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Title: Trichoderma harzianum CDBB-H-1125 and Bacillus subtilis ATCC 6633 for biological control of Rhizoctonia solani in seed Clon 99-39 of Solanum tuberosum L.

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Abstract: In Mexico, one of the most consumed crops is potatoes. There are several bacteria, viruses and phytophatogen fungus that crops are exposed. One of the most important microorganisms is Rhyzoctonia solani, a pathogen fungus that cause several losses in the Mexican and worldwide fields potatoes production. In this work we used an isolated indigenous strain of R. solani from tissue and crops of Solanum tuberosum L. The strain isolated was growth on PDA agar petri dishes and served as an inoculum of the peat moss, a substrate used for growth Clon 99-39 of Solanum tuberosum L. in greenhouse experiments. The antagonists, Trichoderma harzianum CDBB-H-1125 and Bacillus subtilis ATCC 6633 were obtained from an Official Bank Strains Collection of the CINVESTAV, Mexico. T. harzianum was propagated by a solid culture in plastic bags and B. subtilis was propagated by a submerged culture in shake flasks. The experimental greenhouse strategy consisted in an inoculation of seed Clon 99-39 a different concentration of B. subtilis (suspensions with 1 x 106, 1 x 107, 1 x 108 cells by mL), for T. harzianum we used a suspension concentration of 1 x 107, 1 x 108, 1 x109 spores by mL. An also we considered a positive control infected by R. solani and other control with a sterile substrate only. After of an initial inoculum we considered three additional antagonisms add through two months. In the final fresh weight and plant height, the better results were obtained in the crop were we add 1 x 108 cells/mL of B. subtilis, in meantime with T. harzianum, the most promise results were obtained at 1 x 109 spores/mL. But, these parameters were higher when we used a combined formulation of B. subtilis and T. harzianum, especially with 1 x 109 and 1 x 108 spores by mL of T. harzianum and B. subtilis, respectively. Finally, we analyzed the effect of such treatments in the infection severity caused by R. solani in the crop, sized by estimated the infected surface area, such infection were drastically diminished when the crops were inoculated by higher concentration of antagonist; T. harzianum 1 x 109 spores by mL and

B. subtilis 1 x 108 cells by mL or when these were used in a combined fashion.

Dear Editor:

Please find enclosed our manuscript "*Trichoderma harzianum* CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 for biological control of *Rhizoctonia solani* in seed Clon 99-39 of *Solanum tuberosum* L.".

This paper shows the effect of the antagonist *Trichoderma harzianum* and *Bacillus subtilis* on the control of the phytophatogen fungus *Rhizoctonia solani* in *Solanum tuberosum* L in Greenhouse conditions. The results showed that the antagonist enhanced the growth of the plant, increased the fresh weight of the tuber and reduce drastically the severity of the disease caused by *R. solani*.

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All the authors participated in the conception and design of the work. All the authors believe that the manuscript represents valid work; carefully read and fully approve of it. We also warrant that the article is original and that is not submitted anywhere other than your journal. We would of course be ready to provide further information about our data and methods you so desire.

Correspondence about the manuscript should be addressed to MSc Cesar Reyes Reyes at the Universidad Politecnica del Valle de Toluca, and Dr. Luis German Lopez Valdez at the Universidad Autonoma de Chapingo, as indicated in the first page of the manuscript.

Thank you very much for your kind attention. I look forward to hearing from you.

Sincerely yours

The authors

HIGHLIGHTS

- *Bacillus subtilis* ATCC 6633 and *Trichoderma harzianum* CDBB-H-1125 enhances height and yields on crops on seed Clon 99-39 of *Solanum tuberosum* L.
- The highest concentration of spores of *Trichoderma harzianum* CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 diminished the disease injures caused by *Rhizoctonia solani* in seed Clon 99-39 of *Solanum tuberosum* L.

Trichoderma harzianum CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 for biological control of *Rhizoctonia solani* in seed Clon 99-39 of *Solanum tuberosum* L.

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ABSTRACT

In Mexico, one of the most consumed crops is potatoes. There are several bacteria, viruses and phytophatogen fungus that crops are exposed. One of the most important microorganisms is *Rhyzoctonia solani*, a pathogen fungus that cause several losses in the Mexican and worldwide fields potatoes production. In this work we used an isolated indigenous strain of *R. solani* from tissue and crops of *Solanum tuberosum* L. The strain isolated was growth on PDA agar petri dishes and served as an inoculum of the peat moss, a substrate used for growth Clon 99-39 of *Solanum tuberosum* L. in greenhouse experiments. The antagonists, *Trichoderma harzianum* CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 were obtained from an Official Bank Strains Collection of the CINVESTAV, Mexico. *T. harzianum* was propagated by a solid culture in plastic bags and *B. subtilis* was propagated by a submerged culture in shake flasks. The experimental greenhouse strategy consisted in an inoculation of seed Clon 99-39 a different concentration of *B. subtilis* (suspensions with 1×10^6, 1×10^7, 1×10^8 cells by mL), for *T. harzianum* we used a suspension concentration of 1×10^7, 1×10^8, 1×10^9 spores by mL. An also we considered a positive control infected by *R. solani* and other control with a sterile substrate only. After of an initial inoculum we considered three additional antagonisms add through two months.

In the final fresh weight and plant height, the better results were obtained in the crop were we add 1 x 10^8 cells/mL of *B. subtilis*, in meantime with *T. harzianum*, the most promise results were obtained at 1 x 10^9 spores/mL. But, these parameters were higher when we used a combined formulation of *B. subtilis* and *T. harzianum*, especially with 1 x 10^9 and 1 x 10^8 spores by mL of *T. harzianum* and *B. subtilis*, respectively. Finally, we analyzed the effect of such treatments in the infection severity caused by *R. solani* in the crop, sized by estimated the infected surface area, such infection were drastically diminished when the crops were inoculated by higher concentration of antagonist; *T. harzianum* 1 x 10^9 spores by mL and *B. subtilis* 1 x 10^8 cells by mL or when these were used in a combined fashion.

Keywords: *R. solani*; *T. harzianum*; *B. subtilis*; *S. tuberosum*; Biological control, 99-39 Clone

1. Introduction

The State of Mexico is in central Mexico and this Region in one of the main areas of crops potatoes production in Mexico, due at the latitude and specific soil properties of the land, there are at least 11 varieties of *Solanum tuberosum* that are used for crops production. Due the tolerance to low temperatures and its ability to growth over 3,000 high meters near to the volcanoes, the use of the seed Clon 99-39 of *S. tuberosum* is highly recommended. The seed Clon 99-39 is able to produce over 70 ton/hectare (Rubio-Covarrubias et al, 2013), but the fields yields are reduced due to the presence of the phytopathogen fungus, the main disease that affect specifically to the seed Clon 99-39 is *Rhizoctonia solani*.

R. solani is an important fungal pathogen that causes stem canker and black scurf potato (*Solanum tuberosum* L.), and widespread in all the potato growing areas in the world (Frank, 1986). Fungi in the genus *Trichoderma* are important biocontrol agents of several soils borne phytopathogens (Benitez et

al, 2004). *Trichoderma* spp. is well-known biocontrol agent against phytophatogens (Romão-Dumaresq et al, 2012). *Trichoderma* spp. uses different mechanisms for the control of phytopathogens which include mycoparasitism, competence for space and nutrients, secretion of antibiotics and fungal cell wall degrading enzymes (Kubicek et al, 2001; Howell, 2003; Benitez et al, 2004; Harman et al, 2004). In addition, *Trichoderma* could have a stimulatory effect on plant growth (Naseby et al, 2000) as a result of modification of soils conditions. Disease management with biocontrol agents offers a great promise (Prashar *et al*, 2013; Singh and Vyas, 2009). These agents are vital components of sustainable agriculture (Xu et al, 2011), which colonize the rhizosphere and leave no toxic residues as opposed to chemicals (Dubey et al, 2007). More than 100 antimicrobial compounds have been identified from *Trichoderma* spp. (Vinale et al, 2008).

And the other hand, biological control by *Bacillus* spp. involves a number of mechanisms, such as competition, antagonism, systematic resistance induction, and promotion of plant growth. Bacteria that remain in the plant cultivation substrate through the growth of the plants can act directly as phytophatogen fungus antagonists (Velázquez-Ceñedo et al, 2008). *B. subtilis* is well known for the production of antibiotics with an amazing variety of structures (Hu et al, 2007; Stein, 2005). Several strains of *Bacillus* help to promote the health of crops and control diseases by producing antibiotic metabolites, suppressing plant pathogens, others antagonize plant pathogens by competing for nutrients like iron and phosphate, others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake (Gardener, 2004).

In this study, the spores of *T. harzianum* CDBB-H-1125 and cells of *B. subtilis* ATCC 6633 were used for the biological control of *R. solani* in seed Clon 99-39 of *S. tuberosum* L. The spores of *T. harzianum* were obtained by solid cultures with wheat grains in plastic bags that have a filter window for allow oxygen interchange and spores of *B. subtilis* were obtained by a submerged culture in a shake

flasks machine. In greenhouses experiments, the substrate consisted of peat moss and it was infected by *R. solani*, only one treatment was not infected (negative control). The seeds of Clon 99-39 were arrangement in randomized blocks, were each Clon received a bath at different concentrations of spores of *T. harzianum, B. subtilis* or both. We investigated the effect of spore's concentration of *T. harzianum* and *B. subtilis* and their combination in the infected seed tuber by *R. solani* on the yields of crops (fresh weight of the tuber), height of the plants and index severity disease of *R. solani* in seed Clon 99-39 of S. *tuberosum* L.

2. Materials and methods

2.1 Isolation and propagation of *Rhizoctonia solani*

R. solani was isolated from infected crops as described by Goswami et al (2010). The tissue samples were cultivated on PDA (Difco, USA) Petri dish and incubated at room temperature. From petri dishes were obtained pieces of PDA and these were mixing with a potatoes infusion, which were prepared as reported by Aranda (1997). The substrate where the potatoes were growth consisted of peat moss and agrolyte 1:1 (v/v), this previously was autoclaved at 121 °C for 50 minutes, then adjusted at 70 % humidity and incubated for 27 days at room temperature.

2.2 Antagonists microorganisms

We used a strain collection of *Trichoderma harzianum* CDBB-H-1125 and *Bacillis subtilis* ATCC 6633, this strains were kingly donated by the CINVESTAV, Mexico. For the conservation of the strains we used PDA slants for incubated at 30 °C and finally conserved at 4 °C.

2.2 Propagation of antagonists

T. harzianum CDBB-H-1125 was growth in Petri dish on PDA (Difco, USA), the incubation was carried out at room temperature for 12 days, each 12 hours were exposed to the sun light for induce sporulation. For yield the spores we used Tween 80 at 0.1 %. *T. harzianum* was propagated on wheat bran, autoclaved for 50 minutes at 121 °C. We used autoclavable plastic bags with a windows filter (Unicorn Bags, USA) that containing the solid medium; this was inoculated with *T. harzianum* and incubated for 10 days at room temperature. Spores collected from the solid culture were counted by a Newbauer chamber. Spore suspension was prepared by the use of xanthan gum at 1 % and this suspension was used for direct application to the seed Clon 99-39 pots.

