Initial growth, production in consecutive years and biochemical changes on tomato cultivars in organic system with application of Bacillus subtilis

Crecimiento inicial, producción en años sucesivos y alteraciones bioquímicas en cultivares de tomate en sistema orgánico con aplicaciones de Bacillus subtilis

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ABSTRACT

In this study, Bacillus subtilis were applied on seeds, seedlings, and tomato crops for two years in an organic system. The product Serenade® was used on ‘Cardyna’ and ‘Trinidad’ cultivars with the objective of determining the effect of the bacteria on initial plant growth when applied to seeds (50 μL per seed) or the seedlings watered with 100 mL solution at 8 mL L⁻¹ concentration, and tomato yield when applied at the concentration of 4 mL L⁻¹ in the form of a soil drench, foliar sprays and their combination, considering their action in the promotion of growth and also in the induction of resistance allied to possible metabolic deviations. Seed application reduced initial plant growth, while seedling watering at transplanting promoted growth. Chlorophylls a, b and total chlorophyll, carotenoids, and the enzymes phenylalanine ammonia lyase, chitinase, and β1,3 glucanases were stimulated in a variable way among cultivars and applications, indicating the activation of plant defenses. Phenolic compounds, flavonoids, and total sugars were not altered, while the concentrations of reducing sugars were higher due to applications than those of non-reducing sugars. The metabolic changes did not influence the tomato yield.

Keywords: enzymes, Plant-growth promoting bacteria, resistance induction, Solanum lycopersicum L., yield.

RESUMEN

En este estudio se realizaron aplicaciones de Bacillus subtilis en semillas, mudas y en el cultivo del tomate durante dos ciclos en sistema orgánico. Se utilizó el producto Serenade® en los cultivares Cardyna y Trinidad. El objetivo fue determinar el efecto de la bacteria en el crecimiento inicial de las plantas cuando se aplica a las semillas (50 μl por semilla) o a las mudas (100 mL de solución en la concentración de 8 mL L⁻¹), y en la producción de los frutos cuando se aplica en la concentración de 4 mL L⁻¹ en forma de riego, pulverizaciones foliares y su combinación, teniendo en cuenta su acción en la promoción del crecimiento y también en la inducción de resistencia aliada a posibles desvíos metabólicos. La aplicación en las semillas redujo el crecimiento inicial de las plantas, mientras que el riego de las mudas en el momento del trasplante estimuló el crecimiento. Las clorofilas a, b y totales, los carotenoides y las enzimas fenilalanina amonio-lyase, quitinasa y β1,3glucanasas fueron estimuladas de forma variable entre cultivares y aplicaciones, indicando la activación de las defensas de las plantas. Los compuestos fenólicos, flavonoides y azúcares totales no se alteraron, mientras que las concentraciones de azúcares reductores fueron mayores debido a las aplicaciones que las de no reductores. Las alteraciones metabólicas no influyeron en la producción de los tomates.

Palabras clave: bacterias promotoras del crecimiento de las plantas, rendimiento, inducción de resistencia, enzimas, Solanum lycopersicum L.

Introduction

The application of alternative techniques such as the use of beneficial microorganisms in farming becomes relevant for food production. Regarding microorganisms, the use of growth-promoting bacteria and pant-resistance inducers is an option that is compatible with the criteria of sustainability, becoming an alternative for reducing the use of synthetic products (Shaharoona et al., 2008).
Hence, bacteria of the genus *Bacillus* are known to promote plant growth, as the effect reported by Harthmann et al. (2010) when identifying the action of *B. megaterium* on the growth and yield improvement in onions. Besides, *B. subtilis*-based products are used for the biological control of plant diseases (Halfeld-Vieira et al., 2016).

As a result, the activation of plant defenses through the synthesis of proteins such as chitinases and β1,3 glucanases in tomato plants was observed using of *B. subtilis*, indicating that this bacterium induces systemic resistance against certain pathogens (Chowdappa et al., 2013).

Resistance-inducers can be biotic or abiotic products. When applied to plants, they induce defense responses (Durrant and Dong, 2004), resulting in productivity gains as they reduce pathogen severity. However, the physiological cost of inducing resistance may result in changes in the growth and development of the plant, as reported by Karasov et al. (2017), due to the deviation of photoassimilates from the primary to the secondary metabolism of plants, which is related to the synthesis of the defense compounds.

