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Alteraciones bioquímicas y del desarrollo de dos cultivares de tomate bajo la inoculación de diferentes dosis de *Bacillus spp.*

Biochemical and developmental alterations of two tomato cultivars under after inoculation with different doses of Bacillus spp.

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ABSTRACT

The use of plant growth-promoting bacteria contributes to the sustainability of agriculture. Metabolic and morphometric variables, and their correlation with the initial growth of two tomato cultivars, were assessed in this study. This was done to evaluate the effect of seeds inoculated with *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 in two doses: 20% of bacteria suspension as inoculant solution (*Ba*20) and 80% of bacteria suspension as inoculant solution (*Ba*80), equating to 1.6×10^5 and 6.4×10^6 bacteria per seed, respectively. Bacterial inoculation increased the growth of roots at different rates, from 14.56% (total length in the 'Santa Clara I-530' cultivar) up to 197.13% (root volume of the 'Cherry 261' cultivar). Increments in the aerial part also varied from 12.06% (leaves area in 'Santa Clara I-530') up to 88.88% (dry matter in 'Cherry 261'). Increases in the root system with Ba20 were also correlated with an increase in the height and weight of the fresh matter of plant aerial parts. Metabolite concentration (sugars and soluble proteins) was closely related to leaf expansion according to the response of cultivars and doses. Ba20 (1.5×10^5 bacteria per seed) showed plant growth effects on two cultivars of tomato, which is promising for future studies.

Keywords: plant growth-promoting bacteria, bio-fertilizer, metabolites, soluble sugar, soluble protein.

RESUMEM

El uso de bacterias promotoras del crecimiento de planta puede contribuir a la sostenibilidad de la agricultura. Metabólicos y variables morfométricas, y su correlación con el crecimiento inicial de los cultivares de tomate fue analizado en este estudio, con el objetivo de evaluar el efecto de la inoculación de semillas con Bacillus amyloliquefaciens *subs.* plantarum *FZB42, usando dos dosis: 20% de suspensión de bacterias en la solución de inoculante (Ba20) y 80% de suspensión de bacterias en la solución de inoculante (*Ba*80), la concentración estimada es 1,6 x 10^5 y 6,4 x 10^6 bacterias por semilla, respectivamente. La inoculación he proporcionado incremento de crecimiento en las raíces en proporciones diferentes, que van desde 14,56% (longitud total en la variedad 'Santa Clara I-530') a 197,13% (volumen en el cultivar 'Cherry 261'). Mientras que los incrementos de parte aérea variaron de 12,06% (área de las hojas de 'Santa Clara I-530') al 88,88% (materia seca de 'Cherry 261'). Los aumentos del sistema radicular en* Ba*20 se refleja en aumentos en la altura y peso de materia fresca de la parte aérea. Una concentración de metabolitos (azúcares y proteínas solubles) estaba estrechamente relacionada con la expansión de las hojas de acuerdo con la respuesta del cultivar y dosis. El Ba20 (1,6 x 10^5) mostró efecto significativo de la promoción del crecimiento vegetal en dos cultivares de tomate en crecimiento inicial, siendo prometedor para futuros trabajos.*

Palabras clave: *bacterias promotoras del crecimiento vegetal, bio-fertilizantes, metabolitos, azúcar soluble, proteínas solubles.*

Introduction

Sustainable agriculture can be assisted by bacteria that are able to increase plant growth. Several species have been tested in this regard and among them *Bacillus* is notable. Some examples of this includes: *B. subtilis*, which promoted growth in tomato by the production of volatile organic compounds (Tahir *et al*., 2017); *B. cereus, B. macrolides,* and *B. pumilus*, which stimulated development in red pepper (Joo *et al.,* 2004); and *B. megaterium*, *B. safensis*, and *B. simplex*, which increased growth in corn, soybean, and wheat (Akinrinlola *et al*., 2018). Additionally,

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B. velezensis, formerly referred as *B. amyloliquefaciens* subsp. *plantarum* (Dunlap *et al*., 2016), increased growth in tomato seedlings by seed inoculation due to the capacity of auxin production by the bacteria (Szilagyi-Zecchin *et al.,* 2015a). This same species also promoted plant growth in beet, carrot, cucumber, pepper, potato, radish, squash, tomato, and turnip stimulated by volatile compounds released by some strains of this species (Meng *et al*., 2016).

