Venancio *et al. J Pure Appl Microbiol,* **13**(**4**), 1941-1952 | December 2019
Article 5910 | https://doi.org/10.22207/JPAM.13.4.06
Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Lettuce Production under Reduced Levels of N-fertilizer in the Presence of Plant Growth-promoting *Bacillus* spp. Bacteria

Wilson Story Venancio[1], Juliana Marcolino Gomes[2], Andre Shigueyoshi Nakatani[2], Mariangela Hungria[3] and Ricardo Silva Araujo[2]*

[1]Departamento de Fitotecnia e Fitossanidade, Universidade Estadual de Ponta Grossa, Praca Santos Andrade 01, Centro, Ponta Grossa, Parana, Brazil, CEP 84010-330. [2]Total Biotecnologia Industria e Comercio S/A, Rua Emilio Romani, 1190, Cidade Industrial, Curitiba, Parana, Brazil, CEP 81460-020. [3]Embrapa Soja Rodovia Carlos Joao Strass, Distrito de Warta, Londrina, Parana, CEP 86001-970, Brazil.

Abstract

Lettuce consumption has increased worldwide due to the recognition of its nutritional value and to the consumers' search for healthier foods. Increased consumption must be coupled with more efficient production, demanding more nutrients. However, the utilization of chemical inputs in horticulture is decreasing, and biological approaches, such as the inoculation with plant growth-promoting bacteria (PGPB), are becoming more popular. Species of *Bacillus* have long been known promote the growth of plants, including lettuce, in agricultural soils, but little is known about their potential under Brazilian conditions. We have tested the ability of *B. subtilis*, *B. pumilus*, and *B. amyloliquefaciens*, carried in single or combined pre-commercial inoculants, to act as PGPB for field-grown lettuce plants of cultivar Elisa. In our experiments, the PGPB were challenged to promote plant growth when the recommended dose of nitrogen (N) fertilizer was reduced by 50%. Our results demonstrated that all species, either alone or combined, were able to improve plant vigor, the number of leaves, and the average diameter and weight of the lettuce heads, under the test conditions. Average gains in plant vigor and head weight due to all species were of the order of 50% relative to the control (no N fertilizer) treatment and, in most cases, gains due to the presence of the bacteria were superior to those promoted by the full (100%) N fertilization. This is the first report of positive utilization of pre-commercial products containing *Bacillus* for lettuce growth in Brazil.

Keywords: Inoculation; seedlings; plant vigor; PGPB; *Lactuca sativa* L.

***Correspondence:** rsaraujo@totalbiotecnologia.com.br; +(55) (41) 988022413

(Received: 12 October 2019; accepted: 12 November 2019)

Citation: Wilson Story Venancio, Juliana Marcolino Gomes, Andre Shigueyoshi Nakatani, Mariangela Hungria and Ricardo Silva Araujo, Lettuce Production under Reduced Levels of N-fertilizer in the Presence of Plant Growth-promoting *Bacillus* spp. Bacteria, *J Pure Appl Microbiol*., 2019; **13**(**4**):1941-1952. https://doi.org/10.22207/JPAM.13.4.06

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most consumed vegetables in the world. Even though its nutritional value has consistently been underestimated, lettuce is low in calories, fat, and sodium, and its leaves are good sources of dietary fiber, calcium, potassium, phosphorus, iron, magnesium, fluorine, and vitamins A, B1, B2, and C, as well as other health-beneficial bioactive compounds[1]. In Brazil, lettuce is grown in all regions, and it is the green vegetable most consumed by the population.

The development of improved cultivars, and the evolution of management systems, cultural practices, irrigation, planting techniques, and harvest and post-harvest improvements, along with current trends of changes in food consumption have stimulated the intensification of the lettuce crop all around the world[2], and the same has happened in Brazil[3].

Due to the characteristics of its growth cycle, lettuce is very demanding for nutrients, especially potassium, nitrogen, calcium, and phosphorus, among others, and proper nutrition is of utmost importance to guarantee quality production[4]. Although the crop presents a relatively slow growth rate during the first 30 days, nutrient absorption and dry matter accumulation take up and are highly increased from that point on, demanding nutrients[5]. In addition, due to the increased consumption by people, it is mandatory that good quality vegetables are produced all year round[6].

Alternative technologies for sustainable production with reduced inputs of pesticides and fertilizers are currently on high demand[2,7]. One such alternative is inoculation with plant growth-promoting bacteria (PGPB)[8,9]. PGPB comprise a heterogeneous group of beneficial soil bacteria commonly found in the rhizosphere, on the root surface or associated to it, capable of enhancing the growth of plants and protecting them from biotic and abiotic stresses[10]. Such stimuli may result from nutrient mobilization, production of plant growth regulators, control or inhibition of plant pathogens, and bioremediation of soils, among other mechanisms[11-13].

The genus *Bacillus* is composed by aerobic endospore forming bacteria, with many species that occur in agricultural fields, acting as PGPB and contributing directly or indirectly to plant growth[14]. Species of the genus *Bacillus* have been implicated in plant growth promotion, for example, by means of the production of phytohormones, nutrient solubilization and mobilization, production of antibiotics, and induction of plant systemic resistance to pathogens[15-18]. It is believed that the growth-promoting effects of *Bacillus* spp. result from the combined action of different mechanisms. The species *B. amyloliquefaciens*, *B. pumilus*, and *B. subtilis* are probably the best studied PGPB species of the genus[14].

One strain (KPS46) of *B. amyloliquefaciens* that produces auxin, surfactin, and extracellular proteins has successfully enhanced the growth of soybean [*Glycine max* (L.) Merr.] plants[19]. Other authors observed that inoculation of soybean seeds with *B. pumilus* resulted in enhanced plant growth and increased seed protein yield [20]. Saleem et al.[21] postulated that 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing strains of *B. pumilus* may also act as PGPB because the enzyme helps regulate ethylene production by plants under certain stress conditions, thus helping to sustain plant growth. Furthermore, *B. pumilus* strain YSPMK11 was active against the fungal pathogen *Sclerotinia scletotiorum* that causes stalk rot in cauliflower (*Brassica oleracea* var. *botrytis* L.) and which is pathogenic to many other host plants[22].