Therefore, the objective of this study was to evaluate different ways of applying the commercial product Serenade® containing *B. subtilis* in two cultivars of tomato plants through the validation of its effects on the initial growth of the plants with application in seeds and seedlings. Its use was also assessed in the cultivation in an organic system in a protected environment in a high tunnel-type greenhouse. For both experiments, it was used seeds of ‘Cardyna’ and ‘Trinidad’ (HM. Clause®) tomato cultivars with an indeterminate habit, oblong fruits (Italian type) with an average weight of 160 grams, both with multiple tolerances (HM. Clause®), suitable for organic production.

**Experiment with applications in seeds and seedlings**

Sowing was carried out in expanded 200-cell polystyrene trays with unit cell volume corresponding to 18 cm³, filled with a substrate composed of composted poultry litter (Provaso®) combined with composted pine bark in an 1:2 ratio. One seed per cell was distributed at a depth of 1 cm. The seedlings were kept in a nursery with micro-spray timed irrigation until 30 days after sowing (DAS) when they were transplanted to 3.6-L pots filled with the same compounds and proportions.

The treatments consisted of two BS application methods: (i) inoculation of the seeds in 50 μL of BS for each seed, which were left to dry in the shade on paper towels and then were soon seeded, and at 30 DAS were transplanted to the pots; (ii) use of seedlings from non-inoculated seeds, watered with 100 mL of a solution containing 8 mL L⁻¹ of BS, applied to the pots at transplanting; (iii) seedlings from non-inoculated seeds and no application of a solution to the pots as a control. The experimental design was completely randomized, in a 3x2 factorial scheme, with one control and two forms of application of BS in two cultivars of tomato plants, with five replicates composed of two pots with one plant each, totaling ten pots per treatment.

The plants were collected 30 days after transplanting (DAT) when the first inflorescences appeared. The aerial part area was determined using WinRhizo® software coupled to a LA1600 Scanner (Regent Systems, Quebec, Canada), and fresh and dry mass of the leaves, stem, and root were determined.
The applications of the treatments started of 4 mL L⁻¹ of drench and foliar spraying. The concentration of the plant (drench), foliar spraying, and association forms were the following: watering in the lap of two cultivars) with four replications. The application arrangement (control and three forms of application x two cultivars) with four replications. The experimental design was completely randomized with a 4 x 2 factorial arrangement (control and three forms of application x two cultivars) with four replications. The application forms were the following: watering in the lap of the plant (drench), foliar spraying, and association of drench and foliar spraying. The concentration of 4 mL L⁻¹ of BS was used for both application forms. The applications of the treatments started at 21 DAT at a weekly frequency, continuing until the end of the crop, totaling 16 applications in the leaves and drench. An Herbicat® spray coupled to a CO₂ cylinder pressurizing at 40 psi.cm⁻² was used for spraying, providing uniform spraying coverage and avoiding run-off and drift, with a volume of 750 L.ha⁻¹. The drench was performed by distributing 100 mL of the solution near the lap of the plant.

Harvesting started at 106 and 109 days after sowing in the first and second cycles, respectively. All 10-raceme fruits were harvested and evaluated. Each fruit was harvested and evaluated when it was 2/3 reddish colored. The equatorial and longitudinal diameter and the fresh mass were determined. The leaves below each raceme were removed after the harvest of all their respective fruits, a practice that combined the rusticity of the cultivars and the protected environment, avoided the proliferation of foliar pathogens. Thus, no evaluation of incidence and severity was performed.

**Biochemical determination**

The biochemical analyses were carried out at the Biofertilizer Laboratory in the Crop Science Department at Federal University of Paraná (UFPR) in Curitiba, Brazil, and at the Technological Federal University of Paraná (UTFPR) in Dois Vizinhos, State of Paraná, Brazil. The samplings were carried out at the second tomato growth cycle when the leaves were removed at the early hours in the morning, between 7 and 8 hours, at 116 days after sowing, immediately above the 7th raceme, the middle third of the plant. The leaves were fully expanded with no symptoms of pathogen or pest attack or nutritional deficiencies. The leaflets were removed from the extremities, and the medium leaflets were trimmed, frozen, and macerated with liquid nitrogen. Triplicates were used to obtain the means.

