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Efficiency of inoculation by *Bacillus subtilis* on soybean biomass and productivity

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ABSTRACT: The objective of this study was to evaluate the efficiency of *Bacillus subtilis* inoculation in the agronomic performance of the soybean cv. M 9144 RR crop under field conditions. Two field experiments were developed with inoculation by *B. subtilis* in soybeans in two regions. The experiments were conducted in 2015/2016 harvest from December to April. The experiment was performed in a randomized block design with four replicates with and without inoculation by *B. subtilis*. Consequently, biomass and productivity were determined. The *B. subtilis* inoculation increased soybean biomass in both assessment regions. This bacterium showed increased soybean yield in Gurupi and Araguaçu soils (Tocantins, Brazil), with increases above 14.9 and 8.8%, respectively. The inoculation of the strain *B. subtilis* UFT-Bs10 provided biomass increase, nodulation, stand maintenance and increased yield of soybean cv. M 9144 RR in field conditions.

Key words: *Glycine max*; nodulation; plant growth promoting rhizobacteria

Eficiência da inoculação por *Bacillus subtilis* sobre biomassa e produtividade de soja

RESUMO: O objetivo deste estudo foi avaliar a eficiência da inoculação de *Bacillus subtilis* no desempenho agronômico da cultura de soja cv M 9144 RR em condições de campo. Dois experimentos de campo foram desenvolvidos com inoculação por *B. subtilis* em soja em duas regiões. Os experimentos foram conduzidos na safra 2015/2016 de dezembro a abril. O experimento foi realizado em um delineamento em blocos ao acaso com quatro repetições com e sem inoculação por *B. subtilis*. Consequentemente, a biomassa e produtividade foram determinadas. A inoculação de *B. subtilis* aumentou a biomassa de soja em ambas as regiões. Esta bactéria mostrou maior produção de soja nos solos de Gurupi e Araguaçu (Tocantins, Brasil), com aumentos acima de 14,9 e 8,8%, respectivamente. A inoculação da estirpe de *B. subtilis* UFT-Bs10 proporcionou aumento de biomassa, nodulação, manutenção de estande e aumento da produtividade de soja cv. M 9144 RR em condições de campo.

Palavras-chave: *Glycine max*; nodulação; rizobactérias promotoras de crescimento de plantas

Introduction

Soybean crop stands out as one of the strongest agribusinesses in the Brazilian economy. Soybeans have a productivity potential of more than 4000 kg per hectare using an elevated level of technology (Sediyama, 2009). However, several factors limit this high rate of productivity, such as diseases caused by fungi, bacteria, nematodes and viruses. The use of chemicals as fungicides to control of soil diseases is very expensive. Therefore, the integration between biological control techniques and cultural practices to inhibit the pathogen are the best alternatives (Arunasri et al., 2011).

In addition to the ability to use microorganisms with potential for biocontrol of diseases, the use of microorganisms that promote plant growth has been much discussed in the scientific world, where these promoters contribute to increase productivity and biological control. Plant growth promoting rhizobacteria (PGPR) are found in the rhizosphere. These bacteria can be found on the surface or in association with the roots, and they can enhance the direct or indirect growth of the plant (Galdiano Junior, 2011). The most studied genera are *Bacillus, Pseudomonas, Azospirillum* and *Rhizobium* (Graças et al., 2015).

The genus *Bacillus* presents attractive characteristics for studies of biological control of diseases and as plant growth promoters, as well as components of the soil microbial population such as rhizoplane and phylloplane (Lanna Filho et al., 2010; Lima et al., 2014; Zhao et al., 2014). This genus is one of the most important rhizobacteria to increase plant growth, positively influence the germination, development and crop yield due to the production of growth promoting substances, which shows improvement in plant nutrition (Calvo et al., 2010; Gagné-Bourque et al., 2015).

The *Bacillus subtilis* species have a beneficial effect on nodulation (Araújo et al., 2010). They act indirectly by the suppression of diseases and directly by the production or alteration of phytohormones concentration, nitrogen fixation, solubilization of mineral phosphates or other nutrients of the soil, by the increase of the permeability of the roots and siderophores production (Calvo et al., 2010; Gagné-Bourque et al., 2015). Thus, they promote increased crop productivity especially when associated with other management practices, such as fertilization (Lima, 2010). In addition, the beneficial association provides the physiological increase of metabolites, which trigger the sensitivity of the root system to external conditions. These metabolites facilitate the uptake of nutrients, and can lead the seed to rapid germination (Manjula & Podile, 2005).