B. subtilis strain 21-1 has been shown to act both as plant growth promoter and disease suppressor in association with four crops, Chinese cabbage (*Brassica rapa* subsp. *pekinensis)* cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* Miller), and lettuce grown under two different soil conditions[23]. In fact, *B. subtilis* has been demonstrated experimentally to act as PGPB by an array of mechanisms, such as competitive colonization of the rhizosphere, production and secretion of antibiotics, induction of host systemic resistance to pathogens, production of cytokinin-like substances that promote root growth, resulting in increased plant vigor and yield, disease escape, and stress tolerance[24].

Even though bacteria of the genus *Bacillus* are successfully employed as PGPB for various crops in other countries, very little is known about that in Brazil, where the technology of utilization of PGPB other than rhizobia for legumes is still incipient. In this study, we evaluated the ability of strains of *B. amyloliquefaciens*, *B. pumilus*, and *B. subtilis*, prepared as single inoculants or as a mixture of the three species, to promote the growth of field-grown lettuce.

MATERIALS AND METHODS

A collection of 20 bacterial isolates obtained from the soil of a private farm in the town of Lapa (25° 46' 11" S, 49° 42' 57" W, Altitude 908m, Atlantic Forest biome), State of Parana, Brazil was screened for the ability to promote the growth of roots of lettuce seedlings. Four promising isolates were selected for further studies. The isolates are currently deposited at the Culture Collection of Diazotrophic and Plant Growth-Promoting Bacteria of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054).

The four selected isolates were characterized by rep-PCR fingerprinting[25] and sequencing of the 16S rRNA gene. Briefly, genomic DNAs of the four isolates were extracted with Axyprep Bacterial Genomic DNA Miniprep kit (Axygen®, USA). For the rep-PCR analysis, the DNA of each of the isolates was amplified with the BOX-A1R primer (Invitrogen® Life Technologies®, Brazil) using the procedures described elsewhere[26].
The PCR reactions were performed on an Eppendorf® Mastercycler Gradient (Hamburg, Germany). Amplified fragments were separated through electrophoreses at 120 V for 7 h, in gels of 1.5% (w:v) agarose. The gels were stained with ethidium bromide and were visualized and photographed under UV light using a digital camera.

The cluster analysis was performed with the software Bionumerics® 7.5 (Applied Mathematics, Sint-Martens-Latem, Belgium), using the UPGMA algorithm[27] and the Jaccard coefficient[28] with 1.0% tolerance.

For the sequencing of the 16S rRNA gene, the DNAs of the four strains were amplified with the primers fD1 (5´-AGAGTTTGATCCTGGCTCAG-3´) and rD1 (5'-AAGGAGGTGATCCAGCC-3´)[29] and following cycles: 2 min 95°C, 30 X (15s 94°C, 45s 93°C. The amplified products were purified with PureLink® Quick PCR Purification Kit (Invitrogen®, Life Technologies®, Germany). Sequencing was performed on a 3500XL Genetic Analyzer (Hitachi®, Applied Biosystems®, USA), as described before[30] and the gene sequences obtained were deposited in the GenBank.

Field experiments were carried out in three cropping seasons, 2016, 2016/2017, and 2017/2018, at six experimental sites located at the Estacao Experimental Agricola Campos Gerais – EEACG, and one experimental site in a private farm, all in the town of Palmeira, State of Parana, Brazil, with geographic coordinates of 25° 26' 9" S and 50° 01' 01" W, at an altitude of 929 m above sea level. The climate of the region is classified as Cfb (temperate humid with temperate summer), according to K⊻pen[31].

The soil of the experimental areas is classified as Cambisol. Twenty to 30 days before

Table 1. Chemical properties and granulometry of the 0-20 cm layer of the soil at the experimental sites

Sites	Chemical Properties										Granulometry		
	pH ($CaCl_2$)	H + Al	Al	Ca mmol$_c$ dm^{-3}	Mg	K	P (resin) mg dm^{-3}	C g dm^{-3}	CEC* mmol$_c$ dm^{-3}	V♦ %	Clay	Silt g kg^{-1}	Sand
Site 1	4.3	101	11.2	35	17	9.2	98	42	162.2	38	339	242	419
Site 2	4.9	39	0.6	34	11	3.2	50	24	87.2	55	204	88	708
Site 3	5.0	50	< 0.5	53	29	6.8	121	45	138.8	64	228	201	571
Site 4	4.3	9.7	1.7	2.7	1.4	0.3	12.9	35	14.1	31	298	272	430
Site 5	4.4	8.4	0.9	4.0	1.3	0.6	52.6	29	14.3	41	287	318	395
Site 6	4.3	9.0	1.3	2.6	1.2	0.4	23.7	29	13.2	32	252	297	451
Site 7	5.0	4.8	-	4.2	1.8	0.4	50.7	25	11.3	58	265	113	622

*CEC = cation exchange capacity; ♦V = Base saturation planting the experiments, samples from the top 20 cm layer of the soil at the experimental areas were collected for chemical and granulometry analyses (Table 1). Seedbeds were prepared by revolving the soil with a rotary hoe. Some areas had been under fallow, whereas others had crops before planting the experiments (Table 2). Basal fertilizer was applied to the beds at the rates described in Table 2. In addition, nitrogen fertilizer was applied before planting as urea at 200 kg N ha^{-1} (treatments with 100% N) or 100 kg N ha^{-1} (treatments with 50% N). Experimental plots were 0.9 m wide (three rows) by 3 m long, making up a 2.7 m^2 area, with 30 plants $plot^{-1}$. Regular commercial seeds of lettuce cultivar Elisa (58-day cycle, highly resistant to LMV-II) were used in all experiments.