Some of these bacteria, besides producing and providing phytohormones, can metabolize hormone precursors in plants. Hormone concentration is fundamental for the regulation of some physiological processes, and the levels of these hormones modified by microorganisms may lead to variations in plant growth and development (Meng *et al*., 2016). In addition, seeds inoculated with plant growth-promoting bacteria may undergo changes in metabolite production of the primary metabolism, which includes the production of carbohydrates, proteins, and amino acids (Kang *et al.,* 2014).

The changes stimulated by plant growth-promoting bacteria have been explored in agriculture to improve production. In this work, we used tomato (*Solanum lycopersicum* L.), which is cultivated all over the world, and has great economic and social relevance. In addition to, its consumption as a whole fruit, it is consumed in many industrialized forms such as juices, sauces, and pastes, and also in its dehydrated form (FAOSTAT, 2011). Assessing the effect of one strain of plant growth-promoting bacteria may, therefore, contribute to the sustainability of tomato production.

The aim of the present study was to verify biometric and biochemical alterations in the growth of two tomato cultivars inoculated with *Bacillus amyloliquefaciens* subs. *plantarum* FZB42, and to identify its potential as an inoculant or bio-fertilizer.

Material and methods

We used the seeds of two tomato cultivars, 'Santa Clara I-530' (Isla®) and 'Cherry 261' (Isla®), with cycles of 110 and 90 days, respectively, both with undetermined growth rates. Inoculation was performed using *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 (Omex® Agrifluids do Brasil Ltda) registered as strain 10A6 in the *Bacillus* Genetic Stock Center (BGSC, Ohio, U.S.A.). The experiment was conducted at the Federal University of Paraná (UFPR) in the city of Curitiba, Brazil, in a greenhouse located in the Organic Production Research Area.

Seeds were not subjected to chemical treatment and were inoculated with a solution of bacteria at a concentration of 1×10^{11} CFU mL^{-1} without asepsis. Seeds were dried on paper towels without light and immediately sown. Treatments corresponded to 320 µL g^{-1} per seed (Szilagyi-Zecchin *et al.,* 2015a) in the following proportions: *Ba*0 as the control with 100% distilled water; *Ba*20 with 20% bacteria solution and 80% distilled water; and *Ba*80 with 80% bacteria solution and 20% distilled water. The treatments of *Ba*20 and *Ba*80 contained 1.6×10^5 and 6.4×10^6 bacteria per seed, respectively.

Seeds were sown in expanded polystyrene trays with 200 cells filled, with composted bird bedding mixture (Provaso®) and *Pinus* composted bark (Vida Verde®) in a 0.5:3.5 proportion. One seed per cell was placed at a depth of 1 cm. An automatic micro-aspersion irrigation system was used during the day for three minutes every two hours.

Thirty days after sowing, the seedlings were transferred to 2 L plastic vessels filled with the same substrate used in their production. One plant per vessel was maintained, and the vessels were placed in a greenhouse for 30 days. The experimental design was completely randomized with 2 × 3 factorial scheme (tomato cultivars × bacterium doses and control), where each treatment consisted of seven replications totaling 42 plants.

Morphometric analysis

Sixty days after sowing, we evaluated the following variables: area of leaves (cm^2), total root length (cm), volume of roots (cm^3), and volume of the aerial part (cm^3). For this data, we used the computational software WinRhizo® coupled to an LA1600 scanner (Règent Instruments Inc., Canada). Weights (g) of fresh and dry matter of roots, and that of the aerial parts, were obtained by drying the matter in a forced ventilation oven at 60 ºC until a constant weight was reached, before being measured using an analytical precision scale. We also evaluated stem diameter (cm) at the insertion point of the first leaf from the base, the height of the plant (cm) from the base to the insertion point of the last leaf, the number of leaves, and the number of flower buds per plant.

