Co-inoculation With Tropical Strains of *Azospirillum* and *Bacillus* Is More Efficient Than Single Inoculation for Improving Plant Growth and Nutrient Uptake in Maize.

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Abstract

Usage of *Bacillus* and *Azospirillum* as new eco-friendly microbial consortium inoculants is a strategy to increase plant growth and crop yield by improving nutrient availability in agricultural systems. In this study, we designed a multispecies inoculum containing *B. thuringiensis* (strain B116), *B. subtillis* (strain B2084) and *Azospirillum brasilense* (strains A1626 and A2142) to investigate their individual or co-inoculated ability to solubilize and mineralize phosphate, produce indole acetic acid (IAA) and their effect on maize growth promotion in hydroponics and in a non-sterile soil. All strains showed significant IAA production, P mineralization (sodium phytate) and Ca-P, Fe-P (tricalcium phosphate and iron phosphate, respectively) solubilization. In hydroponics, co-inoculation with A1626 x A2142, B2084 x A2142, B2084 x A1626 resulted in higher root total length, total surface area, and surface area of roots with diameter between 0 and 1 mm than other treatments with single inoculant, except B2084. In a greenhouse experiment, maize inoculated with the two *Azospirillum* strains exhibited enhanced shoot dry weight, shoot P and K content, root dry weight, root N and K content and acid and alkaline phosphatase activities than the other treatments. There was a significant correlation between soil P and P shoot, alkaline phosphatase and P shoot and between acid phosphatase and root dry weight. It may be concluded that co-inoculations are most effective than single inoculants strains, mainly between two selected *Azospirillum* strains. Thus, they could have synergistic interactions during maize growth, and be useful in the formulation of inoculants to improve the cropping systems sustainable.

Introduction

Synthetic fertilizers are among the basic principles of plant nutrition management and have been used for decades worldwide to increase the crop production. However, their systematic and uncontrolled use has undesirable effects on soil and water besides promoting CO_2 emissions. Intensive investigations on microorganisms for better adaptation under biotic and abiotic stresses and their plant growth characteristics with fertilizer effects have been undertaken to reduce the use of synthetic nutrients. In this context, microbial inoculant or biofertilizers have received increasing attention, gaining prominence and market scale in agriculture. The development of new microbial inoculants for improve crop production represents a promising environmentally safe and low-cost alternative products for human health and land-saving actions: increasing grain production with less pressure on vegetation native (Parnell et al., 2016).

Microbial inoculants are products that contain microorganisms that act positively on plant growth and promote better adaptation of plants under biotic and abiotic stresses. A new technology with increasing application relies on the use of combined inoculants (co-inoculation), aiming to promote plant growth by synergic mechanisms of different microorganisms that show the great potential of being increasingly used by the farmers (Oliveira-Paiva et al. 2020). The efficiency of co-inoculation is mainly related to the appropriate selection of strains (Santos et al. 2019). Therefore, research is needed to generate knowledge aiming the production of new formulations for commercial inoculants with combined bacteria (Cassán et al. 2015).

Direct interactions of microbial inoculants with host plants include (i) provision of nutrients and minerals, (ii) balancing the hormonal status of plants, and (iii) priming and induction of resistance (Pieterse et al. 2014). Once established within the plant, the ability of the PGPBs to promote plant growth occurs due to several mechanisms, including the acquisition of essential nutrients and modulation of phytohormones (Rodriguez et al. 2004; Correa-Aragunde et al. 2008; Gordillo-Delgado et al. 2016). Although various PGPBs are individually inoculated in plants, more positive and stronger results are obtained inoculating plants with microbial consortia, which contains two or more beneficial microorganisms (Santoyo et al. 2021). Numerous reports in the literature show that these consortia are a feasible strategy for ameliorating drought (Joshi et al. 2020), salinity (Nawaz et al. 2020) and nutrient uptake (Silva et al. 2019), enhancing plant growth under different N levels (Hungria et al. 2013, 2015; Calzavara et al. 2018), and reducing phosphate fertilization (Rosa et al. 2020) of agricultural crops.

Bacillus and *Azospirillum* are among the most predominant bacteria genera in the maize microbiome and present several plant growth-promoting (PGP) traits. The *Bacillus* genus comprises a heterogeneous group of Gram-positive, aerobic and endospore forming bacteria widely distributed in agricultural systems. It consists of approximately 360 species that have distinct physiological, metabolic, and phenotypic characteristics (www.bacterio.net/bacillus.html). Numerous *Bacillus* species are known to enhance nutrient solubilization and facilitate its mobilization in the soil. Our research group reported that maize plants inoculated separately with different *Bacillus* strains could produce indole acetic acid (IAA), solubilizing phosphate, enhance root growth, dry matter, and nutrient accumulation in hydroponics. Under field conditions, these strains increased maize yield and P grain accumulation by around 36 and 58%, respectively, and P grain was increased by 21% in soils with no P added. Even in soils fertilized with triple superphosphate, maize yield increase and P grain accumulation were observed to be around 20% after inoculation when compared to a non-inoculated control (Sousa et al. 2021).

Azospirillum species are widely used as plant inoculants due to their positive effects on plant growth (Okon et al. 2015). Their main mechanisms include nitrogen fixation (Hungria et al. 2006; Helman et al. 2011; Fukami et al. 2018), phosphate solubilization and phytohormone synthesis, especially IAA, which is suggested to be a major actor in promoting plant growth (Spaepen and Vanderleyden 2015; Calzavara et al. 2018; Cassán et al. 2020). *Azospirillum* alters root architecture, increasing root branching and volume, which leads to greater soil exploration, nutrient absorption, and abiotic stress resilience (Yang et al. 2009; Vacheron et al. 2013; Dar et al. 2018). Beneficial results are consistently observed with the inoculation of *Azospirillum* strains in different crops and hence the use of commercial inoculants is spread worldwide (Okon et al. 2015; Hungria et al. 2018; Oliveira et al. 2019; Santos et al. 2021).

