

EFFICACY OF SOME BACTERIAL BIOPRODUCTS AGAINST *SCLEROTIUM CEPIVORUM*

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Abstract

The aim of this paper was to determine the efficacy of some bioproducts based on useful bacteria from the genus *Bacillus* and *Pseudomonas* against the phytopathogenic *Sclerotium cepivorum* fungus which causes white rot in onion crop. The selected bacterial strains for this study were: *Bacillus subtilis* Us.a2, isolated from rhizosphere of green garlic, with deposited DSMZ number 23654; *Pseudomonas chlororaphis* Sal.c2, isolated from rhizosphere of lettuce; *Bacillus pumilus* OS.15 and *Bacillus amyloliquefaciens* OS.17 strains isolated from rhizosphere of onion and *Bacillus subtilis* 98a isolated from wheat straw. The five strains, besides other important biological features such as lithic enzyme production, mobility, action to stimulate plant growth, showed in vitro antagonistic ability against *Sclerotium cepivorum*. In order to be tested in vivo, the strains were formulated as microemulsion (which can be applied as seed treatment) and alginate beads (which can be distributed in rows along with the seeds). The tests were performed in growth chamber conditions by using artificially infected soil. Results showed a moderate or similar efficacy of bioproducts compared with the chemical control Topsin 500 SC.

Key words: bacterial bioproducts, biocontrol efficacy, *Sclerotium cepivorum*

INTRODUCTION

Biological means of useful crop plant microorganisms is one of the directions for reducing the dependence of chemicals products. Biopesticides are widely used in farming because of their advantages: (i) equal or higher efficacy than conventional products when used in integrated control systems; (ii) high applicability hence the use of unqualified staff; (iii) are biodegradable, important feature for the groundwater and surface; (iv) can be successfully used alone or in combination with some pesticides having a synergistic effect; (v) microbial biopesticides are obtained by fermentation and often used as conditioning substrate materials/agricultural bioproducts.

In our country, the first bioproduct was conducted by N. Zinca in 1970 based on avirulent *Agrobacterium vitis* bacteria designed to control the crown-gall of vines.

Subsequently were obtained a series of bioproducts for important soilborne pathogens (*Pythium*, *Rhizoctonia*, *Fusarium*) such as: **Vamfix** (based on spores of mycorrhizal *Glomus* fungi), **Bacillin** (based on *Bacillus subtilis* strains), **Trichosemin** (*Trichoderma viride*), or based on plant growth promoting rhizobacteria such as: **Azostim** (based on *Azospirillum* bacteria), **Nitrostim** (*Bradyrhizobium*), **Nitrofix** (*Rhizobium*).

On the plant protection market in USA there are several bioproducts including bacteria and fungi of genera *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Streptomyces* and *Trichoderma*, *Candida* and *Coniothyrium*. In the last years, there was a slight increase (2.5%) compared to the chemicals products, the highest use (55%) being in the horticulture sector.

The European Union is focused on developing organic farming systems in order to reduce negative side effects that may occur from the use of chemical fertilizers. Some of these bioproducts

are: **Kodiak**, **Companion** (*Bacillus subtilis*), **Actinovate** (*Streptomyces lydicus*), **Mycostop** (*Streptomyces griseoviridis*), **PlantShield** (*Trichoderma harzianum*).

One of the most important soil phytopathogen is *Sclerotium cepivorum* Berk fungus, causing white rot of *Allium* species, being a major limitation factor of onion crop production and the incidence disease in favourable conditions being over 65% [2]. The fungus penetrates the root epidermis and then invades the cortical parenchyma [5] so that, infected plants suffer from water stress and often die before harvesting or of rotting in warehouses. The pathogen persists in soil in the absence of host plants as sclerotia and can survive in this form more than 20 years [1]. Sclerotia remain dormant in soil for 1-3 months and can only germinate in the presence of host plants. The stimulus for germination is the exudates of alkyl-cysteine-sulphoxides which are metabolized by the soil microflora to produce volatile thiols and sulphoxides that trigger dormant sclerotia germination [1, 6]. Biological control of the disease can also be done directly, by adding antagonists or indirectly by changing environmental conditions in favour of selective antagonists [3]. Among antifungal microorganisms active against this pathogen are *Trichoderma viride* and *Coniothyrium minitans* that have proven an over 60% efficacy in reducing the onion bulbs attack. Also, the bacteria from the genus *Bacillus* and *Pseudomonas* were found to have a strongly antagonism against this pathogen [4].

