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Evaluation of Plant Growth Promoting Ability of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR *In Vivo*

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Abstract

Bacillus amyloliquefaciens Bc2 and *Trichderma harzianum* TR were used to evaluate their growth-promoting activity on cultivated strawberries, under laboratory and field conditions, and we have noticed that the percentage of achene germination is important for ones treated with TR (=97%) followed by those treated with Bc2 strain (=90%) and the control (=84%). Inoculations on field showed that on untreated soil with insecticide, TR is effective and allows the development of plants and extends the duration of flowering and fruiting. On treated soil, Bc2 clearly promotes the growth and development of strawberry seedlings and its role as plant growth promoting microorganisms has been proved.

Keywords

Plant Growth Promoting, Strawberry, PGPM, *Bacillus amyloliquefaciens*, *Trichoderma harzianum*

1. Introduction

Current production methods in agriculture, such as the inappropriate use of pesticides and chemical fertilizers, create a long list of environmental and health problems [1] [2]. The rhizosphere is prone to dramatic changes, and the dynam-

ic nature of the rhizosphere creates interactions leading to biological control of plant diseases [3] [4] [5].

Due to a large number of microbial species present in the soil, especially in the rhizosphere, intensive and extensive interactions were established between soil microorganisms and various other soil organisms, including plant roots, and the plants growth stimulation by rhizospheric microorganisms is well established [6].

Increased crop production could be achieved through direct mechanisms of plant growth by PGPM (Plant Growth Promoting Microorganisms), such as the production of growth regulators (auxins, cytokinins and gibberellins), removing the production of stress hormones (ethylene), increase of water and nutrients absorption (with fixing N2 of phosphate solubilizing agents, siderophore producers, etc.). Indirect mechanisms include the inhibition of phytopathogens by production of antibiotics or lytic enzymes of the cell wall, (chitinases) and inducing plant defense mechanisms [7]. A plethora of PGPM has been studied including *Rhizobium*, *Frankia*, *TS*, *Klebsiella*, *Clostridium*, *Nostoc*, *Anabaena*, *Bacillus megaterium*, *Penicillium* sp., and it was reported that *Sclerocystis* sp., *Glomus* sp., *Trichoderma harzianum* and *Trichoderma viride* can improve the growth and yield of various agricultural crops. PGPMs such as *Beauveria bassiana*, *Pseudomonas fluorescens*, *Serratia entomophila*, *Pseudomonas aureofaciens*, *Trichoderma* sp. and *Streptomyces* sp. have biocontrol properties against various pests and diseases [7].

The beneficial plant-microbe interactions in the rhizosphere are the determinant of plant health and soil fertility [8]. Soil microorganisms play an important role in biogeochemical cycles of organic and inorganic soil nutrients and in maintaining plant health and soil quality [8]. It is therefore necessary to increase the efficiency of a meager amount of external inputs using the best combinations of beneficial microbes for sustainable agricultural production. The soil-plant-microbe interactions are complex and the results can influence in many ways the health and productivity of plants [9].

The aim of this work is the evaluation of plant growth promoting ability of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR under laboratory and field conditions.

2. Material and Methods

2.1. Effect on Germination of Strawberry Plant Achenes *In Vivo* under Laboratory Conditions

2.1.1. Microbiological Material

a) *Bacillus amyloliquefaciens* Bc2

Bacillus amyloliquefaciens Bc2 was cultivated in Petri dishes containing the medium Plate Count Agar (PCA) previously autoclaved at 121˚C for 15 minutes. The incubation of the Petri dishes was made at 37˚C in the dark for 24 h. To promote a high biomass production of the bacterial colonies growing on PCA

medium, a colony from the culture was aseptically transferred to an erlenmeyer containing 50 ml of the LBB (Luria Bertani Broth) culture medium. The cultures were incubated in the laboratory at room temperature (25˚C ± 2˚C) with constant agitation on an orbital shaker at 125 rpm for 48 h. The growth and multiplication of bacteria results with changing the appearance of the medium that, light-colored, becomes cloudy.

After 24 h of incubation, 5 ml of the bacterial suspension has been transferred aseptically in sterile tubes and they were centrifuged at 4000 rpm for 10 minutes to separate the colonies of the culture filtrates. The formed supernatant was discarded. Then the colonies were rinsed twice with sterile distilled water [10]. Finally the bacterial colonies were suspended in a tube containing 5 ml of sterile distilled water, stirred and then the suspension was adjusted to a final concentration of 3×10^5 CFU/ml.