The success of *B. subtilis* in promoting plant growth is essentially related to the biological characteristics of this microorganism. *B. subtilis* presents facility for the maintenance of its viability in bioformulates. Thus, the potential for increased productivity as well as the reduction of diseases has become evident for this species (Sarti & Miyazaki, 2013; Lima et al., 2014; Zhao et al., 2014). Therefore, the objective of this work was to evaluate the effect of *Bacillus subtilis* UFT-Bs10 on biomass development, nodulation and yield of soybean (*Glycine max* (L.) Merrill) under field conditions in two regions of Tocantins State in Brazil.

Material and Methods

Two field experiments were carried out by *B. subtilis* inoculation in soybean cv. M 9144 RR, conducted from December 2015 to April 2016. The first experiment was at the experimental station of the Federal University of Tocantins (UFT), Gurupi Campus, located at 11°43'45 "south latitude and 49°04'07" west longitude and 280 m altitude. The second experiment was in an experimental area at the New Frontier Farm (NFF), in the municipality of Araguaçu, in the Southwest region of the Tocantins State, located at 12°55'50'' south latitude and 49°49'35'' west longitude and 278 m altitude. Both regions presented Aw weather (predominantly tropical with dry season), according to classification of Koppen-Geiger. Then, the chemical and physical characteristics of the soils were determined according to Embrapa (2009), and organic matter analysis by the Warlkley-Black process (Table 1). The soil of the experimental area was classified as medium-textured dystrophic Yellow Red Latosol (Embrapa, 2009).

In the UFT experimental station, the soil preparation was performed by the conventional method, where the plowing, harrowing, leveling and furrowing of the soil were done adopting 10 cm furrow depth and 50 cm spacing between rows. The recommended fertilization according to the previous soil analysis was 80 kg of P_2O_5 and 60 kg of KCl per ha. The fertilization was done in line using simple superphosphate ammoniated (3% N and 17% P_2O_5) as phosphorus source and KCl (58% K_2O) as potassium source.

The experiment area at the NFF Station was fertilized before planting based on the soil analysis results, with 150 kg of K_2O, made in the haul 221 kg per hectare of NP with

Table 1. Soil chemical analysis of the experiment at the Experimental Station at the Federal University of Tocantins (UFT), Gurupi, and at the experimental area at the New Frontier Farm (NFF), Araguaçu, TO.

Station	Depth	pH	P	K	Al^{3+}	H+Al	Ca^{2+}	Mg^{2+}	SB	T	V	MO
	(cm)	CaCl	(mg dm^{-3})		(cmol$_c$ dm^{-3})						(%)	(g dm^{-3})
UFT	0-20	4.7	6.23	55.5	0.0	4.13	2.14	0.86	3.1	7.27	43.2	26.7
NFF	0-20	5.1	1.80	59.0	0.0	1.70	1.60	1.20	2.9	4.65	63.4	12.0

Chemical attributes of depth 0-20 cm; pH in water - Ration 1:2.5; P and K - extractor Mehlich 1; Al^{3+}, Ca^{2+} e Mg^{2+} - Extractor KCl (1 mol L^{-1}); H + Al – Extractor SMP; SB Sum of Exchangeable Bases; (T) = Cation exchange capacity at pH 7.0; V = Basis Saturation Index; and MO = organic matter (oxidation: $Na_2Cr_2O_7$ 4N + H_2SO_4 10N).

formulation 05-37 was applied at planting. The planting was done with planter model PP soil, brand Baldan, 11 lines with spacing of 50 cm between rows.

Each experimental plot consisted of 9 lines of 6 meters in length, with spacing between lines of 0.5 m, 1 m between plots and 1 m between blocks, totaling a plot of 24 m². The useful area for determining the productivity was of 8 m², being four lines of 4 m². In both experiments the experimental design was in randomized blocks, with four replications.

The soybean seeds used, in the both experiments, were of the variety M 9144 RR, and inoculated with commercial rhizobium inoculant using peat as vehicle. The inoculant ratio applied was 10 g of the inoculant per kg of seed.

