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SOCIOMICROBIOLOGICAL PROPERTIES OF ANTAGONISTIC BACTERIA ISOLATED FORM BORSÁROS RAISED BOG

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ABSTRACT. The main aim of our study is the development of new antagonistic bacterial based biopreparates. The sociomicrobiological analyses gives information about the bacterial communication forms. These communication forms are important in bacterial antagonism. In this study we analyzed the biofilm formation ability of the isolated bacterial strains in single and co-cultures.

Keywords: *antagonism, soil bacteria, biofilm formation, bacterial communication, bryophyte associated bacteria*

INTRODUCTION

A novel formulation of broad spectrum bacterial biopreparates is the objective of sustainable agriculture. Our main aim is to develop biopreparates, carrying living antagonistic soil bacteria. In order to select bacterial strains for such biopreparates it is important to analyze their ability to colonize plant surfaces [1 and 2].

Antagonistic bacteria are microorganisms which can control the proliferation of plant pathogens [3]. These bacterial strains exert their action by synthesising antimicrobial compounds, such as antibiotics, siderophores, biosurfactants, and antifungal metabolites [1, 4 and 5]. All these metabolites are important in displacement of competing microbial populations. Recent studies show that colonization of the plant surfaces by biocontrol bacteria is more effective when bacteria form microcolonies, called biofilms [1].

Biofilms are multicellular aggregates; bacterial cells being adhered to a surface and each other through an exopolysaccharide matrix [6]. Biofilms are advantageous to bacteria; microorganisms within biofilm are more resistant to environmental stress conditions (e.g. nutrient lack, antibiotics, pH etc). The biofilm forming bacteria also show a higher survival rate, than the planktonic (free-living) organisms [6 and 7]. In biofilm communities can be involved a large number of different bacterial species. This explains the importance of studying biofilm profiles of bacterial co-cultures [8].

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The studied bacterial strains were isolated from Borsáros natural reserve, near Miercurea Ciuc, Harghita County (Romania). In this nutrient poor oligotrophic raised bog the main biomass is given by the *Sphagnum sp.* mosses (*Bryophyta)*. According to Opelt and Berg [9], the *Sphagnum sp.* mosses can be associated with antagonistic bacteria, appropriate to be used as biopreparates [9].

The aim of the current study is to determine the biofilm production ability of the isolated bacterial strains. The best biofilm forming bacterial strains were selected for compatibility study. Those bacteria that showed a good coexistence property were analyzed for their biofilm production in co-cultures.

RESULTS AND DISCUSSION

A total number of sixty six bacterial strains were isolated from Borsáros raised bog. The isolated bacterial strains were sorted in three groups: 22 *Enterobacteriaceae*, 23 *Pseudomonas sp*. and 21 *Bacillus sp*. The selected bacterial strains were studied for their antagonistic properties against plant pathogen microorganisms (*Phytium sp*. and *Erwinia carotovora*) [13].

The biofilm forming capacity of 66 bacterial strains were analyzed. The determination of biofilm production was realized using crystal violet staining, by spectrofluorometric method. Bacterial strains were grown in microtiter plates for 24 h. The optical density of the cell suspension was determined at 620 nm. Biofilms were stained with crystal violet, followed by a dissolving of the bounded dye in absolute ethanol. The optical density of the stained ethanol was measured at 492 nm.

The biofilm forming ability of *Bacillus sp.* strains is presented in Figure 1. The ratio of OD492/OD620 represents the real value of the biofilm production.