Four pre-commercial PGPB inoculants produced by Total Biotecnologia Indתstria e Comיrcio S/A, from Curitiba, State of Paranב, Brazil, were tested. Product A is composed of *Bacillus subtilis*, strain CCTB04, at 1 x 10^8 colony forming units (CFU) ml^{-1}. Product B contains *B. amyloliquefaciens*, strain CCTB06, at 1 x 10^8 CFU ml^{-1}. Product C carries *B. pumilus*, strain CCTB05, at 1 x 10^8 CFU ml^{-1}. Product D consists of a mixture of the three species, *B. subtilis* strain CCTB04, *B. amyloliquefaciens* strain CCTB09, and *B. pumilus*, strain CCTB05, at 1 x 10^8 CFU ml^{-1}. The cropping seasons and areas where each product was tested are shown in Table 2.

Prior to inoculation, 300 ml of each product were diluted to 1,000 L with water, in order to make the final volume necessary to inoculate one hectare, according to the manufacturer's instructions. For inoculation, trays with seedlings were soaked in a volume of diluted inoculant proportional to the area of the tray until the substrate was saturated. Seedlings were then transplanted to the definitive beds and the remaining diluted inoculant was sprayed over the seedlings with the aid of a CO_2-pressurized precision sprayer for agronomic experimentation, with a constant pressure of 35 lb in^{-2} and equipped with an XR 110 02 fan nozzle. Seedlings of the treatments that did not receive bacteria were treated the same way, but with plain water. In the plots, seedlings were planted in a 0.3 m x 0.3 m arrangement, making up a total of 11.1 plants m^{-2}. Plots received either sprinkler or drip irrigation, as necessary.

Treatments consisted of i) non-inoculated, no N fertilizer control (T1), ii) non-inoculated, with 50% of the recommended N fertilizer (T2), iii) non-inoculated, with 100% of the recommended N fertilizer (T3), and iv) inoculated, with 50% of the recommended N fertilizer (T4). The reason for reducing the amount of N fertilizer by 50% in the presence of the inoculants is because Brazilian authorities require that any product that is to be employed in agriculture as plant growth promoter must be able to replace, at least, part of some nutrient. All experiments were set up in a completely randomized block design with six replicates.

Table 2. Cropping seasons, previous crops, products tested, and basal fertilization at each experimental site

Sites♦	Cropping Season	Previous Crop	Products Tested♣				Basal N-P-K fertilizer (kg ha^{-1})
			A	B	C	D	
Site 1	2016	Fallow	X	X	X	NT	00-20-20 (4,000)
	2016/2017	Broccoli	X	X	X	NT	00-20-20 (4,000)
Site 2	2016	Fallow	X	X	X	NT	04-14-08 (2,000)
Site 3	2016/2017	Collard Greens	X	X	X	NT	00-20-20 (2,000)
Site 4	2017/2018	Fallow	NT	NT	NT	X	00-20-20 (2,000)
Site 5	2017/2018	Collard Greens	NT	NT	NT	X	04-14-08 (2,000)
Site 6	2017/2018	Wheat	NT	NT	NT	X	00-20-20 (4,000)
Site 7	2017/2018	Wheat	NT	NT	NT	X	00-20-20 (2,000)

♦Site 1 had experiments both in the 2016 and 2016/2017 seasons.

♣NT – the products were not tested at those sites

When the crop reached the BBCH 49[32] growth stage, eight to ten plants from the central area of each plot were harvested for evaluations. Plant vigor was rated on a scale of 1 (reduced vigor, highly defoliated, unrecoverable plants with compromised nutrition) to 10 (excellent vigor, full of leaves with accentuated growth, appearance as expected for the cultivar). In addition, the number of leaves per plant and the diameter and average weight of the lettuce heads were determined.

All data were subjected to an analysis of homogeneity of the variances and an ANOVA was performed. When significant differences were detected by the ANOVA, treatment means were compared by the Duncan test at $p < 0.05$. All statistical analyses were performed using the SASM – agri[33] software.

RESULTS

Genetic characterization of the strains

DNA profiles obtained in the BOX-PCR analysis indicated that each strain was unique (Fig. 1). High genetic diversity between the strains was highlighted, such that in the dendrogram they were joined at a final level of similarity of less 35% (Fig. 1).

Sequencing of the almost complete 16S rRNA genes confirmed the identity of all four strains as belonging to the *Bacillus* genus. The strains were then identified based on the 16S rRNA genes and the sequences deposited at the NCBI Genbank database, receiving the access numbers as follows: *Bacillus subtilis*, strain CCTB04 (=CNPSo 2720) (MN415973); *B. amyloliquefaciens*, strain CCTB06 (=CNPSo 3202) (MN415976), *B. pumilus*, strain CCTB05 (=CNPSo 3203) (MN415975) and *B. amyloliquefaciens* strain CCTB09 (=CNPSo 3602) (MN415974).

Inoculation with *Bacillus subtilis* (Product A)

Seedling inoculation with *B. subtilis* significantly increased (50%) plant vigor over the control (T1) treatment in all four experiments (Table 3). In two out of the four experiments, *B. subtilis*-inoculated plants (T4) were significantly more vigorous than those that received only 50% of the recommended dose of N fertilizer (T2), whereas in all four experiments they were not significantly different from plants that received 100% of the recommended dose of N fertilizer (T3; Table 3).

All treatments significantly increased the number of leaves relative to the T1 treatment, and in two out of the four experiments T4 induced the production of significantly more (23%) leaves than T2 or T3 (Table 3). All treatments significantly increased the diameter of the lettuce heads over T1, and T4 was significantly superior to T2 and T3 in three and two out of the four experiments, respectively (Table 3). The average weight of the lettuce heads was positively and significantly influenced by all treatments relative to T1 in all four experiments (Table 3). Lettuce heads produced by T4 were significantly (49%) heavier than those form the T2 and T3 treatments in all four and three out of the four experiments, respectively (Table 3). On average, mean values of all parameters evaluated were highest for the T4 treatment (Table 3).

