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BACTERIAL BIOPRODUCTS USED TO CONTROL PHYTOPATHOGENIC SOIL FUNGI COMMON IN FOREST NURSERIES

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Abstract
The aim of this work was to achieve some bioproducts of forest interest by formulating bacterial strains with high biotechnological potential. We also analysed the bioproducts activity *in vitro* and *in planta* against frequently soil phytopathogenic fungi. Two bioproducts, based on *Bacillus subtilis* Bce2 and *Bacillus* sp. 83.2s strains, were formulated and tested in laboratory and solarium conditions. The biological activity of our bioproducts was tested on the following fungal phytopathogens: *Fusarium oxysporum* ZUM 2407*, Rhizoctonia solani* DSM 63002*, Pythium ultimum, Pythium debaryanum* DSM 62946 and an indigenous strain of *Fusarium solani*, isolated from soil planted with spruce seedling (*Picea abies* L., H. Karst.), in Păpăuți nursery, Covasna county. *In vitro* experiments demonstrated that, after formulation, the bioproducts maintained a good antagonistic activity against tested fungal phytopathogens, mainly on *Fusarium* species. The bacterial products applied as soil treatment in naturally infected solarium with edaphic phytopathogenic fungi have led to limiting the attack on spruce seedling.

***Key words*:** bacterial bioproducts, biological activity, edaphic phytopathogenic fungi, spruce seedlings

INTRODUCTION

Various methods to obtain bioproducts based on inoculants microorganisms with multiple actions on crop plants (such as biologically nitrogen fixation, phosphorus solubilisation, microelements biodisponibilization, protection against pest, stimulation of plant growth due to the production of phytohormones and precursors in rhizoplan of crop plants), and on the soil are known. In the last two decades, were tested and evaluated several types of formulations based on polymers (Bashan 1998) which has been shown to be potential carriers of bacteria (Jung et al. 1982).

These types of formulations offer more advantages than first bioproducts conditioned on peat substrate, by conferring more protection for the microorganisms, by encapsulation, to external stress factors, long-term storage at ambient temperature and their progressive soil release when polymers are degraded by the soil microflora action. Alginate is the most common polymer used for bacteria encapsulation in commercial formulations (Cassidy et al. 1996). Conditioning as granular bioproducts on alginate substrate for plant protection is mentioned by many research groups which have developed encapsulation of various microorganisms like mycorrhizal fungi (Arbuscular Mycorrhizal) (Ganry et al., 1982), ectomycorrhizal fungi (Le Tacon et al., 1985), fungi (DeLucca et al., 1990), bacterial antagonists (Aino et al., 1997) and PGPB (Plant Growth Promotion Bacteria) (Trevors et

al.,1992). This encapsulating technology has been used with good results for formulating of PGPB bacteria like *Azospirillum*, *Pseudomonas* and *Bacillus* (Bashan 1986), in order to achieve bioproducts (Bashan 1986; Paul et al., 1993).

This paper presents some achievements on autochthonous bacterial strains with high biotechnological potential and their formulation as bioproducts useful in forest nursery. The bioproducts activity was assessed *in vitro* and *in planta*, against frequently phytopathogenic soil fungi in laboratory and solarium conditions.

MATERIALS AND METHODS

Biological material

Two bacterial strains were used: *Bacillus* sp. 83.2s and *Bacillus subtilis* Bce2, both isolated from soil. These strains were previously identified using Biolog GEN III system (Biolog Inc., Haward, CA) and 16S rDNA sequencing, and selected based on their antagonistic activity against edaphic phytopathogenic fungi that produce infection in forest nurseries and solariums.

The phytopathogenic fungi used in this study were: *Fusarium oxysporum* ZUM 2407*, Rhizoctonia solani* DSM 63002*, Pythium ultimum, Pythium debaryanum* DSM 62946 and a native strain of *Fusarium solani*, isolated from soil planted with spruce seedling (*Picea abies* L., H. Karst.), in Păpăuți nursery, Covasna county.

Cultivation of microorganisms

Bacterial strains were refreshed on Luria-Bertani agar medium (bactotryptone 10g; yeast extract 5g; NaCl 5g; with or without agar-agar 18 g; distilled water up to 1000ml; pH adjusted to 7.4 with NaOH 5M or HCl 1M solutions).