***b*) *Trichoderma harzianum* TR**

The Suspension of TR spores was obtained by adding a volume of 10 ml of sterile distilled water on petri plates containing cultures of TR on PDA (Dextrose Agar), aged 10 days, and gently rubbing the colonies using a sterile Pasteur pipette. The propagule suspension was filtered through a sterile nylon gauze having pores of 102 microns in diameter. The concentration was adjusted to 10^5 spores/ml using Malassez cell.

2.1.2. Plant Material

Strawberry achenes of Sabrina cultivar were collected as follows:

Peeling the surface of fruits, using a spatula, to deposit the achenes on paper and allowed to dry for 72 hours at ambient laboratory temperature.

To study the effect of Bc2 and TR on achenes germination, we have used strawberry achenes previously scarified under sterile conditions in a horizontal laminar flow hood with sulfuric acid (H_2SO_4) for 5 minutes, followed by three washes of 15 minutes with sterile distilled water. A continuation, achenes were soaked in sterile distilled water with slow stirring of 10rpm at laboratory's ambient temperature (25˚C ± 2˚C) for 48 h [11]. Achenes were introduced into each antagonist suspension and also in sterile distilled water (Control), and incubated during ten minutes at laboratory's ambient temperature (25˚C ± 2˚C) then the excess water was absorbed using a double layer of sterile filter paper placed in a Petri dish.

100 achenes were used for each treatment. The control involves placing the disinfected seeds in sterile distilled water. Seeding was performed in a plastic mini-greenhouse (43 cm length and 28 cm of width) containing sandy soil disinfected twice in autoclave at 121˚C for 1 h. In each mini-greenhouse we introduced 100 alkenes for each treatment. Finally the mini-greenhouses were installed in the growth chamber of plant biotechnology laboratory, Faculty of Sciences, Tetouan, Morocco. They are irrigated by tap water every three days. The cultures were maintained under these conditions until the onset of cotyledons to evaluate the effect of each treatment on achenes germination. The ger-

mination percentage of each treatment is noted for each week during three months.

2.2. Effect on the Growth of Strawberry Seedlings under Field Conditions

2.2.1. Strawberry Field: Location and Planting Conditions

The field experiment was conducted during the year 2018/2019 in a field of strawberry cultivar grown by Fortuna from its plantation in November 2018 in the experimental station of the National Institute of Agricultural Research (INRA) in Larache, Morocco (N 35 09 019 and W 006 08 036). Planting conditions were listed in Table 1.

2.2.2. Processing Mode and Application Time

For each antagonist, 1 kg of peat were used as substrate. The latter was placed in autoclavable bags and sterilized three times by autoclaving for 1 h at 121˚C. The contents of the bags were transferred to Plastic lidded boxes (18.2 cm of height, 35 cm breadth and 21.5 of length) previously cleaned (the boxes and lids) by bleach, disinfected by ethanol 92% and placed under UV lamp (Philips Ultra-UV, 30 W/G30T8, 254 nm) in a horizontal laminar flow hood for 20 min. These boxes were inoculated with the antagonist cultures produced on PCA and PDA and subsequently, then they were closed and incubated in the dark at 27˚C for five days for both antagonists. After incubation we have confirmed that the antagonists concentrations were 3×10^5 CFU/g of subtract and 10^5 conidia/g of subtract of Bc2 and TR respectively.

The treatments were carried out only once during the step of planting of strawberry explants. Six grams of the mixture (substrate + antagonist) were mixed with the soil at the roots of the explants at a rate of 60 seedlings per antagonist

Table 1. Terms of planting seedlings of strawberry in the field studied.

Soil treatment before planting	- Treated soil with insecticide ST - Untreated soil SNT
The plastic mulching	black opaque plastic film from 25 to 35 .µm thick and 1m to 1.20 m wide it enables: - Raising the soil temperature in the root zone; - Maintain soil moisture; - Preserving the soil structure; - Avoid fruit stains.
Planting	- Twin ridges on a bank 60 cm wide; - The plants are arranged in staggered rows 20 cm away; - Each trillion is divided into two parts (G treated and untreated soil) each part contains 30 strawberry plants.
Irrigation	Three times per week.
Coverage of small tunnels Nantes	- The tunnel cover film is transparent, a thickness of 100 to 120 microns and a width of 1.5 m.

