteria (log cell number)[1] in the maize roots and non-rhizosphere soil at 41 days after sowing as a function of the inoculation of plant growth-promoting bacteria in three soil acidity levels

Treatments	NFb lactate media (roots)				NFb malate media(roots)				NFb lactate media (soil)				NFb malate media (soil)			
	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean	pH 4.5	pH 5.0	pH 5.5	Mean
Control[2]	5.31	6.73	6.52	6.19	5.95	5.58	6.08	5.87^{b}	6.74	6.03	4.64	5.80	7.68	7.40	7.44	7.51
AZ[3]	6.33	6.14	6.30	6.26	6.18	6.57	6.39	6.38^{ab}	6.04	6.18	7.33	6.52	7.13	7.40	7.32	7.30
HE[4]	6.21	6.00	6.77	6.33	6.72	5.99	6.95	6.55^{ab}	6.73	6.48	6.95	6.72	7.34	7.22	7..16	7.24
AZ+HE[5]	6.92	5.87	6.37	6.39	7.37	6.36	6.65	6.79^{a}	6.61	7.31	6.21	6.71	7.54	7.64	7..23	7.47
Mean	6.19	6.18	6.49		6.56	6.12	6.52		6.53	6.50	6.28		7.42	7.43	7.29	
F test	F value															
Inoculation (I)	0.127^{ns}				3.312*				1.955^{ns}				0.391^{ns}			
Soil pH (pH)	0.673^{ns}				1.650^{ns}				0.257^{ns}				1.198^{ns}			
I × pH	1.704^{ns}				0.961^{ns}				2.243^{ns}				0.206^{ns}			
CV (%)	13.5				11.7				16.7				9.6			
$LSD_{(I)}$	0.94				0.82				1.18				0.78			
$LSD_{(pH)}$	0.74				0.65				0.93				0.61			

[1] Logarithm of the Most Probable Number (MPN) of colony forming units (CFU) of diazotrophic bacteria. [2] Non-inoculated. [3] AZ: Seeds inoculation with *Azospirillum brasilense*. [4] HE: Seeds inoculation with *Herbaspirillum seropedicae*. [5] AZ+HE: Seeds inoculation with *A. brasilense* and *H. seropedicae.*Values represented by the different lower case letters in the column and upper case letters in the lines, show significant differences (Tukey test, $p \leq 0.05$). ns: not significant. * and **: statistical significance at 5% and 1%, respectively, by F test. CV: coefficient of variation. LSD: least significant difference.

associated the two strains of *A. brasilense* and *H. seropedicae*

The soil used in the experiment was collected from the surface layer (0–0.20 m) of a sandy Arenic Hapludult (Red-Yellow Argisol in the Brazilian classification) with 200 g kg^{-1} of clay, 110 g kg^{-1} of silt, and 690 g kg^{-1} of sand. The soil had the following chemical properties: pH (1:2.5 soil/$CaCl_2$ suspension 0.01M) 4.5, 8.2 g dm^{-3} of organic matter, 4.2 mg dm^{-3} of $P_{Mehlich-1}$, 1.5 $cmol_c$ dm^{-3} of Ca, 0.4 $cmol_c$ dm^{-3} of Mg, 0.6 $cmol_c$ dm^{-3} of K, 2.8 cmolc dm^{-3} of H+Al, 0.4 $cmol_c$ dm^{-3} of Al, 5.3 $cmol_c$ dm^{-3} of CEC, 48% of base saturation, 2.5 mg dm^{-3} of $Cu_{Mehlich-1}$, 1.3 mg dm^{-3} of $Zn_{Mehlich-1}$, 48.4 mg dm^{-3} of $Fe_{Mehlich-1}$, and 11.0 mg dm^{-3} of $Mn_{Mehlich-1}$.

The experimental was arranged in a randomized block design, using three levels for the soil acidity factor [pH in $CaCl_2$ of 4.5; 5.0 and 5.5] and four treatments for the seed inoculation factor [control (non-inoculated); inoculation with *Azospirillum brasilense* strain AbV5; inoculation with *Herbaspirillum seropedicae* strain SMR1; and inoculation with two bacteria strains (*A. brasiliense* + *H. seropedicae*)], considering a factorial arrangement (3 × 4). A total of 48 pots were used – four pots per treatment.