B. subtilis ATCC 6633 was growth in Petri dish on PDA (Difco, USA), the incubation was carried out at room temperature for a week, and the cells were collected by Tween at 0.1 %. *B. subtilis* collected from petri dish served as an inoculum and propagated by a submerged culture in an orbital shake flasks machine (Thermo Scientific, USA), the cultures were carried out in flasks of 500 mL of nominal volume with 100 mL of medium agitated at 200 rpm´s and 2.5 cm of shaking diameter. The inorganic medium (Sigma, USA) consisted in g/L: $(NH_4)_2SO_4$ (4.0), K_2HPO_4 (5.32), KH_2PO_4 (6.4), dextrose (10.0), $MgSO_47H_2O$ (0.4), $MnCl_24H_2O$ (0.00785), $CaCl_2$ (0.040), $FeSO_47H_2O$ (0.030). The medium was adjusted at pH 7.2 by NaOH 40 % (Martínez et al, 1997).

The spores of *T. harzianum* and cells of *B. subtilis* were counted by a Newbauer chamber.

2.4. Formulation for *T. harzianum* and *B. subtillis*.

For *T. harzianum* spores formulation we dissolved 1.0 g/L of xanthan (Sigma, USA) in a 1.0 liter of distilled water and mixing for 5 min and then the wheat grains with the fungus spores of *T. harzianum* were washed in it, finally we used a Newbauer chamber for count the total spores by mL.

The desire spore concentration were adjusted by the addition of different weigh of the wheat grains, for example if the weight of the grains were 111 g we obtained 3.4 x 10^9 spores of *T. harzianum* by mL, but when the weight was 11.1 we got only 3.4 x 10^8 spores by mL. The seeds Clon 99-39 of *S. tuberosum* were submerged in each spore concentration for 40 minutes and were spread in pots with the peat moss. We considered four posterior applications of *T. harzianum* spores and each concentration (1 x 10^7 1 x 10^8 and 1 x 10^9 spores by mL) were adjusted at 30 mL. For the formulation of *B. subtillis* we used the same method as described for *T. harzianum*, for the formulation of bacteria we used different volumes of the culture, therefore we considered the highest cell concentration obtained in the shake flasks cultures (1 x 10^{10} cells by mL) of *B. subtillis*.

2.5 Greenhouse experiments

We used the Clon 99-39 (*Solanum tuberosum* L.) kindly donated by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) under of the "Programa de la papa en el Estado de México". The cultures were carried out in plastic pots of 500 g of peat moss. Greenhouse temperature was kept at 22-30 °C and treatments in all experiments were replicated two times in randomized blocks. The experiment begins with the inoculum of seed Clon 99-39 of each treatment and a subsequent application of antagonists each 15 days at their corresponding concentration. All the treatments were irrigated 3 times for a week and density of the pots in greenhouse conditions was kept at 44 plants by m^2.

2.6. Severity index of the *R. solani* in the tuber crop of seed Clon 99-39

We adopted the visual scale reported by Villarreal (2013) and the scale severity index used by Guerrero (1997). The scale considered the infected area by *R. solani* according to the next considerations: Value 1 means nor infected area, Value 2 means since 0.2 from 5 % infected area,

Value 3 means since 5 to 10 % infected area, Value 4 means since 5 to 25 % infected area, Value 5 means since 25 from 50 % infected area and Value 6 means more than 50 % of infected area. We adjusted this area at a complete scale; from 0 % at health crops and 100 % for a total infection.

2.7 Statistical analysis

For search a significant differences between treatments at several concentrations of the antagonist *T. harzianum* y *B. subtilis* over the parameters analyzed were the fresh weight of the tuber Clon 99-39 of *S. tuberosum* L. and height of the plant we used analysis of variance (ANOVA). ANOVA was performed using the GLM procedure in SAS (SAS Institute, 2004). When significant ($P < 0.05$) means separated using Tukey´s protected least significant difference (LSD) test at α level =0.05. For the repeated experiments (i, e., the fresh weight of the tuber Clon 99-39), ANOVA indicated that there was a significant difference between the two experiments. These data were therefore combined and analyzed as a single experiment.

3. Results and discussion

3.1 Effect of the antagonist concentration of *T. harzianum* and *B. subtilis* over the yields of fresh weight of tuber Clon 99-39 and height of the plant

The seeds Clon 99-39 were treated at different antagonist concentrations of *T. harzianum* and *B. subtilis* (Fig. 1a), the final result showed a positive effect over the tuber weight. Fig. 1a shows the effect of the treatments after of 60 days of the experiment in greenhouse farm conditions of seed Clon 99-39. The positive control (R+) only infected by *R. solani* and the negative control (R-), not infected by *R. solani* and nor antagonist inoculants reached a fresh weight of 55 g and 54.6 g, respectively. The treatments with antagonist addition show a stimulatory effect on the biomass accumulation of the tuber

Clon 99-39. For *T. harzianum* the lowest concentration (1 x 10^7 spores by mL) turn out in 80 g, meanwhile at higher concentration (1 x 10^9 spores/mL) the final tuber weight was 85.7 g. The results obtained were according to the reported by Chet (1987), Kleifeld and Chet (1992) where these authors argue that *T. harzianum* is able to act as a mycorrhiza, because there have the capacity to make nutrients soluble and synthetize plant hormone to growth stimulation. In the other hand, the treatments carried out with *B. subtilis* we observed a similar effect. When the spores concentration was higher (1 x 10^8 spores/mL) the weight of the tuber was 86.8 g. (Fig. 1a), but when used a less dosage (1 x 10^6 spores/mL), the tuber reached only 84.2 g. Although there is a clear tendency to enhance the fresh weight of the tuber Clon of *S. tuberosum* L. (Fig. 1a) the statistical analysis of variance showed not significant differences ($P > 0.05$) between inoculant treatments of fresh weigh (Table 1). However the treatment (R-) and (R+) showed significant differences ($P > 0.05$) on the fresh weight of the tuber (Table 1) respect to the treatments where we applied several concentrations of *T. harzianum* and *B. subtilis*.

The same behavior was observed in the final height of the plants, where at 1 x 10^7 spores/mL of *T. harzianum*, result of 35 cm, but when the plant were treated at 1 x 10^9 spores/mL, the plant reached 44.8 cm (Fig. 1b). Meanwhile with *B. subtilis*, in the Fig. 1b shows the effect of the concentration of bacteria over the height of the plant. At the concentration of 1 x 10^6 cells/mL of bacteria, the plant reaches 42 cm height and when the dosage was increased at 1 x 10^8 cells/mL the plant achieves 48.3 cm (Fig. 1b). In this parameter, with 1 x 10^8 cells/mL of *B. subtilis* the tuber Clon reached the highest size, respect to the other treatments. However of the tendency showed in the Fig. 1b there is a not significant differences ($P > 0.05$) respect to the height of the plant between the treatments (Table 1).

In this context, Linderman (2000) argues that *T. harzianum* incite systematic resistance against phytopathogen fungus, which involves micorritic protection. The mechanisms of the systematic

resistance of these fungus is observed in the nutritional effects, competence in the infection sites, morphological changes in the tissues and roots, chemical changes in the plant structure and microbial populations changes in the mycorrhizosphere (Hause y Fester, 2005). In this study, the results are consistent with the reported by Perez et al (2002) whom reported the biologic control of the phytopathogen *Pyrenochaeta lycopersici* by *T. harzianum* and the weight increase of plants and tomatoes fruits. This phenomena has been reported previously by Naseby et al (2000), these authors reported a positive effect by the use of biological control agents such as *T. harzianum*.

Is well known that *B. subtilis* stimulate the vegetal growth by the hormone synthesis induction in the plant, such as cytokinin, ethylene, gibberellins, between others. The genus *Bacillus* has the capacity of releases phospatases for phosphate solubilization, iron reduction and this properties improvement considerably the plant nutrition (Rojas-Solis et al, 2013).

3.2 Effect of the formulate of *T. harzianum* and *B. subtilis* over the weight and height of Clon 99-39 infested with *R. solani*

The Greenhouses experiments with the tuber seed Clon 99-39 were organized in randomized blocks; this arrangement includes nine treatments with three different spore concentrations of *T. harzianum* and *B. subtilis*. In this section we analyze the effect of the combination of *T. harzianum* and *B. subtilis* on the fresh weight and height of the tuber Clon 99-39. In the Fig. 2a we observed the effect of spore concentration of *T. harzianum* at 1 x 10^7 spores/mL (T1) and varying the concentration of *B. subtilis* (from 1 x 10^6 to x 10^8 cells/mL). When the concentration of *B. subtilis* where 1 x 10^6 cells/mL the fresh weight of the tuber was 69 g, whereas at 1 x 10^8 cells/mL, the weight achieved 84.1 gr. We found that increasing *B. subtilis*, the fresh weight of the tuber increasingly. The next level of

concentration of *T. harzianum* (1 x 10^8 spores/mL) and the same concentrations of *B. subtilis* (1 x 10^6, 1 x 10^7 y 1 x 10^8 cells/mL). For the fresh weight increased from 75.5 g to 78.8 g (Fig. 2a), although when *B. subtilis* was increased from 1 x 10^7 to 1 x 10^8 cel/mL, the fresh weight was keeping constant. Finally, we proved the third concentration of *T. harzianum* (1 x 10^9 spores by mL) and several concentrations of *B. subtilis*. In these treatments we achieve the major yields of the fresh weight of tuber Clon 99-39. In the treatment T3B3 we obtained a weight of 79 g, but when the concentration of *B. subtilis* was increased (1 x 10^7 cells/mL) the weight of the tuber was 96 g and a 1 x 10^8 cells/mL of *B. subtilis* the Clon reached 97 grams (Fig. 2a). The Table 1 shows that there are significant differences ($P > 0.05$) between the formulations used in this study, where the best results were obtained with the combination of antagonist T3B2 and T3B3. Whereas the treatment with the less yield was T1B1, its means the minor spore concentration of *T. harzianum* (1 x 10^7 spores by mL) and *B. subtilis* (1 x 10^6 spores by mL).

The results obtained in this study were according with the reports that argue that the combination of *T. harzianum* and *B. subtilis* act a synergic way and therefore the high yields potatoes crops obtained in greenhouse experiments. Although this formulation needs to be proved in fields farms crops. However, Moisy et al (2013) reported that the combination of *T. viridae* and *B. subtilis* in tomatoes crops favored the microflora proliferation in the rhizosphere and increased the deshidrogenase activity therefore increased the nitrogen fixation. These authors reported a significative yield increase in the tomatoes crops. The same combination of *T. harzianum* and *B. subtilis* was used in Ginseng plants, where besides of the antagonist of several phytopathogen fungus, they promote the growth of the seeds and increased the stress tolerance (Liu et al, 2009). In the Fig. 2a we observed that a major spore concentration we obtained the best yields and height of the plant.

In the case of the height of the plant (Fig. 2b) for the same treatments, there is a positive effect on the enhanced height of the plant. For example, for T1 treatment with a combination of different concentrations of *B. subtilis*, the plant reached 43.1 cm and 45.6 cm at 1 x 10^6 and 1 x 10^8 cells/mL, respectively. For a T2 with several concentrations of *B. subtilis* we found that the plant reaches at 46.2 cm at 1 x 10^8 cells/mL, and finally the highest concentration of *T. harzianum* 1 x 10^9 spores/mL (T3) with *B. subtilis* 1 x 10^8 cells/mL (B3), the plant reached 50.0 cm height (Fig. 2b). These results are in concomitant respect to the weight fresh of the tuber Clon 99-39.