Inoculation with *Bacillus pumilus* (Product B)

The presence of *B. pumilus* in the inoculant resulted in significant increases (54%) in plant vigor over T1 in all four experiments (Table 4). In addition, significant increases in plant vigor due to the presence of *B. pumilus* over T2 and T3 were observed in two out of the four experiments (Table 4). The number of leaves was significantly

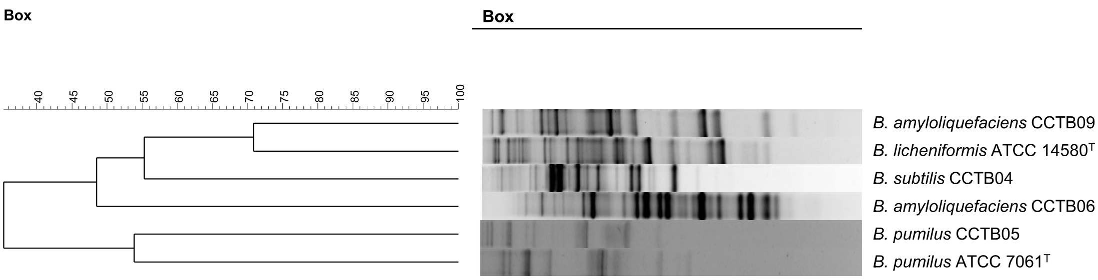

Fig. 1. Rep-PCR fingerprinting and 16S rRNA-based clustering of the isolates employed in this study

Table 3. Plant vigor ratings, numbers of leaves, average head diameter, and average head weight of lettuce plants inoculated with *Bacillus subtilis* strain CCTB 04 (Product A)[§].

	VIGOR					NUMBER OF LEAVES					HEAD DIAMETER (cm)					AVERAGE HEAD WEIGHT (g)				
	2016		2016/17		Ave[£]	2016		2016/17		Ave	2016		2016/17		Ave	2016		2016/17		Ave
	Site 1	Site 2	Site1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3	
T1	5.0c	5.0b	4.7c	4.7b	4.85	44.5b	42.0b	34.3d	54.6c	43.9	30.1c	29.7b	28.9d	28.9d	29.4	189.6c	133.7c	102.8d	246.8d	168.2
T2	7.0b	6.5a	6.2b	5.7a	6.35	50.5a	46.4a	40.5c	60.7b	49.5	32.4b	30.4ab	30.5c	30.5c	30.9	221.8b	163.7b	131.7c	314.2c	207.9
T3	7.7ab	6.3a	7.3a	6.0a	6.80	52.2a	47.6a	46.8b	60.4b	51.8	34.0ab	31.2ab	32.9b	33.2b	32.8	252.8a	162.2b	162.4b	346.6b	231.0
T4	8.3a	7.2a	7.2a	6.5a	7.30	51.3a	48.5a	52.9a	63.9a	54.2	35.2a	32.8a	33.7a	37.5a	34.8	265.5a	186.6a	184.4a	363.0a	249.9
CV %	9.16	10.67	9.99	11.49	---	7.21	5.93	2.78	4.18	---	5.54	6.81	1.92	2.94	---	9.45	5.98	1.92	2.16	---

[§] Values are means of six replicates. Means followed by different letters on the same column are significantly different according to the comparison by the Duncan test at $p < 0.05$, [£] Ave = average across sites and cropping seasons.

Table 4. Plant vigor ratings, numbers of leaves, average head diameter, and average head weight of lettuce plants inoculated with *Bacillus pumilus* strain CCTB 05 (Product B)[§]

	VIGOR					NUMBER OF LEAVES					HEAD DIAMETER (cm)					AVERAGE HEAD WEIGHT (g)				
	2016		2016/17		Ave[£]	2016		2016/17		Ave	2016		2016/17		Ave	2016		2016/17		Ave
	Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3	
T1	5.0c	5.0c	4.7c	4.7b	4.85	44.5b	42.0c	34.3d	54.6c	43.9	30.1c	29.7b	28.9c	28.9d	29.4	189.6c	133.7c	102.8d	246.8d	168.2
T2	7.0b	6.5ab	6.2b	5.7ab	6.35	50.5a	46.4b	40.5c	60.7b	49.5	32.4b	30.4b	30.5b	30.5c	30.9	221.8b	163.7b	131.7c	314.2c	207.9
T3	7.7b	6.3b	7.3a	6.0a	6.80	52.2a	47.6ab	46.8b	60.4b	51.8	34.0ab	31.2ab	32.9a	33.2b	32.8	252.8a	162.2b	162.4b	346.6b	231.0
T4	8.5a	7.5a	7.3a	6.5a	7.45	53.2a	50.6a	53.6a	64.3a	55.4	34.6a	32.8a	33.3a	40.0a	35.2	270.6a	184.9a	189.7a	363.8a	252.2
CV %	8.30	13.21	9.05	15.64	---	7.65	5.26	3.04	3.74	---	5.09	5.87	2.08	2.67	---	9.75	7.19	1.86	2.41	---

[§] Values are means of six replicates. Means followed by different letters on the same column are significantly different according to the comparison by the Duncan test at $p < 0.05$, [£] Ave = average across sites and cropping seasons.

increased by all treatments relative to T1 in all four experiments, and T4 induced the production of significantly more (26%) leaves than T2 and T3 in three and two out of the four experiments, respectively (Table 4).

The diameter of the lettuce heads was significantly increased by all treatments relative to T1 in all but one experiment (Table 4). Once again, T4 induced the production of significantly wider heads than T2 alone, and in three out of the four experiments the results promoted by T4 were equivalent to those promoted by T3 (Table 4). All treatments produced heavier lettuce heads than T1 in all four experiments (Table 4). T4 produced heavier heads over T2 in all four, and over T3 in three out of the four experiments (Table 4). On average, mean values of all parameters were highest for the T4 treatment than for T2 or T3 (Table 4).

