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BACTERIAL BIOCONTROL STRAINS THAT REDUCE RHIZOCTONIA DAMPING-OFF IN TOMATO SEEDLINGS

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Abstract

To suppress the Rhizoctonia damping-off in tomato seedlings we used several antagonistic bacterial strains of Bacillus subtilis, B. amyloliquefaciens, B. pumilus and Mycobacterium alvei. These bacterial strains were previously selected for their in vitro ability to control Rhizoctonia solani and were formulated as alginate pellets. The experiment was performed in a growth chamber on tomato cultivar HEINZ 2274. To compare our results with a standardized fungicide we used a chemical treatment with thiophanate-methil, together with other two experimental controls, a negative one, without treatment and a positive one, uninfected. Results showed that the selected strains of B. amyloliquefaciens, B. pumilus and Mycobacterium alvei expressed a biocontrol activity with 37,5% efficacy in reducing the Rhizoctonia damping-off in tomato seedlings, equivalent to the chemical treatment. However, the 98a strain of Bacillus subtilis expressed 50% efficacy in Rhizoctonia biocontrol.

Keywords: *biocontrol, Rhizoctonia damping-off, tomato*

INTRODUCTION

Worldwide as well as in our country, tomato crop has a high share in the solarium surfaces that produce vegetables, approximately 70-75% of the total surface of solariums for vegetables production [2].

Among the soilborne fungal diseases that attack tomatoes cultivated under greenhouse conditions *Rhizoctonia solani* is one of the phytopathogens causing root and crown rot [8]. *Rhizoctonia*, along with other species such as *Fusarium*, *Pythium* and *Verticillium* can induce damping-off of seedlings and wilt of adult plants [7].

As biocontrol agents *Trichoderma*, *Gliocadium*, *Pseudomonas*, *Paenibacillus* and *Bacillus* genera have already been successfully used for *Rhizoctonia* suppression [4, 5, 9, 12, 13, 14, 15], some of them were formulated in different ways, such as alginate pellets [12].

MATERIALS AND METHODS

Bacterial biocontrol strains

Six bacterial strains were previously selected for their beneficial characteristic in plant protection [3, 16] and used to perform *in vivo* biocontrol of *Rhizoctonia solani* infection in tomatoes. Bacterial strains used in this study were Romanian isolates of *Bacillus subtilis* Us.a2 and 98a strains, *Bacillus amyloliquefaciens* OS.17 and Bw strains, *Bacillus pumilus* OS.15 strain and *Mycobacterium alvei* 82.1s strain. The origin of this strain is presented in table 1. All of these strains were identified using Biolog GEN III, and some of them were also identified through molecular tests like ITS-PCR (OS.15, OS.17, Bw strains), ARDRA technique (Bw strain), sequencing analysis of 16S rRNA gene (Us.a2 strain). Routinely, these strains were grown on LB agar medium at 28°C and formulated as bio-products into sodium alginate beads (photo 1) using Minaxi and Saxena method [11]. Resulted granules had a microbial load of 2×10^7ufc/g bioproduct.

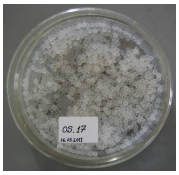

Photo 1. Granular bio-products of bacterial sodium alginate beads

Table 1. Biocontrol bacterial strains used in the experiments

Strain	Source	Provenience
Mycobacterium alvei 82.1s	Isolated from soil, South of Bărăgan area	RDIPP - Bucharest collection
Bacillus subtilis Us.a2	Isolated from garlic rhizosphere, Dolj county	RDIPP - Bucharest collection (DSM 23 654)
Bacillus subtilis 98a	Isolated from wheat straw, South of Romania	RDIPP - Bucharest collection
Bacillus pumilus OS15	Isolated from onion rhizosphere	RDIPP - Bucharest collection
Bacillus amyloliquefaciens OS17	Isolated from onion rhizosphere	RDIPP - Bucharest collection
Bacillus amyloliquefaciens BW	Isolated from soil	Faculty of Biotechnology Bucharest collection

Fungal inoculum

Rhizoctonia solani DSM 63002 strain was routinely grown on Potato-Dextrose-Agar (PDA) for maintenance and *in vitro* tests.

For *in vivo* tests the pathogen was previously cultivated on PDA medium and then multiplied in Roux plates on barley seeds substrate at 25°C for two weeks.