Bacillus and *Azospirillum* strains have been described as efficient PGPBs in different crops, soil, and climatic conditions. For example, the use of a combination of six *Bacillus* strains, either individually or in consortia, enhanced growth in rice and improved plant resilience to abiotic environmental stresses (Joshi et al. 2020). Genes involved in the production of low molecular weight organic acids, P and N metabolism were identified in the genome of *Bacillus* strains (Velloso et al. 2020) and they have also been described as beneficial to plant growth for their efficiency in phosphate solubilization and organic acid production (Gomes et al. 2014; Abreu et al. 2017; Ribeiro et al. 2018). In addition, relevant results regarding *Azospirillum* co-inoculated with different bacterial species have been described in the literature (Gómez-Godínez et al. 2019; Moretti et al. 2020; Rondina et al. 2020; Moreno et al. 2021). There is an increasing adoption of the co-inoculation of *Azospirillum* and *Bradyrhizobium* by soybean farmers in Brazil over the last years. Results of this co-inoculation include higher soybean nodulation, yield, N content in grains, and moderate water restriction tolerance (Cerezini et al. 2016; Rego et al. 2018; Silva et al. 2019). Another successful example of co-inoculation in Brazil is the use of commercial inoculants with two *A. brasilense* strains, Ab-V5 and Ab-V6, which is applied on economically important grasses such as maize, wheat, rice and pastures of brachiaria and co-inoculation of legumes, such as soybean and common bean (Hungria et al. 2010; Hungria et al. 2016; Lopes et al. 2019; Heinrichs et al. 2020). Despite Brazilian long-term tradition on biological nitrogen fixation inoculants, only in 2019 was launched the first commercial inoculant carrying a co-inoculation of two P-solubilizing bacteria (*B. subtilis and B. megaterium*) with great acceptance by the farmers (Santos et al. 2019; Oliveira-Paiva et al. 2020; Sousa et al. 2021). However, there is limited information about the co-inoculation of others *Bacillus* and *A. brasilense* strains and their effect on economically important crops.

Thus, this study aimed to determine the in vitro potential of the two tropical *Azospirillum* and two *Bacillus* strains to solubilize/mineralize P, produce IAA and evaluate the ability of these strains, individually or co-inoculated, to promote nutrient acquisition and maize growth in hydroponics and in soil conditions.

Materials And Methods

Bacterial strains

Four bacterial strains were selected from the Multifunctional Microorganisms Collection from Embrapa Milho e Sorgo. The strains B116 and B2084 can solubilize P and were identified as *B. thuringiensis* and *B. subtillis*, respectively (Oliveira et al. 2009; Abreu et al. 2017; Velloso et al. 2020). The strains A1626 and A2142, two nitrogen-fixing bacteria from the genus *Azospirillum*, were isolated from sorghum stalk and maize rhizosphere soil, respectively. All strains promoted maize growth in hydroponic or field conditions (Sousa et al. 2018, 2021; Velloso et al. 2020).

Identification of Azospirillum species

Bacterial genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega, USA) and amplified with the 16S rDNA primers 8F and 1492R (Turner et al. 1999). PCR reactions were performed with 30 ng of bacterial genomic DNA, 2.5 µL 10X PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 0.4 µM of each primer, 100 µM dNTP, 2.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Invitrogen, USA) in a total volume of 25 µL. PCR was performed with the following conditions: 95°C for 2 min, 30 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C. Finally, reactions were incubated for 10 min at 72°C. The amplification products were purified with the ExoSAP-IT Kit (USB, USA), and sequenced with the primers 8F, 1492R, 515F (Turner et al. 1999) and 902R (Hodkinson and Lutzoni 2009) using Big Dye Terminator v3.1 kit, as recommended by the manufacturer (Applied Biosystems, USA). The samples were analyzed in the automated sequencer ABI PRISM 3500 XL Genetic Analyzer (Applied Biosystems, USA) and DNA sequences were compared using the BlastN program (Altschul et al. 1997). The *16S rRNA* gene sequences were deposited in GenBank under accession numbers MW646094 (A1626) and MW646095 (A2142).

Compatibility test

The bacterial strains were confronted on the same Petri dish to evaluate their compatibility as reported by Cuesta et al. (2012), with modifications. Individual strains were grown in TSB (Trypticase Soy Broth) medium at 28° C overnight. Then, 100 µL of the liquid culture of one strain was spread with a Drigalski loop on the surface of Petri dish containing PDA medium (200 g L^{-1} potato, 20 g L^{-1} dextrose

and 15 g L^{-1} agar). After drying, 25 µL of the culture of another strain were inoculated adding four drops at equidistant points on the PDA surface. The plates were incubated at 28°C ± 2 for 5 days in triplicate and the strain's compatibility was determined by the absence of an inhibition zone.

P solubilization and mineralization

One isolated colony of each strain grown on BDA plates (200 g L^{-1} potato, 20 g L^{-1} dextrose and 15 g L^{-1} agar) was transferred to TSB medium (Trypticase Soy Broth) and incubated overnight at 28º C. After this period, a bacterial suspension in the concentration of 5×10^7 CFU (colony-forming unit) mL^{-1} was transferred, in triplicate, to 100 mL of the NBRIP culture medium (Nautiyal 1999), either individually or co-inoculated with another strain at the same concentration. The NBRIP medium was supplemented separately with 25 g L^{-1} $Ca_3(PO_4)_2$ (Ca-P), 5 g L^{-1} $FePO_4$ (Fe-P) and sodium phytate at 1 g L^{-1}. The samples were incubated at 28°C for nine days at 120 rpm and centrifuged at 5.000 × g for 10 min. The supernatant was filtered on Whatman filter paper No. 42 and the concentration of soluble P was determined by the colorimetric method (Murphy and Riley 1962). Additionally, the pH of the filtrate from all samples, including the controls, was determined.