The aim of this work was to test the efficacy of some bioproducts based on *Bacillus* and *Pseudomonas* useful bacteria formulated as microemulsion and as sodium alginate beads on the growth of *Sclerotium cepivorum* fungal phytopathogen, under controlled conditions.

MATERIAL AND METHOD

Microorganisms

Bacterial strains used in this study were: *Bacillus subtilis* Us.a2 isolated from rhizosphere of green garlic, with deposit number DSM 23654, *Pseudomonas chlororaphis* Sal.c2 isolated from rhizosphere

of lettuce, *Bacillus pumilus* OS.15 and *Bacillus amyloliquefaciens* OS.17 strains isolated from onion rhizosphere and *Bacillus subtilis* 98a isolated from wheat straw, identified by biochemical and molecular tests (Biolog GEN III, ARDRA technique or sequencing analysis of 16S rRNA gene). Bacteria were grown in Luria Bertani broth medium (LB) (bactotryptone – 10g; yeast extract – 5g; distilled water up to 1000 ml; pH adjusted to 7.4 with NaOH 5M or HCl 1M solutions) with a starting inoculum of a 10% aqueous suspension of 1×10^8 cfu/ml, in Erlenmeyer flasks on a rotary shaker (150rpm) at 28°C for 48 h. Biomass was harvested by centrifugation at 3000 rpm for 20 minutes.

Fungal inoculum was the strain DSM 63024 of *Sclerotium cepivorum* which was previously cultivated on PDA medium (decoction from 200g potato, 20 g dextrose, 20 g agar-agar and 1000 ml distilled water, pH 7.0) which was inoculated in Roux plates on barley seeds substrate and incubated 20 days at 20°C. The fungal inoculum was mixed with the soil (previously sterilized by gamma radiation) at a rate of 2% of soil weight (18 g / tray).

In vitro selection of bacterial strains was carried out by testing the antagonistic activity towards different phytopathogenic fungi: (*Botrytis cinerea*, *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407, *Fusarium graminearum* DSM 4527, *Sclerotium bataticola*, *Sclerotium cepivorum* DSM 63024. Bacterial strains (24 h fresh culture) were inoculated on PDA medium by streaking a straight line at 3cm distance towards the calibrate mycelium disk (5mm diameter). Inoculated Petri dishes were incubated at 28°C and analyzed after 48-72 h regarding the inhibition zone (mm). The experiment was repeated three times.

The bacterial strains conditioning procedure was developed in two forms:

1. **microemulsion** consisted in: biomass of sporulated or nonsporulated bacteria as active ingredient – 10 g; sucrose as suspending agent – 5 g; soprofor FL as anionic surface surfactant and dispersant agent – 9.6 g; emulsifier CL3 as non-ionic surface surfactant agent – 2.4 g ; PEG 400 as antifreeze agent – 3.0 g; 5% carboxy-methyl-

celulose solution as thickening agent – 5.0 g; sodium benzoate as stabilizer – 0.2 g; organic solvent – methyl esters of fatty acids in canola or sunflower oil – 30.0 g; distilled water – up to 100 ml.

Preparation of organic phase

In a conditioning recipient with stirring were introduced 30 g solvent, 9.6 g soprofor FL and 2.4 g emulsifier CL3. The mixture was maintained under stirring until obtaining a homogenous solution. Resulted 42 g of emulsifier solution.

Preparation of the aqueous phase

In a 250 ml conditioning gradually recipient with stirring and dropping funnel 10 g biomass, 5 g sucrose, 0.2 g sodium benzoate, 3 g PEG, 5 g carboxy-methyl-celullose of 5% solution and about 80% of the amount of water needed (~ 27 ml) were placed. The mixture was homogenized under stirring for 10 minutes.

Preparation of microemulsion

Over the aqueous phase the organic phase was added under stirring. After adding the organic phase, stirring continued for another 15 minutes. 100 ml of bioproduct resulted as homogenous microemulsion.

2. Alginate beads

Bacteria were cultured in LB liquid medium in a rotary shaker (150 rpm) incubated at 28°C for 48 h up to a final concentration of 10⁸ cfu/ml. Entrapment of bacteria within the alginate beads was carried out under sterile conditions in a laminar flow hood; 20 ml of bacterial culture with 80 ml of 2% (w/v) sodium alginate was aseptically mixed. The mixture was homogenised by stirring gently for 1 h at room temperature and then it was added drop wise with the aid of a 10-ml sterile syringe in CaCl₂ 2% (w/v) solution. The resulting alginate beads (with 3mm diameter) were maintained in the solution at room temperature for 1 h in order to obtain regular solid beads. Afterwards the beads were washed twice with 0.4% sterile NaCl and then stored at 4±1°C up to use.