In both cases, the fresh weight and height of Clon 99-39 were favored with the increase of the concentration of *B. subtilis.* This synergy between two or more antagonistic microorganisms was observed by Morsy et al (2009) whom combined *T. viridae* and *B. subtilis* in tomatoes *(Lycopersicum esculentum* L.) and report the increase of plants survival. Furthermore, the dual inoculation turns out in growth parameters, fruit yield, and nutrients contents of the plants. The same behavior were reported by Zanghlout et al (2007) when combined *T. harzianum* and *B. subtilis* in tomatoes (*L. esculentum* L.) cultivation.

Fig. 3 resume the effect of the combination of the antagonist *T. harzianum* and *B. subtilis* in the fresh weight enhanced of the tuber Clon 99-39, this is corroborate by the fact that the negative control (R-) without *R. solani* and nor antagonist additions and the treatment (R+), only infected with *R. solani* results in low fresh weight of the tuber, but when the antagonist concentration increased in the treatments, these enhanced the biomass accumulation of the tuber Clon, especially when we ads in a combined fashion in the treatment T3B2 and T3B3. Only in the last treatments we found a significant differences ($P>0.05$) in the fresh weight (Table 1).

If we observed all the treatments (Fig. 3), *B. subtilis* promote better the fresh weight of the tuber than *T. harzianum* but the effect was better when we used the antagonist in the mixing way. Several works about *B. subtilis* explain the response systematic induced (RSI) in plants, where the volatile compounds play a relevant role such as the acetoine (3-hydroxy-2-bitanone) reported like a volatile compound as a responsible of turn off the RSI in *Arabidopsis thailiana* (Rudrappa et al, 2010). Other excreted substances by *B. subtilis* include acid indol-3-acetic in maize crops (Idris et al, 2004) and other organic compounds in *A. thailiana* (Gutierrez-Luna et al, 2010). Other study reported by Lagunas et al (2001) demonstrate that the treatment of tomatoes seeds (*Licopersicum sculentum*) with *Bacillus* spp. got stimulate the seed germination around 35 %, the root volume and dry weight 87 and 84 %, respectively. The same behavior were reported by Hernández-Castillo et al (2008) when they applied strains of *Bacillus* spp. in potatoes tubers, in this case, the fresh weight increased notably.

In the Fig. 4 we show the comparison of the treatments of *T. harzianum* and *B. subtilis* on the height of the plant, as we discuss before, the increased of the antagonist concentration seems enhance the growth of the plant. *B. subtilis* (B3) has and strong effect on the growth promote due at the low concentrations used in this study obtained 48 cm height compared with *T. harzianum* (T2) with 41 cm height plant at the same inoculant concentrations. The results were better at highest concentration of *T. harzianum* and *B. subtilis* when used in a combined formulation, specially T3B2 and T3B3 treatments when the plants reached 50 cm height (Fig.4). ANOVA shows a significant difference ($P > 0.05$) between the treatment of T1, R- respect to the rest (Table 1).

In this context, the combined antagonist *T. harzianum* and *B. subtilis* got plants with more height and the dry weight of the Ginseng in 56.11 and 73.3 %, respectively (Liu et al, 2009). Schimiedeknecht *et al*, 2001 used the antagonist *B. subtilis* for a biological control of *Sclerotinia sclerotiorum* in maize and sunflower farming. These authors reported a growth and yield stimulation.

Mudaw and Idris (2014) got better results on the control of chickpea wilt pathogens (*Fusarium oxysporum* and *Fusarium solani*) with the combined use of *Trichoderma* spp. and Bacillus isolates under pot trial conditions. Another study reported more efficacies when used a combination of *Trichoderma* spp. and *Bacillus subtillis* as a biocontrol agents against the *Magnaporthe grisea* in rice (Ali and Nadavajah, 2014), produced higher levels of inhibition of disease incidence and severity compared when used an only single strain.

3.3 Severity disease caused by *R. solani* in Clon 99-39 in Greenhouse experiments

The Fig. 5 shows the effect of different concentrations of *T. harzianum* and *B. subtilis* with the concomitant objective of reduce the superficial damage in the Clon 99-39 caused by *R. solani*. The severity index was estimated by the method reported by Villareal (2013). The severity index was carried out in each one of the 340 seed tuber of the Clon 99-39 yielded and measured trough the direct comparison of the scale reported by Guerrero (1997). However we used a complete scale, for a health tuber we used 0 % of index severity and for 100 % were used for a complete surface infected area of the tuber. The seeds treated with *R. solani* but without antagonist we observed the most remarkable symptoms of the disease. This phenomenon clearly observed the success of our inoculation method of *R. solani* in the peat moss.

In the Fig. 5 we observed that *T. harzianum* was more effective in the growth inhibition of *R. solani* in the surface of the tuber at the end of the experiment. The treatments that were not inoculated with *R. solani* shows less severity of the disease such as the negative control (R-). The best results were found at 1 x 10^9 spores by mL (T3) of *T. harzianum* (index severity of 55 %). Whereas with *B. subtilis*, the best results (B3) were obtained at 1 x 10^8 spores by mL (index severity of 62 %), indicate that at more

concentration of inoculum antagonist, the inhibition was better. Then, the antagonist strains probed here are a serious candidates for reduce the disease symptoms caused by *R. solani* specifically in the surface of tuber Clon 99-39 of *S. tuberosum* L.

It has been established that several species of the genus *Trichoderma* and *Bacillus* have a great antagonism effect over the phytopathogen fungus such as *R. solani*. Several studies describe the use of *B. subtilis* as a biological control agent in individual ads or in combination with other microorganisms such as *Trichoderma* spp. The use of two or more antagonist microorganisms enhanced the efficacy for the control of phytophatogens microorganisms in rice crops (Abeysingne, 2007; Andrei *et al*, 2012; Lixuan *et al*, 2008; Yang *et al*, 2009).

The Fig. 5 shows that *B. subtilis* got a minor effect for reducing the severity symptoms of the disease in the surface of the tuber caused by *R. solani*. Although *B. subtilis* was able to promote high yields of the tuber and height of the plant, but not was too effective such as *T. harzianum* due the appariency of the surface tuber, in this case was not totally healthy. However, Romeiro *et al* (2010) reported that *B. cereus* syntethyze a protein that increase the resistance to the phytopatogen fungus *Corynespora cassicola* and when compared with a commercial fungicide, the number of lesions was almost similar. Ashwini and Srividga (2014) reported that *B. subtilis* produce chitinases, glucanases and cellulases during their antagonist activity against *Colletotrichum gloesporioides* in chilli farming. Chen et al (2013) argues that *B. subtilis* have an antagonist effect due at the biofilms formation; this behavior was observed against *Ralstonia solanacearum* in tomatoes crops under greenhouses conditions. Others authors reported that the antagonist affectivity of *B. subtilis* occurs when the bacteria excrete several molecules, such as micosubtiline (Leclere et al, 2005), subtiline (Zuber et al, 1993), subtilosine A (Babasaki et al, 1985), Tas A (Stover and Driks, 1999) and sublancine (Paik et al, 1988), this molecules

have an a ribosomal origin. There are other molecules with antagonist activity in *B. subtilis* such as bacilisine, clorotetaine, micobalicine (Zuber *et al*, 1993), rizocticcine (Kugler et al, 1990), bacilaena (Patel et al, 1995), dificicline (Zimmerman et al, 1987) and lipopeptides such as surfactine, uterine and fengicines (Zuber et al, 1993).

In other hand, the genus *Bacillus* spp. and *Trichoderma* spp. are able to growth in a wide range of pH´s and all kinds of soils. Furthermore, thanks to the combination of antagonist species, the great wide of secreted metabolites and hydrolytic enzymes that causes parasitism, the synergy of the species for control *R. solani* ensure the success for their control in *S. tuberosum.* Although the Clon 99-39 is one of the most used in central Mexico, there are others Clons and varieties of *S. tuberosum* that are used have a yield above of 25 tons/hectare and the median in Mexico is around 28 ton/hectare. The use of microorganisms of biological control can rise the yields and productivity in the Mexican fields, although the use of the commercial agents such as *T. harzianum* begins the use in the field, there are not widespread use in many areas, therefore the use of the antagonist of *R. solani* is a real promise for increase the productivity in the Mexican fields. However in necessary the development of techniques for a commercial production of the antagonist that have been prove their affectivity against any phytophatogen fungus or bacteria (Harman, 2000).

In the Fig. 6, there is a comparison of the severity of the disease caused by *R. solani* when we used different treatments combinations with *T. harzianum* and *B. subtilis*. The major severity was observed in the positive control (R+), which was only infected by *R. solani*., this result demonstrate the affectivity of the infection of *R. solani* in the peat moss substrate. The treatments with the most visible damage caused by *R. solani* were when we used less spore concentration, for example T1B1 (1×10^7 spores by mL of *T. harzianum* and 1×10^6 cells by mL of *B. subtilis).* But when we increased the

concentrations of the both antagonists, the index severity was reduced significantly, and when we used the highest concentration of antagonist (1 x 10^9 spores by mL of *T. harzianum* and 1 x 10^8 spores by mL of *B. subtilis*) seem a lot to the negative control (R-), which was not infected by *R. solani*. Therefore, the treatment T3B3 was the better results for control the damage caused by *R. solani* under greenhouse conditions (Fig. 6).

The highest concentrations of *T. harzianum* and *B. subtilis* ((1 x 10^9 spores and 1 x 10^8 cells by mL, respectivelly) outcome in the better inhibition of *R. solani* symptoms in the surface of the tuber Clon 99-39. This phenomenon probably to the antibiotic production lipopedtide Iturine A, reported for *B. subtilis* RB14-C (Ohno et al, 1992; Huang et al, 1993), and this metabolite also shows a strong inhibitory activity *in vitro* for a several filamentous fungus (Hiraoka et al, 1992). When this strain was mixing in the soil is able to inhibit the growth of *R. solani* (Asaka and Shoda, 1996). *B. subtilis* is able to growth in a solid and a submerged medium, but when the bacteria come on in contact with the soil, this sporulate immediately (Szczech and Shoda, 2006) and come in latency as spores, in this case in not able to produce antibiotics. Possibly to these phenomena, the low concentrations of *B. subtilis* used in this study don't have a significant antagonist effect against *R. solani*, but when there are ideal conditions, the spores are able to germinate and therefore the antibiotic production begins. Shoda (2000) reported this phenomenon in the rhizosphere of the plants and this is the place where the stolon of the plant potatoes causes to swell and growth in the roots. In our case, the affectivity of *B. subtilis* against *R. solani* could be at the fact that the Clon 99-39 suffers a bath where the antagonist was able to join to the seed and this could be increased by the use of the xanthan gum in the process. Furthermore, in the seed begin the development of the root and stalk and a posteriori in the development of the tuber, ensue the presence of the *T. harzianum* and *B. subtilis* thought the yields of the crops.