In order to reach enough biomass, bacterial strains were inoculated in LB liquid culture medium, and grown for 1- 2 days at 28^0C, under continuous aeration, till reaching a 10^9cfu / ml concentration. After incubation, the axenically grown cultures of bacteria were harvested by centrifugation at 3750 rpm for 20 minutes, at 10^0C and the bacterial suspension at 10^9 cfu/ml were prepared in sterile phosphate saline buffer.

Bacterial formulation

The bioproducts were formulated by mixing five parts of bacterial suspension (10^9 cfu/ml), three parts of 5% sodium alginate solution, one part of yeast plasmolysate, four parts of kaolin and one part of skim milk powder. The resulted mixture was homogenised and dribbled in 0.25 M $CaCl_2$ solution. The resulted beads were aseptically dried in the laminar flow at 20^0C. The granular bioproducts were than grinded as powder. The wettable powder obtained was aseptically packed in polyethylene bags, 5g / bag, and used as soil treatment for $10m^2$ of area, at softwood crop in solarium.

Bioproducts viability

During storage, at 7, 14 and 30 days, bioproduct samples were analysed in order to establish the germs viability and the maintenance of bacterial biocontrol ability. Aseptically collected samples were grown on LB culture medium, and incubated at 28^0C for 48 hours. Then, the purity and the concentration of microorganisms were analysed. The test was performed in three replicates.

Bacterial load evaluation

A quantity of 100 mg beads were placed in 20ml of sterile phosphate salin buffer (PBS) and incubated overnight at 28^0C with 150 rpm stirring to dissolve and release of microbial cells.

Subsequently, serial dilutions were performed, from which 100µl suspension of 10^{-2}, 10^{-6} and 10^{-8} dilutions were taken and distributed on nutrient agar medium. After 18 hours of incubation at 28^0C, the developed isolated colonies were counted in order to quantify the microbial load in the bioproducts.

Testing the biological activity of bacterial bioproducts in laboratory conditions

Tests were carried out in Petri dishes on potato – dextrose – agar culture medium (PDA: 200 g potato decoct, 20 g dextrose, 20 g agar-agar and 1000 ml distilled water, pH 7.0) using the double cultures technique. The fungal inoculum consisted in mycelial disks, 5mm diameter, derived from cultures in the active stage of growth. Petri dishes were central inoculated with mycelial disks and subsequently co-inoculated with granular bioproducts. In each test plate, three bacterial beads were placed equidistant to each other and at 2 cm from the fungal plugs. Control plates were simultaneously performed. After co-inoculation, the test plates were incubated at 26^0C and analysed in order to assess the inhibitory activity of fungal mycelium, after 48 hours, 4 and 7 days, respectively. The experiment was carried out in three replicates. The inhibitory activity of bacterial bioproducts was evaluated using the formula of Lee et al. (2008):

$$\% \text{ inhibition} = (SC) / C \times 100$$

where: C is the average of mycelium growth in the control, and S is the average of mycelium growth in the direction of bacterial bioproduct, mycelia increases being expressed in centimetres.

Test of the biological activity of bacterial bioproducts in solarium conditions

The wettable powder bioproducts were applied in solarium on spruce seedling (*Picea abies* L., H. Karst.) in the second year of growth. Treatments of 3.5 g of bioproduct / m^2 soil were used. The bioproducts were applied in plots heavily attacked by *Fusarium*, by uniform spraying on soil surface, using 20L / 3mp. The experiment was performed in Păpăuți nursery, Forestry District Comandău, Covasna County.

RESULTS AND DISCUSSIONS

Generally, to increase the bacterial survival rate to desiccation and improve their compatibility with plant protection products, several procedures were described for the production of bioinoculants with controlled release, designed for soil treatment. These formulation methods have the advantage that enable a continuously release of the bacteria after application, which ensures a better colonization of plant rhizosphere and rhizoplan (figure1).

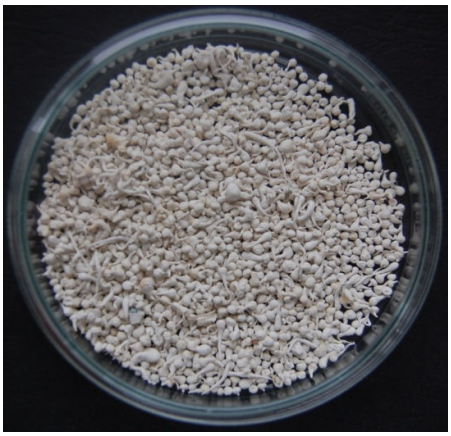


Figure 1. Granular type of bacterial bioproducts

The main advantages of the inoculants formulated as alginate beads are their non-toxic nature, fast degradation in soil and especially, their gradually release (Bashan 1998). The functionality of this formulation method has been experimented previously and was shown to provide satisfactory results.