Test of the antagonistic capacity preservation was achieved *in vitro* on PDA medium by inoculating phytopathogenic fungus (disk of 5 mm diameter, from a five-

old days culture on PDA incubated at 22°C) and bioproducts in the same Petri dish (9 mm diameter). After 5 days of incubation at 28°C, linear growth of tested pathogen was recorded. The percent of growth inhibition was calculated using the following formula:

$$\%IG = \frac{GC - GT}{GC} \times 100$$

where: IG = inhibition of growth; GC = growth in control and GT = growth in treatment

The preservation of the biological qualities of bioproducts was tested *in vivo* under controlled conditions on the efficacy against *Sclerotium cepivorum* phytopathogen which attacks mostly the seedlings, causing severe damages. The experiment was carried out in growth chamber on seeds of onion (*Allium cepa*) cultivar RIJNSBURGER, disinfected in a first stage with 70% ethanol and in the second stage with 4% sodium hypochlorite solution. 30 seeds/variant were planted in soil previously sterilized by gamma ionizing radiation, and artificially infected with *Sclerotium cepivorum* phytopathogenic fungus at a rate of 18 g inoculum/tray. The alginate beads bioproducts were applied in rows along with the seeds (6 beads / row, 18 beads / tray) and those conditioned as microemulsion were applied as seed treatment by immersion for 20 minutes. Chemical treatment was performed using soil TOPSIN®500 SC at a dose of 0.25% and row sowing application (5 ml / row, 15 ml / tray).

Experimental variants were: V1 – *Pseudomonas chlororaphis* Sal.c2 vs. *Sclerotium cepivorum*; V2 – *Bacillus subtilis* Us.a2 vs. *S. cepivorum*; V3 – *B. subtilis* 98a vs. *S. cepivorum*; V4 – *Bacillus pumilus* OS.15 vs. *S. cepivorum*; V5 – *Bacillus amyloliquefaciens* OS.17 vs. *S. cepivorum*; V6 – Chemical control TOPSIN 500 SC vs. *Sclerotium cepivorum*; V7 – Negative control with *Sclerotium cepivorum*; V8 – Positive control – sterilized soil.

After four weeks, the plants were analyzed in terms of specific symptoms of disease like yellowing and wilting of the leaves, particularly browning and growth of white fluffy mycelium at the base of the stem. Roots that showed no characteristic symptom of the disease were considered healthy (photo 4).

Efficacy data were calculated according to Abbot's formula and represent the mean of three replicates.

RESULTS AND DISCUSSIONS

Selection of the bacterial strains

Table 1 shows the activity of bacterial strains *in vitro* their antagonistic activity on the

growth of phytopathogenic mycelium fungi studied (observations at three days of incubation at 28°C for *Botrytis cinerea*, *Fusarium graminearum*, *Fusarium oxysporum* f. sp. *radicis lycopersici* and *Sclerotium bataticola* fungi, five days of incubation at 22°C for *Sclerotium cepivorum* fungus respectively, according to Manka & Manka method [7] (Photo 1).

Table 1. *In vitro* antagonistic activity of tested bacterial strains against to different phytopathogenic fungi (3-5 days of incubation at 22-28°C)

Bacterial strains	<i>Botrytis cinerea</i>	<i>Fusarium graminearum</i>	<i>Fusarium oxysporum</i> f. sp. <i>radicis lycopersici</i>	<i>Sclerotium bataticola</i>	<i>Sclerotium cepivorum</i>
<i>Bacillus pumilus</i> OS.15	+++	++++	+++	+++	+++
<i>Bacillus amyloliquefaciens</i> OS.17	++++	+++	++++	++++	+++
<i>Bacillus subtilis</i> Us.a2	+++	+++	+++	+++	++++
<i>Pseudomonas chlororaphis</i> Sal.c2	+++	+++	+++	+++	++++
<i>Bacillus subtilis</i> 98a	+++	+++	+++	+++	++++

Legend: ++++ = very strong inhibition of mycelium growth and formation of propagation organs; +++ = strong inhibition of mycelium growth; ++ = moderate inhibition of mycelium growth