Inoculation with *Bacillus amyloliquefaciens* (Product C)

B. amyloliquefaciens significantly increased plant vigor relative to T1 (51%) in all four experiments, and relative to T2 in two out of the four experiments; no differences relative to T3 were observed (Table 5). The number of leaves was significantly increased by all treatments, relative to T1, in all four experiments, and T4 (27% increase) was superior to T2 or T3 in three out of the four experiments (Table 5).

The diameter of the lettuce heads was significantly increased by all treatments relative to T1 in all but one of the experiments; in addition, T4 was superior to T2 in three, and to T3 in two out of the four experiments, respectively (Table 4). All treatments induced the production of heavier lettuce heads relative to T1, and positive significant effects of T4 could be observed over T2 in all four experiments, whereas T4 was superior to T3 in three out of the four experiments (Table 5). The mean values of all parameters evaluated were highest in response to the T4 treatment, when compared to T2 or T3 (Table 5).

Inoculation with Bacillus subtilis, *B. pumilus*, and *B. amyloliquefacients* (Product D)

The triple inoculant, consisting of the mixture of the three species of *Bacillus*, was effective to significantly (39% increase) improve plant vigor over T1 in all four experiments (Table 6). When compared to T2 or T3, significant differences were observed in only one experiment, probably due to a higher degree of variability among data (Table 6). All treatments significantly improved the number of leaves relative to T1 in three out of the four experiments, and T4 was significantly better than T2 or T3 in two of them (Table 6).

The diameter of the lettuce heads was significantly improved by the inoculant relative to T1; however, in most cases no significant differences were detected between T4 and T2 or T3 alone (Table 6). All treatments resulted in significantly heavier lettuce heads in three out of the four experiments, in comparison to T1 (Table 6). T4 was significantly superior to T3 in two, and superior to T2 alone in all four experiments, respectively (Table 6). On average, mean values of all parameters were highest in response to T4 than all other treatments.

Overall, the parameters that were most positively influenced by all inoculants tested were plant vigor and the average weight of the lettuce heads.

DISCUSSION AND CONCLUSION

Modern agriculture is challenged to be able to feed an ever-increasing population with quantity and quality products. In order to attain these objectives, intensification of agricultural practices has become necessary, frequently compromising the environment[34]. In recent years, efforts have been made to reduce chemical inputs into horticultural systems, with the concomitant utilization of soil microorganisms with varying modes of interaction with plants[35]. Such microorganisms are collectively known as plant growth-promoting bacteria (PGPB), acting to increase nutrient- and water-use efficiency and uptake capacity, and to alleviate biotic stresses[36].

We have evaluated the ability of the species *Bacillus subtilis* (strain CCTB04), *B. pumilus* (strain CCTB05), and *B. amyloliquefaciens* (strains CCTB06 and CCTB09), either as single inoculants or a mixture of the three species, to promote the growth and production of lettuce under reduced N fertilization. In our experiments, all isolated species, as well as their mixture, contributed to improve all parameters evaluated relative to the controls, and the gains obtained with inoculation of the PGPB were superior to those obtained when the full dose of N fertilizer was applied. The

Table 5. Plant vigor ratings, numbers of leaves, average head diameter, and average head weight of lettuce plants inoculated with *Bacillus amyloliquefaciens* strain CCTB 06 (Product C)§.

	VIGOR					NUMBER OF LEAVES					HEAD DIAMETER (cm)					AVERAGE HEAD WEIGHT (g)				
	2016		2016/17		Ave£	2016		2016/17		Ave	2016		2016/17		Ave	2016		2016/17		Ave
	Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3		Site 1	Site 2	Site 1	Site 3	
T1	5.0c	5.0b	4.7c	4.7b	4.85	44.5b	42.0c	34.3d	54.6c	43.9	30.1c	29.7b	28.9d	28.9d	29.4	189.6c	133.7c	102.8d	246.8d	168.2
T2	7.0b	6.5a	6.2b	5.7ab	6.35	50.5a	46.4b	40.5c	60.7b	49.5	32.4b	30.4ab	30.5c	30.5c	30.9	221.8b	163.7b	131.7c	314.2c	207.9
T3	7.7ab	6.3a	7.3a	6.0a	6.80	52.2a	47.6b	46.8b	60.4b	51.8	34.0ab	31.2ab	32.9b	33.2b	32.8	252.8a	162.2b	162.4b	346.6b	231.0
T4	8.0a	7.0a	7.7a	6.5a	7.30	53.9a	51.8a	52.6a	64.7a	55.8	35.4a	32.9a	34.0a	38.2a	35.1	262.7a	188.5a	188.6a	369.7a	252.4
CV%	8.35	12.56	9.05	13.15	---	8.06	5.73	3.04	3.98	---	5.28	7.10	2.08	3.22	---	9.97	7.50	1.86	2.09	---

§ Values are means of six replicates. Means followed by different letters on the same column are significantly different according to the comparison by the Duncan test at $p < 0.05$,£ Ave = average across sites and cropping seasons.

Table 6. Plant vigor ratings, numbers of leaves, average head diameter, and average head weight of lettuce plants inoculated with a mixed inoculant containing *Bacillus subtilis* strain CCTB 04, *B. pumilus* strain CCTB05, and *B. amyloliquefaciens* strain CCTB 09 (Product D)§, 2017/2018 season.