In vitro indole acetic acid (IAA) production

The production of tryptophan-dependent IAA molecules was measured by the colorimetric method according to Patten and Glick (1996). Each strain was grown in 50 mL of liquid TSB culture medium supplemented with 1.0 mg mL^{-1} of DL-tryptophan and incubated at 30°C for five days at 100 rpm in the dark. After centrifugation for 10 min at 5,500 rpm, 0.1 mL of the supernatant was mixed with 0.1 mL of the Salkowski reagent (Loper and Schroth 1986) and incubated for 20 min in the dark. The concentration of IAA molecules in the supernatant was determined by the colorimetric measurement at 540 nm (Labsystems Multiskan, USA) in triplicate and compared to a standard curve.

Maize plant growth under hydroponic conditions

The hydroponic experiment was conducted with 11 treatments (four individual strains, six co-inoculations and one negative control (non-inoculated) (Table 1), arranged in a completely randomized design with three replicates with five maize seedlings each.

Table 1
Identification of the treatments with *Azospirillum brasilense* and *Bacillus* spp. strains.

Strains	Identification
Non-inoculated	NaCl 0.85% (w/v)
B116	*Bacillus thuringiensis*
B2084	*B. subtilis*
A1626	*Azospirillum brasilense*
A2142	*A. brasilense*
B116 x B2084	*B. thuringiensis* + *B. subtilis*
B116 x A1626	*B. thuringiensis* + *A. brasilense*
B116 x A2142	*B. thuringiensis* + *A. brasilense*
B2084 x A1626	*B. subtilis* + *A. brasilense*
B2084 x A2142	*B. subtilis* + *A. brasilense*
A1626 x A2142	*A. brasilense* + *A. brasilense*

For the preparation of microbial inoculants, the strains were grown individually in liquid TSB culture medium at 28°C and 150 rpm for three days. After the incubation period, cultures were centrifuged at 6,000 rpm for 10 min. Bacterial suspensions were adjusted to absorbances equal to or higher than 1, at wavelength of 550 nm, to obtain a concentration of 10^7 CFU mL^{-1} after resuspension in 2.0 L of 0.85% (w/v) NaCl.

The maize seedlings were evaluated as described by Sousa et al. (2021). Maize seeds were surface disinfested with 0.5% (v/v) sodium hypochlorite for five minutes, washed and soaked for four hours in deionized water and transferred to germination paper rolls. After seed germination for three days, uniform seedlings were transferred to trays containing eight liters of half strength Hoagland´s nutrient solution pH 5.65 (Liu et al. 1998) and acclimatized for seven days. After acclimatization, the roots were incubated for six hours at room temperature with the bacterial suspension, prepared as described previously. In the control, the plants were incubated in 0.85% (w/v) saline solution. The trays with maize seedlings were manually agitated at frequent intervals to facilitate contact of the bacteria with the roots. After the incubation period, the inoculum excess was removed by gentle shaking and the seedlings were incubated in nutrient solution. The nutrient solution was changed every three days and the plants were kept for another ten days in a growth chamber with constant aeration at controlled day/night temperatures (27/20°C) with a 12 h photoperiod and light intensity of 330 µmol photons m^{-2} s^{-1}. In all experiments, roots were separated from the shoot and photographed with a digital camera (Nikon D300S SLR). The obtained images were analyzed with the softwares RootReader2D and WinRhizo v. 4.0 (Regent Systems, Canada) to measure traits related to root morphology, such as total root length (L), total root surface area (SA), and surface area of roots with diameters between 0–1 mm (SA1), 1–2 mm (SA2) and larger than 2 mm (SA3) (cm^2) (Sousa et al. 2012). Dry weight measurements were carried out for roots and shoots, which were placed separately in paper bags, dried in a forced circulation oven at 65°C and weighed on a precision scale until constant weight.

Greenhouse experiment

For the greenhouse experiment, 11 treatments described for hydroponics (Table 1) were arranged in a completely randomized design, with four replicates (Table 1). Pots containing 5 kg of a Latossolo Vermelho, very clayey texture (Typic Haplustox, Brazilian savanna) were used, with the following chemical and physical characteristics in the top soil (0–20 cm): pH-water = 5,24; Al = 0,4; Ca = 2,5; Mg = 0,2 ($cmol_c$ dm^{-3}); CEC (cation exchange capacity) = 11,8 $cmol_c$ dm^{-3}; P = 2,2; K = 30,3 (mg dm^{-3}); V (base saturation) = 23,2 % and clay content = 740 g kg^{-1}.

Twenty days before sowing, soil acidity correction was carried out based on chemical analysis. The liming requirement was calculated to reach a base saturation of 70% with the application of 6.0 Mg ha^{-1} of dolomitic limestone (43% CaO, 14% MgO, 80% PRNT) and 1.0 Mg ha^{-1} of phosphogypsum (17% Ca, 14% S). Irrigation was performed to maintain soil humidity at 80% of the field capacity. For soil fertilization, urea (90 kg ha^{-1} of N), triple superphosphate (TSP) (450 kg ha^{-1} of P_2O_5), 500 kg ha^{-1} of KCl and 50 kg ha^{-1} of commercial formulation of micronutrient FTE – fritted trace elements (9.0% Zn, 1.6% B, 0.8% Cu, 3.0% Fe, 2.6% Mn and 0.1% Mo) were applied.