(30 of treated soil and 30 on untreated soil). The control in our study was a ridge treated with distilled water instead of the studied antagonists. The observation was carried out by noting the length of the petiole, number of leaflets, flowers and fruit of each plant each month after planting. We gave clues for each parameter as follows:

- Ridge 1: treated by *Trichoderma harzianum* TR
- Ridge 2: treated by *Bacillus amyloliquefaciens* Bc2
- Ridge 3: treated with water (Control)
- ST: treated soil with insecticide
- SNT: untreated soil
- T1 to T6: From December 2018 to May 2019.

3. Results

3.1. Effect of *Bacillus amyloliquefaciens* Bc2, and *Trichoderma harzianum* TR on the *In Vivo* Germination of Strawberry Achenes

The chronological germination tracking shows that after about the second week of seedlings, the cotyledons of treated and untreated achenes begin to appear.

The germination percentage (**Figure 1** and **Figure 2**) seed treated with TR, was higher than in those treated with Bc2 and it remains slightly higher than control achenes (Soaked in water).

Note that *Bacillus* sp. and *Trichoderma* sp. included in the category of PGPMs (Plant Growth Promoting Microbes), the microorganisms that promote plant growth and resistance through the production of PGR (Plant Growth Regulators), a phosphate solubilizing can improve the effectiveness of fertilizers, allow the production of phosphates. Produce siderophores, which plays a role in the induction or increase the resistance of plants against phytopathogens.

3.2. Effect of *Bacillus amyloliquefaciens* Bc2 and *Trichoderma harzianum* TR on the Growth of Strawberry Grown under Field Conditions

The inoculated plants with Bc2 and TR showed increasing productivity compared to uninoculated plants. The two antagonists have had a different effect on the development of strawberry seedlings. The average length of the petioles increases with time, in the month of May (T6) we observed that the length reached 16.87/23.20; 17.06/22.77 and 16.90/13.67 cm for plants treated with TR and Water Bc2 ST/SNT respectively (**Figure 3**).

We noticed that the length of the petiole, number of leaflets, flowers and fruits per plant inoculated by TR on untreated soil are higher than those on soil treated, the opposite was observed on plants inoculated by Bc2 on treated soil are higher those on untreated soil (**Figures 3-6**).

The average number of leaflets increases with time, in the month of May (T6) we observed that the average number reached 42.67/60.70; 68.20/62.00 and

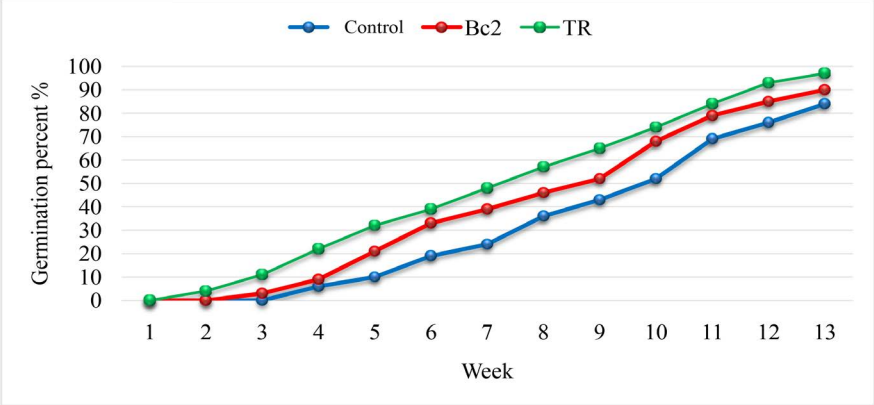

Figure 1. Germination percent of strawberries achenes soaked in Bc2, TR and distilled water (Control).

Figure 2. 9 weeks old seedlings (Left) and 3 months old seedlings transferred to a pot (right).