At the UFPR, for the extraction of chlorophyll $a$ (663nm), chlorophyll $b$ (647 nm), and carotenoids (470 nm), the methodology described by Pompelli et al. (2013) was followed using acetone and calcium carbonate and spectrophotometer reading (BEL 2000 UV/VIS). Reducing and non-reducing sugars were determined using the DNS method. The phenolic compounds were determined by the adaptation of the Prussian Blue method. The flavonoids contents were determined using the adapted method of Eghdami and Sadeghi (2010), which is based on the formation of complexes that react with aluminum.
At the UTFPR, protein determination and phenylalanine ammonia-lyase activity (PAL), chitinase, and β1,3 glucanase enzymes were performed. The adaptation of the method described by Bradford (1976) in which the complete protein reading was performed in a 590-nm spectrophotometer using bovine serum albumin as standard to determine the total protein concentration. The determination of phenylalanine ammonia-lyase activity (PAL) was performed based on the difference in absorbance resulting from the conversion of phenylalanine to trans-cinnamic acid. For the determination of chitinase and ß-1,3-glucanase activities, the enzymatic activity of chitinase was assessed by the Busso and Mazaro (2015) method. For the spectrophotometric determination of ß-1,3-glucanase activities in the extracts, bright blue curdlan-remazol (Sigma Aldrich® - 4 mg mL⁻¹) was used as the substrate.

**Statistical analyses**

Data were tested for homogeneity of the variances using the test of Bartlett and submitted to ANOVA. When significant, means were compared by the test of Tukey (p < 0.05%; p < 0.01%), using Assistat® 7.7 Beta software.

**Results and discussion**

**Experiment with application in seeds and seedlings**

The results showed that BS watering at seedling transplant stimulated plant growth in both cultivars (Table 1), and consequently, the accumulation of fresh (Table 2) and dry (Table 3) mass in a variable manner between the cultivars over the initial growth.
The increase in the vegetative development of PGPB-treated plants, as observed in the BS watering treatment, identified by the increase in the aerial part area, particularly at the ‘Trinidade’ cultivar (Table 1), may be related to the cellular elongation promoted by the auxines, considering that Bacillus may be the producers of this hormone (Szilagyi-Zecchin et al., 2015).

According to Walia et al. (2014), auxin is quantitatively the most abundant hormone secreted by PGPB besides being the main factor responsible for the higher growth of plants submitted to treatments with these microorganisms, both roots and aerial part, as shown in Table 2. Moreover, the mean values of the fresh mass of leaves, stems, and roots increased through watering with BS. Among cultivars, ‘Trinidade’ presented the most significant values of the fresh mass of leaves, stems, and roots.

Adesemoye et al. (2008) report that after 60 DAS, a period equivalent to that in this study, the dry mass of tomato plants treated with B. subtilis increased significantly, corroborating what was observed in the present study with ‘Trinidade,’ which showed an increase in the dry mass of the stems in BS watering treatment (Table 3).

However, application to the seeds at sowing did not alter the fresh mass of the plants compared to the control (Table 2). Nevertheless, it reduced growth, especially in the ‘Cardyna’ (Table 1) cultivar. It also reduced leaf dry mass accumulation in both cultivars, the dry mass of the stem in ‘Cardyna’ and the root in ‘Trinidade’ at values lower than those observed in control (Table 3). Szilagyi-Zecchin et al. (2015) reported that application of Bacillus amiloliquefaciens on seeds promoted positive effects but stressed that excessive concentration of the bacterium might reduce plant growth. So, it is likely that the use of 50 μL of the Serenade® commercial product for each seed with no dilution was excessive, and its dilutions should be tested in further studies.

Applications in consecutive years in organic system

Data analysis did not identify interactions between cultivars and forms of BS application and changes in mass, number, and yield of fruits of the cultivars in the two cycles, either.