Bacterial load

The bacterial concentration in the granular bioproducts was quantified at one month after formulation. The microbial load in bioproducts was quantified by counting the isolated colonies developed on the nutrient agar plates (table 1).

Bioproducts stability in time

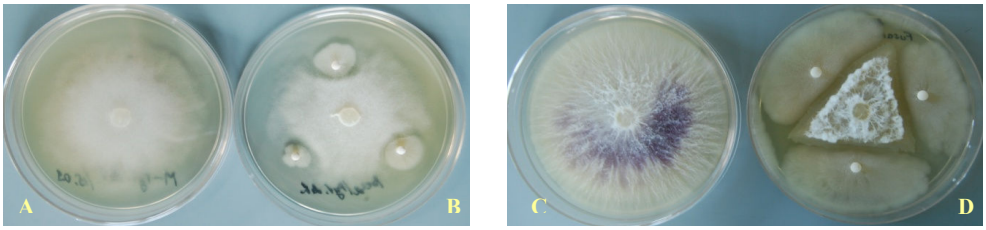
After being stored at room temperature, the bioproducts revealed that the bacterial concentration has an adequate value to initiate the biological activity on softwood seedlings by growth stimulation and protection against phytopathogenic fungi attack.

As shown in table 2, the survival level of germs ranged in very close limits with slight decreasing tendency in time, without affecting the product quality.

The data showed that the concentrations of *Bacillus* bioproducts formulated as alginate beads revealed that bacterial inoculums survived throughout storage at room temperature (22^0C), without causing dramatic declines of titre.

In vitro ***evaluation of bioproducts biological activity***

The *in vitro* test results showed that granular bioproducts had antagonistic activity at optimum parameters against phytopathogenic fungi tested (figure 2, table 3).



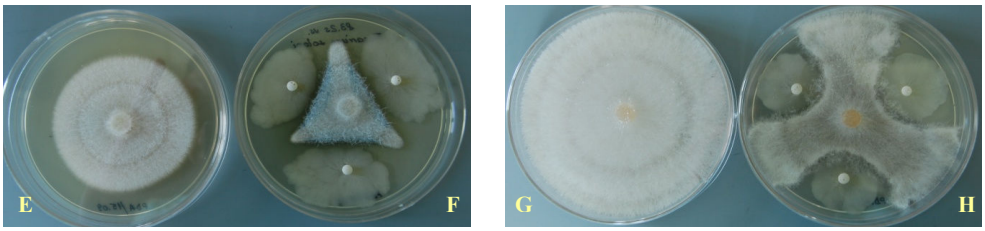


Figure 2. *In vitro* antagonistic activity of bacterial bioproducts
A. *Pythium debaryanum* control; **B**. *Bacillus subtilis* Bce2 bioproduct vs. *Pythium debaryanum*; **C**. *Fusarium oxysporum* control; **D**. *Bacillus* sp. 83.2s bioproduct vs. *Fusarium oxysporum;* **E**. *Fusarium solani* control; **F**. *Bacillus* sp. 83.2s bioproduct vs. *Fusarium solani;* **G**. *Rhizoctonia solani* control; **H**. *Bacillus subtilis* Bce2 bioproduct vs. *Rhizoctonia solani.*

The bacterial bioproducts had a good *in vitro* efficacy against tested fungal phytopathogens, mainly against *Fusarium* species, from 54.9 to 62.5%, even after seven days of co-cultivation.

Considering the bioproducts antagonistic activity in laboratory conditions, by applying soil biological treatments in solarium naturally infected with *Fusarium solani,* we expect a limitation of the attack level on spruce seedlings (figure 3).

Figure 3. Preparation and application of bacterial treatments:
A. bacterial bioproduct; **B**. wettable powder dissolving in water; **C**. distribution of treatment solutions in spraying containers; **D.** treatments appliance; **E.** plant aspect after treatment.

According to the data we received from the Păpăuți forest nursery, Covasna county, in the treated variants the infections caused by phytopathogenic soil fungi stagnated and the spruce seedlings presented a good phytosanitary health (figure 3).

