Universidade Federal Rural do Semi-Árido
Pró-Reitoria de Pesquisa e Pós-Graduação
http://periodicos.ufersa.edu.br/index.php/sistema

ISSN 0100-316X (impresso)
ISSN 1983-2125 (online)

DIVERSITY AND GROWTH-PROMOTING ACTIVITIES OF *Bacillus* sp. IN MAIZE[1]

FABIO FERNANDO DE ARAÚJO[2*], ELLEN CRISTINA SOUZA[2], RENATO TADEU GUERREIRO[2], LUCIANA MACHADO GUABERTO[2], ADEMIR SERGIO FERREIRA DE ARAÚJO[3]

ABSTRACT - The aim of this paper was to evaluate biochemical activities related to promotion of plant growth of isolates of the genus *Bacillus* originated from areas under maize cultivation, and to evaluate the genetic similarity among the isolates using PCR-based RAPD markers. Several strains of the genera *Bacillus* were isolated from twenty distinct maize production areas of the State of São Paulo, Brazil. Forty isolates were obtained and characterized as antagonistic to plant pathogenic fungi, production of auxin (IAA), phosphate solubilization *in vitro* and root colonization. The potential for phosphate solubilization was not identified in any of the isolates. In addition, the majority of the isolates did not show any antagonistic effect against *Fusarium oxysporum* and *Colletotrichum truncatum*. Based on root colonization, six *Bacillus* isolates were selected to evaluate their growth-promotion activities in maize. All the six isolates increased root growth, while only one isolate did not promoted shoot growth and nutrient uptake in plants when compared to control. Low genetic similarity among the selected isolates was detected by RAPD analysis using eleven primers. Our results showed the utility of the *in vitro* selection criteria used in this study for screening of *Bacillus* sp. with plant growth-promoting activity, as they may reduce the number of *Bacillus* isolates required at the final screening stage in field.

Keywords: Rhizobacteria. *Zea mays*. Plant nutrition. RAPD-PCR.

DIVERSIDADE E ATIVIDADE PROMOTORA DE CRESCIMENTO DE ISOLADOS DE *Bacillus* sp. EM MILHO

RESUMO - O objetivo do estudo foi o de avaliar atividades bioquímicas relacionadas à promoção de crescimento de plantas em isolados do gênero *Bacillus,* originários de áreas de cultivo de milho, bem como avaliar a similaridade genética entre as bactérias com o uso de marcadores RAPD-PCR. Para isto foram isoladas bactérias de solo, sob cultivo de milho, em vinte regiões produtoras do estado de São Paulo, Brasil. No laboratório, 40 isolados bacterianos foram caracterizados como pertencentes ao gênero *Bacillus* e selecionados quanto ao potencial antagônico a fungos, produção de auxinas, solubilização de fosfatos e colonização radicular. Não foi encontrado atividade de solubilização de fosfato em nenhum isolado, como também a maioria dos isolados não apresentou antagonismo contra *Fusarium oxysporum* and *Colletotrichum truncatum*. Fundamentando-se no potencial de colonização radicular, seis isolados de *Bacillus* foram selecionados para avaliação de promoção de crescimento de milho. Todos os seis isolados aumentaram o crescimento das raízes e apenas um não promoveu o crescimento da parte aérea e acúmulo de nutrientes nas plantas quando comparados à testemunha. As análises de RAPD, com emprego de onze "primers", detectaram baixa similaridade genética entre os isolados selecionados. Os resultados obtidos neste trabalho mostram que os critérios de seleção *in vitro* utilizados neste estudo podem ser úteis em programas de seleção de isolados de *Bacillus* sp como promotores de crescimento em milho, pois podem contribuir para redução do número de isolados bacterianos para etapa da triagem final em condições de campo.

Palavras-chave: Rizobactérias. *Zea mays.* Nutrição vegetal. RAPD-PCR.