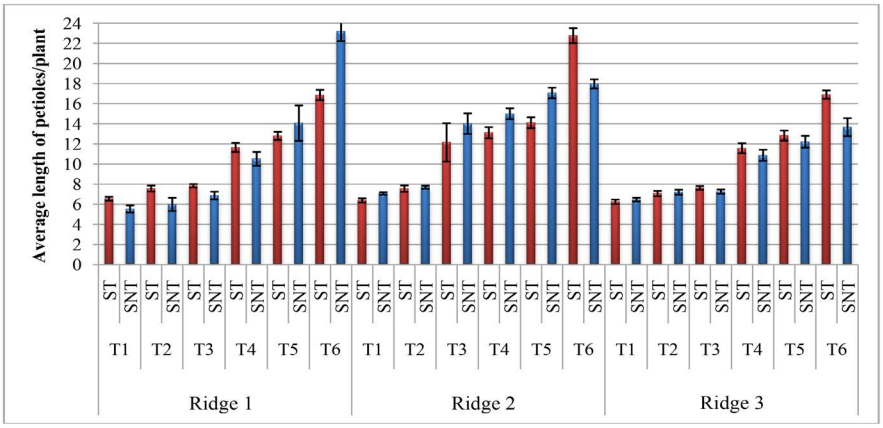

Figure 3. Average length of petioles by cm per plant in ridges processed by TR; Bc2 and control each month.

52.30/43.30 leaflets per plant for the plants treated with TR; Water and Bc2 on ST/SNT respectively (**Figure 4**).

Regarding the average number of flowers and fruits, allowed us to note that the rate of flowering and fruiting begins to decrease from the month March (T4)

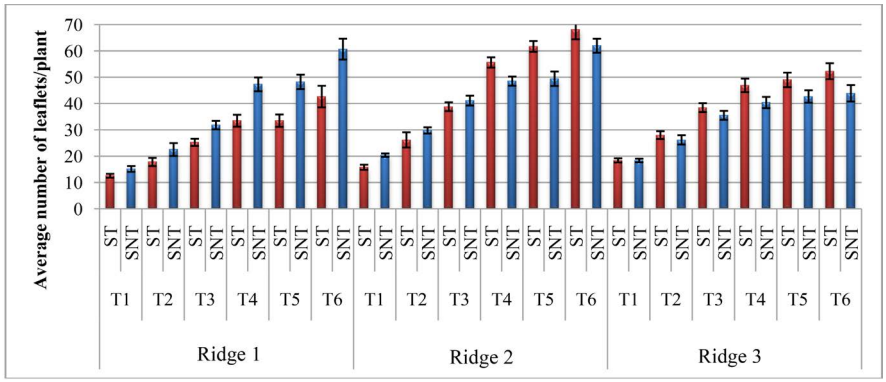

Figure4. Average number of leaflets per plant in ridges processed by TR; Bc2 and control each month.

for the treated plants Bc2 and water from the month of April (T5) for the plants treated with TR (**Figure 5** and **Figure 6**).

4. Discussion

Soil, the source of primary food production, is an ecosystem composed of many different microorganisms. The green revolution, the use of chemical fertilizers, fungicides and insecticides, has a side effect which is the soil fatigue. Microbiological products and techniques can help establish a sustainable agriculture [12].

The results obtained in this study, we found that the percentage of germination of strawberry achenes *in vivo* inoculated by TR and Bc2 are superior to that of inoculated achenes by water, but TR remains the most effective.

Observations of inoculations under field conditions allowed us to note that inoculation by TR on untreated soil remains effective for the development of plants and extend the duration of flowering and fruiting. And the inoculation by Bc2 on treated soil acts effectively for the development of strawberry seedlings.

It has been shown that certain strains of Trichoderma stimulate plant growth by producing compounds that promote plant growth (PGP) [13] [14] [15] [16], although the biological control features and PGP are rarely found together. PGPMs are unpredictable and are influenced by environmental factors [17] [18]. It is believed that PGPMs mechanisms result in various direct effects on plants, reduced microbial activity and inactivated toxic compounds in the root zone [19]. Trichoderma species may also mitigate a wide range of abiotic stresses such as salinity, temperature and drought; they can improve the efficiency of photosynthesis, improve nutrient absorption and greatly increase the efficiency of nitrogen use in crops. All these attributes can help improve the characteristics of the PGPMs often evident during inoculation [19] [20] [21] [22]. The strains stimulate the plant growth by production of plant growth promoting compounds that remain to be defined [15] [16] [23], most likely by the combination of one or more remarkably diverse range of secondary metabolites and proteins such as pyrones, the peptaibols and terpenes [24] produced by Trichoderma.