Bacillus subtilis UFT-Bs10 from Microbiology Laboratory at UFT was used in the experiments. This isolate was obtained from cerrado soil in crop areas in the State of Tocantins, and identified according to methodology by analysis of the fatty acid (Sasser, 2001) and genetic profile by sequencing the 16S rRNA region (Morgulis et al., 2008): NC000964.3 GenBank access.

The inoculum was prepared in LB medium and incubated for 48 h at 30 °C in a shaker at 120 rpm. The mean inoculum concentration of *B. subtilis* used was 2 x 10^8 CFU mL^{-1}.

The treatments were with and without inoculation by *Bacillus subtilis.* The treatment with liquid inoculant composed of *B. subtilis* was applied to the sowing, directly in the planting line with 2 L per hectare dosage.

In both experiments, the phytotechnical and phytosanitary management were carried out during the development of the crop according to Henning (2009) recommendations. To control the weeds, only one application of the post-emergent herbicide was performed with a dose of 50 mL per pump and using two 20 L cost pumps, totaling 100 mL of the product was performed. The insecticide Intrerpred 240 SC was used to for the control of the soybean caterpillar (*Anticarsia gemmatalis* Hübner) and the soybean looper (*Pseudoplusia includens* Walker). A dose of 15 mL per pump was used and two applications were applied in a total of 30 mL of product. Therefore, the Lancer 750 SP product, with a dose of 250 g, per costal pump and 2 applications totaling 500 g was used to control the cucurbit beetle (*Diabrotica speciosa* Germar) and southern green stink bug (*Nezara viridula* Linnaeus).

Two biomass evaluations were performed during the experiment, at 25 and 56 days after planting (DAP) at UFT and at 20 and 60 DAP at the NFF, being considered the stages V3 and beginning of R3 of the culture occurring the differences in days in the evaluations of the two sites, to quantify the root, shoot and total biomass, as well as the number of nodules and dry mass of the nodules.

The samples were collected from three plants located on the lines before the border, within the plot area, to avoid compromising the area of each plot, where the aerial and root parts were separated, identified and stored in paper bags.

The roots, nodules and aerial parts were dried in an oven in order to obtain dry weight and then weighed in an electronic scale to obtain the dry mass of aerial part (DMAP), root dry mass (RDM), total dry mass (TDM), number of nodules (NN) and nodule dry mass (NDM).

The harvest was carried out in the useful area of the experimental plot, then it was threshed and weighed to estimate the productivity (14% corrected humidity) per hectare and the count of the final stand.

The survival of plants was determined by the percentage of survival of the plants in relation to the expected stand of 12 plants per linear meter. The efficiency (E%) of inoculation by *B. subtilis* in stand maintenance was calculated using the equation: E% = {1 - [Ti / Tc}] × 100, where E% = treatment efficacy; Ti = mean of the final stand in the treatment inoculated by *B. subtilis* (%); Tc = mean of the final stand in the control treatment (%).

In both experiments, the analysis of variance and the Duncan test at 5% of probability were done using the statistical program ASSISTAT (Silva, 2008).

Results and Discussion

In the experiment at the Federal University of Tocantins Experimental Station, the first soybean harvest was carried out 25 days after planting (DAP), at the V3 stage, where biomass evaluations (DMAP, RDM and TDM) did not present a significant difference between the treatments (Table 3). The second harvest was performed 56 DAP, where the treatment with *B. subtilis* inoculation had a superior result ($p < 0.05$) for DMAP, RDM and TDM production compared to the control. The number of nodules (NN) and nodule dry mass (NDM) at 25 DAP did not differ significantly between treatments. However, the treatment by *B. subtilis* at 56 DAP was superior ($p < 0.05$) than control in the NN and NDM evaluations (Table 2).

The initial stand variable (25 DAP) showed no significant difference between the treatments. However, the final stand 50 DAP was superior ($p < 0.05$) for treatment with *B. subtilis* (Table 3). Thus, survival did not have a significant difference and the efficiency of *B. subtilis* inoculation was 4.8%. The productivity was higher ($p < 0.05$) for *B. subtilis* inoculation than control. Therefore, a yield of 43.1 bags ha^{-1}, 14.9% higher than the control with 37.5 bags ha^{-1}, corresponding to an increase of 5.6 bags ha^{-1} (Table 3).