In this context, we ensures that antagonist species used in this study, are able to keep near of the root for could act as antagonist of *R. solani* and promote the factor growth liberation in the root of the plant and in the tuber, this phenomena has been reported by Weller (1998), Zheng and Sinclair (2000). Know well the population dynamic of the microorganisms in the rhizosphere is useful to understand the physical mechanisms of the antagonist activity of *T. harzianum* and *B. subtilis* against *R. solani* in the development of the plant and the tuber. Furthermore, the complete treatment consisted in four additional applications of the spores in the plants thought two months. Our results are according with the reported by Reddy and Rahe (1989a), Safiyasov et al (1995), Sailaja et al (1987), Zheng and Sinclair (2000), Manjula and Podile (2001), where these authors reported that treatment of the seeds of *S. tuberosum* with *B. subtilis* increased significantly the growth of the tuber and reduced the effect of the phytopathogen fungus. Milus and Rothrock (1993) reported that the treatment of oats seeds with *B. subtilis* and *B. pumilus* reduced significantly the effect of the pathogen microorganisms when is compared with the control treatment.

We concluded that the combined use of *T. harzianum* and *B. subtilis* were successful for antagonist of the phytopathogen fungus of *R. solani* in the seed Clon 99-39 of the *S. tuberosum* L. The dual combination of these antagonists increased significantly the weight of the tuber and the height of the plant. And these microorganisms reduce significantly the index severity of the disease caused by *R. solani* although the symptoms still appear in the crop tuber.

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Figure legends

Table 1. Treatments of the randomized blocks

Treatment	Dosage (Spores or cells by mL) of antagonists microorganisms	Nomenclature	Fresh weight of the tuber (ANOVA Tukey, $p < 0.05$)	Height of the plant (ANOVA Tukey, $p < 0.05$)
1	Contaminated by *R. solani*	R+	a	b
2	Sterile substrate only	R-	a	a
3	*T. harzianum* 1.0×10^7	T1	b	a
4	*T. harzianum* 1.0×10^8	T2	b	b
5	*T. harzianum* 1.0×10^9	T3	b	b
6	*B. subtilis* 1×10^6	B1	b	b
7	*B. subtilis* 1×10^7	B2	b	b
8	*B. subtilis* 1×10^8	B3	b	b
9	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^6	T1B1	b	b
10	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^7	T1B2	b	b
11	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^8	T1B3	b	b
12	*T. harzianum* 6.6×10^8 and *B. subtilis* 1×10^6	T2B1	b	b
13	*T. harzianum* 1.0×10^8 and *B. subtilis* 1×10^7	T2B2	b	b
14	*T. harzianum* 6.6×10^8 and *B. subtilis* 1×10^8	T2B3	b	b
15	*T. harzianum* 6.6×10^9 *B. subtilis* 1×10^6	T3B1	b	b
16	*T. harzianum* 1.0×10^9 and *B. subtilis* 1.0×10^7	T3B2	c	b
17	*T. harzianum* 1.0×10^9 and *B. subtilis* 1×10^8	T3B3	c	b

Same letter means a not significant difference ($p < 0.05$), ANOVA Tukey

Different letter means a significant difference ($p < 0.05$), ANOVA Tukey

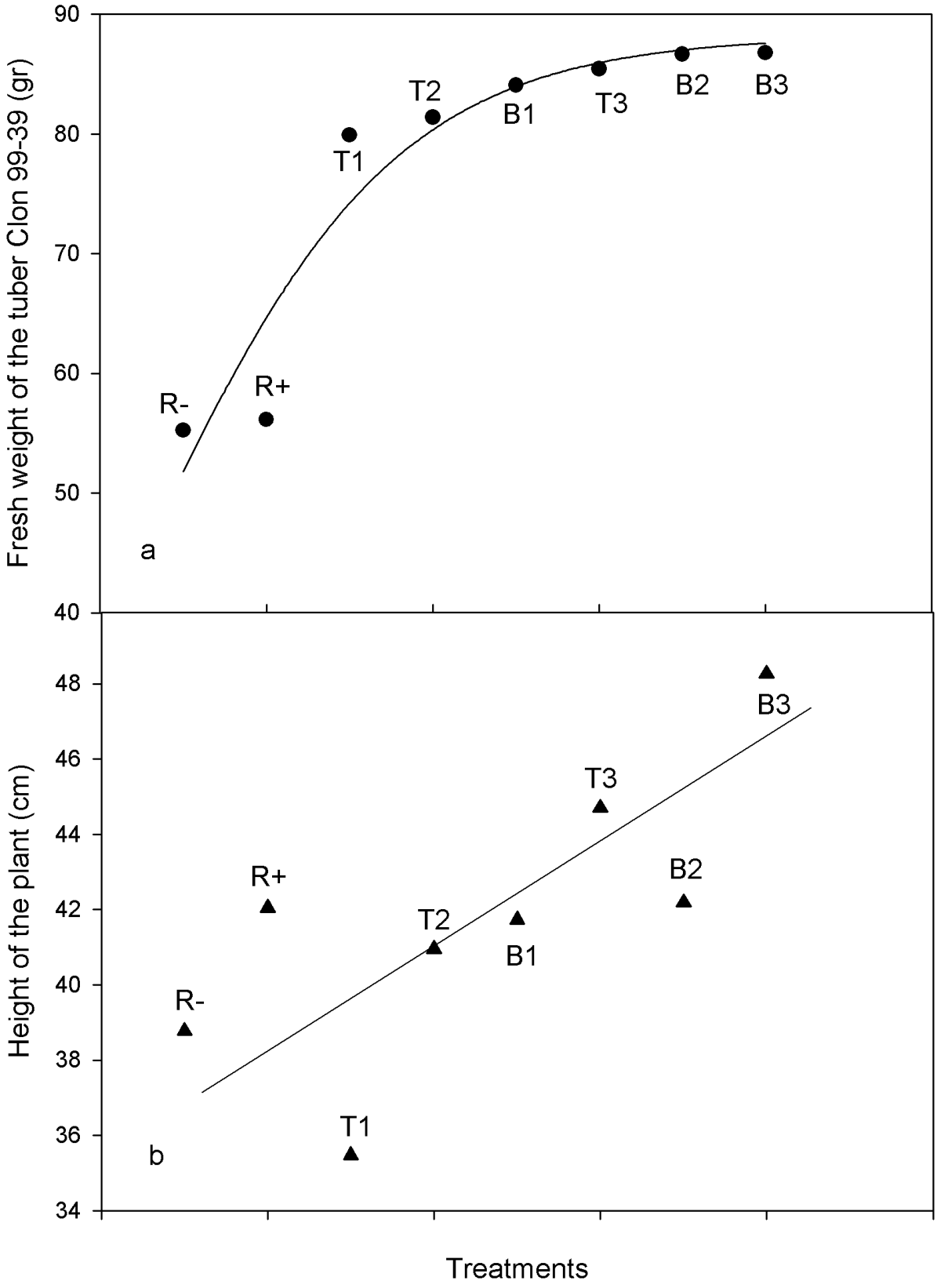

Fresh weight of the tuber Clon 99-39 (gr)
90
80
70
60
50
40
R-
R+
T1
T2
B1
T3
B2
B3
a
Height of the plant (cm)
48
46
44
42
40
38
36
34
B3
T3
R+
B2
T2
B1
R-
T1
b
Treatments

Fig. 1. Comparison of the different treatments with single antagonist concentration of *T. harzianum* or *B. subtillis* on the fresh weight of Clon 99-39 of *S. tuberosum* and height of the plant. **Fig. 1a**. Effect of the antagonist concentration on the final fresh weight of the Clon 99-39: for *T. harzianum* T1 (1 x 10^7 spores/mL), T2 (1 x 10^8 spores/mL), T3 (1 x 109 spores/mL); for *B. subtilis* B1 (1 x 10^6 cells/mL), B2 (1 x 10^7 cells/mL), B3 (1 x 10^8 cells/mL); R+ belongs to the infected potatoes by *R. solani* (positive control) and R- correspond to negative control (not infected and nor antagonist presence). **Fig. 1b**. Effect of the antagonist concentration on the final height plant of the Clon 99-39: Same treatments as described in Fig. 1a. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

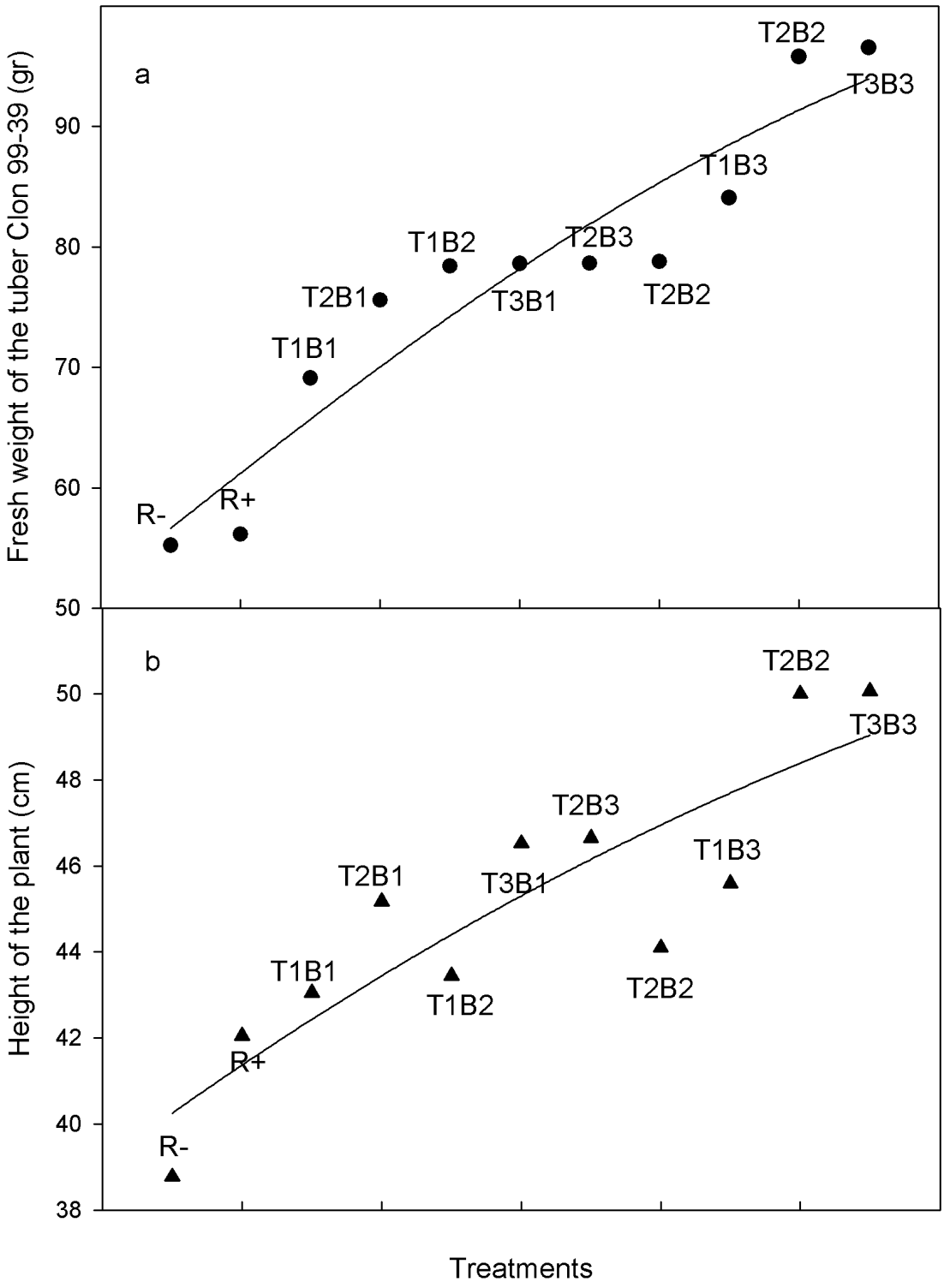

a
b
Fresh weight of the tuber Clon 99-39 (gr)
Height of the plant (cm)
Treatments
R-
R+
T1B1
T2B1
T1B2
T3B1
T2B3
T2B2
T1B3
T3B3
90
80
70
60
50
48
46
44
42
40
38

