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PLANT GROWTH PROMOTING BACTERIA WITH ANTIFUNGAL ACTIVITY

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Abstract

The aim of the present study was to analyse the potential of two plant beneficial bacteria, Cp.b4 and 75.1s, to prevent fungal pathogens proliferation and improve tomato seedlings growth. The bacterial strains were selected from the RDIPP microbial collection, due to their antifungal activity towards solanaceous plant pathogens. Both strains were previously identified as Bacillus cereus/thuringiensis based on their biochemical profile with the Biolog GEN III system. Growth chamber tests on tomato seedlings have shown that biological treatments, with the above-mentioned bacterial strains, induced a better vegetative growth to the seedlings, and increased the photosynthetic capacity of the plants. To prevent early blight caused by Alternaria sp. in solanaceous plants, the ability to inhibit conidia germination was analysed. Biological treatments with bacterial suspension reduced the number of germinated conidia by 4.98 fold (when using 75.1s strain) and by 2.57 fold (when using Cp.b4 strain). The study also revealed that mixed treatments of bacteria suspension along with a low dose of fungicide inhibit conidia germination, being comparable with the chemical control, Mycoguard 500 SC, applied at the recommended dose of 0.2%.

Key words*: PGPB, tomatoes, early blight prevention.*

INTRODUCTION

Tomato culture is an important vegetable grown in greenhouses and open fields. The culture has numerous pests and diseases in all stages of vegetation and post-harvesting. Moreover, new races of pathogens and emerging pests make plant protection more difficult. Therefore, there is a continuous interest in preventing pathogenic infections and increasing the productive potential of the plant.
Among plant pathogens, *Alternaria* spp. (like *A. solani*, *A. alternata* or *A. tomatophila*) are ubiquitous fungal phytopathogens, which can induce early blight in various plant species, including tomatoes and other edible plants (Thomma, 2003; Woudenberg et al., 2015). These pathogens infect plants in various growth stages, including vegetable fruits during ripening, causing fruit rot. It survives and overwinters as saprophyte on plant debris. The leaf infections occur on cool, wet weather, and fruit rot symptoms are usually associated with a predisposing injury due to sunscald, frost, or blossom end rot (calcium deficiency) (Chaerani and Voorrips, 2006; Kennelly et al., 2012). It is, therefore, important to maintain a good physiological status during plant growth in order to prevent infections. Moreover, microbial inoculation with plant growth promoting bacteria having biocontrol activity could improve plants health and vigour in order to prevent pathogenic infections (Wang et al., 2008; Jagadeesh and Jagadeesh, 2009; Zahoor et al., 2017).
Considering these, we studied two plant beneficial bacteria, Cp.b4 and 75.1s, regarding their biocontrol potential in preventing *Alternaria* sp. and other fungal pathogens proliferation. Microbial ability to improve tomato seedlings growth was also evaluated.

MATERIALS AND METHODS

Bacterial strains

Two bacterial strains were used in this study, 75.1s and Cp.b4, both from the RDIPP microbial collection. These two strains were

isolated from soil of Bărăgan area (75.1s) and onion rhizosphere from Dolj county (Cp.b4). Bacteria were stored at -80°C, in Luria Bertani (LB) medium supplemented with 30% glycerol. Routinely, they were grown on LB agar, at 28°C.

Bacterial identification procedure

Bacteria were identified with the Biolog GENIII system, using protocol B for Gram-positive, spore forming, fast growing bacilli, according to the manufacturer's guidelines (US Patent 5,627,045). Therefore, each bacterial strain was grown on Biolog Universal Growth medium, at 33°C, for not more than 18 hours. Isolated colonies were then homogenised in B type inoculation fluid, at 97% turbidity, detected in 590 nm wavelength light. Biolog GEN III Microplates, having 96 wells, were filled with bacterial suspension, disposing 100 µl in each well. These special plates are preloaded with 71 different carbon sources and 23 chemicals, which allow several biochemical reactions a single test. Inoculated plates were maintained at 33°C. Within 20 to 48 hours of incubation, plates were spectrophotometrically analysed at 590 nm and 750 nm wavelengths, at the Biolog Microstation Reader. The readings were processed with the MicroLog3 software, which identifies the microbial strains based on their phenotypic pattern.

Enzymatic assays

Bacterial ability to produce hydrolytic enzymes was tested on different growth substrates (Sicuia et al, 2015). Bacterial ability to solubilize tricalcium phosphate was tested on Pikovskaya agar medium. Chitinase production was determined on Roberts and Selitrennikoff (1988) medium with colloidal chitin from crab shell. Cellulase activity was tested on carboxymethil cellulose containing medium, and revealed with 0.1% Congo red. Lipase activity was evaluated on Tween 80 supplemented medium (mTMB), and protease production was tested on slim milk agar.