	VIGOR					NUMBER OF LEAVES					HEAD DIAMETER (cm)					AVERAGE HEAD WEIGHT (g)				
	Site 4	Site 5	Site 6	Site 7	Ave£	Site 4	Site 5	Site 6	Site 7	Ave	Site 4	Site 5	Site 6	Site 7	Ave	Site 4	Site 5	Site 6	Site 7	Ave
T1	4.7c	4.8b	3.7c	4.5b	4.42	24.3b	36.5d	23.6d	37.8ns	30.6	21.3b	24.1c	18.5b	19.8b	20.9	81.3c	147.9d	42.6c	122.8b	168.2
T2	6.3b	6.7a	4.7ab	4.7ab	5.60	27.6a	41.8c	26.0c	39.6	33.8	23.5a	26.6b	21.0a	21.3ab	23.1	124.4b	187.1c	78.2b	132.6b	207.9
T3	6.5ab	6.7a	4.1bc	5.1ab	5.60	29.3a	45.1b	28.1b	40.6	35.8	24.2a	29.5a	22.0a	23.3a	24.7	139.1a	236.6b	82.9b	164.1a	231.0
T4	7.0a	6.3a	5.5a	5.8a	6.15	29.5a	47.8a	29.9a	42.0	37.3	24.2a	29.2a	22.0a	23.8a	24.8	137.9a	267.8a	89.5a	165.4a	252.4
CV %	8.21	19.30	15.18	19.36	---	7.01	3.93	5.04	9.21	---	7.53	3.10	5.76	6.33	---	7.07	6.58	6.44	11.52	---

§ Values are means of six replicates. Means followed by different letters on the same column are significantly different according to the comparison by the Duncan test at $p < 0.05$; ns = not significantly different. £ Ave = average across sites.

bacteria were especially efficient to improve plant vigor and the weight of the lettuce heads, both with an average 49% increase over the control.

One strain of *B. subtilis* (BS 21-1) has been previously demonstrated to improve plant height and leaf width of four different crops, including lettuce, and to help suppress soft rot of Chinese cabbage and lettuce, when plants were challenged by pathogen inoculation[23]. We have not detected any disease incidence in our experiments, but the positive responses of plant growth, expressed as increased plant vigor, numbers of leaves and average weight of lettuce heads could be attributed to the strain of *B. subtilis* we have employed. Other authors[37] have verified an average 30% increase in the weight of lettuce heads produced with inoculation of *B. subtilis*, which was associated with the production and secretion of cytokinin and auxin by the tested strain. We have not evaluated the production of plant-growth hormones by our strain, but since this biochemical trait is common among strains of *B. subtilis*[38], the increases we observed in our experiments may be related to it.

Gains in plant vigor and average head weight upon inoculation with *B. pumilus* were slightly higher than for *B. subtilis*. These results may be related to the ability of strain CCTB 3203 to produce indole-acetic acid, which is not produced by *B.* subtilis strain CCTB 2720 (data not shown). Furthermore, Adesemoye et al.[39] have demonstrated that one strain (T4) of *B. pumilus* is able to increase N uptake by tomato plants that received only 80% of the recommended dose of N fertilizer. We have not determined the N content in the leaves of our plants, but our results strongly suggest that the strain we employed was able to stimulate N uptake by the lettuce plants even when only 50% of the N fertilizer dose was applied, as the presence of the PGPB improved the results over the treatment with 50% N alone. In addition, strains of *B. pumilus* that produce the enzyme ACC-deaminase have been shown to sustain root and plant growth and development due to the inhibition of ethylene synthesis by the plants under stress conditions[21]. We have not determined if strain CCTB05 produces ACC-deaminase, but if we consider that reduced levels of an important nutrient such as N in the soil represent an abiotic stress for the plant, it is reasonable to speculate that the positive results we observed may have been due to the enzymatic activity of the bacteria, especially since ACC-deaminase-producing strains of *Pseudomonas fluorescens* have been sown to perform better as PGPB under reduced levels of soil nitrate[40].

Inoculation with *B. amyloliquefaciens* also resulted in gains over 50% in plant vigor and average head weight over the control. Earlier studies[19] demonstrated that this species produced indoles, lipopeptides and proteins that stimulated soybean growth both in the laboratory and in the greenhouse. Since this species produces auxins[17], which are known to favor the development of secondary roots and root hairs, improving water- and nutrient-uptake and plant growth[41], the positive results we observed may be due to hormone production by the strain we used. Furthermore, strains of *B. amyloliquefaciens* have been implicated in the induction of plant systemic resistance to pathogens[42]. Since lettuce is quite susceptible to foliar diseases and no measures for disease control have been adopted in our experiments, it is possible that the presence of the inoculated bacteria may have helped plants suppress any casual incidence of pathogens.

The combination of all three species of *Bacillus* also promoted gains in plant vigor and average head weight. Mixed inoculants containing bacilli have been successfully employed in experiments before. For example, Adesemoye et al.[43] have successfully employed a mixture of *B. pumilus, B. amyloliquefaciens* and mycorrhizal fungus that allowed a 25% reduction in the rate of fertilization for tomato. As mentioned before[14], several species of the genus *Bacillus* have been implicated in plant growth promotion by a diversified array of mechanisms. For example, *B. subtilis* has 4 – 5% of its genome devoted to producing antimicrobial compounds[38]. *B. pumilus* and *B. amyloliquefaciens* have also been shown to produce substances with antimicrobial activity[45,46]. The antimicrobial activity of these species may account for protection against pathogens and the presence of two or more species in the inoculants may bring complementary effects.

In our experiments, all three species of *Bacillus* tested, as well as their mixture were effective to promote growth of lettuce, with a concomitant 50% reduction in the amount of

N fertilizer applied to the crop. This is the first report of such data in Brazil, supporting the recommendation of the products for lettuce inoculation.

ACKNOWLEDGEMENTS

The authors acknowledge support from Total Biotecnologia Industria e Comercio S/A, for providing the products for test and for planting and conducting the experiments, as well as the staff of Estacao Experimental Agricola Campos Gerais, Universidade Estadual de Ponta Grossa, for planting and conducting the experiments, handling samples and analyzing data.

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

FUNDING

The work presented here has been fully funded by Total Biotecnologia Industria e Comercio S/A.

AUTHORS' CONTRIBUTIONS

WSV, JMG and ASN have contributed to set up and conduct experiments, as well as to collect samples and analyze data. MH has performed the genetic characterizations of the strains and has written the results of such analyses and provided Figure 1. RSA has contributed with aid in data analyses and manuscript preparation. All authors have helped review the manuscript before submission for publication.