Fig. 2. Comparison of the different treatments with formulations of *T. harzianum* and *B. subtilis* combinations over the fresh weight of Clon 99-39 of *S. tuberosum* and height of the plant. **Fig. 2a**. Effect of the antagonist concentration on the final fresh weight of the Clon 99-39, combinations: T1B1 (1×10^7 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T1B2 (1×10^7 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *Bacillus subtilis*), T1B3 (1×10^7 spores/mL of *T. harzianum*; $\times 10^8$ cells/mL of *B. subtilis*), T2B1 (1×10^8 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T2B2 (1×10^8 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *B. subtilis*), T2B3 (1×10^8 spores/mL of *T. harzianum*; 1×10^8 cells/mL of *B. subtilis*), T3B1 (1×10^9 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T3B2 (1×10^9 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *B. subtilis*), T3B3 (1×10^9 spores/mL of *T. harzianum*; 1×10^8 cells/mL of *B. subtilis*); R+ belongs to the infected potatoes by *R. solani* (positive control) and R- correspond to negative control (not infected and nor antagonist presence). **Fig. 2b**. Effect of the antagonist concentration on the final height plant of the Clon 99-39: Same treatments as described in Fig. 2a. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

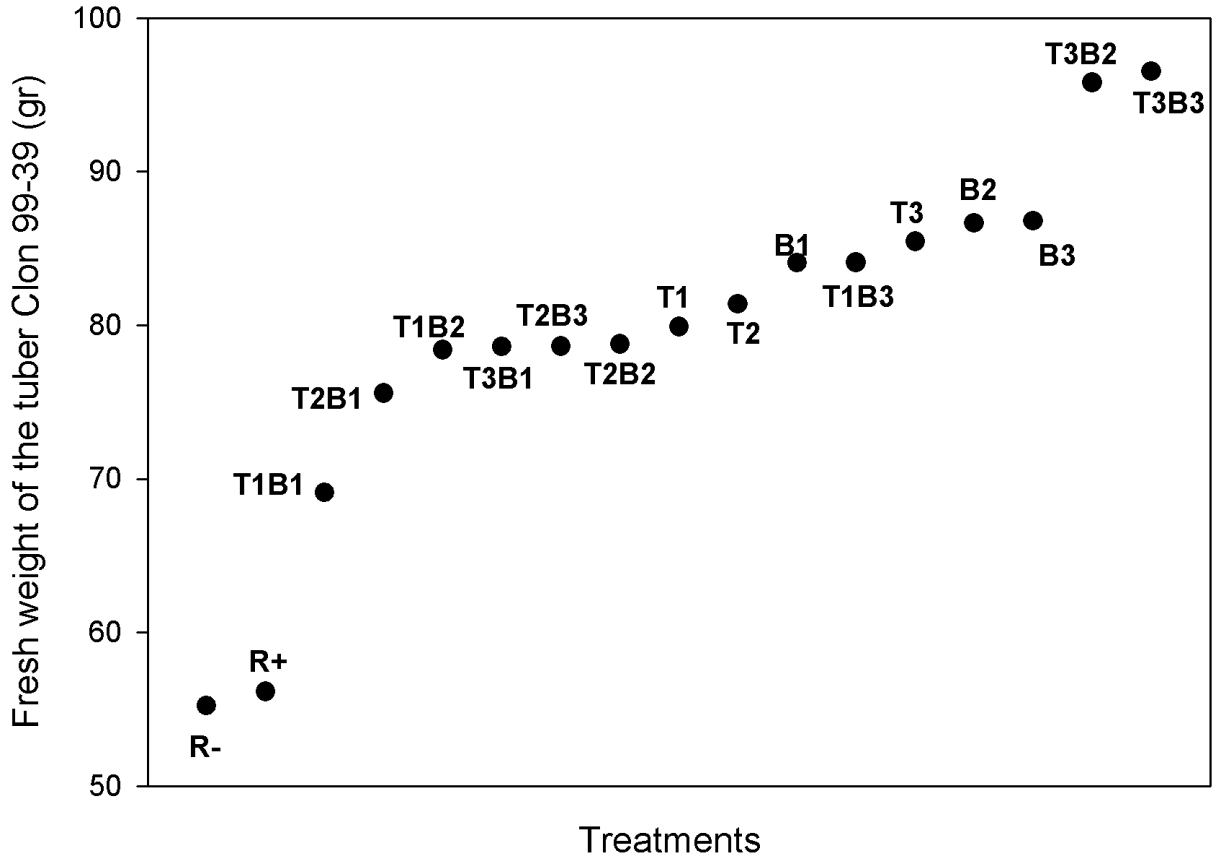


Fig. 3. Comparison of the treatments of *T. harzianum* and *B. subtilis* over the fresh weight of Clon 99-39 of *S. tuberosum*. The Fig. 3 shows the enhanced of the growth by the antagonist over the fresh weight of the plant, the treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks thought two months of the experiment.

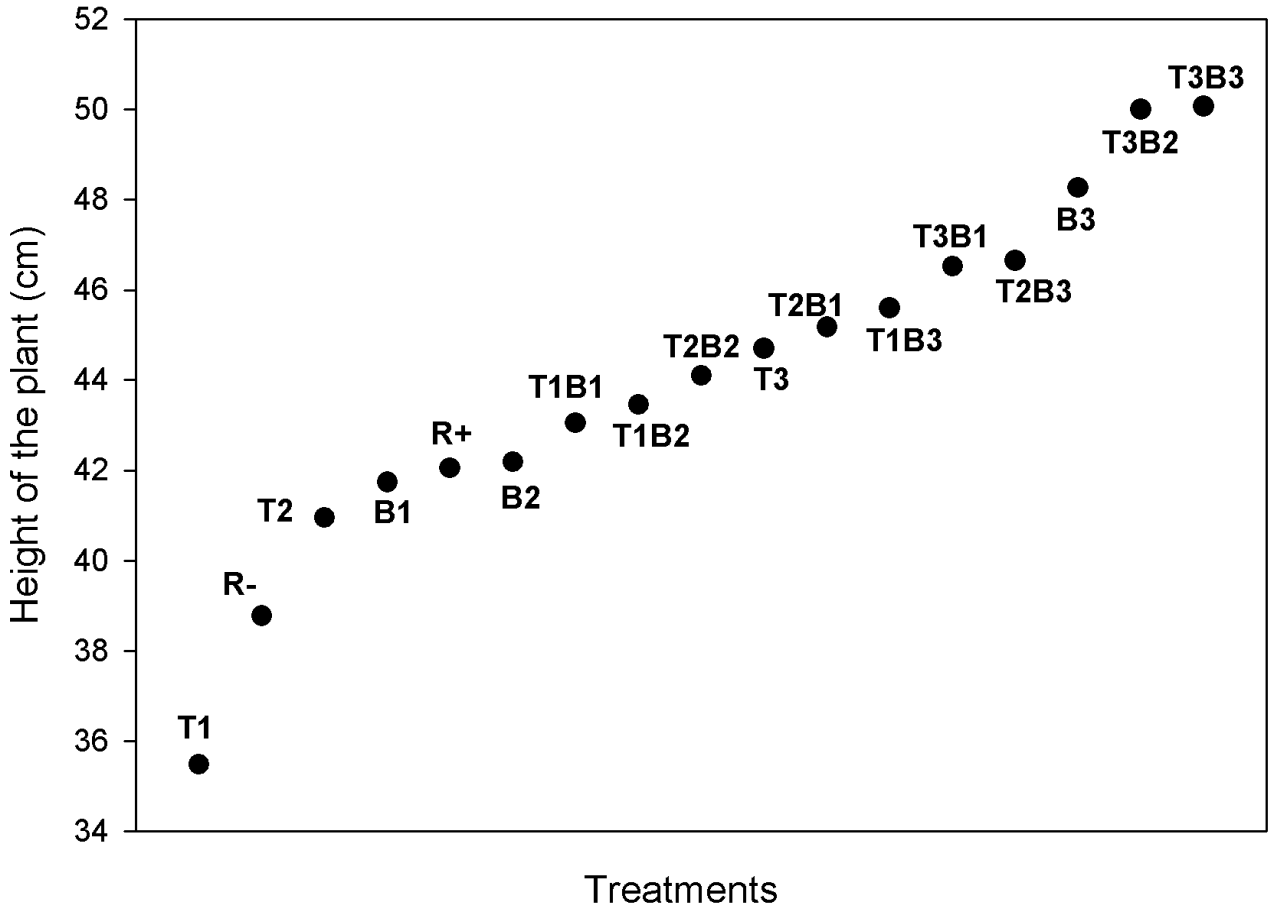


Fig. 4. Comparison of the treatments of *T. harzianum* and *B. subtilis* on the height of the plant of Clon 99-39 of *S. tuberosum*. The Fig. 5 shows the enhanced of the growth by the antagonist over the height of the plant, the treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks thought two months of the experiment.

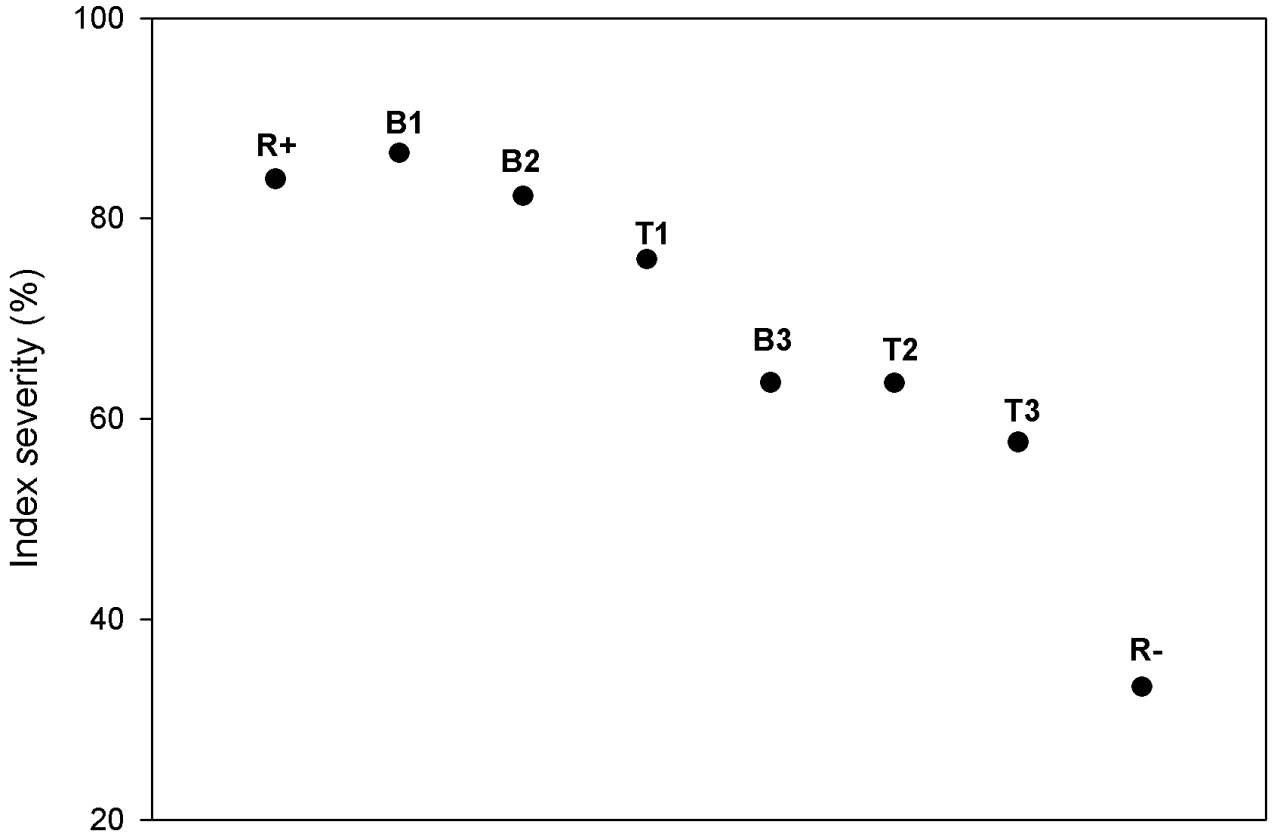


Fig. 5. Comparison of the combination of antagonist of *T. harzianum* and *B. subtilis* on the index severity of the *R. solani* in the surface of Clon 99-39 of *S. tuberosum*. The treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