Antifungal assay

The biocontrol activity of the studied/ mentioned bacterial strains was evaluated *in vitro*, using the dual culture technique, on Potato-Dextrose-Agar medium. The antifungal assay was performed against five plant pathogens, *Alternaria* sp. (new isolate from tomato plant), *Botrytis cinerea* (new isolate from tomato fruit), *Fusarium oxysporum* f. sp. *radicis lycopersici* ZUM 2407, *Pythium debaryanum* DSM 62946 and *Rhizoctonia solani* DSM 63002.

Bacterial ability to inhibit conidia germination was also studied against *Alternaria* sp. Tests were performed *in vitro*, in 2 ml tubes, on Potato-Dextrose-Broth. Fungal spore suspension was prepared in 10^5 conidia/ml, and bacteria in 10^8 cfu/ml. A chemical control was also included within the study. Mycoguard® 500SC commercial product, based on chlorothalonil 500 g/l, was used at the recommended concentration (0.2%) for *Alternaria* spp. control (according to the pesticide factsheet). Bacterial treatments were tested individually and in combination with half of the chemical dose. This was carried out in order to see if there is any possibility in reducing the dose of pesticides for *Alternaria* spp. prevention. Samples were incubated at 25°C, optimal for conidia germination (Troncoso-Rojas and Tiznado-Hernández, 2014). The observations were made after 72 hours of incubations, using a light microscope. The test was performed in triplicates and repeated twice.

Plant growth promotion

This test was performed on tomato seedlings. Three experimental variants were studied, two biological treatments, 75.1s and Cp.b4 respectively, comparing to an untreated control. Bacterial suspensions of 10^8 cfu/ml were first applied as seed treatment, by immersion, for 20 minutes. The second treatment was applied to the soil, after transplantation, with 2 ml of bacterial suspension near each plant. Seedlings were transferred in 10 cm diameter pots. Plants were grown in controlled conditions using a Sanyo MLR351H growth chamber, in 16 hours photoperiods, at 24ºC/light, 18ºC/dark, 14000 lx and 70% RH.

Biometric observations were made six weeks after transplantation. From each experimental variant, eight plants were analysed. Root length was determined through direct measurement. Fresh weight of total aerial growth and roots were separately weighted. Dry weight was determined after 3 hours of soaking at 105°C.

Assimilatory pigments were quantified through specific analytical methods. One gram of freshly harvested leaves, from different plants of each experimental variant, were used in order to quantify chlorophyll a (chl a), chlorophyll b (chl b), xanthophyll and carotenoids. Pigments were extracted in acetone (99.99%) at 4°C, overnight. The chlorophyll, xanthophyll and carotenoids content were quantified according to Lichtenthaler and Welburn (1987). Thereafter, the filtered extracts were spectrophotometrically analysed at 662 nm, 645 nm and 470 nm, respectively. The absorbance measurements were used for assimilatory pigments quantification. Their concentration was calculated, in mg/g of fresh weight, using Lichtenthaler's equations.

RESULTS AND DISCUSSIONS

Bacterial identification

The identification was made based on bacterial phenotypic fingerprint obtained in the GEN III Microplates. These plates started out colourless. However, during incubation, an increased respiration, due to carbon source utilization or bacterial growth, reduced the tetrazolium redox dye, forming a purple color in specific wells, giving a phenotypic fingerprint for each bacterial strain (Figure 1). The color intensity was compared with the negative control for carbon source utilization assays, and positive control for chemical sensitivity assays. The phenotypic pattern was compared to Biolog's extensive species library, and the identification was given at specie level. Both strains were identified as *Bacillus cereus*/*thuringiensis*.

Figure 1. Phenotypic profile of *Bacillus cereus*/*thuringiensis* Cp.b4 on Biolog GEN III microplate under B type protocol (after 20 hours of incubation at 33°C)

Enzymatic characterisation

The bacterial strains were able to produce chitinase, carboxymethil cellulase (CMC-ase), caseinase, and lipase enzymes (Figure 2). Some of these enzymes are involved in the biocontrol strategy, degrading insect and fungal cell wall structure (Veliz et al., 2017).

Chitinase activity was detected on colloidal chitin media containing bromocresol purple as pH indicator dye (pH 4.7). Therefore, the breakdown of chitin into N-acetyl glucosamine is modifying the pH from acid (4.7) towards alkaline, thus changing the colour of the pH indicator dye, from yellow to purple. As a result of chitinase activity, a purple zone was generated around the bacterial colonies after 5 days of incubation on the specified medium (Figure 2 a). Regarding CMC and casein hydrolysis, the enzyme producing strains were surrounded by a clear hallo due to the cellulase and protease activity. The CMC-ase production was revealed by flooding the plates with 0.1% Congo red solution for 15 minutes, followed by 1M sodium chloride rinses (Figure 2 b, c). The hydrolysis of Tween 80 from mTMB medium, due to lipase activity, generated a white precipitate around bacterial colonies as a result of calcium soap production (Figure 2 d).