***In vitro* antagonistic tests**

The antagonistic activity of the isolates was revealed on PDA medium, using the dual culture technique. To see if the antifungal activity is maintained after formulation as sodium alginate beads, the bioproducts were *in vitro* tested against *Rhizoctonia solani* using the mentioned method. The test was performed in Petri dish where the fungal inoculum (plugs of 5 mm diameter) was placed in the middle of the plate and the bacterial inoculum was either streaked with fresh culture or, in case of granules, placed at 2 cm from the fungal colony, on both sides.

Fungal growth inhibition was visually appreciated according to Manka and Manka method [10]. Antagonistic activity was biometrically evaluated using a modified version of Islam *et al.* [6] calculation, through which they determined the percentage of inhibition efficacy:

$$E\% = \frac{RC - RI}{RC} \times 100$$

where: E (%) = antagonistic efficacy or pathogenic inhibition of growth; RC = radius of mycelia in the control (mm), RI = radius of the mycelia growth towards the interaction zone with the antagonism (mm).

***In vivo* growth chamber experiment**

The test was performed on tomato seedlings (*Lycopersicon esculentum* Mill.) cv. Heintz 2274 sensitive to *Rhizoctonia solani* attack. Tomato seeds were disinfected in two steps, first with 70% ethanol and then with 4% sodium hypochlorite.

The soil used was "FLORIMO" universal peat containing: Shagnum peat, black peat, earthworm humus, clay, river sand at pH 6,5-7. This was previously sterilized by gamma irradiation (25kGy).

The soil infection was done by mixing the fresh culture of *Rhizoctonia solani* at 2% rate (w/w) with the sterilized soil. The test was performed in plastic trays of 30cm/19cm/7cm. Thirty tomato seeds were sown per tray.

The alginate beads bioproducts were applied at the same time with sowing, near the seed, six pellets to 10 seeds.

For the chemical control variant we used Topsin 500SC fungicide based on thiophanate methyl, in 0,14% concentration and dose of 100µl/seed. As a negative control we used untreated plants grown in infested soil. Plants grown in gamma sterilized soil, without treatments or artificial infection, served as positive control.

Experimental variants are presented in table 2. Each variant had three repetitions and each repetition 10 plants.

Table 2. Experimental variants from the *in vivo* test on *Rhizoctonia solani* suppression in tomato seedlings

Experimental variants		Treatment characteristics
V1	*Mycobacterium alvei* 82.1s	Biological treatments with bacterial products and *Rhizoctonia solani* infected soil
V2	*Bacillus subtilis* Us.a2	
V3	*Bacillus subtilis* 98a	
V4	*Bacillus amyloliquefaciens* Bw	
V5	*Bacillus pumilus* OS.15	
V6	*Bacillus amyloliquefaciens* OS.17	
V7	Positive control	Uninfected soil
V8	Negative control	*Rhizoctonia solani* infected soil
V9	Chemical control	Chemical treatment with thiophanate methyl and *R.solani* infected soil

The experiment was performed in a growth chamber (Sanyo MLR-351H) with 16 hours photoperiod, at 24ºC/light, 16ºC/dark, 16000 lx luminance and 70% RH. During the experiment soil was moistened with tap water, every two days.

After 3 weeks of growth, plants were analyzed for *Rhizoctonia* infection symptoms (photo 4). Results were statistically analyzed and

treatments efficacy (%) was calculated with ABBOT formula.

RESULTS AND DISCUSSION

In vitro antagonistic activity against *Rhizoctonia solani*

Tested bacterial strains showed an antagonistic efficacy of 59 to 80% against *Rhizoctonia solani.* Results from the fungal inhibition after 5 days of interaction at 25°C are presented in table 3.

Table 3. *In vitro* evaluation of the bacterial strains antagonistic activity against *Rhizoctonia solani* (after 5 days of incubation at 25°C)

Bacterial strains	*Rhizoctonia solani* inhibition with bacterial biomass	
	Growth inhibition (according to Manka and Manka, 1992)	Antagonistic efficacy (according to Islam *et al.*, 2009)
Mycobacterium alvei 82.1s	+ + +	70%
Bacillus subtilis Us.a2	+	59%
Bacillus subtilis 98a	+ + + +	80%
Bacillus amyloliquefaciens Bw	+ + + +	77%
Bacillus pumilus OS.15	+ + +	71%
Bacillus amyloliquefaciens OS.17	+ + +	69%

Legend: + + + + = very strong inhibition of the fungal growth; + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; − = no inhibition of the fungal growth.

Photo 2. *In vitro* antagonistic activity of bacterial strains vs. *Rhizoctonia solani* (5 days of incubation at 25°C)
a – *Bacillus pumilus* OS.15; **b** – *R.solani* control; **c** *Bacillus amyloliquefaciens* OS.17.