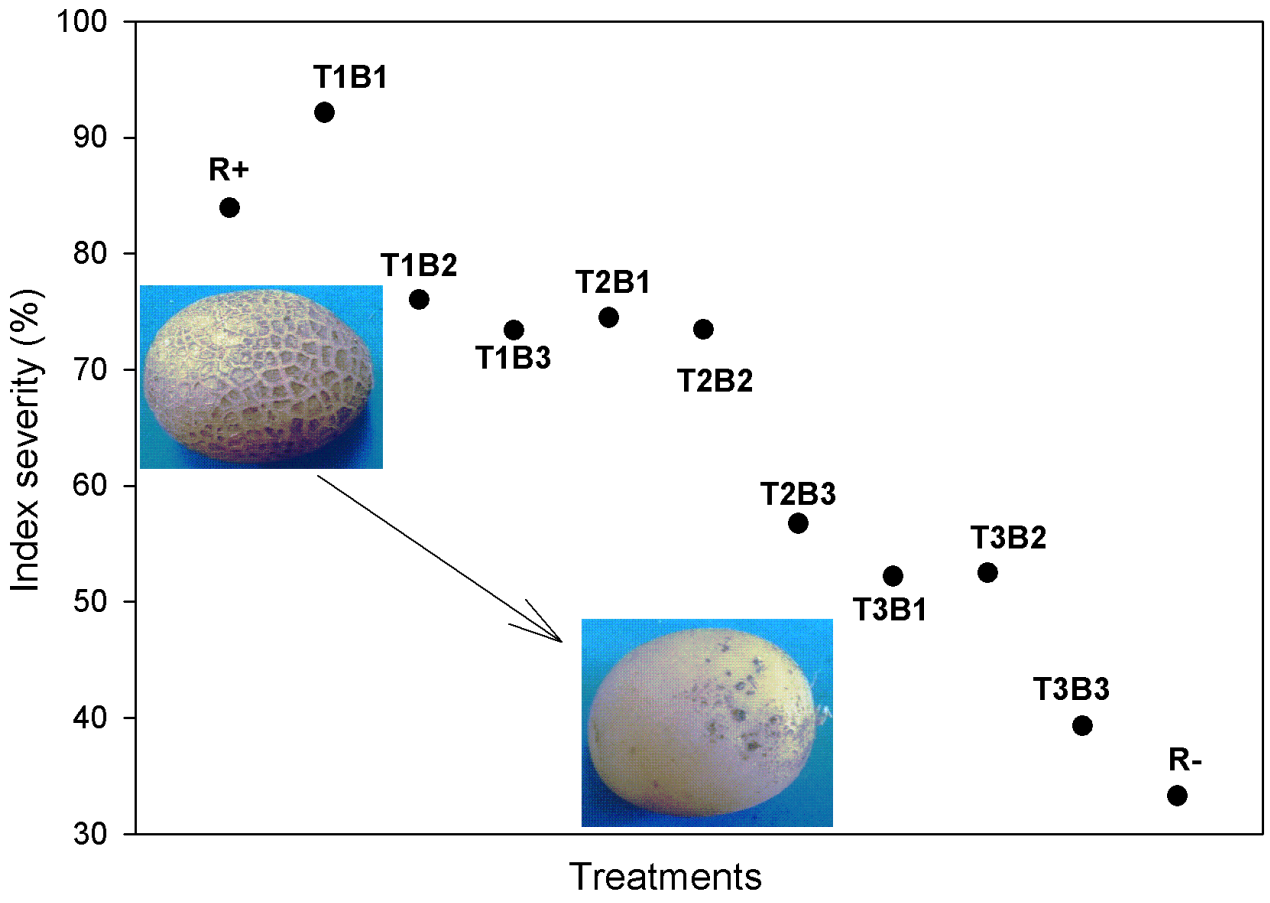


Fig. 6. Comparison of the treatments of *T. harzianum* and *B. subtilis* on the index severity of the *R. solani* in the surface of Clon 99-39 of *S. tuberosum*. The treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

HIGHLIGHTS

- *Bacillus subtilis* ATCC 6633 and *Trichoderma harzianum* CDBB-H-1125 enhances height and yields on crops on seed Clon 99-39 of *Solanum tuberosum* L.
- The highest concentration of spores of *Trichoderma harzianum* CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 diminished the disease injures caused by *Rhizoctonia solani* in seed Clon 99-39 of *Solanum tuberosum* L.

Graphical Abstract

Seed Clon 99-39

Infected with *R. solani* only

Infected with *R. solani* and treated with *T. harzianum* and *B. subtilis*

Tables

Table 1. Treatments of the randomized blocks

Treatment	Dosage (Spores or cells by mL) of antagonists microorganisms	Nomenclature	Fresh weight of the tuber (ANOVA Tukey, $p < 0.05$)	Height of the plant (ANOVA Tukey, $p < 0.05$)
1	Contaminated by *R. solani*	R+	a	b
2	Sterile substrate only	R-	a	a
3	*T. harzianum* 1.0×10^7	T1	b	a
4	*T. harzianum* 1.0×10^8	T2	b	b
5	*T. harzianum* 1.0×10^9	T3	b	b
6	*B. subtilis* 1×10^6	B1	b	b
7	*B. subtilis* 1×10^7	B2	b	b
8	*B. subtilis* 1×10^8	B3	b	b
9	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^6	T1B1	b	b
10	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^7	T1B2	b	b
11	*T. harzianum* 6.6×10^7 and *B. subtilis* 1×10^8	T1B3	b	b
12	*T. harzianum* 6.6×10^8 and *B. subtilis* 1×10^6	T2B1	b	b
13	*T. harzianum* 1.0×10^8 and *B. subtilis* 1×10^7	T2B2	b	b
14	*T. harzianum* 6.6×10^8 and *B. subtilis* 1×10^8	T2B3	b	b
15	*T. harzianum* 6.6×10^9 *B. subtilis* 1×10^6	T3B1	b	b
16	*T. harzianum* 1.0×10^9 and *B. subtilis* 1.0×10^7	T3B2	c	b
17	*T. harzianum* 1.0×10^9 and *B. subtilis* 1×10^8	T3B3	c	b

Same letter means a not significant difference ($p < 0.05$), ANOVA Tukey

Different letter means a significant difference ($p < 0.05$), ANOVA Tukey

Figure

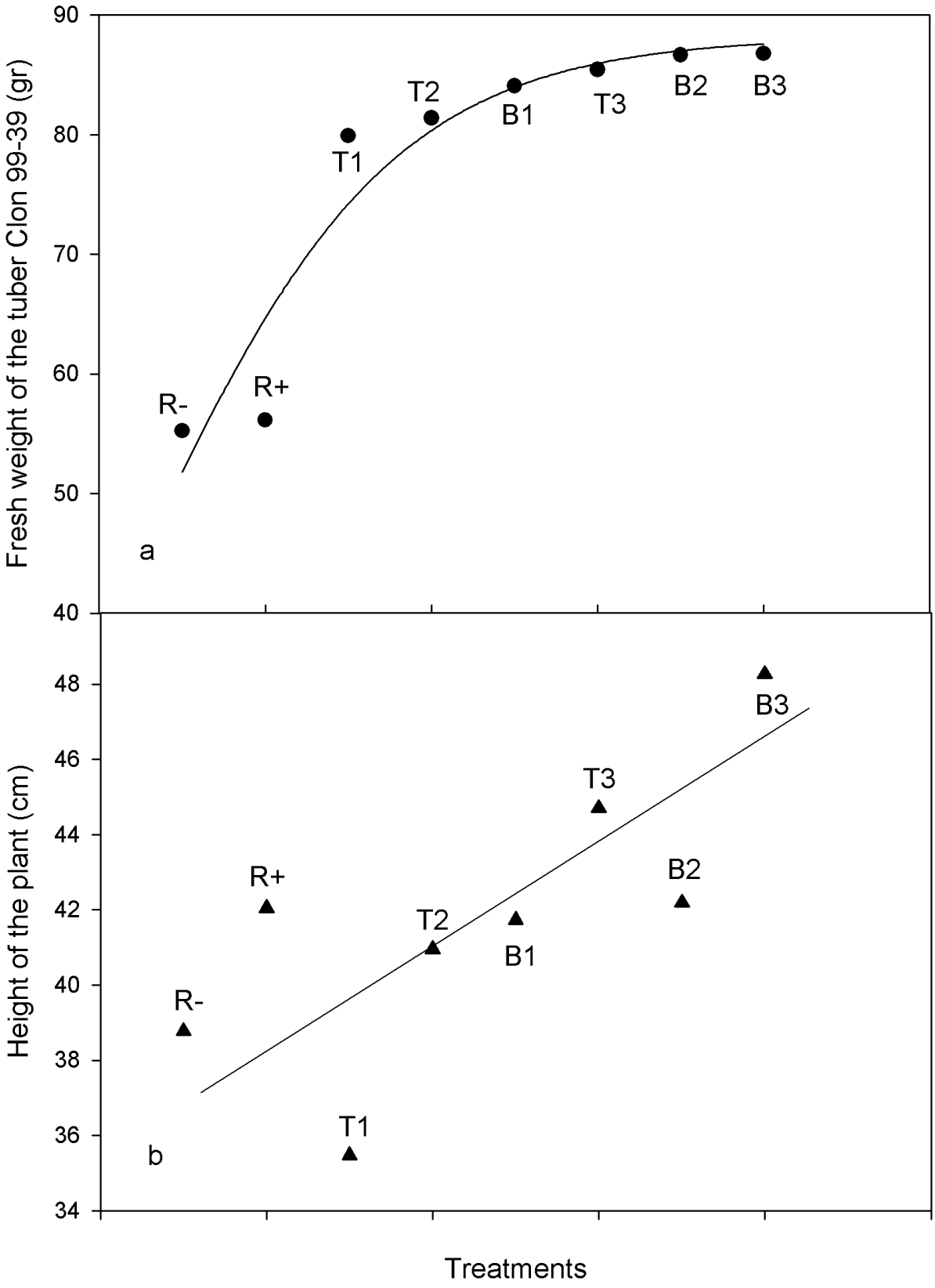

Fresh weight of the tuber Clon 99-39 (gr)
90
80
70
60
50
40
R-
R+
T1
T2
B1
T3
B2
B3
a
Height of the plant (cm)
48
46
44
42
40
38
36
34
R-
R+
T1
T2
B1
T3
B2
B3
b
Treatments