In the experiment at the Experimental Station of New Frontier Farm, the first evaluation 20 DAP had a significant difference for the variables DMAP, RDM, TDM, NN and NDM, all higher ($p < 0.05$) for the treatment with *B. subtilis* than the control. In the second evaluation at 60 DAP also the treatment with inoculation of *B. subtilis* was also superior ($p < 0.05$) for the variables DMAP, RDM, TDM and NDM, there was no significant difference for NN (Table 4).

The initial stand variable 20 DAP and the final stand at 60 DAP had a significant difference between the treatments, where the treatment inoculated by *B. subtilis* was superior ($p < 0.05$) than the control (Table 5). The survival of the plants did not present significant difference, where the efficiency

Table 2. Dry mass of aerial part (DMAP), root dry mass (RDM), total dry mass (TDM), number of nodules (NN) and nodule dry mass (NDM) in soybean M 9144 RR inoculated by *Bacillus subtilis* UFT-Bs10 and cultivated at the Federal University of Tocantins Experimental Station, Gurupi, TO. Harvest 2015/2016.

Treatments	DMAP	RDM	TDM	NN	NDM
		(g)			(mg)
			25 DAP (V3 Stage)		
B. subtilis UFT-Bs10	0.80 a	0.23 a	1.03 a	14.8 a	64.3 a
Control	0.77 a	0.23 a	1.00 a	8.7 a	40.5 a
CV (%)	18.5	16.5	17.8	35.7	35.2
			56 DAP (R3 Stage)		
B. subtilis UFT-Bs10	14.1 a	2.23 a	16.33 a	18.3 a	192.5 a
Control	8.10 b	1.52 b	9.60 b	11.4 b	120.0 b
CV (%)	19.6	11.4	12.3	20.2	18.6

Averages followed by the same lowercase letter in the columns do not differ by Duncan's test at 5%. DAP = Days after planting, CV = Coefficient of variation.

Table 3. Initial stand (IS), final stand (FS), survival, efficiency (% E) and productivity of soybean M 9144 RR inoculate by *Bacillus subtilis* UFT-Bs10 at the Federal University of Tocantins Experimental Station. Harvest 2015/2016.

Treatments	IS - 25 DAP	FS - 50 DAP	% Survival	% E	Productivity	Yield
	(plants m^{-2})				(kg ha^{-1})	(bags ha^{-1})
B. subtilis	144.7 a	115.8 a	80.0 a	4.8	2585.4 a	43.1 a
Control	140.3 a	110.3 b	78.6 a	-	2250.1 b	37.5 b
CV (%)	8.7	7.8	12.1	-	8.6	8.6

Averages followed by the same lowercase letter in the columns do not differ by Duncan's test at 1 and 5%. Survival: percentage of plant survival in relation to the expected stand of 12 plants per linear meter. DAP = Days after planting, %E = efficiency of inoculation by *B. subtilis* in stand maintenance in relation to control, Yield Bags 60 kg ha^{-1}, CV = Coefficient of variation.

Table 4. Dry mass of aerial part (DMAP), root dry mass (RDM), total dry mass (TDM), number of nodules (NN) and nodule dry mass (NDM) in soybean M 9144 RR inoculated by *Bacillus subtilis* UFT-Bs10 and cultivated at New Frontier Farm Experimental Station, Araguaçu, TO. Harvest 2015/2016.

Treatments	DMAP	RDM	TDM	NN	NDM
		(g)			(mg)
			20 DAP (V3 Stage)		
B. subtilis	0.70 a	0.33 a	1.03 a	22 a	172 a
Control	0.53 b	0.20 b	0.73 b	10 b	60 b
CV (%)	10.4	15.2	10.5	15.4	15.0
			60 DAP (R3 Stage)		
B. subtilis	13.66 a	2.55 a	16.21 a	67 a	361 a
Control	11.09 b	2.31 b	13.40 b	53 a	224 b
CV (%)	10.6	12.2	11.8	12.0	13.0

Averages followed by the same lowercase letter in the columns do not differ by Duncan's test at 5%. DAP = Days after planting, CV Coefficient of variation.

Table 5. Initial stand (IS), final stand (FS), survival, efficiency and productivity of soybean M 9144 RR inoculate by *Bacillus subtilis* UFT-Bs10 at the FNF Experimental Station. Harvest 2015/2016.