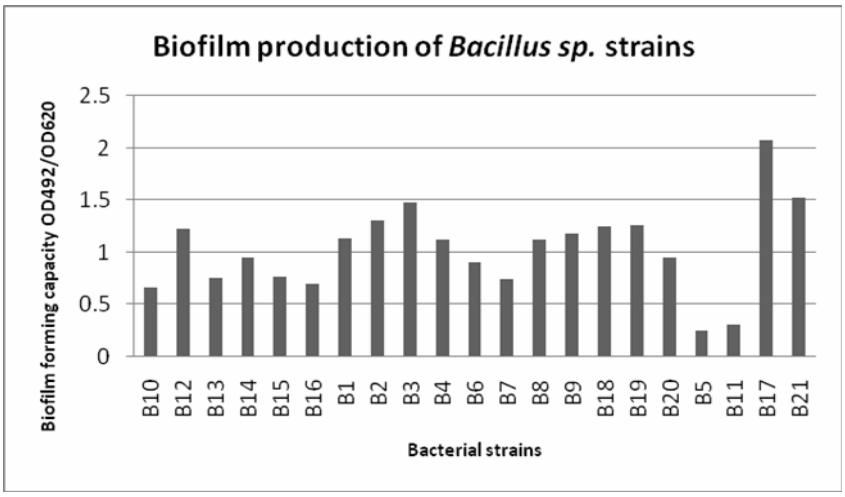

Figure 1. Biofilm production ability of *Bacillus sp.* Strains

From the analyzed bacterial strains a number of 3 (B3, B17 and B21) were selected for further study. These bacterial strains proved to be good biofilm producers.

From the analyzed *Pseudomonas sp.* a number of 6 strains (P6, P8, P10, P11, P18 and P19) were selected for compatibility study (Figure 2).

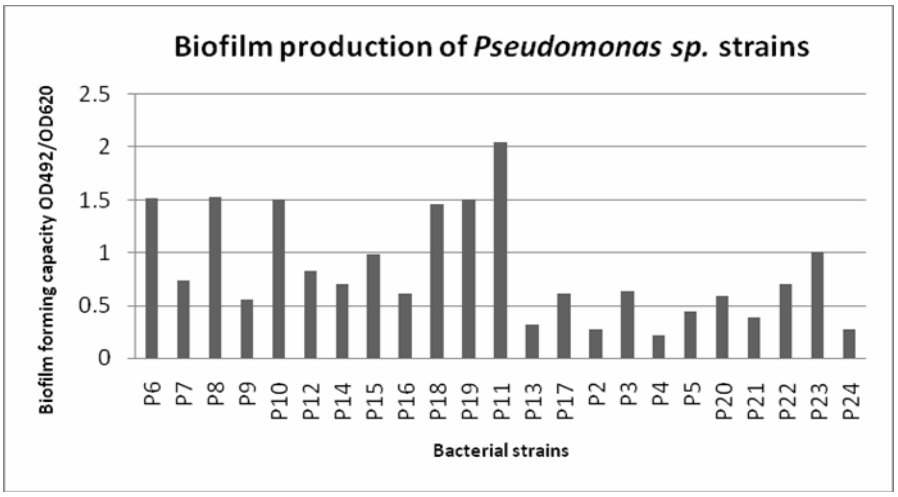

Figure 2. Biofilm production ability of *Pseudomonas sp.* bacterial strains

We also selected 2 strains of *Enterobacteriaceae* for further analysis. In Figure 3 the real values of the biofilm quantity (OD492/OD620) are presented. The good biofilm forming bacteria, selected for further analysis are: E2 and E20.

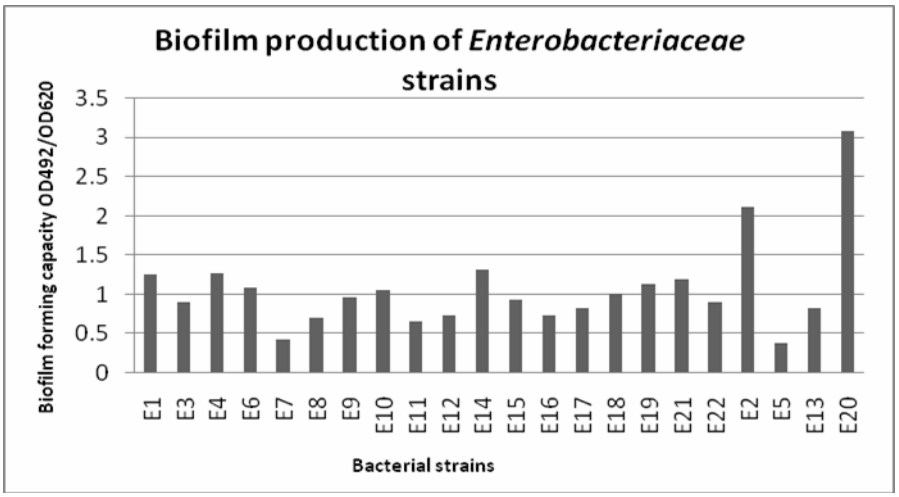

Figure 3. Biofilm production ability of *Enterobacteriaceae* bacterial strains

Compatibility test was realized with agar diffusion assay for the selected biofilm producing bacterial strains. Figure 4 shows the compatibility of E2 bacterial strain with P6, P11, P18, P19 and B3 bacterial strains. Inhibition zone formed in case of P6 bacteria refers that this strain inhibits the proliferation of E2 bacterial strains. In case of P11, P18, P19 and B3 bacterial strains the inhibition zone is not visualized.