CONCLUSIONS

- Within this study were obtained two bioproducts based on *Bacillus* sp. 83.2s and *Bacillus subtilis* Bce2;
- The formulation method enabled to achieve high quality bioproducts, which maintained the *in vitro* bacterial biocontrol activity towards phytopathogenic fungi*;*
- Conditioning form was appropriate since it allowed the survival of the bacteria at room temperature;
- Laboratory experiments have shown a good maintenance of the bacterial biocontrol traits against the tested phytopathogenic fungi;
- The bacterial bioproducts showed a better efficacy against *Fusarium* species, including *F. solani* isolated from the spruce seedlings substrate;
- The *Bacillus* bioproducts, applied as soil treatments into naturally infected solarium, limited the attack level of soil borne fungal phytopathogens on spruce seedlings.

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REFERENCES

BASHAN,Y., HOLGUIN,G. (1998). Proposal for the division of plant growth-promoting Rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol Biochem* 30, 1225-1228.

JUNG, G., MUGNIER, J., DIEM, H.G., DOMMERGUES, Y.R. (1982). Polymerentrapped *Rhizobium* as an inoculant for legumes. *Plant Soil* 65, 219-231.

CASSIDY, M.B., LEE, H., TREVORS, J.T. (1996). Environmental applications of immobilized microbial cells: a review. *J Ind Microbiol* 16, 79-101.

AINO M, MAEKAWA Y, MAYAMA S, KATO H, 1997. Biocontrol of bacterial wilt of tomato by producing seedlings colonized with endophytic antagonistic pseudomonads. In: Ogoshi A, Kobayashi K, Homma Y, Kodama F, Kondo N, Akino S (eds) Plant growth-promoting Rhizobacteria -present status and future prospects. Faculty of Agriculture, Hokkaido University, Sapporo, pp 120-123.

BASHAN, Y. (1986). Alginate beads as synthetic inoculant carriers for the slow release of bacteria that affect plant growth. *Appl Environ Microbiol* 51, 1089-1098.

DeLUCCA, A.J., CONNICK, W.J. Jr, FRAVEL, D.R., LEWIS, J.A., BLAND, J.M. (1990). The use of bacterial alginates to prepare biocontrol formulations. *J Ind Microbiol* 6, 129-134.

GANRY, F., DIEM, H.G., DOMMERGUES, Y.R. (1982). Effect of inoculation with *Glomus mosseae* on nitrogen fixation by field grown soybeans. *Plant Soil* 68, 321-329.

LEE, Y.S., KIM, J., SHIN, S.C., LEE, S.G., PARK, I.K. (2008). Antifungal activity of Myrtaceae essential oils and their components against three phytopathogenic fungi. *Flavour and Fragrance Journal*, 23, 23-28.

LE TACON, F., JUNG, G., MUGNIER, J., MICHELOT, P., MAUPERIN, C. (1985). Efficiency in a forest nursary of an ectomycorrhizal fungus inoculum produced in a fermentor and entrapped in polymeric gels. *Can J Bot* 63, 1664-1668.

PAUL, E., FAGES, J., BLANC, P., GOMA, G., PAREILLEUX, A. (1993). Survival of alginate - entrapped cells of *Azospirillum lipoferum* during dehydration and storage in relation to water properties. *Appl Microbiol Biotechnol* 40, 34-39

TREVORS, J.T., ELSAS, J.D. van, LEE, H., OVERBEEK, L.S. van. (1992). Use of alginate and other carriers for encapsulation of microbial cells for use in soil. *Micron Releases* 1, 61-69.

Table 1

Determination of microbial load in the alginate beads bioproducts (after 30 days of storage at room temperature)

Bacterial bioproduct	initial concentration <cfu/g>	Bacterial load after 30 days <cfu/g>
Bce2	$1,3\times10^{9}$	$8,3\times10^{8}$
83.2s	$1,3\times10^{9}$	$7,8\times10^{8}$

Table 2

Survival of the beneficial bacteria conditioned as bioproducts (x 10^{8} cfu) during storage at room temperature

Bioproduct	0	7 days	14 days	30 days
Bacillus sp. 83.2s	13,4	10,9	9,4	7,8
Bacillus subtilis Bce2	13.2	10.4	9.8	8.3

Table 3

Evaluation of bioproducts inhibitory activity against phytopathogenic fungi

Phytopathogenic fungi	Co-cultivation (days)	Bacterial bioproducts	
		Bacillus subtilis Bce2	*Bacillus* sp. 83.2s
Pythium debaryanum	2	39,3%	41,2%
Pythium ultimum	2	22,9%	30,8%
Rhizoctonia solani	4	48,7%	64,3%
Fusarium oxysporum	7	54,9%	57,1%
Fusarium solani	7	62,5%	56%