The bacterial inoculation was prepared as follows: cells from 50 mL cultures incubated for 72 h in LB medium were harvested by centrifugation at 10,000 × g for 10 min, resuspended in a 0.85% (w/v) NaCl solution and the optical densities were adjusted to 1.0 absorbance at 540 nm, corresponding to 10^8 cells mL^{-1}. Subsequently, the bacterial suspension was added in a sterilized mineral coal in the proportion of 30% (w/v) of liquid inoculant (10 mL pot^{-1} for individual strain or 5 mL pot^{-1} of each strain for co-inoculation). The inoculant (bacteria + mineral coal) was pelletized as a seed coat onto maize seeds, using 4% (w/w) cassava starch gum as adhesive. Strains were inoculated on five maize seeds (cultivar AG 7098), leaving three plants per pot eight days after sowing. The side-dress fertilization was performed with urea (90 kg N ha^{-1}) at 20 days after sowing.

The plants were harvested at 45 days after sowing, and roots and shoots were separated and dried in a forced air circulation oven at the temperature of 65º C until constant weight to obtain dry matter. Then, the plant material was ground in a Wiley mill, and chemical analyses were conducted for determining the N, P and K concentration in shoots and roots in the Laboratory of Plant Chemical Analysis at Embrapa Milho e Sorgo using ICP-OES (Nogueira and Souza 2005). N, P and K content were calculated by multiplying the N, P and K concentrations with the dry weight, which was performed separately for roots and shoots. The rhizosphere soil was collected for determination of available phosphorus content, extractor Melich-1 (Silva 2009) and acid and alkaline phosphatase activity.

Phosphatase activity in soil

The determination of phosphatase activity was performed according to the methodology described by Tabatabai and Bremner (1970). For acid phosphatase analysis, the sample's pH was adjusted to 6.5 with 1 M HCl, and for alkaline phosphatase activity, the pH was corrected to 11 with 1 M NaOH. The p-nitrophenol (PNP) concentration was determined in triplicate by a colorimetric measurement at 540 nm (Labsystems Multiskan, USA) and compared to a standard curve.

Statistical analysis

The data was submitted to variance analysis using the software SISVAR 5.6 (Ferreira 2011) and the means were compared by the LSD test at 5% level of probability.

Results

Bacillus **and** Azospirillum **identification and strains compatibility**

The strains A1626 and A2142 were molecularly identified as *A. brasilense.* Both *Bacillus* and *Azospirillum* strains were able to coexist, since no inhibition zone was observed at the intersection between strains, indicating the possibility of using these strains together in an inoculant.

Bacillus **and** Azospirillum **strains were capable to solubilize Ca-P, Fe-P and mineralize sodium phytate**

We observed a significant decrease ($p < 0.05$) in the pH of the medium after nine days of growth for all strains and P sources (Table 2). A significant difference in solubilized Ca-P values was observed for all treatments except A2142, ranging from 2.98 (A2142) to 46.19 mgP L^{-1} (A1626) (Table 2). In general, the co-inoculation of B116 x A2142 and B2084 x A2142 presented higher Ca-P solubilization values than when these strains were evaluated separately. Similarly, the different strains showed significant solubilization of Fe-P. Moreover, B116, B2084, B116 x A1626 and A1626 x A2142 significantly solubilized more Fe-P than the other strains. On the other hand, the B116 and B2084 strains were more efficient in Fe-P solubilization when cultivated separately than together. *Bacillus* strains B2084 and B116 x B2084 mineralized more P after growing in medium containing sodium phytate (Table 2). A negative correlation of -0.76 ($p < 0,05$); -0.55 and − 0.16 was observed for Ca-P, Fe-P and sodium phytate solubilization/mineralization and pH, respectively.

Table 2

Phosphorus solubilized and mineralized and pH after nine days of growing at 28 °C in liquid culture containing tricalcium phosphate (Ca-P), iron phosphate (Fe-P) and sodium phytate and tryptophan-dependent indole-3-acetic acid (IAA) like molecules production by *Azospirillum brasilense* e *Bacillus* spp. strains.

Treatments	Ca-P		Fe-P		Sodium phytate		IAA
	P (mgP L^{-1})	pH	P (mgP L^{-1})	pH	P (mgP L^{-1})	pH	(µg.mL^{-1})
Non-inoculated	0.09 g*	6.36 a	0.11 e	6.47 a	0.15 g	6.58 a	0.00 e
B116**	25.52 de	4.04 b	42.95 ab	4.80 cde	12.48 d	4.72 b	49.54 c
B2084	29.05 cd	4.07 b	45.38 ab	4.82 bcd	25.00 b	4.80 b	30.16 d
A1626	46.19 a	4.09 b	23.64 d	4.66 ef	3.97 f	3.75 g	65.78 a
A2142	2.98 g	4.00 b	31.89 c	4.87 bc	5.60 ef	4.57 bc	52.10 b
B116 x B2084	13.82 f	3.98 b	33.66 c	4.73 cde	29.00 a	4.41 cd	-
B116 x A1626	31.29 c	4.06 b	46.23 a	4.47 g	6.52 ef	4.04 ef	-
B116 x A2142	33.27 bc	4.03 b	43.88 ab	4.48 g	6.77 e	4.35 cd	-
B2084 x A1626	24.28 e	3.97 b	33.78 c	4.52 fg	5.70 ef	4.26 de	-
B2084 x A2142	37.41 b	3.99 b	40.28 b	4.96 b	16.49 c	4.31 d	-
B1626 x A2142	29.64 cd	3.96 b	41.44 ab	4.69 de	6.26 ef	3.95 fg	-
CV (%)	10.90	2.40	9.01	1.88	15.16	3.14	

*Means followed by the same letter did not differ significantly by LSD test ($p < 0.05$).