Biochemical analysis

We collected samples from the middle of the third and fourth leaves, counting from the top to the bottom. Leaves were collected between 9 and 10 a.m., 60 days after sowing, and then macerated with liquid nitrogen until a fine powder was obtained. Values are expressed in μg of metabolite per g of fresh leaf matter.

Chlorophylls and carotenoids were extracted according to the methods of Lichtenthaler (1987) with 80% acetone in distilled H_2O added to 0.1% CaCO3 (w/v). Readings were taken at 663 nm, 647 nm, and 470 nm. The equations described by Lichtenthaler and Buschmann (2001) were applied.

Reducing sugars were determined according to Miller (1959), and total sugars were determined according to the methods described by Maldonade *et al.* (2013). The standard curve was obtained with glycose at 1 mg mL^{-1} (5.5 mM), considering values between 50 and 800 µg mL^{-1}. Non-reducing sugars were calculated by subtracting the reducing sugars from the total sugars.

Extraction of soluble proteins was performed according to Du *et al.* (2010), and the colorimetric reaction was performed according to Bradford (1976). The standard curve was constructed using bovine serum albumin (BSA) at 0.2% (w/v), with values between 28 and 140 µg mL^{-1}.

Statistic

Data were subjected to ANOVA, and means were compared at 5% and 1% significance levels. Pearson's correlation test was also performed considering all the described variables. All statistical analyses were performed using the software Assistat 7.7 Beta (Silva and Azevedo, 2002).

Results and Discussion

Morphometric analysis

There was an interaction between doses and cultivars for all variables referred to the root system (Table 1). In the cultivar 'Cherry 261' (Ch261) the

Table 1. Morphometric analysis of plants of two tomato cultivars inoculated with *Bacillus amyloliquefaciens* subs. *plantarum* FZB42 (*Ba*) in a greenhouse trial. The analysis was performed 60 days after sowing. (GA) General Average, (CV) Coefficient of Variation. Santa Clara I-530 (SC530) and Cherry 261 (Ch261) cultivars.

	SC530	Ch261	SC530	Ch261	SC530	Ch261	SC530	Ch261
				Root				
	Volume (cm^3)		Length (cm)		Fresh weight (g)		Dry weight (g)	
*Ba*0	1.61^{aA}	1.74^{cA}	625.29^{bB}	402.62^{cB}	1.76^{bA}	1.56^{cA}	0.08^{aA}	0.05^{cB}
*Ba*20	1.80^{aB}	5.17^{aA}	716.36^{aB}	904.54^{aA}	2.50^{aB}	4.51^{aA}	0.10^{aB}	0.13^{aA}
*Ba*80	1.21^{bB}	4.42^{bA}	594.54^{bB}	809.52^{bA}	1.20^{cB}	3.67^{bA}	0.06^{bB}	0.11^{bA}
GA	1.54	3.78	645.40	705.56	1.82	3.25	0.08	0.10
CV%	10.49		7.08		15.58		16.30	
				Shoot				
	Flower bud number		Stem diameter (cm)		Plant height (cm)		Leaf number	
*Ba*0	0.00	4.14^{b}	3.09	3.74	26.41^{aA}	17.69^{bB}	6.65^{aB}	7.48^{bA}
*Ba*20	0.00	10.71^{a}	3.11	3.87	27.73^{aA}	24.97^{aA}	6.65^{aB}	10.15^{aA}
*Ba*80	0.00	11.50^{a}	2.85	3.81	21.20^{bB}	25.07^{aA}	5.45^{bB}	10.33^{aA}
GA	0.00	8.78	3.02^{B}	3.81^{A}	25.12	22.58	4.25	9.32
CV%	14.32		9.02		11.27		6.92	
	Area (cm^2)		Volume (cm^3)		Fresh weight (g)		Dry weight (g)	
*Ba*0	612.56^{bA}	361.32^{cB}	13.69^{aA}	4.38^{bB}	9.25^{bB}	20.75^{bA}	0.83^{bA}	0.36^{bB}
*Ba*20	686.46^{aA}	764.26^{aA}	14.00^{aA}	10.73^{aB}	11.67^{aB}	28.77^{aA}	1.02^{aA}	0.68^{aB}
*Ba*80	382.50^{cA}	617.68^{bA}	6.83^{bA}	8.05^{aA}	5.62^{cB}	28.61^{aA}	0.45^{cB}	0.82^{aA}
GA	560.51	360.98	11.51	7.72	8.85	26.04	0.77	0.62
CV%	13.30		29.75		12.39		17.01	