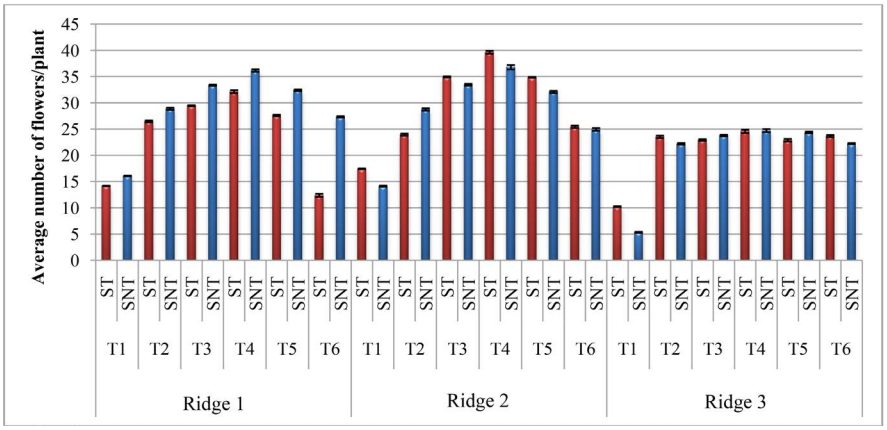

Figure 5. Average numbers of flowers per plant in ridges processed by TR; Bc2 and witness each month.

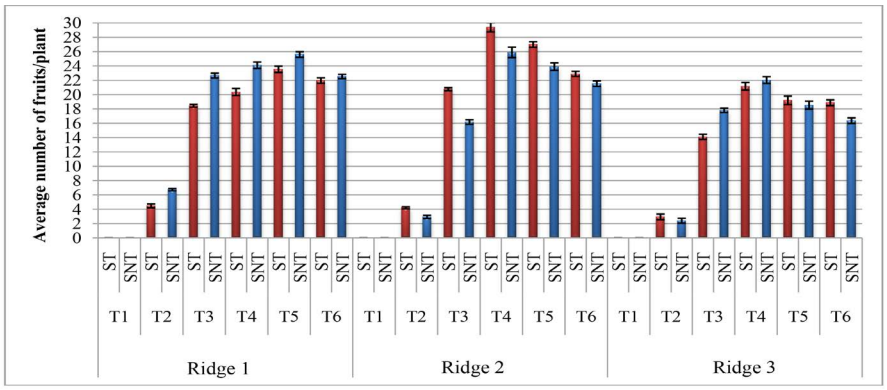

Figure 6. Average number of fruit per plant in ridges processed by TR; Bc2 and witness each month.

Strains of Trichoderma are more effective at colonizing and promoting plant growth if they are enriched in organic substrate (for example, as a bio-organic fertilizer) and then applied [25] [26]. Therefore, the mode of application of this PGPM, alone or in combination with organic compost, was taken into account in this study.

It is generally accepted that the Trichoderma strains should colonize plant roots before stimulating growth [27]. Therefore, the results of the biomass and yield of tomato in a study involved the SQR-T037 *T. harzianum* which had the ability to recognize and adhere to the roots of the plant [28]. Some researchers have suggested, Trichoderma strains could be defined as opportunistic avirulent symbiotic organisms of plants that is able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi to stimulate plant growth [19] [29]. Research has shown that Trichoderma species were able to quickly colonize niches left vacant by other organisms and because of TasHyd1 gene that had an important role in root colonization of Trichoderma plants [30] [31].

The bacteria having potential activity of the growth stimulation include species of Pseudomonas, Bacillus, Stenotrophomonas, Serratia and Arthrobacter

[32] [33] [34] [35] [36]. [32] reported for the first time two volatile compounds (2,3-butanediol and acetoin) that triggered stimulation of plant growth.

It was reported that the plant growth promoting rhizobacteria produce plant growth regulators such as auxin, gibberellins, cytokinins and ethylene [33] [37] [38] [39], and by increasing the availability of nitrogen, potassium and iron in soil [40] [41] [42] have previously reported production of IAA by *B. subtilis* spp. The plant growth promoting activity induced by *Bacillus* spp. has been demonstrated in many species of plants, including melons, beans, tomato, tobacco, and the model plant *Arabidopsis thaliana* [43].

*Bacillus amyloliquefaciens*FZB42 is a gram positive bacterium and a model for the study of plant-microbe interactions. This specie is commercially used as biofertilizer and biocontrol agent [44].

According to most studies on strawberries, the PGPMs showed a positive effect on their growth and development [45]-[50].

5. Conclusions

We conclude that, the studied strains of *B. amyloliquefaciens* and *Trichoderma harzianum* significantly not only protect strawberry plants from phytopathogens infection but also improve the plant growth parameters.

In the context of the PGPMs study, further work needs to be done to confirm the effectiveness of these microorganisms.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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