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*Autor para correspondência.
[1]Recebido para publicação em 16/02/2011; aceito em 17/09/2011.
[2]Faculdade de Ciências Agrárias, UNOESTE, Rod. Raposo Tavares, km 572, 19067-175, Presidente Prudente - SP, fabio@unoeste.br, ellensouza@gmail.com.br, tadeuguerreiro@ig.com.br, luciana.guaberto@gmail.com; asfaruaj@yahoo.com.br
[3]Centro de Ciências Agrárias, UFPI, Caixa Postal 2004, 64000-000, Teresina - PI;
asfaruaj@yahoo.com

INTRODUCTION

The use of plant growth-promoting rhizobacteria (PGPR) in sustainable agriculture has been increased in the last decades in several regions of the world. Various bacteria genus are included in PGPR group, such as *Pseudomonas, Azospirillum*, *Bacillus*, *Enterobacter*, *Xanthomonas* and *Serratia* (KHALID et al., 2004). The mechanisms of action of PGPR may initially be linked to inhibition of soilborn plant pathogens and thereby stimulate plant growth indirectly (GUPTA et al., 2000). It is often difficult to recognize the mechanisms and relate directly to promotion of plant growth, since more than one mechanism is produced by bacteria (ARAÚJO et al., 2005).

The mode of action through which the genus *Bacillus* used in agriculture for biological control of plant pathogens has been related to the production of antibiotics (ARAUJO et al., 2005). The results found with *Bacillus* sp. in control of plant diseases and the perspectives of their use as plant growth-promoter prompted others studies using seed inoculation procedures (KAVITHA et al., 2005; ARAUJO, 2008).

The interaction of these bacteria with plant roots in the soil environment and the production of microbial metabolites as plant growth regulators has addressed by several authors (ARAUJO et al., 2005; MAHAFFEE; BACKMAN, 1993). The presence of plant growth regulators in rhizosphere is an important factor for enhancing plant development. The main phytohormones synthesized by plant-associated microrganisms are gibberellins, auxins and cytokinins (TSAVKELOVA et al., 2006).

It is very important to find novel strains of *Bacillus* and to know their potential for producing substances that can improve plant growth. However, there are few studies about evaluation of *Bacillus* strains on plant growth promotion in Brazil (BENEDUZI et al., 2008). The aims of this paper were to (i) find novel *Bacillus* strains from diverse maize fields; (ii) to evaluate their plant growth promoting activities and (iii) to assess the genetic relationship among the *Bacillus* isolates using RAPD-PCR analysis.

MATERIALS AND METHODS

Soil samples were collected, at 0-20 cm depth, in twenty sites (six cities) under maize cultivation at São Paulo State, Brazil (22°07'04" S and 51º22' W, 472 m). The samples were refrigerated until the time of analysis. The soils of this region are classified as ultisols and locations (cities) were georeferenced (Table 1). *Bacillus* genus was isolated according to Buchanan et al. (1975). In brief, ten grams of soil were mixed with 90 ml of distilled water and used for the bacterial isolation procedures. The soil suspensions were pasteurized (20 min, 70 ^{o}C) to select bacteria resistant to high temperature. These bacteria were plated onto nutrient agar (NA) medium and incubated for 48 h at 28 ^{o}C. Typical *Bacillus* colonies were isolated and characterized according to Araújo et al. (2005). Through this procedure, forty *Bacillus* isolates, representing the different sites sampled, were characterized. All isolates were evaluated for their potential antagonism to plant pathogens, capacity of indol-acetic acid (IAA) production and ability to solubilize phosphate.

The potential antagonism to plant pathogens was evaluated according Araujo et al. (2005). For this, we used the plant pathogenic fungi *Fusarium oxysporum* and *Colletotrichum truncatum* obtained from laboratory of plant pathology (Unoeste, São Paulo, Brazil). The fungi were multiplied in culture medium potato agar for seven days. After this period discs (Φ 5 mm) containing the mycelium were introduced in four equidistant points in Petri dishes with PDA medium, 24 hours before the inoculation of *Bacillus* isolates in the center of the plate, each isolate was evaluated in triplicate. The plates were incubated at 28 C for seven days. After this period, we observed the formation of zone of inhibition between the bacterial isolate and the phytopathogen.