Fig. 1. Comparison of the different treatments with single antagonist concentration of *T. harzianum* or *B. subtillis* on the fresh weight of Clon 99-39 of *S. tuberosum* and height of the plant. **Fig. 1a**. Effect of the antagonist concentration on the final fresh weight of the Clon 99-39: for *T. harzianum* T1 (1 x 10^7 spores/mL), T2 (1 x 10^8 spores/mL), T3 (1 x 109 spores/mL); for *B. subtilis* B1 (1 x 10^6 cells/mL), B2 (1 x 10^7 cells/mL), B3 (1 x 10^8 cells/mL); R+ belongs to the infected potatoes by *R. solani* (positive control) and R- correspond to negative control (not infected and nor antagonist presence). **Fig. 1b**. Effect of the antagonist concentration on the final height plant of the Clon 99-39: Same treatments as described in Fig. 1a. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

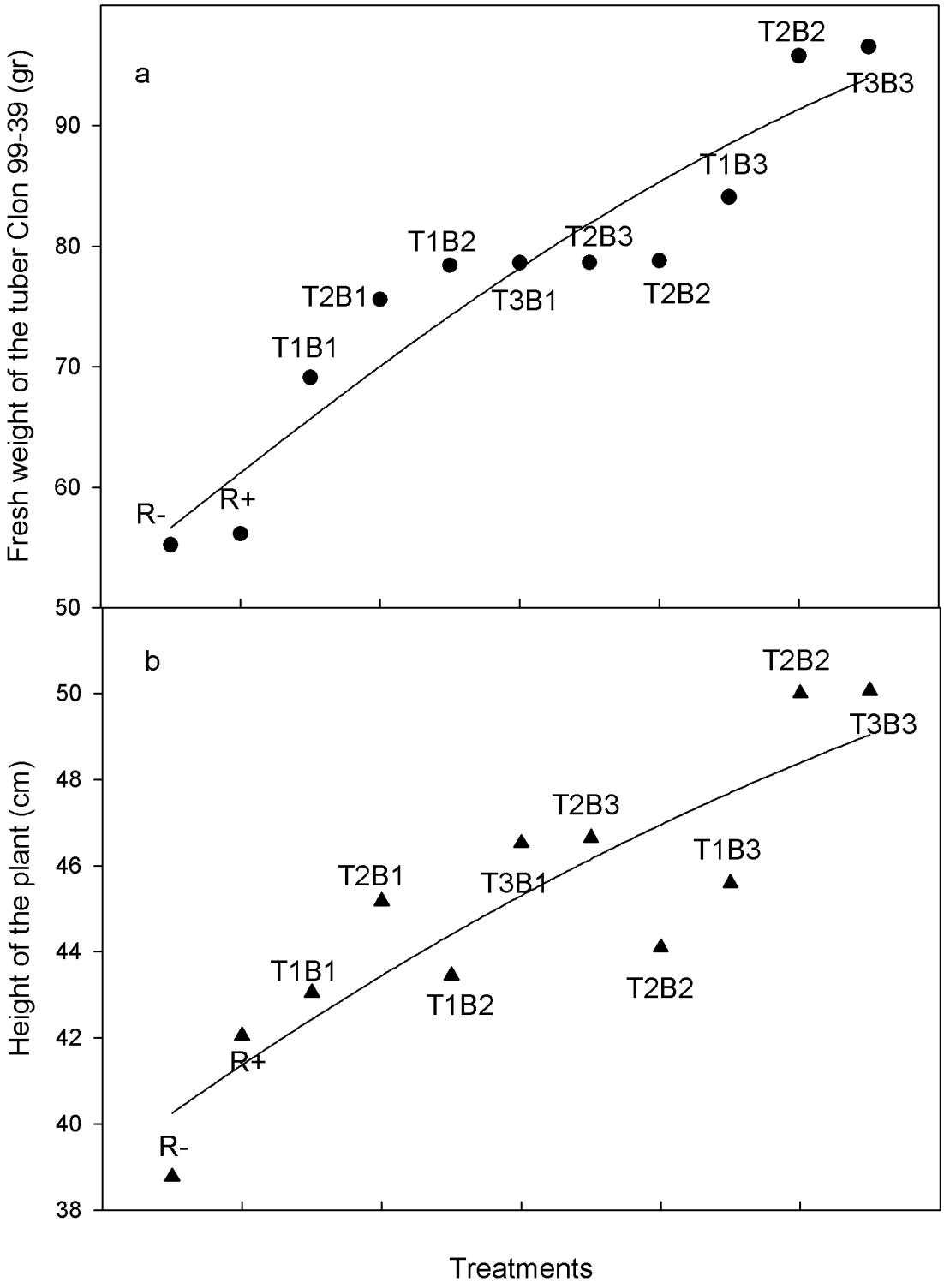

a
b
Fresh weight of the tuber Clon 99-39 (gr)
Height of the plant (cm)
Treatments
R-
R+
T1B1
T2B1
T1B2
T3B1
T2B3
T2B2
T1B3
T3B3
90
80
70
60
50
48
46
44
42
40
38

Fig. 2. Comparison of the different treatments with formulations of *T. harzianum* and *B. subtilis* combinations over the fresh weight of Clon 99-39 of *S. tuberosum* and height of the plant. **Fig. 2a**. Effect of the antagonist concentration on the final fresh weight of the Clon 99-39, combinations: T1B1 (1×10^7 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T1B2 (1×10^7 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *Bacillus subtilis*), T1B3 (1×10^7 spores/mL of *T. harzianum*; $\times 10^8$ cells/mL of *B. subtilis*), T2B1 (1×10^8 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T2B2 (1×10^8 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *B. subtilis*), T2B3 (1×10^8 spores/mL of *T. harzianum*; 1×10^8 cells/mL of *B. subtilis*), T3B1 (1×10^9 spores/mL of *T. harzianum*; 1×10^6 cells/mL of *B. subtilis*), T3B2 (1×10^9 spores/mL of *T. harzianum*; 1×10^7 cells/mL of *B. subtilis*), T3B3 (1×10^9 spores/mL of *T. harzianum*; 1×10^8 cells/mL of *B. subtilis*); R+ belongs to the infected potatoes by *R. solani* (positive control) and R- correspond to negative control (not infected and nor antagonist presence). **Fig. 2b**. Effect of the antagonist concentration on the final height plant of the Clon 99-39: Same treatments as described in Fig. 2a. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

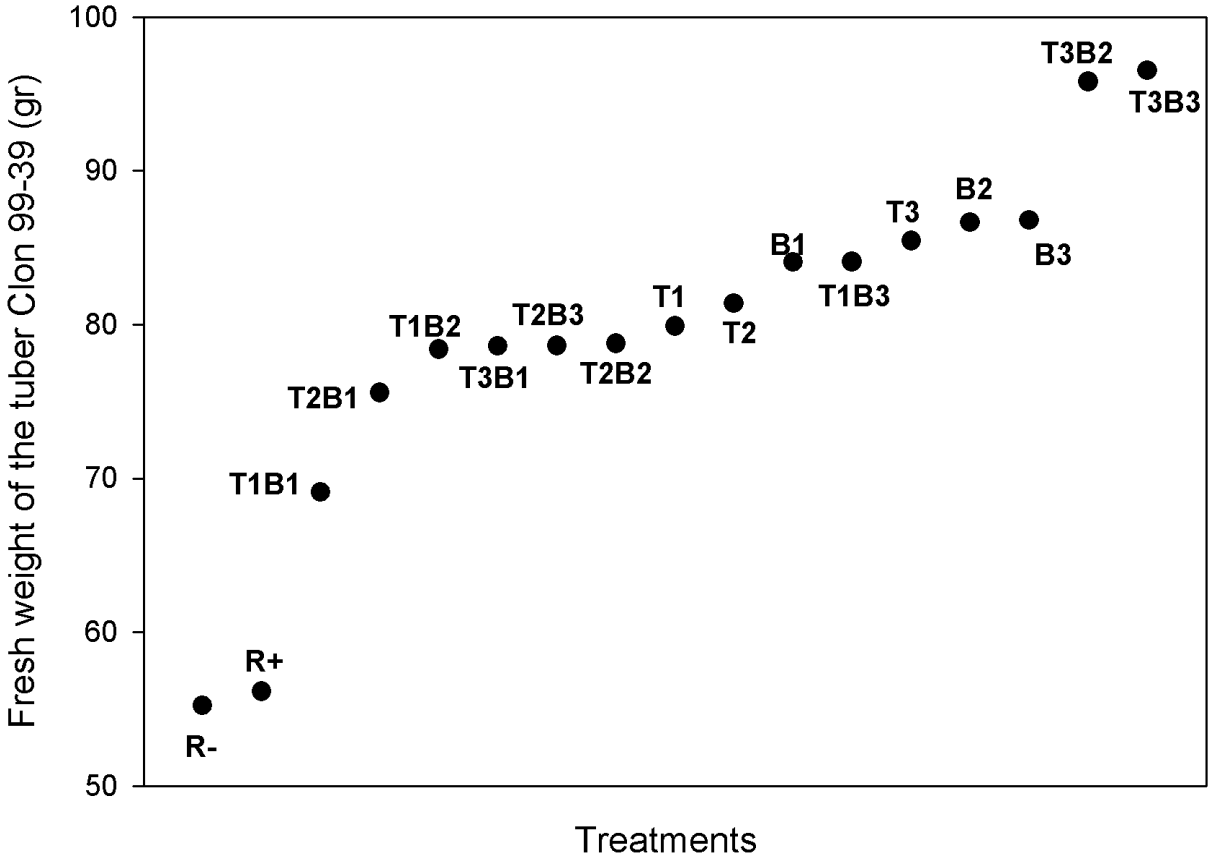
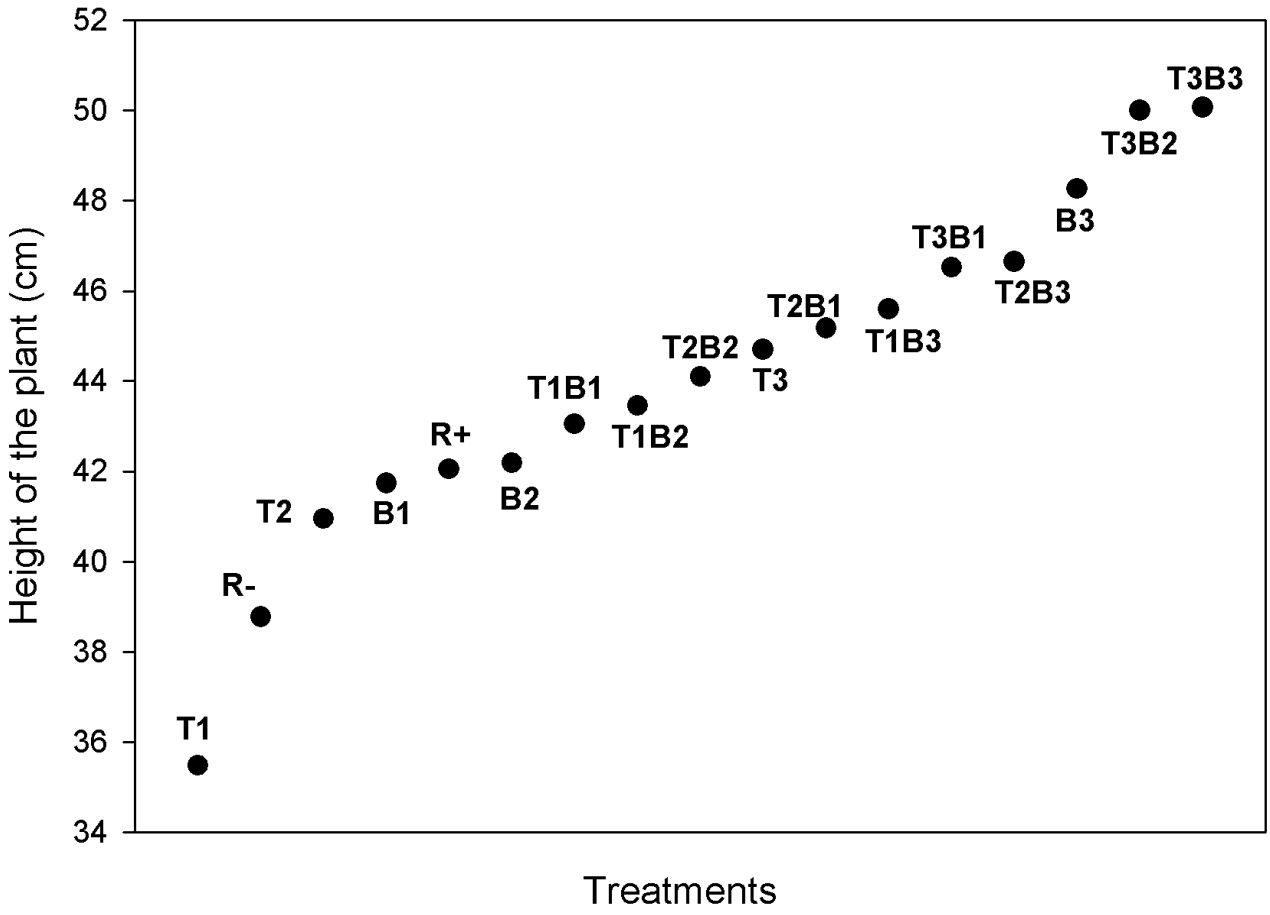