Soil pH calibration, fertilization and seed inoculation

A previous study was carried out for 21 days to obtain the neutralization fitting curve and then calculate the lime rate to be used to obtain the different soil pH values. Four subsamples received the application of dolomitic lime rates in amount equivalent for 0, 30, 60, 90 and 120% neutralization of the soil potential acidity. These subsamples were incubated at 80% of field capacity until constant pH for 21 days. After this period, soil subsamples were air-dried, crushed, and sieved to pass a 2-mm mesh screen. Soil pH in 0.01 mol L^{-1} $CaCl_2$ was determined potentiometrically in a 1:2.5 (soil:$CaCl_2$ solution) suspension using a combined calomel reference glass electrode and pH meter. The following soil pH values were obtained: $pH_1 = 4.4$, $pH_2 = 5.7$, $pH_3 = 6.9$, $pH_4 = 7.5$, and $pH_5 = 7.7$ units. Thus, the different soil pH values (i.e., 4.5, 5.0 and 5.5 pH) were obtained using the following equation: [$pH = 4.78 + 3.706LR - 1.162LR^2$; R^2: 0.99], where LR is lime rate (in g kg^{-1} of soil). Therefore, the lime rates applied to increase the soil pH to 5.0 and 5.5 were, respectively, of 0.79 and 2.70 g per pot (13 dm^3). After liming, soil was moistened to reach 80% water retention capacity and incubated for 30 days. The soil pH values were of 4.5±0.1; 5.1±0.1 and 5.5±0.1, respectively, for the application of 0; 0.79 and 2.70 g pot^{-1} of lime. The basic fertilization was performed with applying 300 mg dm^{-3} of P as simple superphosphate (18% P_2O_5; 25% CaO and 12% S), 100 mg dm^{-3} of K as potassium chloride (60% K_2O) and 40 mg dm^{-3} of S as gypsum (13% S and 18% Ca). The fertilizer amount applied was performed according to the recommendations for greenhouse crops as described by Alvarez and Fonseca (1990), with modifications. The exceptions were the omissions of N and micronutrients application. Maize seeds were inoculated with 4.0 mL of inoculant (AbV5 strain and/or SMR1 strain) for each thousand seeds and then maintained at rest for twelve hours in the shade and at temperature of 25 °C. In treatment with the inoculation of the two bacteria strains, 2 mL of each inoculant were applied. The control treatment received the 4 mL application of distilled water. Inoculants were provided by the Biochemistry and Molecular Biology Laboratory, Paraná Federal University (UFPR), Curitiba, Brazil and had concentration of 10^7 CFU mL^{-1}. The inoculation was performed in plastic bags properly sterilized in a laminar flow hood, after having been submitted to 30 minutes of UV germicidal lamp with the aim of reducing the number of microorganisms on surfaces and air.Six seeds of maize (*Zea mays* L., Pioneer 30F53H hybrid) were sown in pots, and three days after seedling emergence, they were thinned to three plants per pot. This hybrid was used because of its preference for the cultivation by farmers in South Brazil. The soil water content was maintained near at the field capacity with two daily irrigations.

Biometric measurements and N, P and K concentration

Maize plant height (from the soil surface to the apex of the plants) was measured at 14, 21, and 41 days after plant sowing (DAS). At 41 DAS, the plants in all treatments were harvested and separated into roots, stems and leaves. The plant parts were removed carefully and washed with deionized water, dried for four days at 65 ºC, and then weighed. The stem diameter was measured (mm) using an electronic caliper. The leaf area (LA, dm^2 $plant^{-1}$) was determined using the following equation proposed by Benincasa (2003): LA = [(LAs × TDML)/DMs], where LAs is the leaf area of the sample collected, TDML is the total dry matter of leaf and DMs is the dry matter of the sample collected. Maize leaves was ground, digested in nitric-perchloric acid, and P was determined by colorimetry, K concentration were determined by flame photometry, N by sulfuric acid digestion and Kjeldahl distillation, as previously described (EMBRAPA, 2009).

Gas exchange and chlorophyll measurements

Leaf gas exchange was monitored with an infrared gas analyzer (LI-6400XT, LICOR, Inc. USA), in the maize growing stage of four developed leaves – V4 (41 DAS). Measurements of leaf CO_2 assimilation rate (*A*, in μmol CO_2 m^{-2} s^{-1}), transpiration rate (*E*, in mmol H_2O m^{-2} s^{-1}) and stomatal conductance (g_S, mol m^{-2} s^{-1}) were taken in the morning period from 9:00 to 11:00 hours. Water-use efficiency – WUE (*A*/*E*) was also calculated. The gas exchange parameters were determined at 400 μmol mol^{-1} of [CO_2] and 1,500 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD), with standard deviation of 0.7313. The mean photons density of the external environment provided by the apparatus was 1,142.35 μmol m^{-2} s^{-1}, 36.55% relative humidity, and air flow of 499.44 mL per minute.

Chlorophyll readings were made using a SPAD meter (SPAD 502® Konica Minolta) at 41 DAS, on the fully expanded leaves from lower leaves (SPAD 1), central leaves (SPAD 2) and top leaves (SPAD 3) of the three maize plants in each pot.