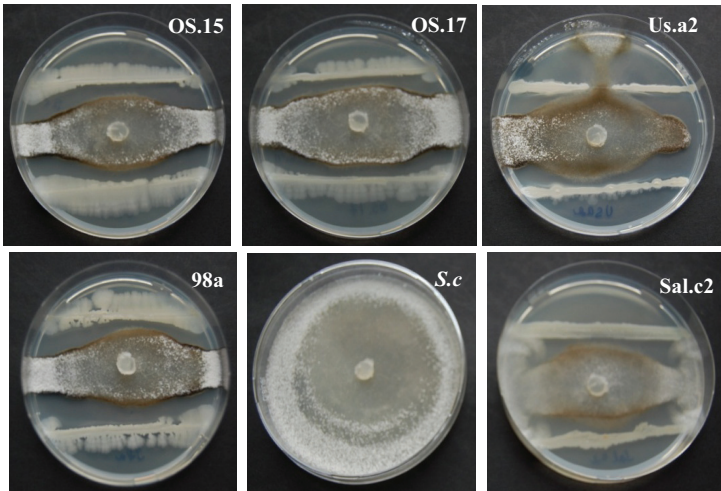


Photo 1. *In vitro* antagonism of bacterial strains vs. *Sclerotium cepivorum* (5 days of incubation at 22°C)

The bacterial strains, tested *in vitro* regarding their antagonistic activity produced a series of antifungal metabolites which inhibited the growth of the phytopathogens studied, causing large aria of inhibition (>5mm equivalent to ++++; >3mm equivalent to +++).

The conditioning of the selected bacterial strains was performed in order to obtain bioproducts (Photos 2), which were afterwards tested, both *in vitro* and *in vivo* for establishing the biological control agent qualities.

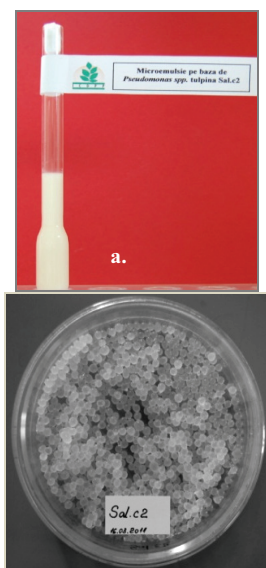


Photo 2. Bacterial bioproducts: a. Microemulsion formulation, b. Alginate beads formulation

Verification of preserving bio-antagonistic capacity

The results presented in table 2, indicated that both types of bioproducts based on the bacterial strains previously selected showed a strong inhibitor effect against *Sclerotium cepivorum* pathogen growth *in vitro*. Thus, bioproducts like alginate beads based on *Bacillus pumilus* OS.15 and *Bacillus subtilis* 98a strains showed the greatest percentage of inhibition (95%) to the growth of the pathogen followed by *Bacillus amyloliquefaciens* OS.17 bioproduct which had an inhibitory effect on percentage of 87.5% (Photo 3).

Microemulsion form of conditioned strains has also preserved the biological qualities of antagonists, showing inhibitory effect on fungus growth with 85% (*Bacillus subtilis* 98a) and 70-75%, respectively (Photo 4).

Table 2. *In vitro* antagonistic effect exerted by the bioproducts on *Sclerotium cepivorum* growth (5 days of incubation at 22°C)

Bioproducts vs <i>Sclerotium cepivorum</i>			
Alginate beads	inhibition (%)	Microemulsion	inhibition (%)
<i>Bacillus pumilus</i> OS.15	95	<i>Bacillus pumilus</i> OS.15	75
<i>Bacillus amyloliquefaciens</i> OS.17	87.5	<i>Bacillus amyloliquefaciens</i> OS.17	75
<i>Bacillus subtilis</i> Us.a2	75	<i>Bacillus subtilis</i> Us.a2	70
<i>Pseudomonas chlororaphis</i> Sal.c2	75	<i>Pseudomonas chlororaphis</i> Sal.c2	70
<i>Bacillus subtilis</i> 98a	95	<i>Bacillus subtilis</i> 98a	85