Fig. 3. Comparison of the treatments of *T. harzianum* and *B. subtilis* over the fresh weight of Clon 99-39 of *S. tuberosum*. The Fig. 3 shows the enhanced of the growth by the antagonist over the fresh weight of the plant, the treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks thought two months of the experiment.



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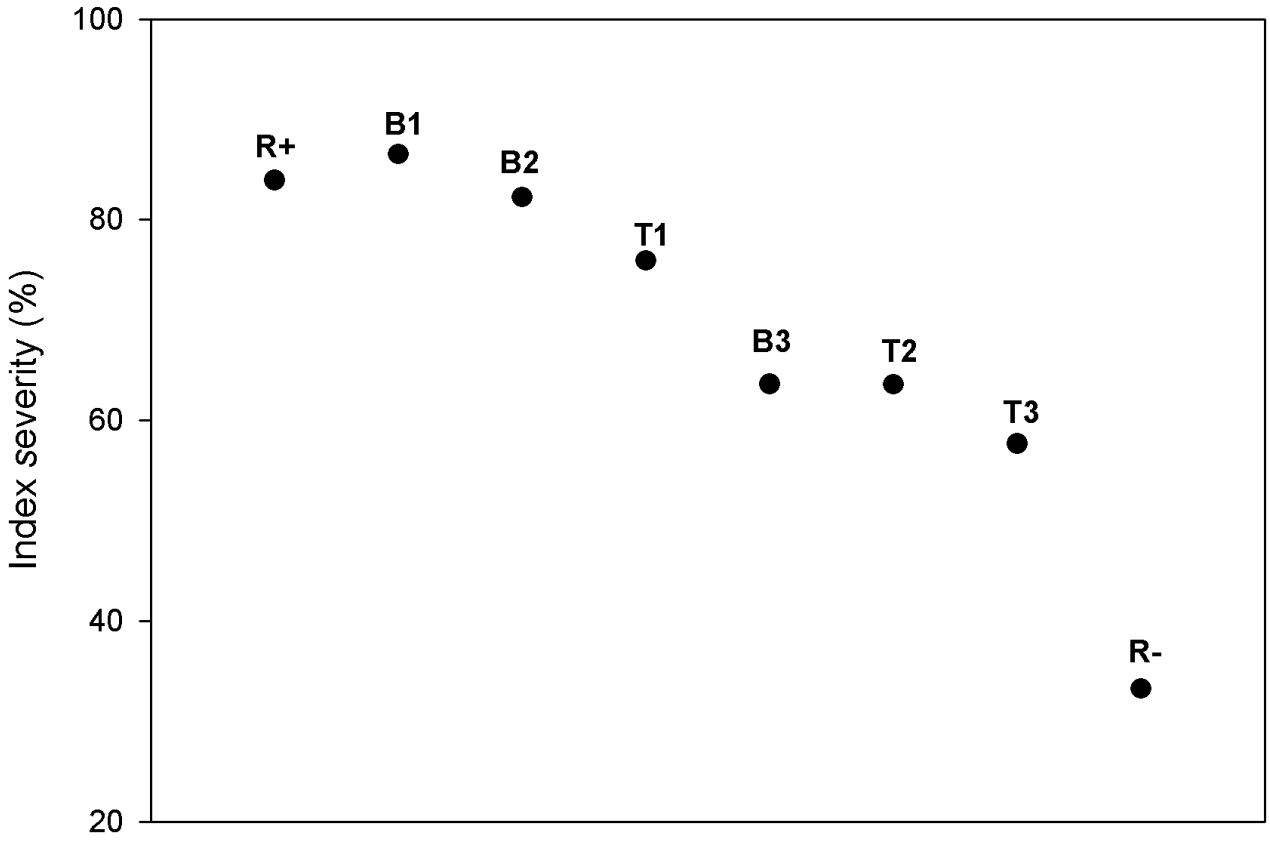

Fig. 5. Comparison of the combination of antagonist of *T. harzianum* and *B. subtilis* on the index severity of the *R. solani* in the surface of Clon 99-39 of *S. tuberosum*. The treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

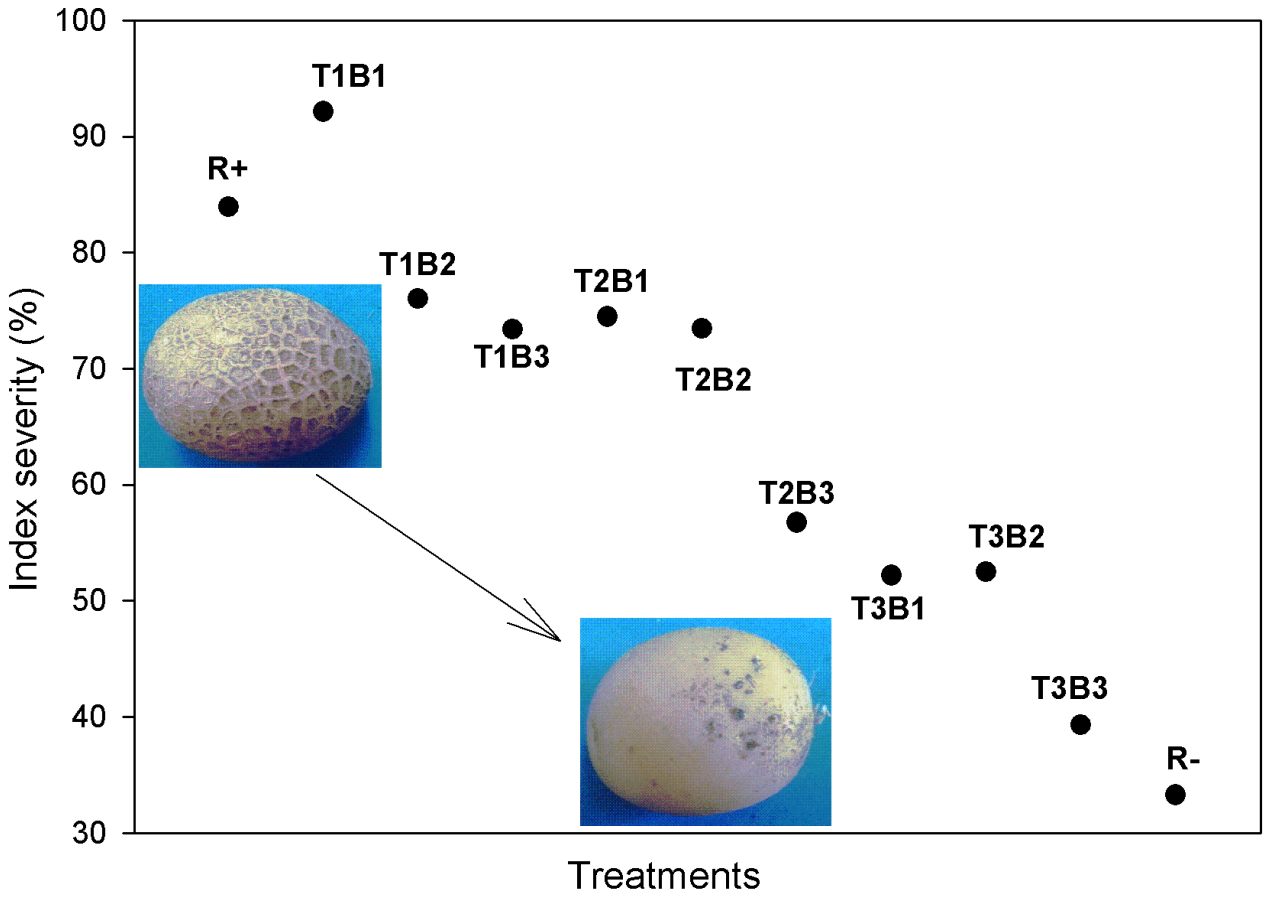

Fig. 6. Comparison of the treatments of *T. harzianum* and *B. subtilis* on the index severity of the *R. solani* in the surface of Clon 99-39 of *S. tuberosum*. The treatments are described in the Table I. The seed of Clon 99-39 was inoculated at the beginning of the experiment and each two weeks through two months of the experiment.

HIGHLIGHTS

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- The highest concentration of spores of *Trichoderma harzianum* CDBB-H-1125 and *Bacillus subtilis* ATCC 6633 diminished the disease injures caused by *Rhizoctonia solani* in seed Clon 99-39 of *Solanum tuberosum* L.

Graphical Abstract

Seed Clon 99-39

Infected with *R. solani* only

Infected with *R. solani* and treated with *T. harzianum* and *B. subtilis*