Treatments	IS - 25 DAP	FS - 50 DAP	% Survival	% E	Productivity	Yield
	(plants m^{-2})				(kg ha^{-1})	(bags ha^{-1})
B. subtilis	130.5 a	130.0 a	99.6 a	6.6	3,330 a	55.5 a
Control	125.0 a	122.0 b	97.6 a	-	3,060 b	51.0 b
CV (%)	8.5	8.1	7.6	-	8.8	8.8

Averages followed by the same lowercase letter in the columns do not differ by Duncan's test at 1 and 5%. Survival: percentage of plant survival in relation to the expected stand of 30 plants m^{-2}. DAP = Days after planting, %E = efficiency of inoculation by *B. subtilis* in stand maintenance in relation to control, Yield Bags 60 kg ha^{-1}, CV = Coefficient of variation.

of the use of *B. subtilis* was of 6.6%. The productivity was higher for *B. subtilis* inoculation (3330 kg ha^{-1}) than the control (3060 kg ha^{-1}). 55.5 bags ha^{-1} were produced in the inoculated treatment, being 8.8% higher than the control with 51.0 bags ha^{-1}, corresponding to an increase of 4.5 bags ha^{-1} (Table 5).

The positive effects observed for root and area biomass (Tables 2 and 4) may be related to the ability of the *B. subtilis* UFT 201 strain to produce plant phytohormones growth and nutrient solubilization. *B. subtilis* species produce phytohormones during their development, which also stimulate root growth as verified by Araújo et al. (2005) on soybean. The growth promotion caused by soil microorganisms occurs due to the action of several factors still unclear, which may involve plant hormones production, vitamins synthesis or conversion of useful materials for plants, mineral absorption and translocation, and pathogen control (Aguiar et al., 2013).

The effect of increased nutrient availability provided by *B. subtilis* inoculation was found by Canbolat et al. (2006) in wheat and barley, which suggested that the *Bacillus* strains used had potential to increase plant growth. Tsavkelova et al. (2006) reported that *B. subtilis* isolates can also guide plant hormonal regulation in which root growth is controlled by the auxin, gibberellin and cytokinin synthesis. All these effects may have a direct effect on the increase of biomass and, consequently, on productivity as observed in the present study, where there was a significant increase with the inoculation of *B. subtilis* UFT-Bs10 in relation to the control without inoculation (Tables 3 and 5).

Rhizospheric microorganisms mainly the genus *Bacillus*, have large potential in the biological control of phytopathogenic nematodes as well as growth promoters (Fernandes et al., 2013). According to Yao et al. (2006), *B. subtilis* has been used commercially for biocontrol of plant diseases, as well as to increase crop productivity. These effects may have been reflected in the initial and final stand, where the treatment with *B. subtilis* inoculation presented higher values than the control in both experiments (Tables 3 and 5).

This study presented results related to the capacity to promote soybean growth in the field. The positive effect of the application of *B. subtilis* UFT-Bs10 on the development and yield of soybean in fields of for two regions showed the capacity of adaptability and resistance of this bacterium to different conditions. Araújo (2008) observed that the inoculation of corn and cotton seeds by *B. subtilis* showed potential to increase the growth of both crops.

The successful use of *B. subtilis* in plant growth is related to the biological characteristics of this microorganism, which expresses facilities for maintaining its viability in bioformulates and their potential to increase biomass and productivity, as well as the reduction of soil diseases, which reflect on stand maintenance.

The quantitative results obtained from the promotion of growth may be different due to the PGPR and plant interaction, where the plant variety can influence the colonization of the roots by the production of substances that stimulate a certain microorganism (Yaryura et al., 2008), as well as the substances produced by the PGPR. Despite the technological advances, this complex interaction is still poorly understood (Hardoim et al., 2015) and this explains the lack of reliable commercial products for this purpose.

The application of *B. subtilis* as plant growth and biocontrol promoters is one of the most important strategies for the agricultural sector, due to the emerging demands to reduce dependence on chemical fertilizers, and the need to develop sustainable agriculture, where inoculation of this bacterium promotes the highest yield and protection of the crops in the field.

Conclusions

The inoculation of the strain *B. subtilis* UFT-Bs10 provided biomass increase and nodulation soybean cv. M 9144 RR in field conditions.

The inoculation of the strain *B. subtilis* UFT-Bs10 provided maintenance of the stand and increased yield of cv. M 9144 RR in field conditions.

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