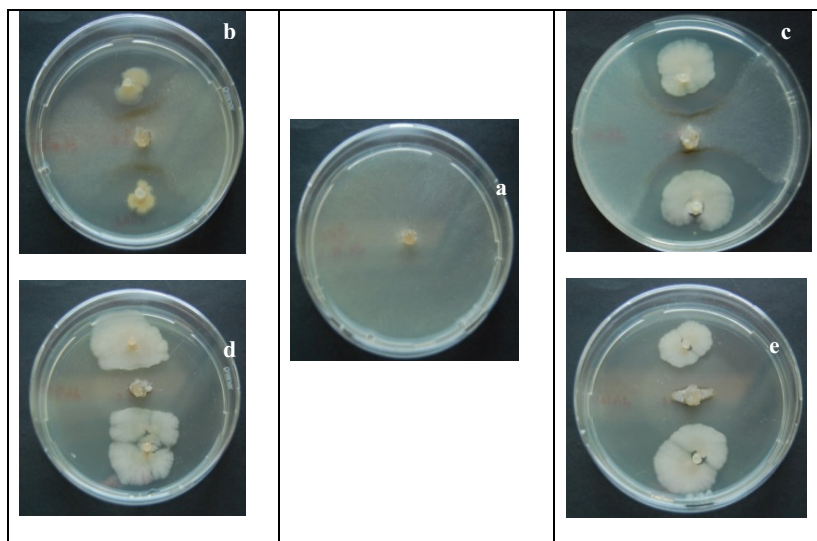


Photo 3. *In vitro* antagonistic activity of alginate beads bioproducts against *Sclerotium cepivorum* pathogen (5 days incubation at 22°C): a – fungal control; b – *Bacillus subtilis* Us.a2; c – *Bacillus amyloliquefaciens* OS.17; d – *Bacillus subtilis* 98a; e – *Bacillus pumilus* OS.15)

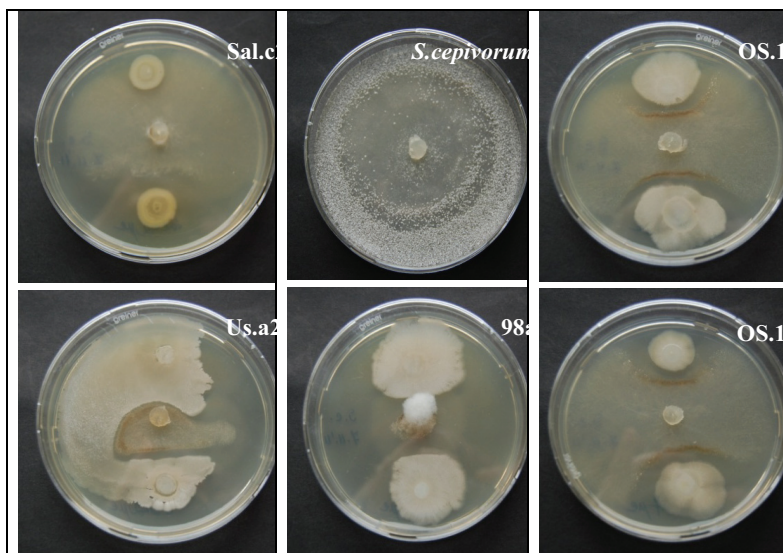


Photo 4. *In vitro* antagonistic activity of microemulsion bioproducts vs. *Sclerotium cepivorum* pathogen (4 days of incubation at 22°C)

***In vivo* preservation of the biological qualities of the bacterial bioproducts**

The efficacy of the bioproducts against *Sclerotium cepivorum* in onion seedlings is represented graphically (figures 2, 3).

Among the microemulsion bioproducts applied as seed treatment the best efficacy was recorded in OS.15, 98a and OS.17 treatment variants (80-90%) with similar or very close values to the chemical control (90%). Also, Us.a2 and Sal.c2 bioproducts showed a protective but moderate effect on seedlings (40-50%) compared to chemical control (figure 2).

Administration of alginate beads showed reduced efficacy compared to the chemical control, mean values being recorded in OS.17,

98a and Us.a2 treatment variants (50-60%). Disease characteristic symptoms were noticed only in Salc2 treated variant which showed the lowest efficacy (10%) (figure 3).

The differences between efficacy values recorded for the two types of formulation of bioproducts mentioned above were significant because of the conditioning form. Thus, conditioning the bacterial strains as alginate beads allowed a slow release of microorganisms in soil, which led to a weaker colonization of seedling roots and, consequently, a lower protective effect from chemical control.

In the case of microemulsion, the bacteria were applied directly, as seed treatment causing a better colonization of plant roots and significant protection against *Sclerotium cepivorum* attack.