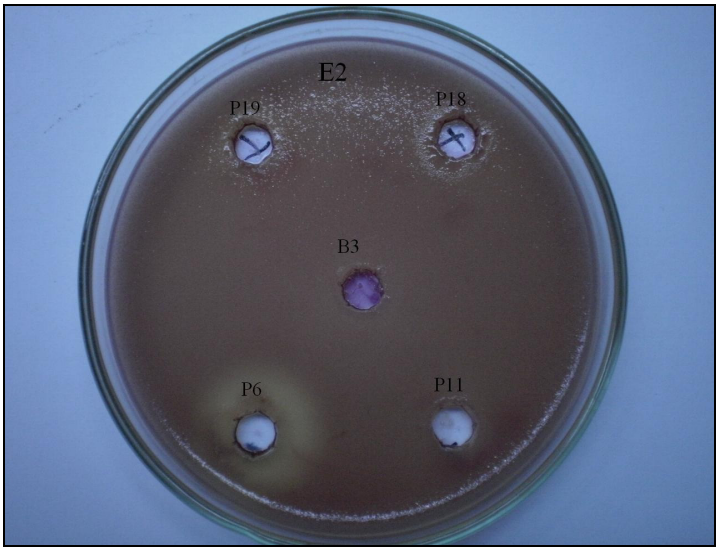

Figure 4. Compatibility test with agar diffusion assay, P6 bacterial strain inhibits the proliferation of E2 bacterial strain

We found 8 similar cases, when bacterial strains inhibited the proliferation of each other. Inhibition zones were formed between bacterial strains as follows: P6-P8, P6-P11, P6-E20, P10-P6, P18-P11, P19-P6, P19-P11 and B17-P8. P6 bacterial strain, isolated as *Pseudomonas sp.* showed the highest number of antagonistic activity.

In order to develop a bacterial based biopreparate it is essential, to analyze the biofilm formation ability of bacterial co-cultures. We selected the following bacterial strains: P10, P18, P19, B3, B17, B21, E2 and E20. In Figure 5 are shown the biofilm production ability of the co-cultures compared with the single cultures. The grey columns represent the biofilm forming ability of the single bacterial strains; the black column represents the biofilm production of the co-cultures. In case of 5 samples the biofilm forming ability of the co-cultures showed a higher value, than in the case of single bacterial strains. B3, P10, E2 are the bacterial strains that reinforce the biofilm production in co-cultures.

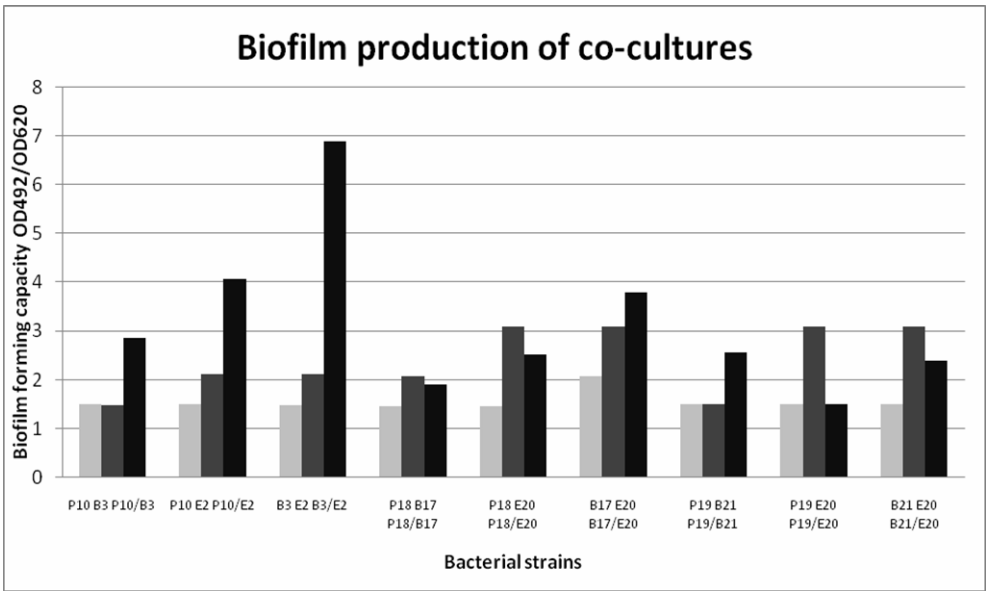

Figure 5. Biofilm production of co-cultures

CONCLUSIONS

Preliminary study was made in order to develop antagonistic bacterial based biopesticides. Compatibility test and biofilm formation ability of single strains and co-cultures of isolated antagonistic bacterial strains were studied.