The determination of IAA was carried out in Petri dishes containing TSB (Trypticase Soybean Agar) supplemented with dextrose and L-tryptophan medium. The amount of auxin expressed as IAA was estimated by spectrophotometry (KHALID et al., 2004), at 550 nm, using Salkowski colouring reagent (EHMANN, 1977). The method described by Carneiro et al. (1994) was used to identify isolates able to solubilize phosphates. The solubilization of P by isolates was quantified using insoluble tricalcium phosphate in medium containing (per L) 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 1.5% agar (pH 7.0). The colonies formed on the plates with clear halos were considered solubilizing.

According to the potential antagonism to plant pathogens and capacity of high indol-acetic acid (IAA) production was verified the ability of colonization of maize roots, as additional criterion for recommendation and selection of bacteria as plant growth promoters (BENIZRI et al., 2001). For this was conducted the root colonization test in tubes with agar-water, as described by Romeiro (2007). After this we selected six strains (1B, 2B, 3A, 4B, 5A and 6B) for further studies.

DNA from these strains was directly extracted from bacterial cultures by the method described by Doyle e Doyle (1990). DNA was electrophoresed on 1.0% agarose ethidium bromide gel. For the RAPD-PCR analysis, DNA was amplified using 11 primers with following sequences 5'-3': A1 (CAGGCCCTTC), A2 (TGCCGAGCTG), A11 (CAATCGCCGT), A18 (AGGTGACCGT), C2 (GTGAGGCGTC), C5 (GATGACCGCC), C7 (GTCCCGACGA), C8 (TGGACCGGTG), C11 (AAAGCTCGTC), C13 (AAGCCTCGTC) and C20

(ACTTCGCCAC) previously selected from OPERON Technologies Inc. The total volume of reaction was 25 µL, containing 18.4 µL of Mili Q water, 2.5 µL of buffer (10 X), 2 mM $MgCl_2$, 200 µM of dNTP, 10 pmols of primer (oligonucleotide), 1 U of *Taq* DNA polymerase and 1.0 µL DNA (100 ng). The reaction was carried out in MJ thermocycler-100 PTC (MJ Research, Cambridge, Mass.) programmed for initial denaturation at 94 ºC for 5 min, 48 cycles consisting of: 92 ^{o}C for 30 s, 37 ^{o}C for 1 min and 30 s at 72 ºC for extension. The products of amplification were submitted to electrophoresis (150V/120mA) in 1.5% agarose gel in TBE (Tris-borate-edta) buffer, containing ethidium bromide (0.5 mg L^{-1}). The gel was visualized and imaged using chemilmager® system (Fig. 2). Similar procedure was done for references strains of *Saccharomyces cerevisiae*, *Aspergillus niger* and *Bacillus subtilis* PRBS-1 (ARAÚJO et al., 2005). Using the NTSYS-PC v.2.0 program (ROHLF, 1998), a genetic similarity matrix was created with Jaccard coefficient of similarity. The genetic similarity matrix was subjected to cluster analysis with an unweighted pair-grouped method with arithmetic average (UPGMA) to generate a dendrogram.

The selected strains were evaluated in a plant growth promotion experiment with maize in a greenhouse. The experiment was conducted in plastic pots (15-cm diameter) containing 5 kg of non sterile soil samples(utilsols) with pH ($CaCl_2$ 1 mol L^{-1}) 6.0; 6 g dm^{-3} of organic matter; 38 mg dm^{-3} of $P_{soluble}$; 3.6 $mmol_c$ dm^{-3} of K; 24 $mmol_c$ dm^{-3} of Ca; 8 $mmol_c$ dm^{-3} of Mg. Maize seeds (Hybrid RG selegraos) were surface-sterilized in 70% ethanol for 2 min and 1.2% sodium hypochlorite for 10 min, and rinsed 10 times in sterile tap water. Three seeds were sowed and inoculated with 0.1 mL of an aqueous suspension of each isolate (10^8 cfu mL^{-1}) obtained by scraping the cells on plates with solid media. The soil moisture was maintained at 60% of field capacity. The treatments investigated were: 1) negative control (CONT); 2) plants were inoculated with isolates 1B, 2B, 3A, 4B, 5A and 6B. The experiment was arranged in a completely randomized design with four replicates.