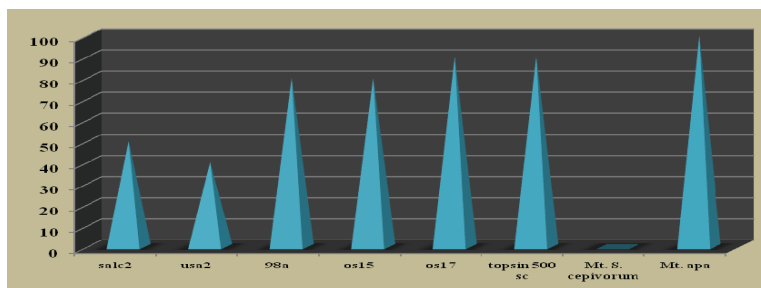


Fig. 2. The efficacy of microemulsion bioproducts in controlling *Sclerotium cepivorum* fungus on onion crop (24°C, 70% humidity)

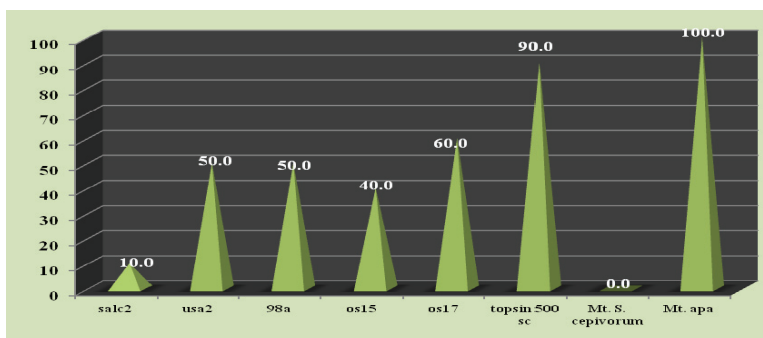


Fig. 3. The efficacy of alginate beads bioproducts in controlling *Sclerotium cepivorum* fungus on onion crop (24°C, 70% humidity)

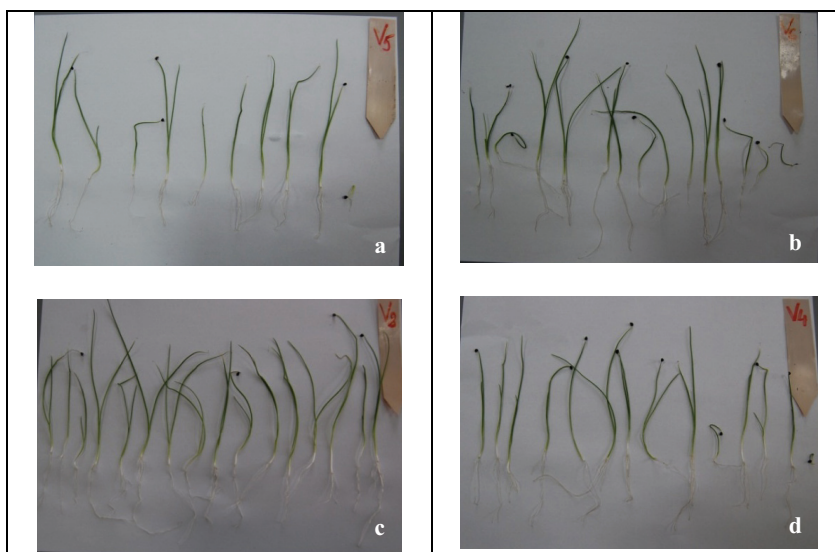


Fig. 4. The influence of the biological treatments on onion seedlings in growth chamber conditions: **a** - *Bacillus amyloliquefaciens* OS.17; **b** - Chemical control TOPSIN 500 SC; **c** - positive control; **d** - *Bacillus pumilus* OS.15

CONCLUSIONS

Five bacterial strains with high antagonistic activity *in vitro* against various phytopathogenic fungi were selected and formulated as microemulsion and sodium alginate beads.

In vitro bioassay showed that our bacterial bioproducts preserved antagonistic capacity against *Sclerotium cepivorum* fungus.

Conditioning methods used, allowed the development of high quality bioproducts which showed the property to preserve very well the biological qualities of the microorganisms.

In vivo results have shown the beneficial effects of the microorganisms formulated and the high efficacy in seedlings protection against the *Sclerotium cepivorum* attack.

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