** Sample identification are according to Table 1.

CV – coefficient of variation

Bacillus **and** Azospirillum **produced in vitro IAA-like molecules**

Tryptophan-dependent IAA molecule production was observed in all the strains and ranged between 30.16 (B2084) and 65.78 (A1626) (Table 2).

Bacterial inoculation enhanced root growth, nutrient content, and dry weight under hydroponic condition

Maize seedlings were grown in nutrient solution to verify the effect of the inoculation of the isolated and co-inoculated strains on plant growth, root morphology and nutrient content. Overall, treatments showed a significant ($p < 0.05$) increase compared to the non-inoculated control regarding all evaluated traits, except for surface area of roots with diameter between 1 and 2 mm (Tables 3 and 4). Co-inoculation with A1626 x A2142, B2084 x A2142, B2084 x A1626 and B2084 resulted in higher root total length, total surface area and surface area of roots with diameter between 0 and 1 mm than other treatments than single inoculant, except B2084 (Table 3). In addition, co-inoculation with A1626 x A2142 significantly outperformed all other treatments for shoot dry weight (SDW) and shoot N content (Table 4) and it was superior to single inoculation of these strains for SDW, shoot N, P and K content. *Bacillus* strain B2084 inoculated separately or co-inoculated with B116, A1626 and A2142 showed superior performance when compared to the non-inoculated control for shoot dry weight and shoot N content (Tables 3 and 4).

Table 3
Means for root morphology traits of maize seedlings 10 days after inoculation with *Azospirillum brasilense* e *Bacillus* spp. strains grown under hydroponic conditions.

Treatments	L (cm)	SA (cm^2)	SA1 0-1 mm (cm^2)	SA2 1–2 mm (cm^2)	SA3 > 2 mm (cm^2)
Non-inoculated	403.46 de*	133.67 d	35.40 d	68.49 abcd	16.84 c
B116**	399.51 de	140.83 cd	33.44 d	71.07 abcd	22.38 bc
B2084	617.81 a	195.11 a	70.10 a	79.05 ab	25.98 ab
A1626	405.08 de	140.88 cd	44.67 cd	57.44 de	24.93 ab
A2142	427.15 cd	147.33 cd	46.97 cd	57.91 de	27.70 ab
B116 x B2084	491.21 bc	164.47 bc	55.92 bc	65.42 bcde	26.84 ab
B116 x A1626	364.87 de	138.56 d	36.35 d	60.66 cde	28.23 ab
B116 x A2142	350.21 e	131.19 d	34.94 d	51.82 e	32.17 a
B2084 x A1626	559.15 ab	181.42 ab	61.77 ab	76.25 abc	25.25 ab
B2084 x A2142	576.10 a	188.45 a	63.18 ab	76.14 abc	29.20 ab
A1626 x A2142	631.67 a	202.42 a	71.98 a	82.00 a	28.56 ab
CV (%)	9.47	8.83	16.38	13.65	17.46

L total root length (cm), SA total surface area (cm^2), SA1 surface area of roots with diameter between 0 and 1 mm (cm^2), SA2 surface area of roots with diameter between 1 and 2 mm (cm^2), SA3 surface area of roots with diameter > 2 mm (cm^2).

*Means followed by the same letter did not differ significantly by LSD test ($p < 0.05$).

** Sample identification are according to Table 1.

CV – coefficient of variation

Table 4

Means for shoot and root dry weight and N, P, K content of maize 10 days after inoculation with *Azospirillum brasilense* and *Bacillus* spp. strains grown under hydroponic conditions.

Treatments	SDW	N	P	K	RDW	N	P	K
		Shoot						
	g	g.g^{-1} plant dry weight			g	g.g^{-1} plant dry weight		
Non-inoculated	0.160 f*	2.89 e	5.12 de	31.32 de	0.060 c	0.83 c	0.64 b	3.99 d
B116**	0.177 def	3.16 de	4.64 cde	34.07 bcd	0.067 bc	0.86 c	0.82 ab	5.42 cd
B2084	0.203 bcd	3.76 bc	5.49 cd	39.79 bc	0.087 a	1.00 ab	0.93 a	6.41 abc
1626	0.167 ef	3.03 de	3.63 f	26.61 e	0.070 bc	0.90 bc	0.85 ab	5.96 bc
A2142	0.180 def	3.20 de	4.34 ef	33.51 cd	0.077 ab	0.90 bc	0.85 ab	6.20 bc
B116 x B2084	0.220 b	4.05 b	5.72 cd	36.67 bcd	0.067 bc	0.91 abc	0.98 a	7.91 a
B116 x A1626	0.190 cde	3.29 de	4.36 ef	34.10 bcd	0.087 a	0.94 abc	0.92 a	7.21 ab
B116 x A2142	0.197 bcd	3.39 cd	4.21 ef	31.09 de	0.080 ab	0.96 abc	0.90 a	6.87 abc
B2084 x A1626	0.210 bc	3.82 b	6.23 bc	36.31 bcd	0.080 ab	0.91 abc	0.85 ab	7.01 ab
B2084 x A2142	0.217 bc	3.97 b	6.89 ab	40.73 ab	0.080 ab	1.04 a	1.00 a	7.10 ab
A1626 x A2142	0.253 a	4.67 a	7.55 a	46.83 a	0.080 ab	0.91 abc	0.94 a	6.94 abc
CV(%)	8.08	7.03	12.28	11.09	11.02	8.67	15.79	13.97
SDW shoot dry weight, RDW root dry weight								
*Means followed by the same letter did not differ significantly by LSD test ($p < 0.05$).								
** Sample identification are according to Table 1.								
CV – coefficient of variation								

Azospirillum **co-inoculation improves shoot and root traits in greenhouse**

A pot experiment was conducted in a greenhouse to determine the performance of maize inoculated with the strains in soil fertilized with triple superphosphate. After 45 days, the co-inoculation treatments significantly ($p < 0.05$) improved shoot and root traits (Table 5). Maize inoculated with the A1626 x A2142 strains exhibited enhanced shoot dry weight up to 21.3%, shoot P and K content up to 30.8% and 13.8%, respectively, root dry weight up to 44.7%, and root N and K content up to 32.2% and 23.9%, respectively. The B116 and the consortium B116 x A1626 presented higher shoot P content and B116 x A2142 presented higher P root content. P shoot and acid and alkaline phosphatase activities were also higher in the A1626 x A2142 inoculum than single inoculations and of these strains beyond non-inoculated control (Table 5). Treatments B116 and B2084 x A2142 also presented higher alkaline phosphatase activity.