Table 1. Antagonism to phytopathogenic fungi, production of IAA e P-solubilization by *Bacillus* isolates of different sites under maize cultivation in São Paulo, Brazil.

Isolates	Site (localization)	Antagonism to *Colletotrichum truncatum*	Antagonism to *Fusarium oxysporum*	IAA (µg ml^{-1})	P-solubilization
1B	Taciba (22º23'23"S and 51º17'05"W)	positive	positive	3.28±0,42	negative
2B	Pirapozinho (22º16'31" S and 51º30'W)	negative	negative	10.0±1,23	negative
4B	Birigui (21º17'19"S and 50º20'24"W)	negative	positive	7.28±1,43	negative
5A	Penapólis (21º25'12"S and 50º04'40"W)	negative	negative	21.3± 4,45	negative
6B	Castilho (20º52'20"S and 51º29'15"W)	negative	positive	7.68±1,87	negative
3A	Nova Granada (20º32'02" S and 49º18'51"W)	negative	positive	4.96±1,01	negative

Fifty days after sowing, all plants were harvested and dried at 65 ^{o}C to constant weight for shoot and root dry mass determination. N, P and K contents were estimated according to Malavolta (1997). Data obtained from the different treatments were statistically analyzed by SISVAR software using Scott-Knott test at $P < 0.05$.

RESULTS AND DISCUSSION

The results of phosphate solubilization, potential antagonism to plant pathogens and capacity of IAA production are shown in Table 1. None of isolates examined were able to solubilize phosphate. The ability of bacteria to solubilize phosphates is important for agriculture as it may enhance the availability of phosphorus for plants (BENEDUZI et al., 2008). However, in this study, we did not found isolates capable of solubilizing phosphate, which was not expected since tropical soils have large concentrations of phosphorus-solubilizing bacteria (ARAUJO; SANTOS JR., 2009).

Four out of the six examined isolates shared antagonism to *Fusarium oxysporum* (1B, 4B, 6B and 3A) and only one was antagonic to *Colletotricum truncatum* (1B). This antifungal activity is important for biological control of plant pathogens in rhizosphere and can indirectly promote plant growth (KLOEPPER; SCHORTH, 1981).

All six isolates were able to produce IAA. Two isolates produced more than 10 µg ml^{-1}. In particular, the isolate 5A was the most efficient IAA producer, with 21.3 µg ml^{-1}. Our results showed that the different isolates varied greatly in their efficiency for producing auxin. According to MIRZA et al. (2001), IAA production by PGPR can vary among different species and isolates, and it is also influ-