The importance of biofilm production was demonstrated by Simoes et al. [10]. Biofilms are protecting area for bacteria, allowing bacterial strains to survive in hostile environments. In case of soil bacteria – such as plant growth promoting and biocontrol bacteria- biofilms play key role in the colonization of root surfaces and survival in the harsh environment [1]. According to Maheswari [11] the biofilm formation on the plant surfaces protects the plant against phytopathogen microorganisms, trough resistance mechanisms such as Quorum sensing or antibiotic production. Although microtiter plate assay is a very easy method to measure the quantity of the bacterial biofilm, in case of soil bacteria is not commonly used.

Rinaudi et al. [12] studied biofilm formation ability of soil bacterial strain *Sinorhizobium meliloti* on different nutritional conditions. According to their results the biofilm formation in nutritionally limited conditions increases. This indicates a survival strategy of bacteria, colonizing the root surface by bacteria has the advantage to increased capture of nutrients that may be absorbed to the surface. In our study the analyzed sixty six bacterial strains were isolated from

a nutrient poor environment. From bacterial strains isolated as *Pseudomonas sp.*, *Bacillus sp.* and *Enterobacteriaceae,* 11 strains proved to be good biofilm producers. These bacterial strains can be categorized as good surface colonisers. These bacterial strains were further analyzed for their compatibility.

One of the analyzed bacterial strains (P6 strain) proved to be the less compatible with the other strains. Five bacterial strains (B3, B21, P8, P11 and E2) showed a good coexistence property.

Liu et al. [8] compared biofilm formation of two bacterial strains under single culture and co-culture. During the study was proved that under co-culture conditions bacterial biofilm form more densely then in single culture conditions. We analysed the biofilm forming capacity of 9 bacterial pairs. In case of 5 pairs the biofilm forming capacity proved to be better then in single cultures. These strains could be good root surface colonizing bacteria.

Further analyses - such as the ability of signal molecules production, studying the antimicrobial products secreted by bacterial strains, optimization of culturing conditions, testing the isolated bacterial strains on plants - are necessary for these bacterial strains to be used as potential biopreparates.

EXPERIMENTAL SECTION

Bacterial strains

Bacterial strains were isolated from Borsáros raised bog, form the surface, the tissues and the rhizosphere of *Sphagnum sp.* The moss samples were collected in summer of 2010. For bacterial isolation, three different selective growth medium was used: King B agar (proteose peptone 20 g/L, glycerol 10 ml/L, di-potassium hydrogen phosphate 1,5 g/L, magnesium sulphate* $7H_2O$ 1,5 g/L, agar 20 g/L) for *Pseudomonas sp.*, Nutrient agar (peptone 5 g/L, sodium chloride 5 g/L, yeast extract 2 g/L, meat extract 1 g/L, agar-agar 15 g/L) for *Bacillus sp.* and MacConkey agar (peptone form casein 17 g/L, peptone from meat 3 g/L, sodium chloride 5 g/L, lactose 10 g/L, Bile salt mixture 1,5 g/L, neutral red 0,03 g/L, crystal violet 0,001 g/L, agar-agar 13,5 g/L) for selection of *Enterobacteriaceae* bacterial strains. The isolates were tested of their antifungal (Phytium sp.) and antibacterial (Erwinia carotovora) activity [13].