DATA AVAILABILITY

All datasets generated and analyzed during this study are included in this manuscript. Raw data are available upon request from Total Biotecnologia Industria e Comercio S/A.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Kim MJ, Moon Y, Tou JC, Mou B, Waterland NL. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *Journal of Food Composition Analysis*, 2016; **49**: 19-34. https://doi.org/10.1016/j.jfca.2016.03.004
2. Barriere V, Lecompte F, Nicot P, Maisonneuve B, Tchamitchian M, Lescourret F. Lettuce cropping with less pesticides. A review. *Agronomy for Sustainable Development*, 2014; **34**: 175-198. https://doi.org/10.1007/s13593-013-0158-5
3. Resende FV, Saminoz TCO, Idal MC, Souza RB de, Clemente FMV. Cultivo de alface em sistema organico de production. Brasilia, DF, Embrapa: *Circular Tecnica 56*. (in Portuguese), 2007.
4. Bertossi APA, Thomazini A, Fonseca AS da, Amaral JFT. do. Nutritional efficiency of phosphorus in lettuce. *Journal of Agricultural Science*, 2013; **5**: 125-131. https://doi.org/10.5539/jas.v5n8p125
5. Zambon FRA. Nutricao mineral da alface (*Lactuca sativa* L.), 1982; pp. 316-348 In Muller J.J.V., Casali V.W.D., (eds.), Seminirios de Olericultura (in Portuguese). 2nd ed. Vinosa: Universidade Federal de Vinosa.
6. Oshe S, Dourado-Neto D, Manfron PA, Santos OS. Qualidade de cultivares de alface produzidas em hidroponia. *Scientia Agricola* (in Portuguese), 2001; **58**: 181-185. https://doi.org/10.1590/S0103-90162001000100027
7. Vale M, Seldin L, Aranjo FF, Lima R. Plant growth promoting rhizobacteria: fundamentals and applications, 2010; pp. 21-43 In Maheshwari DK, (ed.), Plant growth and health promoting bacteria. Berlin: *Springer*. https://doi.org/10.1007/978-3-642-13612-2_2
8. Bashan Y, de-Bashan LE, Prabhu SR, Hernandez J. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1988 – 2013). *Plant and Soil*, 2014: **378**: 1-33. https://doi.org/10.1007/s11104-013-1956-x
9. Hungria M, Mendes IC. Nitrogen fixation with soybean: the perfect symbiosis?, 2015; pp. 1005-1019. In de Bruijn FJ (ed.), Biological Nitrogen Fixation. Hoboken (NJ): Wiley & Sons. https://doi.org/10.1002/9781119053095.ch99
10. Souza R, Ambrosini A, Passaglia LMP. Plant growth-promoting bacteria inoculants in agricultural soils. *Genetics and Molecular Biology*, 2015; **38**: 401-419. https://doi.org/10.1590/S1415-475738420150053
11. de-Bashan LE, Hernandez JP, Bashan Y. The potential contribution of plant growth-promoting bacteria to reduce environmental degradation – A comprehensive evaluation. *Applied Soil Ecology*, 2012; **61**: 171-189. https://doi.org/10.1016/j.apsoil.2011.09.003
12. Nehra V, Choudhary M. A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture. *Journal of Applied and Natural Science*, 2015; **7**: 540-556. https://doi.org/10.31018/jans.v7i1.642
13. Yasmin S, Zaka A, Imran A, Zahid MA, Yousaf S, Rasul G, Arif M, Mirza MS. Plant growth promotion and suppression of bacterial leaf blight in rice by inoculated bacteria. *PLos ONE*, 2016; **8**: 1-19. https://doi.org/10.1371/journal.pone.0160688
14. Kumar A, Prakash A, Johri BN. *Bacillus* as PGPR in crop ecosystem, 2011; pp. 37-59. In Maheshwari

DK (ed.), Bacteria in agrobiology: crop ecosystems. Berlin Heidelberg: Springer Verlag. https://doi.org/10.1007/978-3-642-18357-7_2