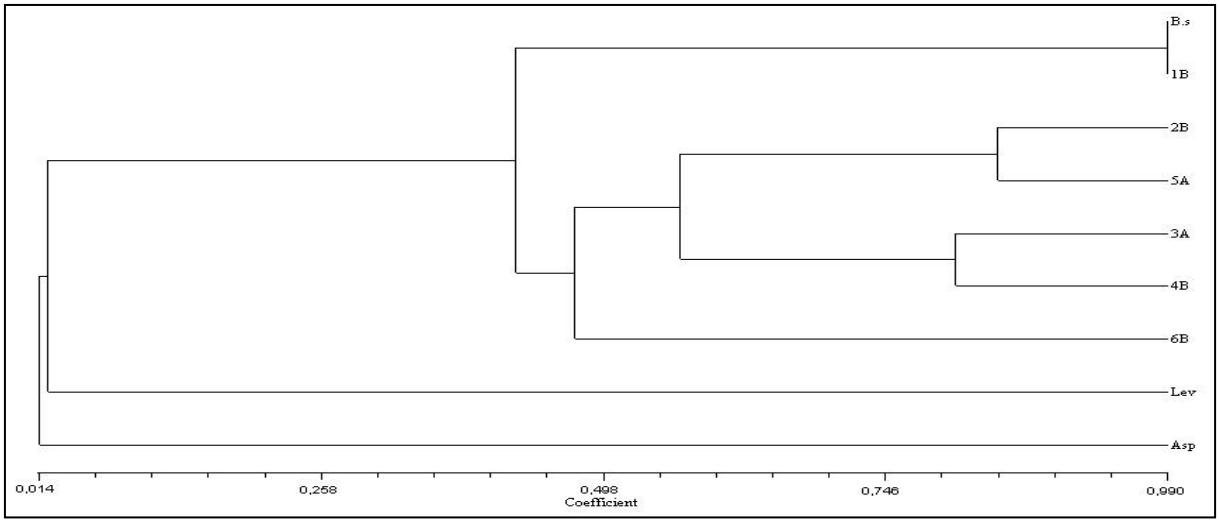

Figure 1. Dendrogram of genetic relationship among six *Bacillus* isolates (1B, 2B, 3A, 4B, 5A, 6B), *B.subtilis* PRBS-1 (B.s.), *Sacharomyces cerevisae* (lev) and *Aspergillus niger* (Asp). Using Jaccard coefficient of similarity.

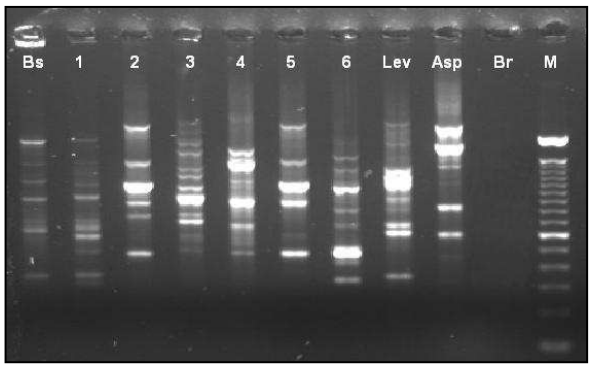

Figure 2. RAPD-PCR profiles of *Bacillus* isolates obtained with primer A11, *Bacillus* isolates 1 (1B); 2 (2B); 3 (3A); 4(4B); 5(5A); 6(6B), Bs (*Bacillus subtilis* PRBS-1); Lev (*Saccharomyces Cerevisiae*); Asp (*Aspergillus niger*); Br (negative control) M- 100 pb DNA marker. Electrophoresis (150V/120mA) in 1.5% agarose gel in TBE buffer, containing ethidium bromide (0.5 mg L^{-1}).

enced by culture condition, growth stage and substrate availability. The quantity of IAA produced by isolates 2B and 5A are within the range found in others studies (BENEDUZI et al., 2008). Taking into account the results found with these isolates 2B and 5A, making it possible to indicate both isolates as efficient IAA producers.

The isolate 1B exhibited antagonism against two pathogenic fungi (*Fusarium oxysporum* and *Colletotricum truncatum*) and produced only a small quantity of IAA, indicating its showing lower efficiency to promote plant growth as compared with others isolates. This result showed the need for simultaneous screening of isolates for IAA production and antifungal activity in order to select effective PGPR for development of biofertilizers.

The clustering (Figure 1) showed low similarity (above50%), where there four clusters with different genetic profiles were observed. *Bacillus subtilis* strain PRBS-1 showed 100% of similarity with isolate 1B, while two other clusters were observed with two isolates each one: 2B and 5A; 3A and 4B. The isolates 2B and 5A consists of isolates without antagonism to plant pathogens and high ability to produce IAA, while 3A and 4B consist of isolates with antagonism to plant pathogens and low ability to produce IAA.