Table 5
Dry matter, plant content of N, P, K and soil characteristics after inoculation with the *Azospirillum brasilense* e *Bacillus* spp. strains in maize grown under greenhouse with triple superphosphate (TSP) fertilization.

	Shoot				Root				Soil		
Treatments	SDW	N	P	K	RDW	N	P	K	Acid Phosphatase	Alkaline Phosphatase	P Mehlich 1
	(g.pot^{-1})	(mg.pot^{-1})			(g.pot^{-1})	(mg.pot^{-1})			µg p-nitrophenol h^{-1} g^{-1} soil		mg/dm^3
Non-inoculated	18.10 b*	635.08 a	37.76 cd	79.48 abc	6.65 bcd	90.51 b	9.23 cd	15.98 ab	500.90 bcde	220.74 bcd	29.20 c
B116**	19.89 ab	677.37 a	45.44 ab	78.76 abc	9.37 ab	82.29 b	14.49 a	18.33 ab	552.98 abc	260.50 a	43.16 b
B2084	18.62 b	622.30 a	37.50 bc	83.29 abc	5.63 c	88.65 b	8.08 d	13.37 b	435.27 def	236.20 abc	27.96 c
B1626	19.70 ab	668.62 a	40.08 bcd	84.39 abc	8.26 abc	102.00 ab	8.45 d	16.56 ab	535.90 bcd	221.08 bcd	20.52 c
B2142	19.58 ab	642.68 a	37.29 c	76.88 bc	7.66 abc	104.71 ab	9.90 bcd	17.28 ab	462.77 cdef	198.23 d	16.55 c
B116 x B2084	20.67 ab	709.59 a	40.30 bcd	74.25 c	7.16 abc	104.01 ab	10.06 bcd	14.79 ab	573.81 ab	251.65 ab	19.86 c
B116 x B1626	19.72 ab	669.92 a	44.78 ab	89.10 ab	6.34 cd	94.69 ab	9.13 cd	14.69 ab	374.96 f	235.41 abc	58.27 a
B116 x B2142	19.87 ab	684.52 a	42.07 bc	89.10 ab	8.87 abc	102.65 ab	13.87 ab	17.08 ab	462.15 cdef	216.04 cd	19.05 c
B2084 x B1626	19.64 ab	667.00 a	44.06 abc	81.82 abc	6.39 cd	86.46 b	8.68 d	13.38 b	532.56 bcd	230.49 abc	64.24 a
B2084 x B2142	20.44 ab	683.65 a	44.03 abc	85.43 abc	6.29 cd	78.38 b	7.74 d	13.07 b	392.98 ef	255.01 a	23.06 c
B1626 x B2142	21.96 a	714.87 a	49.40 a	90.41 a	9.62 a	119.63 a	13.01 abc	19.79 a	655.06 a	258.38 a	55.17 ab
CV(%)	9.34	9.91	11.40	11.19	26.87	20.04	28.30	26.63	15.68	9.87	22.50

SDW shoot dry weight, RDW root dry weight

*Means followed by the same letter did not differ significantly by LSD test ($p < 0.05$).

** Sample identification are according to Table 1.

CV – coefficient of variation

The highest concentration of available P in the soil after 45 days of maize inoculation was observed in the treatments B116, B116 x A1626, B2084 x A1626 and A1626 x A2142 (Table 5) compared to the non-inoculated control. The combinations of A1626 x A2142 and B2084 x A1626 provide more soil P release than inoculant carrying these isolated strains. There was a significant correlation between available P in the soil and shoot P (r = 0.69, $p = 0.02$). In addition, there were positive correlations between acid phosphatase (ACP) activity and root dry weight (r = 0.64, $p = 0.03$), and shoot K (r = 0.61, $p = 0.04$). Alkaline phosphatase (ALP) activity also correlated with shoot P (r = 0.67, $p = 0.04$).

Azospirillum co-inoculation outperformed in the principal component analysis

Considering hydroponic and greenhouse experiments, a principal component analysis (PCA) was performed with the root morphology, dry weight, and nutrient content traits (Fig. 1). The first principal component (PC1) explained 43.3% and the second principal component (PC2) explained 22.2% of the phenotypic variation observed for the analyzed traits (Fig. 1). The non-inoculated treatment, B116, A1626, A2142,

B116 x A1626 and B116 x A2142 were in the left quadrant, showing smaller root systems, dry weight, and nutrient content. Treatments in the right quadrants, highlighting B2084, B2084 x A1626, B116 x B2084 and B2084 x A2142, showed higher root surface area, dry weight, and nutrient content. Moreover, the co-inoculation of A1626 x A2142 outperformed all other treatments in the right quadrant presenting not only higher growth in nutrient solution but also higher biomass and nutrient content in greenhouse conditions.