The average fruit mass of both cultivars ranged from 107.52 to 115.61 g in their first cycle, and

| Table 3. Dry mass of leaves stems, and roots of tomatoes of ‘Cardyna’ and ‘Trinidade’ cultivars submitted to the Bacillus subtilis (Serenade®) application methods in the form of seed inoculation, seedling watering, and control with no application at 60 DAS. |
|--------------------------------------------------|----------------|----------------|----------------|----------------|
|                                                   | Seeds inoculation | Seedling watering | Control       | Cultivars      |
| Leaf dry matter (g)                               |                 |                 |                |                |
| Cardyna                                           | 4.80           | 5.43           | 5.45           | 5.23 b         |
| Trinidad                                          | 5.92           | 6.84           | 6.59           | 6.45 a         |
| Mean (T)                                          | 5.36 B         | 6.13 A         | 6.02 A         |                |
| C.V (%)                                           | 2.71           |                 |                |                |
| Stem dry matter (g)                               |                 |                 |                |                |
| Cardyna                                           | 2.99 Cb        | 5.10 Ab        | 4.18 Bb        |                |
| Trinidad                                          | 4.48 Ba        | 5.89 Aa        | 4.63 Ba        |                |
| Mean (T)                                          |                 |                 |                |                |
| C.V (%)                                           | 2.73           |                 |                |                |
| Root dry matter (g)                               |                 |                 |                |                |
| Cardyna                                           | 0.88 Bb        | 1.03 Ab        | 0.80 Bb        |                |
| Trinidad                                          | 1.01 Ba        | 1.21 Aa        | 1.11 ABa       |                |
| Mean (T)                                          |                 |                 |                |                |
| C.V (%)                                           | 5.85           |                 |                |                |

Means followed by the same lower-case letter in the columns and upper-case letter in the rows are not different from each other by the test of Tukey (p < 0.05).
from 98.32 to 113.06 g in their second cycle, with an average number of fruits per plant ranging from 48.02 to 52.22 between cycles and cultivars. The average fruit yield in this study varied from 4.8 to 5.4 kg per plant, which is equivalent to the production obtained by Luz et al. (2007) in the conventional system, from 3.0 to 5.0 kg per plant, and 4.0 kg in the organic system.

**Biochemical changes**

Babu et al. (2015) reported that PGPB might promote increases in plant chlorophyll content, as observed in leaf applications of BS, stimulating the synthesis of chlorophylls and also of carotenoids. However, the drench and its spraying combination did not alter the pigment contents, nor was there any interaction between forms of BS application and cultivars. By comparing the cultivars, ‘Cardyna’ presented higher levels of chlorophylls and carotenoids (Table 4), indicating a characteristic of the genotype as no interaction with the forms of BS application was found.

Alteration in the pigments promoted by BS in ‘Cardyna’ were not sufficient to promote the increase in the sugar content in the leaves. On the contrary, the foliar spraying diminished the contents of reducing sugar on cultivars, while drench and the combination of the applications decreased the content of non-reducing sugars (Table 5). The content of total sugars was not influenced by the manners in which BS was applied, nor did it vary significantly among cultivars, with mean values of 7352 μg g in ‘Cardyna’ and 6961 μg g in ‘Trinidade’.

The plant-bacteria association may provide the activation of several metabolic pathways of sucrose, which is the primary non-reducing sugar, combined with glucose and fructose, which is one of the reasons for stimulating the growth of plants, according to Kang et al. (2014). The authors reported that plants treated with bacteria from the PGPB group obtained an increase in the contents of chlorophyll, total sugars, and reducing sugars. However, in the present study, the chlorophylls’ increased contents did not reflect any increase in the sugars.

Drench application stimulated the accumulation of non-reducing sugars in ‘Trinidade’, while drench + spraying, on the other hand, caused a reduction in the contents. Consequently, of the total amount of the sugars of the plants, the greater fraction was that of reducing sugars.

Mazaro et al. (2009) state that the increase in the reducing sugars may be related to the increase in the metabolic activity through the induction of compound production related to the expression of resistance against diseases. However, this is not characterized in this work as the higher content of reducing sugars also occurred in the control group, possibly relating to the role of the leaf as a source of photoassimilates for nearby fruits.