Most Probable Number (MPN) of diazotrophic bacteria

Samples of roots and non-rhizosphere soil were collected at 15 and 41 DAS. Root samples (1 g) were washed in distilled water, chopped into pieces 100 mm long, macerated in 9 mL of salt solution (0.85 % NaCl), and submitted to successive serial dilutions (10^{-2} to 10^{-7}) using the same salt solution. Soil samples (10 g) were submitted to successive serial dilutions (10^{-1} to 10^{-7}) in a salt solution (0.85 % NaCl). Then, 0.1 mL aliquots of the two diluted suspensions were inoculated onto semi-solid culture media known to favor the growth of certain diazotrophic species, but also permit the growth of two diazotrophic species studied. The media used were as follows: NFb malate (selective for *Herbaspirillum seropedicae*) and NFb lactate (selective for *Azospirillum brasilense*), with three replications. The inoculated media were kept for 7 days in growth chambers at 30 ºC. The population of diazotrophics was estimated using the Most Probable Number (MPN) technique (Döbereiner et al., 1995).

Data statistical analyses

Data were analyzed by ANOVA, and the means of soil acidity levels and seed inoculation treatments were compared by Tukey test at the 0.05 level of confidence. All analyses were performed using Sisvar 5.1 software for Windows (Statistical Analysis Software, UFLA, Lavras, MG, BRA) (Ferreira, 2011).

Conclusions

Seed inoculation with two diazotrophic bacteria strains (AbV5 and SMR1) improved the leaf area, stem diameter and relative chlorophyll content, but had no effect on dry matter yield of maize plants, even with the changes of soil acidity levels. Seed inoculation with *Herbaspirillum seropedicae* enhances the nitrogen concentration in the leaf tissue of maize under soil acidity conditions and without the nitrogen supply, indicating increase in the biological nitrogen fixation.
Inoculation of diazotrophic bacteria in acidic sandy soil (4.5 to 5.0 pH) resulted in higher phosphorus concentration in the leaf tissue of maize, indicating that there was an increase of phosphorus solubilization promoted by diazotrophic bacteria.
Soil acidity resulted in the stomatal closure of maize plants, reducing the leaf CO_2 assimilation rate, leaf transpiration rate and water use efficiency. Leaf CO_2 assimilation rate was not affected by the maize seed inoculation with plant growth promoting bacteria.The NFb Lactate (selective for *A. brasilense*) and NFb Malate (selective for *H. seropedicae*) culture media were not effective in differentiating of the two diazotrophic species studied and *Herbaspirillum seropedicae*, for both the root and soil samples.

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References

Alvarez VVH, Fonseca DM (1990). Definition of phosphorus levels to determine the phosphate maximum adsorption capacity and the response curves for greenhouse experiments. R Bras Ci Solo. 14(1): 49-55.

Araujo FF, Foloni JSS, Wutzke M, Melegari AS, Rack E (2013) Hybrids and varieties of corn inoculated with *Herbaspirillum seropedicae.* Semin-Cienc Agrar. 34:1043-1054.

Baldani JI, Baldani VLD (2005) History on the biological nitrogen fixation research in graminaceous plants: Special emphasis on the Brazilian experience. An Acad Bras Cienc. 77: 549-579.

Baldani VLD, Alvarez MAB, Baldani JI, Döbereiner J (1986) Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat and sorghum. Plant Soil. 90: 35-46.

Baldani JI, Caruso L. Baldani VLD, Goi SR, Döbereiner J (1997). Recent advances in BNF with non-legume plants. Soil Biol Biochem. 29:911-922.

Bashan Y, Holguin G, De-Bashan LE (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural and envioronmental advances (1997-2003). Can J Microbiol. 50: 521-577.

BENINCASA MMP (2003) Análise de crescimento de plantas: noções básicas. Jaboticabal: FUNEP, 42p.

Boddey RM, Oliveira OC, Urquiaga S, Reis VM, Olivares FL, Baldani VLD, Döbereiner J (1995) Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. Plant Soil. 174: 195-209.

Campanharo M, Lira Junior MA, Nascimento CWA, Stamford NP, Freire FJ, Costa JVT (2010) Soil acidity on nitrogen fixation in common beans. R Bras Cienc Agrár. 5: 285-290.

Chanway CP, Nelsona LM, Holl FB (1988) Cultivar specific growth promotion of spring wheat by co-existent Bacillus species. Can J Microbiol. 34: 925-929.

CONAB (2014) Companhia Nacional de Abastecimento: Indicadores da Agropecuária. Brasília, 90 p.

Dartora J, Guimarães VF, Marini D, Sander G (2013) Adubação nitrogenada associada à inoculação com *Azospirillum brasilense* e *Herbaspirillum seropedicae* na cultura do milho. R Bras Eng Agríc Ambiental. 17: 1023–1029.

Döbereiner J, Baldani VLD, Baldani JI (1995) Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. Itaguaí: Embrapa – CNPAB, 60p.