In vitro evaluation of the bio-products antagonistic activity revealed that sodium alginate beads formulation maintains the bacterial antifungal properties. Results with the bio-products antagonistic activity are listed in table 4. Sodium alginate beads formulation of our selected bacterial strains showed an antagonistic efficacy of 58 to 77,3% against *Rhizoctonia solani* fungal growth. Results from the *in vitro* test revealed that our selected bacterial strains maintained their antagonistic activity and showed a strong inhibitory effect against *Rhizoctonia solani* growth (photo 3).

Table 4. *In vitro* evaluation of the bacterial bio-products antagonistic activity against *Rhizoctonia solani* (after 5 days of incubation at 25°C)

Bacterial bio-products	*Rhizoctonia solani* inhibition with bacterial bio-products	
	Growth inhibition (according to Manka and Manka, 1992)	Antagonistic efficacy (according to Islam *et al.*, 2009)
Mycobacterium alvei 82.1s	+ +	64,8%
Bacillus subtilis Us.a2	+	58%
Bacillus subtilis 98a	+ + + +	77,3%
Bacillus amyloliquefaciens Bw	+ + +	74,5%
Bacillus pumilus OS.15	+ + +	68%
Bacillus amyloliquefaciens OS.17	+ + +	67,5%

Legend: + + + + = very strong inhibition of the fungal growth; + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; − = no inhibition of the fungal growth.

Photo 3. *In vitro* antagonistic activity against *Rhizoctonia solani* by the selected bacterial strains after formulation as sodium alginate beads:
a – *Bacillus pumilus* OS.15; **b** – *R.solani* control; **c** *Bacillus amyloliquefaciens* OS.17.

In vivo* biological control of *Rhizoctonia

Symptoms: *Rhizoctonia* seedlings damping-off may occur before and/or after emergence. In pre-emergence damping off, the seeds fail to emerge after sowing; they became mushy, turn brown, and decompose as a consequence of seed infection. On seedlings stage, infection occurs on roots, hypocotyls and plant's crown. Infected seedlings form small lesions, pale brown and soft (photo 4). In time, root and crown lesions fuse and form large areas. Plants became stunted comparing with non-affected ones. In severe attack, plants collapse and the entire root is rotted. Tested bacterial strains showed an antagonistic efficacy of 59 to 80% against *Rhizoctonia solani.*

The highest disease incidence was recorded in the negative control, where plants were grown without treatment in the artificial infected soil and where *Rhizoctonia* infection was evaluated with a frequency of 36,4%. According to Abbot's algorithm, for efficacy evaluation of the treatments, results regarding the attack level in the negative control revealed the maximum disease incidence. Using this algorithm we found that thiophanate methyl treatment used as chemical control for *Rhizoctonia* suppression revealed a 37,5% efficacy protection. Similar defense capacity against the pathogenic infection was found when using biological treatments with *B. pumilus* OS.15, *B. amyloliquefaciens* OS.17 and Bw, and *Mycobacterium alvei* 82.1s. However, the highest efficacy protection (50%) was found in *B. subtilis* 98a treatment. Biological treatment with *B. subtilis* Us.a2 strain showed the lowest biocontrol activity, in this experimental variant treatment was evaluated at 12,5% efficacy. Tomato seedlings from the control trays and biological treatments are illustrated in photo 5 and 6 respectively. Treatments efficacy is illustrated in figure 1.

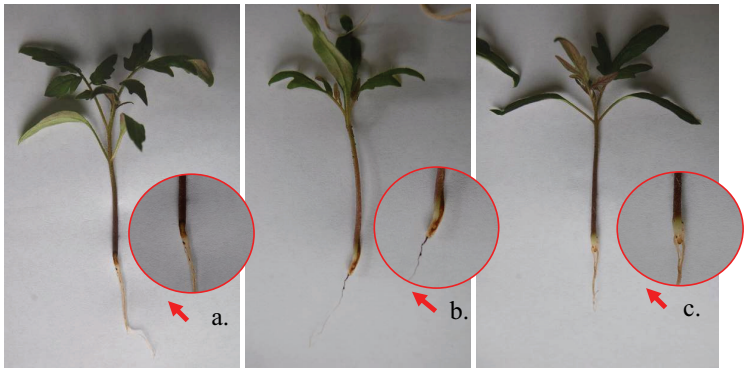

Photo 4. *Rhizoctonia* disease symptoms on tomato seedlings: a. browned hypocotyls; b. root and crown lesions; c. rotten root broken down from the plant