Discussion

In the present investigation, two *Azospirilum* (A1626 and A2142) and two *Bacillus* strains (B116 and B2084) isolated from tropical maize and sorghum were selected based on plant growth promoting traits for a co-inoculation assay. The choice of the strains was based in our research group's previous studies, considering that the individual inoculation of *Bacillus* strains B116 and B2084 significantly increased the dry weight of maize seedlings in hydroponics and maize yield in the field (36 and 12%, respectively) (Sousa et al. 2021). The individual inoculation of *Bacillus* strains B116 significantly increased the sorghum yield and phosphorus grain uptake in the field (19% and 36%, respectively) (Mattos et al., 2021) and individual inoculation of B2084, increase the millet biomass and P content (Ribeiro et al., 2008). Strain B116, identified as *B. thuringiensis* (Lana et al., 2020), was isolated from the maize rhizosphere, being efficient in biofilm and exopolysaccharide production, and phosphate solubilization (Oliveira et al. 2009; Velloso et al. 2020). Strain B2084 is an endophytic bacterium isolated from maize leaves and identified as *B. subtilis* by 16S rDNA sequencing (Sousa et al. 2021) and by protein profile using MALDI-TOF mass spectrometry (Lana et al. 2020). In addition, the *Azospirillum* strains (A1626 and A2142) increased maize root surface area in hydroponics (Sousa et al. 2018) and show promising results in maize inoculation under field experiments (unpublished results).

In this study, all the *Bacillus* and *Azospirillum* strains significantly solubilized Ca-P and Fe-P, and mineralized sodium phytate. The co-inoculation of *Bacillus* and *Azospirillum* presented higher Ca-P solubilization values than when these strains were evaluated separately. Probably, the organic acids produced together by these strains made this effect possible. In our studies, the strain B2084 was the largest producer of gluconic acid in a ranking of 55 strains (Abreu et al. 2017) which has been associated as the main mechanism of solubilization of Ca-P. In fact, soil bacteria increase P solubility because the majority of these microorganisms can secrete organic acids (gluconic acid), which help decrease rhizosphere pH by decoupling the connection between calcium and phosphate. They can compete or even replace phosphate sorbed on the surfaces of soil clays and chelate Ca, Al and Fe, thus avoiding the precipitation of phosphate (Sharma et al. 2013).

The hydroponics results demonstrated that *Bacillus* and *Azospirillum* co-inoculation influenced root morphology, shoot and root dry weight, N, P and K content. (Table 3). The co-inoculation of A1626 x A2142 was responsible for an increase of nutrient content and plant biomass (Table 4), showing that a larger root system leads to greater growth and improved nutrient uptake more than single strains. Our results showed a positive and significant high correlation between root surface area, root length, shoot/root dry weight, and root/shoot nutrients (N, P and K), indicating that a maize plant with a larger root system, can translocate more nutrients to the shoot and enhance its vegetative growth. Accelerated plant growth during the early stages of crop development leads to greater stress resistance and yield (Sousa et al. 2021). Overall, a larger root surface area increases water and nutrient acquisition and leads to an increase in the allocation of resources, enhancing plant resistance and biomass accumulation (Contesto et al. 2008; Combes-Meynet et al. 2011; Vacheron et al. 2013).

Synergy among *Azospirillum*, *Bacillus* and other bacterial species has been described in the literature (Poonguzhali et al. 2005; Felici et al. 2008; Wang et al. 2019). Gómez-Godínez et al. (2019) reported the effect of a multispecies inoculum of PGPBs containing *Rhizobium phaseoli*, *Sinorhizobium americanum* and *A. brasilense* nitrogen-fixing strains and other PGPB such as *B. amyloliquefaciens* and *Methylobacterium extorquens* on the growth of one-month-old maize plants. The multispecies inoculum exerted a greater beneficial effect on maize plants than the effect obtained with single bacteria. The authors observed that *Azospirillum* nitrogen fixation was lower than observed with the multispecies inoculum. Interestingly, they hypothesized that biofilm formation induced by root exudates in *Bacillus* and *Azospirillum* forming aggregates may provide favorable nitrogen-fixing conditions by protecting bacteria from oxygen, or the consortia may provide nutrients that stimulate *Azospirillum* nitrogen fixation.

In another study, inoculation with *A. brasilense* and *B. subtilis* increased the stem diameter and shoot N content of maize plants when N fertilization was not used at the sowing. The authors concluded that the observed increases indicate a synergy between the two microorganism species, demonstrating that, in this case, their combined inoculation in maize reduced the need for N fertilization at sowing (Moreno et al. 2021). Combined inoculation of *Azospirillum* and a P-solubilizer (*B. megaterium* var. *phosphaticum*) showed higher shoot length, root length, 1,000 grain weight, and rain yield in rice than in the individual inoculation of *Azospirillum* (Arangarasan et al. 1998).

Azospirillum and *Bacillus* strains are known to be efficient IAA producers. In fact, all strains produced high concentrations of this phytohormone, especially the *Azospirillum* strains, which resulted in positive effects on root growth. Various plant species inoculated with IAA producing bacteria reported increased root growth and/or enhanced formation of lateral roots and root hairs, which further increases

the surface area and length of lateral and adventitious roots (Mohite, 2013; Joshi et al. 2020). In general, plant-associated bacteria use the tryptophan present in plant exudates as a precursor for IAA biosynthesis. In addition, several *Bacillus* strains are IAA producers and can promote the growth of different crops (Kim et al. 2017; Bahadir et al. 2018; Robles Montoya et al. 2019). Recently our group sequenced the genome of the *Bacillus* B116 strain and genes related to IAA biosynthesis were described (Velloso et al. 2020).