The RAPD-PCR analysis (Figure 2) confirmed that the isolate 1B showed great similarity with the strain of *B. subtilis* AP-3 reported as a producer of antibiotic and hormone plant (ARAUJO et al., 2005). The fungal antagonism and auxin production may be the cause of the greater similarity between the isolates themselves and also with the strain of *B. subtilis* evaluated. The poor similarity were also encountered in other study with RAPD-PCR analysis of isolates within the same species of *Bacillus* (MITEVA et al., 1999). Matarante et al. (2004) using RAPD-PCR and AFLP reported that the dissimilarity between isolates *B. subtilis* and *B. pumilus* varied from 0.12 to 1.00. In this study we tested eleven primers, out of which two provided large number of bands that can be useful in genetics analysis of *Bacillus* (OLIVE; BEAN, 1999). Nevertheless, further investigations using large number of isolates and more RAPD-PCR primers are needed for a better understanding of the taxonomic relationship of the representative isolates of the species *Bacillus* sp with fungal antagonistic and producing hormones in tropical soils.

The inoculation of maize with novel isolates resulted in a significant increase in root and shoot dry mass production and in plant height when compared with the control (Figure 3). The exceptions were the isolates 1B and 4B that did not promote significant increase in shoot dry mass and plant height, respectively. Increases in root growth by application of PGPR are reported (HOLGUIN; GLICK, 2001; SILVA et al., 2007). Improved root growth is important due to the resulting increase in the volume of soil explored. For example, treatment of clipped soybean