The induction of defense activates mechanisms that already exist in the plant and may involve the synthesis of β-1,3-glucanase enzymes, chitinase, 

| Application forms                  | Chlorophyll a | Chlorophyll b | Total chlorophyll | Carotenoids |
|------------------------------------|---------------|---------------|-------------------|-------------|
| Control                            | 0.225 b       | 0.360 b       | 0.140 b           |             |
| Foliar sprays                      | 0.274 a       | 0.170 a       | 0.444 a           | 0.183 a     |
| Drench+ foliar sprays              | 0.207 b       | 0.144 ab      | 0.352 b           | 0.140 b     |
| Drench                             | 0.218 b       | 0.156 ab      | 0.375 b           | 0.149 b     |

| Cultivars                          | Chlorophyll a | Chlorophyll b | Total chlorophyll | Carotenoids |
|------------------------------------|---------------|---------------|-------------------|-------------|
| Cardyna                            | 0.250 a       | 0.162 a       | 0.421 a           | 0.164 a     |
| Trinidad                           | 0.212 b       | 0.135 b       | 0.353 b           | 0.141 b     |
| C.V.%                              | 7.82          | 11.03         | 8.78              | 6.57        |

Means followed by the same lower-case letter in the columns are not different from each other by the test of Tukey ($p < 0.05$).
phenylalanine ammonia-lyase, polyphenoloxidase, and the rise in the concentration of phenolic compounds and flavonoids in plants (Cavalcanti et al., 2006).

The BS treatments did not influence the accumulation of phenolic compounds in tomato plants. Also, the difference between cultivars was not significant (Table 6), despite the greater accumulation reported by Ownley and Windham (2010) as frequently related to resistance induction processes. The phenolic compound values ranged

| Reducing sugars (µg/g) | Drench | Foliar spray | Drench + spray | Control |
|-----------------------|--------|--------------|----------------|---------|
| Cardyna               | 5803 Aa| 4420 Ba      | 5341 ABb       | 5930 Aa |
| Trinidad              | 4329 Bb| 5083 Ba      | 6810 Aa        | 5223 Ba |
| C.V.%                 | 9.03   |              |                |         |

| Non-reducing sugars (µg/g) | Cardyna | Trinidad | C.V.% |
|---------------------------|---------|----------|-------|
| Drench                    | 1619 Bb | 2483 Aa  | 12.57 |
| Foliar spray              | 2100 ABa| 1747 Ba  |       |
| Drench + spray            | 1666 Ba | 896 Cb   |       |
| Control                   | 2259 Aa | 1768 Bb  |       |

Means followed by the same lower-case letter in the columns and upper-case letter in the rows are not different from each other by the test of Tukey ($p < 0.05$).

Table 6. Mean values of total free proteins (mg g$^{-1}$), phenylalanine ammonia-lyase (PAL) expressed as UAbs/min/mg protein, chitinase expressed in U.E.mg.protein, and β1,3 glucanases in U.E/mg, in leaves of ‘Cardyna’ and ‘Trinidad’ tomatoes, submitted Bacillus subtilis (Serenade®) application in the form of watering, foliar spraying, watering combined with foliar spraying, and control with no application.

| Proteins (mg/g) | Cultivars | Treatments | Means |
|----------------|-----------|------------|-------|
|                | Cardyna   | ns         | 1.70 a|
|                | Trinidad  | ns         | 1.46 b|
| C.V.%          | 11.82     |            |       |

| PAL           | Drench    | Foliar spray | Drench+ spray | Control |
|---------------|-----------|--------------|---------------|---------|
| Cardyna       | 0.0504 ABa| 0.0611 Aa    | 0.0470 Bb     | 0.0436 Ba |
| Trinidad      | 0.0515 Ba | 0.0533 Ba    | 0.0757 Aa     | 0.0466 Ba |
| C.V.%         | 11.43     |              |               |         |

| β1,3glucanases| Cardyna   | 0.0134 Aa   | 0.0127 ABb    | 0.0110 Ba  | 0.0083 Cb |
|---------------|-----------|-------------|--------------|-----------|-----------|
| Trinidad      | 0.0129 Ba | 0.0165 Aa   | 0.0122 Ba    | 0.0123 Ba |
| C.V.%         | 8.97      |             |              |           |