Note: Values followed by different letters in each column between doses and in each line between cultivars are significantly different ($p \leq 0.01$) in the Scott-Knott test.

two doses stimulated volume increase and *Ba*80 inoculation promoted growth of approximately 300%, while in the cultivar 'Santa Clara I-530' (SC530), volume decreased when inoculated with this treatment. Total root length increased twice in Ch261 with both bacterial concentrations and SC530 only presented an increase in root length after *Ba*20 inoculation.

In addition to volume and length, the values of fresh and dry matter of the roots showed differences between cultivars, highlighting *Ba*20 as the dose that promoted the highest increases. SC530 plants inoculated with *Ba*80 showed lower fresh and dry matter when compared to the control, indicating high sensitivity to the dose. Conversely, the two doses in Ch261 showed twice the weight gain compared to the control.

The promotion of root growth following inoculation with *Bacillus* strains was also reported by Kang *et al.* (2014) using *B. megaterium* in mustard plants, where they found an increase of 21.79% in root length, and by Cendales *et al.* (2017) who tested *B. subtilis* and observed significant increases in length and fresh weight of stems and roots in tomato seedlings after 37 days. Szilagyi-Zecchin *et al.* (2015b) reported that *Bacillus* sp. promoted a 65.1% increase in the root length of corn plants 30 days after inoculation, linking this effect to the auxin released by *Bacillus* sp. strains.

In this study, the increases observed in the roots are probably related to auxinic stimulation provided by *B. amyloliquefaciens* subsp. *plantarum* FZB42, as this strain can produce indole compounds (Szilagyi-Zecchin *et al.,* 2015a) and auxins, such as indole acetic acid (IAA). These play a fundamental role in cell elongation and root development.

Once synthesized, the auxin produced by microorganisms is released and can enter the root cells in sufficient quantity to modify development and normal plant growth (Sukumar *et al*., 2013). Therefore, the differences in root growth between Ch261 and SC530 may be associated with the release of microbial indole compounds. IAA released by bacteria stimulates increases in root growth, but if it is not in ideal concentrations, it can inhibit plant growth depending on the number of compounds and the sensitivity of the plant tissue (Ali *et al.,* 2010; Duca *et al*., 2014). This is as we observed in the root growth of SC530 inoculated with *Ba*80.

There was no correlation between cultivars and doses for stem diameter, but the cultivars did show differences between them (Table 1). The variables of the aerial parts of plants, such as the number, volume, and area of leaves; plant height; and the weight of fresh and dry matter, showed correlation between doses and cultivars.

On average, the control in Ch261 exhibited 4.14 mature flower buds (totally developed buds, open or partially open), while plants inoculated with *Ba*20 and *Ba*80 had 10.71 and 11.5 mature buds, respectively. Therefore, according to the Solenaceae phenological growth stage identification key (Feller *et al.,* 1995), the growth stage in the control plants was 5 (based on the appearance of flowers), while inoculated plants were already in growth stage 6, with the plants fully flowered and showing open flowers. This indicates that inoculation accelerated plant development.

Plant height results showed that SC530 plants treated with *Ba*80 were shorter than the control, while Ch261 plants were taller than the control when inoculated with *Ba*20 or *Ba*80 as a probable response to the bacterial indole compounds. These can promote or inhibit plant growth - for example, the IAA produced by bacteria acts together with the endogenous plant supply, and thus, the impact of bacterial IAA is closely related to the sensitivity of the tissues (Ali *et al.,* 2010).

We observed the same tendency for the number of leaves, in which SC530 inoculated with *Ba*80 had the lowest number, while the control and *Ba*20 inoculation did not differ. The opposite was observed in Ch261, which presented approximately three more leaves when inoculated with both doses. Similar results were reported by Ahmed and Hasnain (2010), who described the effect of *Bacillus* strains increasing the number of leaves in *Solanum tuberosum* by approximately 33%.