15. Gutierrez-Manero FJ, Ramos B, Probanza A, Mehouachi J, Talon M. The plant growth promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberelins. *Physiologia Plantarum*, 2001; **111**: 206-211. https://doi.org/10.1034/j.1399-3054.2001.1110211.x
16. Whipps JM. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, 2001; **52**: 487-512. https://doi.org/10.1093/jxb/52.suppl_1.487
17. Idris EES, Iglesias DJ, Talon M, Borriss R. Tryptophan-dependent production of Indole-3-Acetic Acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Molecular Plant-Microbe Interactions*, 2007; **20**: 619-626. https://doi.org/10.1094/MPMI-20-6-0619
18. Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, 2009; **321**: 305-339. https://doi.org/10.1007/s11104-009-9895-2
19. Buensanteai N, Yuen GY, Prathuangwong S. The biocontrol bacterium *Bacillus amyloliquefaciens* KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plants. *Journal of Agricultural Science*, 2008; **41**: 3-4.
20. Stefan M, Dunca S, Olteanu Z, Oprica L, Ungureanu E, Hritcu L, Mihasan M, Cojocaru D. Soybean (*Glycine max* [L.] Merr.) inoculation with *Bacillus pumilus* RS3 promotes plant growth and increases seed protein yield: relevance for environmentally-friendly agricultural applications. *Carpathian Journal of Earth and Environmental Sciences*, 2010; **5**: 131-138.
21. Saleem M, Arshad M, Hussain S, Bhatti AS. Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *Journal of Industrial Microbiology and Biotechnology*, 2007; **34**: 635-648. https://doi.org/10.1007/s10295-007-0240-6
22. Kaushal M, Kumar A, Kaushal R. *Bacillus pumilus* strain YSPMK11 as plant growth promoter and biocontrol agent against *Sclerotinia sclerotiorum*. *3 Biotech*, 2017; **7**: 90-98. https://doi.org/10.1007/s13205-017-0732-7
23. Lee SW, Lee SH, Balaraju K, Park KS, Nam KW, Park JW, Park K. Growth promotion and induced disease suppression of four vegetable crops by a selected plant growth-promoting rhizobacteria (PGPR) strain *Bacillus subtilis* 21-1 under two different soil conditions. *Acta Physiologiae Plantarum*, 2014; **36**: 1353-1362. https://doi.org/10.1007/s11738-014-1514-z
24. Kilian M, Steiner U, Krebs B, Junge H, Scmiedeknecht G, Hain R. FZB24 *Bacillus subtilis* – mode of action of a microbial agent enhancing plant vitality. *Pflanzenschutz Nachrichten Bayer*, 2000; **1**: 72-93.
25. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, 1994; **5**: 25-40.
26. Kaschuk G, Hungria M, Andrade D, Campo R. Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. *Applied Soil Ecology*, 2006; **32**: 210-220. https://doi.org/10.1016/j.apsoil.2005.06.008
27. Sneath PH, Sokal RR. 1973. Numerical taxonomy: The principles and practice of numerical classification. W. H. Freeman and Company, San Francisco.
28. Jaccard P. The distribution of the flora in the alpine zone. *New Phytologist*, 1912; **11**: 37-50. https://doi.org/10.1111/j.1469-8137.1912.tb05611.x
29. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 1991; **132**: 697-703. https://doi.org/10.1128/jb.173.2.697-703.1991
30. Delamuta JRM, Menna P, Ribeiro RA, Hungria M. Phylogenies of symbiotic genes of *Bradyrhizobium* symbionts of legumes of economic and environmental importance in Brazil support the definition of the new symbiovars pachyrhizi and sojae. *Systematic and Applied Microbiology*, 2017; **40**: 254-265. https://doi.org/10.1016/j.syapm.2017.04.005
31. Alvares CA, Stape JL, Sentelhas PC, Moraes Goncalves JL, Sparovek G. Koppen's climate classification map for Brazil. *Meteorologische Zeitschrift*. 2013; **22**: 711-728. https://doi.org/10.1127/0941-2948/2013/0507
32. Meier U. (ed.). Growth stages of mono- and dicotyledonous plants. BBCH Monograph. Quedlingburg: Julius K hn-Institut (JKI), 2018.
33. Canteri MG, Althaus RA, Virgens Filho JS, Giglioti EA, Godoy CV. SASM-Agri: Sistema para analise e separacao de medias em experimentos agricolas pelos metodos Scoft-Knott, Tukeye Duncan *Revista Brasileira de Agrocomputao* (in Portuguese), 2001; **1**(2): 18-24.
34. Stoate C, Baldi A, Beja P, Boatman ND, Herzon I, van Doorn A, de Snoo GR, Rakosky L, Ramwell C. 2009. Ecological impacts of early 21st century agricultural change in Europe – a review. *Journal of Environmental Management*, 2009; **91**: 22-46. https://doi.org/10.1016/j.jenvman.2009.07.005
35. Ruzzi M, Aroca R. Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Scientia Horticulturae*, 2015; **196**: 124-134. https://doi.org/10.1016/j.scienta.2015.08.042
36. Armada E, Portela G, Roldan A, Azcon R. Combined use of beneficial soil microoorganism and agrowaste residue to cope with plant water limitation under semiarid conditions. *Geoderma*, 2014; **232**: 640-648. https://doi.org/10.1016/j.geoderma.2014.06.025
37. Arkhipova TN, Veselov SU, Melentiev AU, Marttynenko EV, Kudoyarova GR. Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. *Plant and Soil*, 2005; **272**: 201-209. https://doi.org/10.1007/s11104-004-5047-x
38. Idris EES, Bochow H, Rosss H, Boriss F. Use of *Bacillus subtilis* as biocontrol agent. 6. Phytohormone action of culture filtrate prepared from plant growth promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. *Journal of Plant Diseases and Protection*, 2004; **111**: 583-597.
39. Adesemoye AO, Torbert HA, Kloepper JW. Increased plant uptake of nitrogen from ^{15}N-depleted fertilizer

using plant growth-promoting rhizobacteria. *Applied Soil Ecology*, 2010; **46**: 54-58. https://doi.org/10.1016/j.apsoil.2010.06.010

40. Shaharoona B, Naveed M, Arshad M, Zahir ZA. Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield and nutrient use efficiency of wheat (*Triticum aestivum* L.). *Applied Microbiology and Biotechnology*, 2008; **79**: 147-155. https://doi.org/10.1007/s00253-008-1419-0
41. Aloni R, Aloni E, Langhans M, Ullrich CI. Role of auxin in regulating *Arabidopsis* flower development. *Planta*, 2006; **223**: 315-328. https://doi.org/10.1007/s00425-005-0088-9
42. Kloepper JW, Ryu CM, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology*, 2004; **94**: 1259-1266. https://doi.org/10.1094/PHYTO.2004.94.11.1259
43. Adesemoye AO, Torbert HA, Kloepper JW. Plant growth-promoting rhizobacteria allowed reduced application rates of chemical fertilizers. *Microbial Ecology*, 2009; **58**: 921-929. https://doi.org/10.1007/s00248-009-9531-y
44. Stein T. *Bacillus subtilis* antibiotics: structures, synthesis and specific functions. *Molecular Microbiology*, 2005; **56**: 845-847. https://doi.org/10.1111/j.1365-2958.2005.04587.x
45. Peypoux F, Bonmatin JM, Wallach J. Recent trends in the biochemistry of surfactin. *Applied Microbiology and Biotechnology*, 1999; **51**:553-563. https://doi.org/10.1007/s002530051432
46. Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *Journal of Bacteriology*, 2004; **186**: 1084-1096. https://doi.org/10.1128/JB.186.4.1084-1096.2004