Biofilm formation assay

Biofilm formation assay were conducted according to the methods of Tamás et al. [14]. Bacterial strains were grown in Nutrient broth, for 24 h cultures. For each culture dilution series were made (1 ml of bacterial strain was loaded in 9 ml of Nutrient broth). 200 μl of bacterial suspension was

loaded in a 96 well microtiter plate (SPL). 200 μl of sterile Nutrient broth was used as a control. The optical density of the cell suspension was measured before and after the incubation at 620 nm (Fluorostar Optima, BMG Labtech, microtiter plate reader). After 24 h incubation at 28 °C, cells were washed twice with 300 μl distilled water (StatFax 2600). Biofilms were stained 40 minutes with 300 μl crystal-violet solution (0.1%). Wells were washed two times with 300 μl of demineralised water. The bounded crystal-violet dye was dissolved in 300 μl ethanol (96%). 200 μl of solubilised ethanol solution was transferred into a new microtiter plate. The optical density of the wells was measured at 492 nm. The biofilm formation assay was repeated eight times.

The biofilm formation study of the co-cultures was performed as described above. The selected bacterial strains were as follows: P10, P18, P19, B3, B17, B21, E2 and E20. Bacterial cells were grown in pairs in Nutrient broth. The bacterial pairs were as follows: P10-B3, P10-E2, B3-E2, P18-B17, P18-E20, B17-E20, P19-B21 and B21-E20.

Agar diffusion assay

The compatibility test was performed for the best biofilm forming 11 bacterial strains. Bacterial strains were cultured for 24 h, at 28 °C. After the incubation bacterial cultures were diluted in sterile distilled water, until the optical density at 660 nm (OD_{660}) was 0.3. Nutrient agar, containing 1 % TTC (triphenyl tetrazolium chloride) was tempered at 40-50 °C, and inoculated with the test organisms. The inoculated medium was poured in sterile Petri dishes. After solidification, wells of 5 mm diameter were cut, with the use of sterile glass tubes (5 wells in each Petri dish, 2 Petri dishes for every bacterial strain).Test bacterial cells, were grown in Nutrient broth for 1 week, at 28 °C. After incubation the cells were centrifuged at 5000 RPM for 30 minutes, and separated with membrane filters to obtain the bacteria free supernatant. 100 μl of supernatant was added in each well. Red coloration of the nutrient medium refers to the growth of bacterial cells. Appearance of inhibition zones around the wells refers to antagonistic property of the tested bacterial strain. We tested the compatibility of each bacterial strain with the other 10 strains.

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REFERENCES

1. M.A. Molina, J.L. Ramos, M. Espinosa-Urgel, *Reviews in Environmental Science and Biotechnology*, **2003**, *2,* 101.
2. Al-Taweil H.I., Osman M.B., Hamid A.A., Yusoff W.M.W., *American Journal of Agricultura land Biological Sciences*, **2009**, *4*(1), 79.
3. D. Chandler, G. Davidson, W.P. Grant, J. Greaves, G.M. Tatchell, *Trend sin Food Science & Technology*, **2008**, *19*, 276.
4. B.R. Glick, Y. Bashan, *Biotechnology Advances*, **1997**, *15* (2), 356-360.
5. M. Shoda, *Journal of Bioscience and Bioengeneering*, **2000**, *89*(6), 516.
6. B.E. Ramey, M. Koutsoudis, S.B. Bodman, C. Fuqua, *Current Opininon in Microbiology*, **2004**, *7*, 604.
7. E.D. Elder, A.S. Fenech, A.D. Zink, T.P. Bradley, A.L. Fuller, *Proceedings of the 1995 Georgia Water Resources Conference*, **1995**, 386.
8. L. Liu, L. Chu, Q. Liu, C. Wang, Y. Xia, X. Peng, *African Juornal of Microbiology Research*, **2010**, *4*(3), 180.
9. K. Opelt, G. Berg, *Applied and Environmental Microbiology*, **2004**, *70* (11), 6569.
10. M. Simoes, L.C. Simoes, M.J. Vieira, *Food Science and Technology*, **2010**, *43*, 575.
11. D.K. Maheshwari, "Plant Growth and Health Promoting Bacteria", Springer, Münster, **2010**.
12. L. Rinaudi, N.A. Fujishige, A.M. Hirsch, E. Banchio, A. Zorreguieta, W. Giordani, *Research in Microbiology*, **2006**, *157*, 869.
13. S. Szentes, Gy. Mara, Sz. Lányi, G.L. Radu, *Scientific Bulletin of Mineral Resources and Environment Faculty, North University of Baia Mare,* **2010**, *16*(2), 54.
14. É. Tamás, Gy. Mara, É. Laslo, É. György, B. Ábrahám, Sz. Lányi, *Studia UBB Chemia*, **2009**, Special Issue No. 2, 51.