Inoculation with *Ba*20 increased the area and volume of the leaves in both cultivars. In SC530, they decreased by approximately half when inoculated with *Ba*80. Meanwhile, Ch261 practically doubled its leaf area and volume under inoculation. Working with an auxin-producing strain of *Bacillus* sp., Szilagyi-Zecchin *et al.* (2015b) found an increase of 39.4% in the leaves of maize plants. Thus, the increase in the area and volume of leaves is associated with the auxin compounds released by *B. amyloliquefaciens* subsp. *plantarum* FZB42, which interacts with the tissues of each cultivar tested here.

In addition, considering the aerial parts of plants, we observed a similar pattern of reaction for

the weight of dry and fresh matter, in which *Ba*20 increased and *Ba*80 decreased weights in SC530. In Ch261, however, significant increases were observed under both doses. It is important to note that bacterial concentration reflects the quantity of microbial IAA that each plant was exposed to, and therefore, it determines whether the bacteria are promoting or inhibiting plant growth (Duca *et al.,* 2014). In general, SC530 inoculated with *Ba*80 presented lower results for almost all morphometric variables, except stem diameter and root length (Table 1), demonstrating that excessive doses can cause a decrease in plant growth. However, other factors cannot be ruled out, such as the presence and interaction with other metabolites besides auxin, which also contribute to plant growth inhibition.

Biochemical analysis

Levels of chlorophylls and carotenoids did not show any variation after inoculation (data not shown).

There was no interaction between cultivars and doses of inoculation for sugars, but differences between doses were indicated by the test of means (Table 2). Leaves of SC530 at both doses showed a decrease in total sugar content, and in Ch261, a decrease was observed in *Ba*20. Both cultivars increased in reducing sugar according to the dose. For non-reducing sugars, there were no differences in Ch261, whereas values decreased according to the dose in SC530.

The decrease in reducing sugars seen in Ch261 treated with *Ba*20 (Table 2) could be due to the use of these carbohydrates in cell wall building, which is associated with the increase in leaf volume (Table 1). In the same way, leaves of SC530 treated with *Ba*80 presented more reducing sugars than the other treatments, which was associated with the lowest area and volume of the leaves. Therefore, reducing sugar content is related to the expansion of leaves, which is corroborated by the negative correlations observed between morphometric and biochemical variables (Table 3).

We observed that the smaller the area of leaves and the weight of dry matter of the aerial part, the higher the resulting concentrations of carbohydrates, with regard to the minor expansion of leaves. This reduction in leaf development may be linked to excess auxin imposed by bacteria, as observed by Keller *et al.* (2004), who applied exogenous auxin at high concentrations in common bean (*Phaseolus vulgaris*) and *Arabidopsis*, and also found a reduction in the leaf area of these plants.

Other compounds linked to leaf size are proteins, and the analysis of total soluble proteins indicates that their concentrations are also intimately bound to leaf expansion and mass quantity, once the metabolites are expressed in $\mu g\ g^{-1}$ of fresh matter (Table 2). Therefore, we observed that the treatments with greater development of the aerial part (Ch261 *Ba*20 and *Ba*80 with 28.77 g and 28.61 g fresh weight, respectively) showed less soluble proteins. Conversely, the treatment with minor plant development (SC530 *Ba*80 with 5.62 g fresh weight) presented more protein per *g* of matter. Finally, the lower the leaf area and weight of dry matter of the aerial part, the higher the resulting concentration of protein.

Increases in the growth of roots and aerial components were, therefore, verified in both cultivars inoculated with *B. amyloliquefaciens* subs. *plantarum* FZB42 in *Ba*20. These increases varied in roots from 14.56% (total length, SC530) to 197.13% (root volume, Ch261) and in the aerial part from 12.06% (leaf area, SC530) to 88.88% (dry matter, Ch261). Comparing the root system and aerial parts by linear correlation analysis,

Table 2. Biochemical analysis of leaves of two tomato cultivars inoculated with *Bacillus amyloliquefaciens* FZB42 (*Ba*) in a greenhouse trial. The analysis was performed 60 days after sowing. (CV) Coefficient of Variation. Santa Clara I-530 (SC530) and Cherry 261 (Ch261) cultivars.