Means followed by the same lower-case letter in the columns and upper-case letter in the rows are not different from each other by the test of Tukey ($p < 0.05$).
from 3990 mg·g$^{-1}$ in ‘Cardyna’ to 4127 mg·g$^{-1}$ in ‘Trinidade.’ Likewise, contents of flavonoid did not express differences between the application forms or cultivars, with ‘Cardyna’ presenting 1875 mg·g$^{-1}$ and ‘Trinidade’ with 1873 mg·g$^{-1}$. Therefore, it can be inferred from the results obtained in this study that the tomato plants did not receive any stimuli for the increase in the phenolic compounds with the BS applications.

In general, increases in total free proteins may be associated with resistance induction processes related to increased specific enzymes (Lawrence et al., 2000). However, the content of these proteins in tomato leaves was not influenced by BS applications. By comparing the tomatoes, ‘Cardyna’ presented a higher value than ‘Trinidade,’ identifying a characteristic of the cultivar. As no alteration occurred in the content of free proteins, BS treatments promoted a rise in phenylalanine ammonia-lyase (PAL) (Table 6), an enzyme that affects the synthesis of salicylic acid, phenylpropanoids, flavonoids, and phytoalexins (Gerasimova et al., 2005). Despite acting on the metabolic pathway of flavonoid synthesis, the increment in PAL did not reflect any increase in phenolic compounds, as already reported.

According to Olivares et al. (2015), foliar spraying with PGPB may promote an increase in PAL activity. Combined with BS applications, the increments varied according to the manner of application and cultivar, showing some interactions. The highest value was found in ‘Cardyna’ when submitted to foliar spraying, while in ‘Trinidade,’ it occurred when drench was combined with spraying (Table 6).

Besides the changes in PAL, the presence of chitinases, enzymes that stimulate the hydrolysis of the β1,4 bonds of the N-acetylglucosamines, components of chitin in fungal cell walls, was increased in ‘Cardyna’ through drench with BS, while in ‘Trinidade’, the chitinase was increased by foliar spraying.

According to Lawrence et al. (2000), there is a synergism between chitinase and β1,3 glucanases. Moreover, BS treatments promoted increases in the concentrations of both enzymes in the leaf tissue of tomato plants. The increase in β1,3 glucanases occurred variably between treatments and cultivars, where drench promoted the highest value for ‘Cardina’, and foliar spraying for ‘Trinidade,’ indicating that BS stimulated the accumulation of the enzyme even in plants that did not show any disease symptoms.

The changes in the activity of PR-proteins such as PAL, β1,3 glucanases, and chitinases may allow the monitoring of the resistance status of plants when exposed to pathogens (Choudhary et al., 2007). In this study, the incidence of diseases in plants was not identified because of the growth conditions and cultivars, so the enzymatic changes refer to BS applications.

By considering that the same substrates of the primary metabolism that activate pathways of plant growth and development such as phosphoenolpyruvate in the route of glycolysis or erythrose-4-phosphate of pentoses are also responsible for supplying the secondary metabolism. Such as phenylpropanoids whose key enzyme is PAL, Carvalho (2012), stated that plants stimulated to activate defense mechanisms even without the presence of pathogens, may present metabolic costs resulting in the reduction in production.

Nevertheless, although the activation of defense mechanisms related to PR-proteins with variable BS applications between treatments and cultivars, the production and characteristics of ‘Cardyna’ and ‘Trinidade’ fruits in two crop cycles were not affected, being equivalent to the genetic potential of the cultivars and the results obtained in other studies.

**Conclusions**

The application of Bacillus subtilis in the seeds reduced the initial growth of the plants while, watering of the seedlings at transplanting stimulated it. In the cultivations, variability between cultivars, and application forms, the chlorophylls, a, b and total chlorophylls, the carotenoids, and the enzymes related to the defense of the plants were stimulated. Phenolic compounds, flavonoids, and total sugars were not altered. The concentrations of reducing sugars were higher than those of non-reducing sugars. The metabolic changes did not influence the yield of tomato plants in the organic system.
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