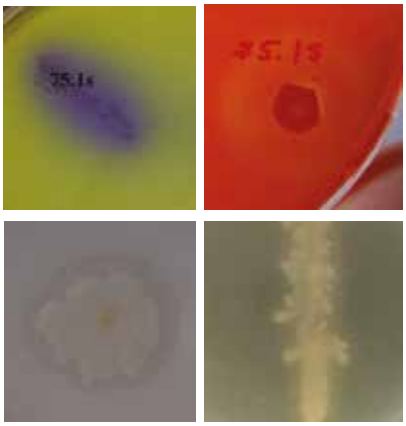

Figure 2. Bacterial hydrolytic activity:
a. chitinase, b. CMC-ase, c. protease, d. lipase

The bacterial ability to solubilize tricalcium phosphate from Pikovskaya agar medium indicates the bacterial potential in increasing plant growth through nutrient availability. Secondarily, an improved phosphorus uptake is also improving plant resistance to the phytopathogenic attacks.

Antifungal activity

The antifungal potential of the studied bacterial strains was revealed, by dual culture assay, against (new isolate from tomato plant), *Botrytis cinerea* (new isolate from tomato fruit), *Fusarium oxysporum* f. sp. *radicis lycopersici* ZUM 2407, *Pythium debaryanum* DSM 62946 and *Rhizoctonia solani* DSM 63002 (Table 1).

We consider that the antifungal potential of these strains is related with the lytic enzyme production and direct competition for nutrients and niche.

Table 1. Antifungal spectrum of the studied *Bacillus cereus/thuringiensis* strains

Microbial strains	75.1s	Cp.b4
Alternaria sp.	+ + +	+ + +
Botrytis cinerea	+ +	+ + +
Fusarium oxysporum f. sp. *radicis lycopersici*	+ +	+
Pythium debaryanum	+	+
Rhizoctonia solani	+ + +	+ + +

Where: + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; – = no inhibition of the fungi

Fungal cell lysis associated with protoplasmatic content likings were seen at the microbial interaction zone between *Botrytis cinerea* and both bacterial strains (figure 3 a, c). Hyphal growth deformations were also seen in *Fusarium oxysporum* and *Rhizoctonia solani* at the interaction zone with the biocontrol bacteria (figure 3 b). The bacterial strains induced swelling of the fungal cells of *Fusarium oxysporum* (figure 3 c), and mycelia dehydration in *Botrytis cinerea* after prolonged incubation (figure 3 d). Similar aspects were previously described on plant pathogenic fungi when *Bacillus* spp. treatments were used for biological control (Huang et al., 2012; Boiu-Sicuia et al., 2017a, b).

Regarding the bacterial potential to inhibit conidia germination, we noticed that all tested treatments reduced the number of germinated conidia of *Alternaria* sp. (Table 2). The most efficient treatments were the chemical control and the mixed applications of biocontrol bacteria and chemical pesticide in half dose. Among bacterial treatments, 75.1s had a better biocontrol effect than Cp.b4 strain.

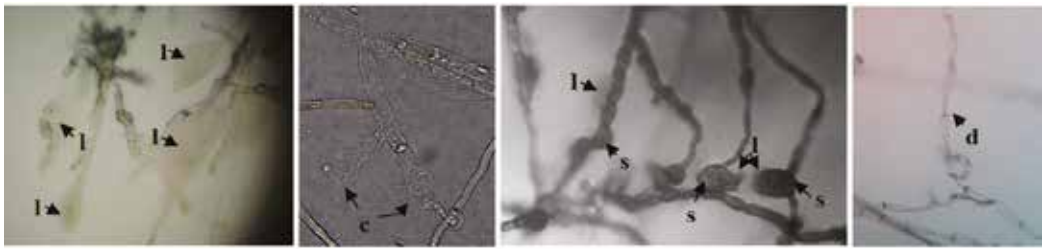

Figure 3. Fungal growth deformations and lysis due to biocontrol bacteria:
A, D = *Botrytis cinerea*, B = *Rhizoctonia solani*, C = *Furarium oxysporum* f.sp. *radicis lycopersici*
l = cell lysis and cytoplasm likings, c = mycelial curling, s = fungal cell swelling, d = mycelia dehydration