	SC530	Ch261	SC530	Ch261	SC530	Ch261	SC530	Ch261
	Total soluble sugars**		Reducing sugars		Non-reducing sugars		Total soluble proteins	
*Ba*0	9036.36[a]	2660.61[a]	1177.78[b]	1143.43[a]	7858.58[a]	1517.17[a]	460.57[b]	599.14[a]
*Ba*20	7939.39[b]	2436.36[b]	1060.61[b]	894.95[b]	6878.78[b]	1541.41[a]	462.00[b]	349.14[b]
*Ba*80	7175.76[b]	2824.24[a]	2391.92[a]	1335.35[a]	4783.84[c]	1488.89[a]	687.71[a]	394.86[b]
CV%	10.17	6.42	8.06	12.39	10.83	11.04	8.46	19.24

Note: Values followed by different letters in each column are significantly different ($p \leq 0.05$) in the Scott-Knott test, ** $p \leq 0.01$.

Pearson's coefficients showed positive values for both cultivars inoculated with *Ba*20 (Table 3).

Thus, increases in the root system with the *Ba*20 dose reflected an increase in plant height and weight of fresh matter of the aerial part and, for the Ch261 cultivar, the *Ba*80 dose provided a positive correlation between these variables. In contrast, negative correlations were observed in SC530 for *Ba*80 dose, and the growth of aerial parts was not correlated with the growth of roots. The percentage gain highlighted the positive effect on plant growth, and the positive linear correlations confirmed these growth stimulations on the aerial parts and roots of both cultivars treated with *Ba*20.

Besides all these aspects, it must be considered that the experiment was conducted with distinct tomato genotypes, and the variances in the cycle of growth concerning these genetic differences may influence their reaction to the inoculation. Ch261 had a shorter cycle (90 days to harvest) and already had flower buds, while SC530 had a 110-day cycle and did not flourish when the experiment ended. Consequently, they did not have the same physiological status.

Conclusions

Inoculation with *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 promotes plant growth in tomato cultivars 'Santa Clara I-530' and 'Cherry 261'. The *Ba*20 dose improved growth results for the aerial part of the plant as well as the root system. SC530 is more sensitive to the inoculation dose

Table 3. Correlation analysis between morphometric characteristics and metabolites of two tomato cultivars inoculated with *Bacillus amyloliquefaciens* subs. *plantarum* FZB42 (*Ba*) in a greenhouse trial (*p-value* > 0.0077). The analysis was performed 60 days after sowing.

Doses and Cultivars			r Pearson	p-value
*Ba*20 - Santa Clara I-530				
Root		Shoot		
length	x	height	0.9635	0.0364
length	x	fresh weight	0.9872	0.0128
*Ba*80 - Santa Clara I-530				
Root		Shoot		
length	x	leaves area	–0.7462	0.0537
length	x	dry weight	–0.9464	0.0513
Metabolites		Shoot		
total sugar	x	leaves area	–0.9575	0.0425
total sugar	x	dry weight	–0.9923	0.0077
reducing sugar	x	leaves area	–0.9575	0.0425
reducing sugar	x	dry weight	–0.9923	0.0077
non-reducing sugar	x	leaves area	–0.9575	0.0425
non-reducing sugar	x	dry weight	–0.9923	0.0077
protein	x	dry weight	–0.9909	0.0091
*Ba*20 - Cherry 261				
Root		Shoot		
length	x	height	0.9632	0.0367
dry weight	x	height	0.9726	0.0274
length	x	fresh weight	0.9511	0.0491
dry weight	x	fresh weight	0.9827	0.0173
*Ba*80 - Cherry 261				
Root		Shoot		
volume	x	leaves number	0.9575	0.0424
dry weight	x	leaves number	0.9513	0.0487

because with the *Ba*80 inoculation it resulted in less development in the aerial parts and the roots. Inoculation with *Ba*20 (*B. amyloliquefaciens* FZB42 at 1.5×10^5 bacteria per seed) is a promising treatment to enhance the sustainable production of tomato in terms of initial plant growth promotion. These findings also stimulate future studies aiming to verify the action of plant growth-promoting bacteria on yield.

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