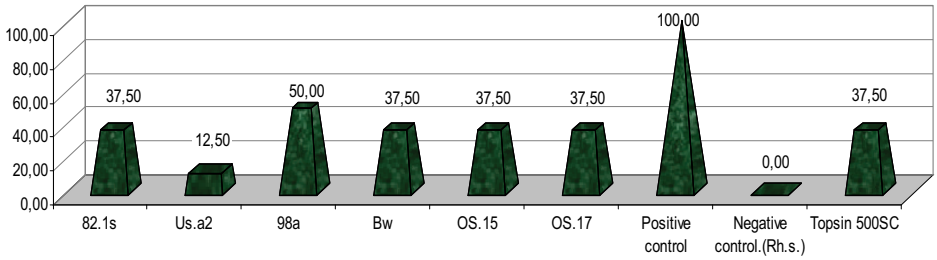

Fig. 1. Treatments efficacy in reducing *Rhizoctonia* damping-off in tomato seedlings

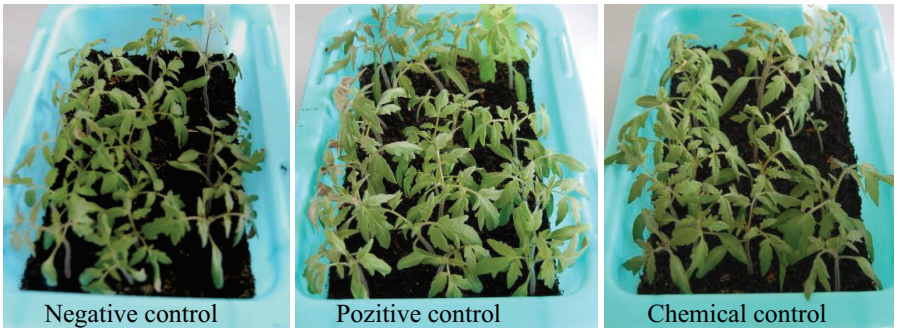

Photo 5. The experimental trays of the three controls with tomato seedlings obtained in growth chamber conditions

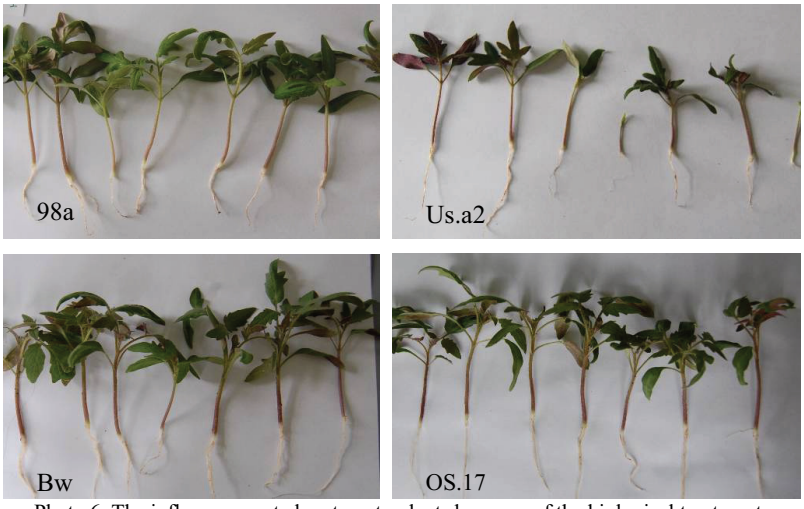

Photo 6. The influence exerted on tomato plants by some of the biological treatments after three weeks from seeding

CONCLUSIONS

Six bacterial strains with high antagonistic activity *in vitro* against various phytopathogenic fungi including *Rhizoctonia solani* were selected and formulated as granular bio-products.

In vitro bioassay showed that selected bacterial strains preserved their antagonistic properties against *Rhizoctonia solani* when formulated as sodium alginate beads.

In vivo evaluation of *Rhizoctonia* attack suppression revealed that biological treatments with some of the tested bacterial strains (82.1s;

OS.15; OS.17; Bw) are comparable with standard chemical treatment of thiophanate methyl.

Growth chamber experiments showed that biological treatment with *Bacillus subtilis* 98a strain can reduce the attack level of *Rhizoctonia solani* infection by half in tomato seedlings.

In vivo tests on tomato seedlings revealed five bacterial strains with good biocontrol activity in reducing *Rhizoctonia* damping-off.

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