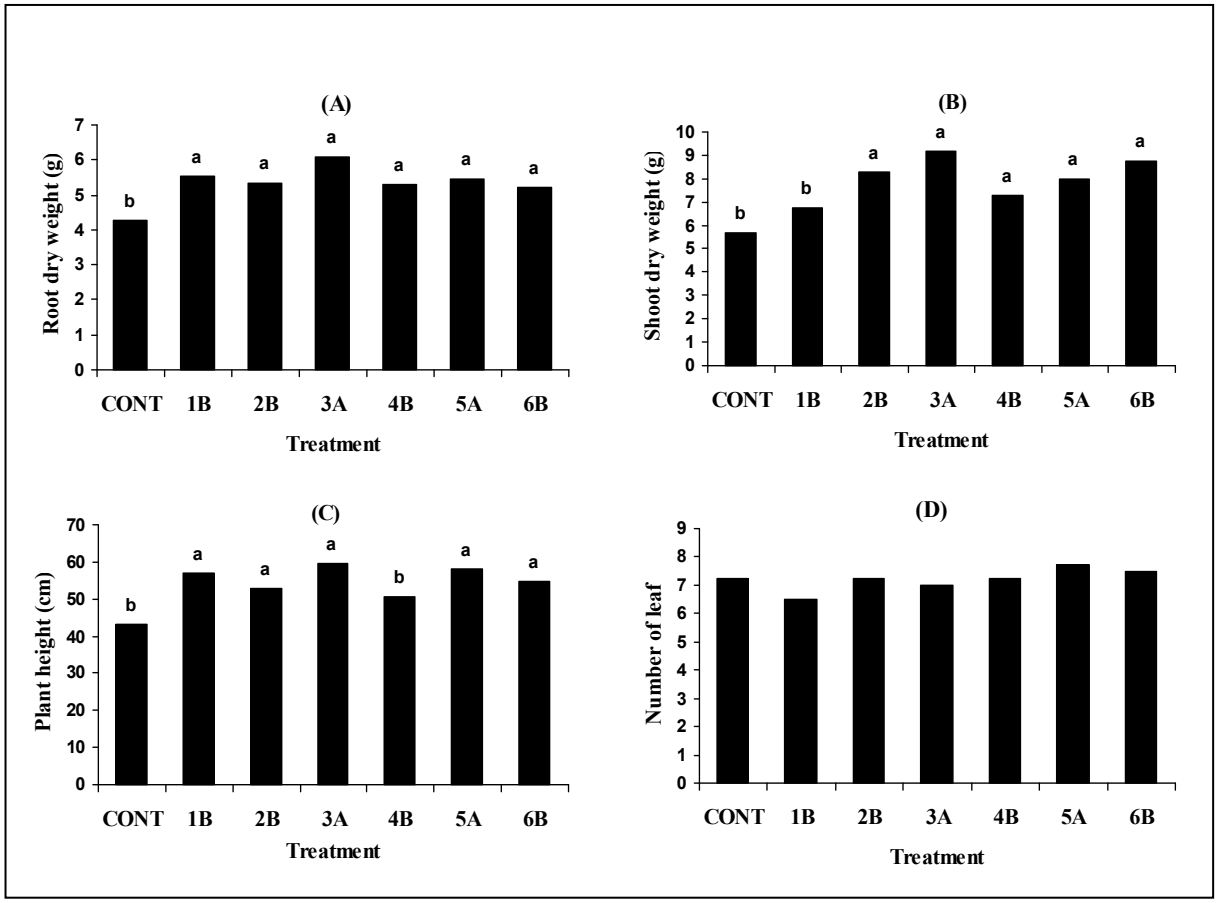

Figure 3. (A) root dry mass, (B) shoot dry mass, (C) plant height and (D) number of unfolded leaf, measured in maize inoculated with *Bacillus* isolates 50 days after sowing. Treatments sharing the same letter are not significantly different at Scott-Knott (P<0.05).

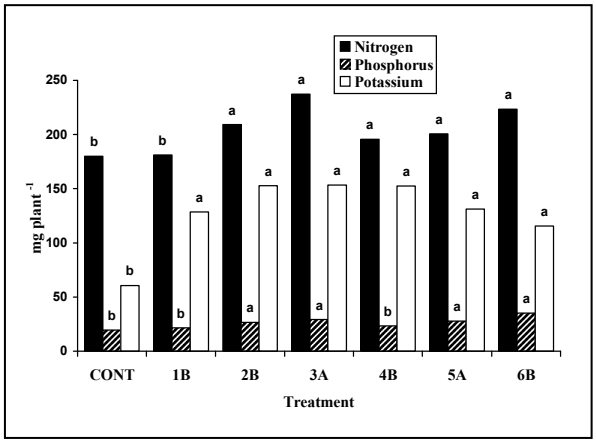

Figure 4. Accumulation of nutrients (N, P and K) in maize shoots 50 days after inoculati, on with *Bacillus* isolates. Treatments sharing the same letter are not significantly different at Scott-Knott (P<0.05).

roots with *Azospirillum brasilense* Sp7 caused a 63% increase in root dry mass and resulted in more than 10-fold increase in total root length (MOLLA et al., 2001).

Several studies have reported that the production of the phytohormones is implicated in the plant growth promoted by PGPR. Most commonly, IAA producing PGPR are believed to increase root growth and root length (BARBIERI, 1986), resulting in greater root surface area which enables the plant to access more nutrients from soil. All six isolates were able to produce IAA; therefore, this production of IAA may have promoted increased growth of roots and shoots, in agreement with the results in other grasses such as wheat (KHALID et al., 2004) and rice (BENEDUZI et al., 2008).

Out of the six examined isolates, five (2B, 3A, 4B, 5A and 6B) contributed to increase N content in the maize aerial part, while four isolates (2B, 3A, 5A and 6B) contributed to increase P content (Figure 4). The higher accumulation of plant nutrients in maize has been reported in a study under controlled condition and inoculation with *B. subtilis* (ARAUJO, 2008). Marques et al. (2010) reported that ammonium production by *Bacillus* isolates tested was positively related to N accumulation in *Zea mays*. The increase in K content, observed in plants inoculated with all isolates, suggested an additional positive effect of rhizobacteria that may also considered in future selection of rhizobacteria in order to improve nutrient uptake by plants. Previously, Sheng; He (2006) reported production of organic acids by *Bacillus edaphicus* with high ability to mobilize metals and K fraction of mineral soil.

Also, our results are in agreement with Adesemoye et al. (2008) that observed greater root and shoot growth due to the higher K uptake promoted by *Baccilus subtilis*.

CONCLUSION

The examined *Bacillus* showed to be promising isolates for the evaluation of PGPR. The *in vitro* selection criteria used in this study may be useful in screening programs for growth-promoting *Bacillus* sp., reducing the number of isolates necessary at the final stages of the screening tests with plants.

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