Table 2. Inhibition of *Alternaria* sp. conidia germination

Experimental variant	Germinated conidia	Inhibition of germinated conidia	Inhibition of germinating filaments
	(%)		
Alternaria sp. - Untreated control	32.14	-	-
Chemical control 0.2% Mycoguard	2.7	91.6	86.7
Cp.b4 biocontrol treatment	12.5	61.1	98.7
Mixed treatment Cp.b4 & 0.1% Mycoguard	2.33	92.8	99.1
75.1s biocontrol treatment	6.46	79.2	98.7
Mixed treatment 75.1s & 0.1% Mycoguard	3.33	89.6	99.1

The germination filaments were measured using the APS Assess 2.0 software, and the filaments length was quantified compared to conidia dimensions. Concerning this, the

biocontrol and mixt treatments had a better influence to inhibit the growth of germination filaments in *Alternaria* sp. Although the chemical control reduced the number of germinated conidia, the germination filaments were longer than the other treatments (Table 2).

Plant growth promoting activity

Tomato seedlings were grown in controlled conditions. Six weeks after transplantation, several biometric observations were made (Table 3). Root length, in cm, was measured, fresh and dry weight of total aerial growth and roots were separately determined as grams of plant tissue per single plant.

Growth chamber tests on tomato seedlings have shown that biological treatments increase plant vigour. Plant growth promotion was observed both on aerial parts and root systems when seedlings were treated with beneficial bacteria.

Table 3. Biometric parameters of bacterial inoculated tomato seedlings

Biometric parameters	Untreated control	Bacterial inoculated plants	
		Cp.b4	75.1s
Root length (cm)	13.6	17.9	15.7
Aerial fresh weight (g/plant)	5.7675	9.1043	7.2463
Aerial dry weight (g/plant)	0.5391	0.9630	0.7778
Root fresh weight (g/plant)	0.6345	1.0500	0.7952
Root dry weight (g/plant)	0.0380	0.0819	0.0639

An increase in root length and weight was achieved for the bacterial inoculated plants (Figure 4), and a positive correlation of the root system with the shoots was revealed. Between the two tested bacterial strains, Cp.b4 was more efficient than 75.1s in plant growth promotion (Table 3).

Figure 4. Tomato roots: a) untreated control; b) 75.1s treated seedlings; c) Cp.b4 treated seedlings

A positive influence of the bacterial treatments on tomato seedlings was also seen when the assimilatory pigments were quantified (Table 4).

Table 4. Assimilatory pigments in the leaves of tomato seedlings

Biometric parameters	Untreated control	Bacterial inoculated plants	
		Cp.b4	75.1s
	mg/ g of fresh weight		
Chlorophyll a	2.435	3.041	3.052
Chlorophyll b	0.949	1.231	1.168
Xanthophyll and Carotenoids	0.487	0.580	0.610

Regarding the chlorophyll content, bacterial treatments slightly increased chl a and chl b content compared to the untreated control, with no significant differences among the applied treatments. Moreover, for xanthophyll and carotenoids, the pigments content was a little higher in treated plants. These results showed an increased photosynthetic capacity in bacterial inoculated seedlings.

CONCLUSIONS

Two bacterial strains of *Bacillus cereus*/ *thuringiensis*, Cp.b4 and 75.1 s, were analysed in this study. Both strains demonstrated *in vitro* biocontrol activity and plant growth promotion properties on tomato seedlings.

Both strains produced chitinase, carboxymethil cellulase, caseinase, and lipase enzymes. Due to their high metabolic activity this biocontrol strains expressed a wide antimicrobial action against *Alternaria* sp. (new isolate from tomato plant), *Botrytis cinerea* (new isolate from tomato fruit), *Fusarium oxysporum* f. sp. *radicis lycopersici* ZUM 2407, *Pythium debaryanum* DSM 62946 and *Rhizoctonia solani* DSM 63002. The biocontrol strains also inhibited conidia germination in *Alternaria* sp., showing a high potential in prevent early blight infections. Biological treatments with bacterial suspension reduced the number of germinated conidia by 4.98 fold (when using 75.1s strain) and by 2.57 fold (when using Cp.b4 strain) than the untreated control. Mixed treatments of bacteria suspension and low dose (0.1%) of chemical fungicide strongly inhibit conidia germination, being comparable with the

chemical control, Mycoguard 500SC, applied at the recommended dose of 0.2%.

Regarding plant growth promotion activity, the mentioned strains, applied as seed and soil treatment, increased tomato root length, biomass fresh and dry weight, and plant photosynthetic capacity. The bacteria also revealed the ability to solubilize tricalcium phosphate from Pikovskaya agar medium indicating an increasing potential in improving nutrient availability for the plants.

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