Döbereiner J, De-Polli H (1980) *Diazotrophic rhizocoenoses*. Stewart DP, Gallon JR. In: Nitrogen fixation. London: Academic Press, 301-334.

EMBRAPA (2009) Empresa Brasileira de Pesquisa Agropecuária: Manual de análises químicas de solos, plantas e fertilizantes. Brasília, 628 p.

EMBRAPA (1980) Empresa Brasileira de Pesquisa Agropecuária. Projeto “Racionalização do uso de insumos”. Sub-Projeto “Pesquisa em racionalização de fertilizantes e corretivos na agricultura”. Brasília, 78p.

Fancelli AL, Dourado Neto D (2000) Produção de milho. Guaíba: Agropecuária. 360 p.

Ferreira DF (2011) Sisvar: A computer statistical analysis system. Cienc Agrotec. 15(6): 1039-1042.

Guimarães TG, Fontes PCR, Pereira PRG, Alvarez VVH, Monnerat PH (1999) Relations among chlorophyll contents determined by a portable meter and nitrogen forms in leaves of tomatoes cultivated in two soil types. R Bras Eng Agríc Ambiental. 58: 209-216.

Hartwig I, Oliveira AC, Carvalho FIF, Bertan I, Silva, JAGJ, Schmidt DAM, Valério IP, Maia LC, Fonseca DAR, Reis CER (2007) Associated mechanisms of aluminum tolerance in plants. Semin-Cienc Agrar. 28: 219-228.

Hungria M, Campo RJ, Souza EMS, Pedrosa FO (2010) Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. Plant Soil. 331: 413-425.

IBGE (2013) Instituto Brasileiro de Geografia e Estatística. Levantamento Sistemático da Produção Agrícola março de 2013. 26: 1-84.

Inagaki AM, Guimarães VF, Rodrigues LFOS, Silva MB, Diamante MS, Rampim L, Mioranza TM, Duarte Júnior JB (2014). Phosphorus fertilization associated to inoculation of maize with diazotrophic bactéria. Afr J Agric Res. 9(48): 3480-3487.

Malavolta E (1979) ABC da adubação. In: (ed) Agronômica Ceres, 4rd edn São Paulo.

Moreira FMS, Silva K, Nóbrega RSA, Carvalho F (2010) Diazotrophic associative bacteria: diversity, ecology and potential applications. Com Sci. 1: 74-99.

Moreira FMS, Siqueira JO (2006) Microbiologia e bioquímica do solo. Lavras: UFLA, 729 p.

Oliveira ALM, Canuto EL, Silva EE, Reis VM, Baldani JI (2004) Survival of endophytic diazotrophic bacteria in soil under different moisture levels. Braz J Microbiol. 35: 295-299.

Pedraza RO (2008) Recent advances in nitrogen-fixing acetic acid bacteria. Int J Food Microbiol. 125:25-35.

Reis Júnior FB, Machado CTT, Baldani AT, Sodek L (2008) Inoculação de *Azospirillum amazonense* em dois genótipos de milho sob diferentes regimes de nitrogênio. R Bras Cienc Solo. 32: 1139-1146.

Rodrigues LFOS, Guimarães VF, Silva MB, Pinto Junior AS, Klein J, Costa ACPR (2014) Características agronômicas do trigo em função de *Azospirillum brasilense*, ácidos húmicos e nitrogênio em casa de vegetação. R Bras Eng Agríc Ambiental. 18: 31-37.

Rufini M, Ferreira PAA, Soares BL, Oliveira DP, Andrade MJB, Moreira FMS (2011) Simbiose de bactérias fixadoras de nitrogênio com feijoeiro-comum em diferentes valores de pH. Pesq Agropec Bras. 46: 81-88.

Sala VMR, Cardoso EJBN, Freitas JG, Silveira APAD (2008) Novas bactérias diazotróficas endofíticas na cultura do trigo em interação com a adubação nitrogenada, no campo. R Bras Cienc Solo. 32: 1099-1106.

Stamford NP, Santos CERS, Stamford Júnior WP, Dias SL (2004) Biofertilizantes de rocha fosfatada com *Acidithiobacillus* como adubação alternativa de caupi em solo com baixo P disponível. Revista Analytica. 3: 48-53.

Steiner F, Zoz T, Pinto Junior AS, Castagnara DD, Dranski JAL (2012) Effects of aluminum on plant growth and nutrient uptake in young physic nut plants. Semin-Cienc Agrar. 33(5): 1779-1788.

Taiz L, Zeiger E (2013) Fisiologia vegetal. 5th ed. Porto Alegre: ArtMed, 954p

Walpola BC, Yoon MH (2013) Phosphate solubilizing bacteria: Assessment of their effect on growth promotion and phosphorous uptake of mung bean (*Vigna radiata* [L.] R. Wilczek). Chilean J Agric Res. 73: 275-281.