Significant increases in maize shoot and root biomass and enhanced shoot nutrient content (N, P and K) were observed after co-inoculation with the two *Azospirillum* strains under the greenhouse treatment (Table 5). Interestingly, shoot N content was increased, probably due to the bacterium's capacity to fix N_2. On the other side, the IAA mediated increase in the root system resulting in improved growth as well as P and K uptake. Even in soils fertilized with triple superphosphate, co-inoculation with *Azospirillum* strains increased shoot and root dry weight by around 20% and 44%, respectively, compared to the non-inoculated control.

The present study also revealed that the *Bacillus* + *Azospirillum* and *Azospirillum* + *Azospirillum* co-inoculations influenced the rhizosphere P availability, and alkaline (ALP) and acid (ACP) phosphatase activity under the greenhouse treatment. There were also positive correlations between increases in P shoot and P available in the soil, P shoot and ALP, and between root dry weight and ACP. Altogether, these factors lead to an increased early root growth and could enhance grain yield (Mahanta et al. 2018). Phosphatases are enzymes released by plants and microorganisms that contribute to the cleavage of organic P to supply P to the soil solution and to the plants (Abou-Baker et al. 2011). ALP is deemed to be principally derived from soil microbes (Wei et al. 2019). Considering that approximately 80% of P in Brazilian soils in agricultural areas is organic (Novais et al. 2007), constituting an important reservoir of this nutrient, the role of phosphatases is relevant, as the organic form of P is not available directly to the agricultural crops. In this sense, soil phosphatase activity can be a good indicator of the organic P mineralization potential and biological activity of soils (Chen, 2003). In addition, our results also showed an increase in the available rhizosphere P and a high correlation with P shoot in the inoculated treatments, mainly when the strains B116 x A1626, B2084 x A1626 and A1626 x A2142 were co-inoculated. It confirms that the advantage of the combined use of strains is to complement the action of each one with extra properties or to increase a type of function, increasing the effect in a synergistic way (Santoyo et al. 2021).

Interestingly, the *Azospirillum* A1626, shared by these inoculants, also showed higher Ca-P solubilization in relation to *Bacillus* strains, which can explain the P rhizosphere availability reflected in the high maize shoot biomass and P shoot accumulation, where this strain was co-inoculated with other *Azospirillum* or *Bacillus* strains. The inoculation of A1626 together with A2142 also increased the activity of soil ALP and ACP in relation to the single inoculation and non-inoculated control, demonstrating that these enzymes may also have influenced the higher P acquisition and biomass gain in the treatments where A1626 was present. While strain A1626 is endophytic, isolated from sorghum stalk, theA2142 was isolated from maize rhizosphere, which can represent an advantage on the combined use of these strains, as they can colonize different niches inside the plant and in the rhizosphere. Thus, the distinction between free-living soil bacteria, the rhizosphere population, and endosymbionts of a host plant may represent a true continuum, with microbes able to move between the soil, the rhizosphere, and inside the root (Farrar et al. 2014).

Some works report that *Azospirillum* is a PGPB because of its hormonal effect (Rodriguez et al. 2004; Turan et al. 2012; Spaepen and Vanderleyden 2015; Fukami et al. 2017; Singh et al. 2017), but our results showed that A1626 also solubilized phosphate, increasing maize growth. Rosa et al. (2020) also observed that the inoculation of sugarcane with *B. subtilis* and *A. brasilense* along with the application of 45 kg P_2O_5 ha^{-1} improved available P soil content more than four times and the P total accumulation in the entire plant in comparison to non-inoculated treatments. Based on previous works which addressed P-solubilization potential by PGPR inoculation in plants, Granada et al. (2018) consider that an average reduction of 33% in P-fertilization could be achieved with the use of highly efficient P-solubilizing bacterial isolates as crop inoculants.

It may be concluded that the combination of two strains exerted a beneficial effect on maize plants that was greater than the effect obtained with single bacteria, because the co-inoculation of *A. brasilense* + *A. brasilense* and *B. subtilis* + *A. brasilense* strains showed a positive effect on maize shoot and root dry weight, shoot N, P and K content in hydroponics and greenhouse treatments. In addition, the combined inoculants reported in this work increased phosphatase activity and available rhizosphere P.

Further studies on the long-term survival of the inoculants are required to verify the beneficial effects of these bacterial strains on nitrogen fixing, soil phosphate mobilization and on maize yield under field conditions. These studies could be useful in the formulation of new inoculants, improving the cropping systems into which they can be most profitably and ecofriendly applied. These bio-based combined inoculants could be made easily available to farmers representing a promising environmentally safe and low-cost alternative to reducing synthetic fertilizers in agriculture.

Declarations

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Ethics approval - The experiments reported in this study did not involve human participants and/or animals.

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Figures

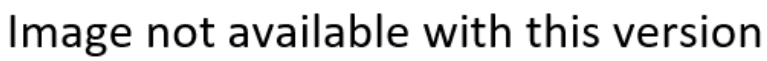

Figure 1

Principal component analysis (PCA) for traits measured in hydroponic and greenhouse conditions. Greenhouse: (g_SDW) shoot dry weight, (g_N_S) shoot N content, (g_P_S) Shoot P content, (g_K_S) shoot K content, (g_RDW) root dry weight, (g_N_R) root N content, (g_P_R) root P content, (g_K_R) root K content, (g_Acid_P) acid phosphatase, (g_Alk_P) alkaline phosphatase. Hydroponics: (SDW) shoot dry weight, (N_S) shoot N content, (P_S) shoot P content, (K_S) shoot K content, (RDW) root dry weight, (N_R) root N content, (P_R) root P content, (K_R) root K content, (RL) root length, (RSA) root surface area, (RSA1) surface area of root with diameter 0-1 mm, (RSA2) surface area of root with diameter 1-2 mm, (RSA3) surface area of root with diameter >2 mm. The proportion of the total variance explained by each principal component (PC1 and PC2) is shown on their respective axis.