RESEARCH ON THE ROLE OF MICROBIAL CONSORTIUM IN BIOSYNTHESIS OF HUMIC PRECURSORS BASED ON SECONDARY EXOMETABOLITES

MATEI S., MATEI* GABI-MIRELA, DUMITRU SORINA, IGNAT P.
National Research-Development Institute for Soil Science Agrochemistry and Environment-Bucharest, Bd. Mărăști 61, 011464, Sector 1, Bucharest, Romania;
*e-mail: so_matei602003@yahoo.com

Keywords: exometabolites, humic precursors, microbial consortia, enzymes, polysaccharide

ABSTRACT

The ability of exometabolites formation by secondary metabolism of edaphic microorganisms is genetically determined and antibiotic properties and their biologically activity accompanies this skill, also. Microbial isolates from different soil types and vermicompost were selected based on their ability to excrete soluble secondary metabolites in growth medium, and identified as belonging to the genera Bacillus, Pseudomonas, Lactobacillus, Streptomyces, Fusarium, Aspergillus and Botrytis. The qualitative and quantitative influence of the number of microbial isolates or the type of microbial consortia on the content of secondary exometabolites with a composition close to humic acids precursors has been assessed by circular chromatography analysis, enzymes activities and polysaccharide biosynthesis.

INTRODUCTION

Exometabolites interactions in soil are poorly understood but are important in global biogeochemical cycle of carbon, influencing especially soil organic matter. The study of potential metabolic interactions within microbial communities from different consortia may improve the understanding of soil organic matter dynamics. A study of the exometabolites released by microbial consortia may be relevant under changes in environmental conditions, also.

The formation of exometabolites by metabolism of edaphic microorganisms is genetically determined and is an identifying criterion. These soluble secondary metabolites, can have different colors and confer protection against light and UV radiation, can absorb oxygen in redox processes and these skills accompany antibiotics and specific enzymatic properties, as well.

In soil, for microbial growth, the nutrients suffer catalytic conversion into a new biomass and in extracellular products, a large part of them can form the humus fraction of soil organic matter and it could thought to be of microbial origin (Schmidt, 2011).

Microbial compounds released in soil can become an alternative effective source of carbon supply thereof, are produced by a variety of microorganisms (bacteria, fungi, yeasts, protozoa) and belong to structural groups as varied as carotenoids, anthraquinones, chlorophylls, flavins, quinones, violaceins (Kenini and Gupta, 2011).

Enzymes cellulases refer to a group of enzymes (including exoglucanase, endoglucanase and β-glucosidase) acting together to hydrolyze cellulose. A large number of microorganisms (bacteria, fungi and actinomycetes) are known to degrade cellulose or similar compounds, with the cellulase enzymes biosynthesized by their secondary metabolism, in glucose and other active ingredients which improve cell permeability (Nagaraju et al., 2009). Microbial cellulases degrade cellulose to glucose in synergistic action because of similar structure for catalytic domain and cellulose connecting linkers.

Laccases are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor and play a role in the formation/degradation of lignin. These enzymes have the potential to cross link polymers, polysaccharides, to increase the oxidation rate of free SH groups of proteins or to oxidize peptide bound tyrosine. Also, the polyphenol complexes are formed by laccases because these delay the oxidation of polyphenols and stabilize the chemical structure.

The antimicrobial activity of microbial exometabolites was tested on algae, cyanobacteria, bacteria or fungi (Carmichael, 1992; Smith and Doan, 1999; Jaki et al., 2000; Rainer and Furkert, 2006, Grata and Nabrdalik, 2014). Antifungal activity was confirmed against soil fungus *Aspergillus ochraceus* as a human pathogen producing mycotoxins and is adapted to varied ecological niches (Meenupriya, et al. 2011). The influence of exometabolites from associated microorganisms revealed regulatory effects on dynamics of enzymes activity (Timokhina, 2009).

Microbial metabolites can modulate plantlets root growth through the production of secondary metabolites, enzymes and phytohormones. The effects are an increase of the number and length of lateral roots and root hairs. Also, exometabolites influence plant nutrition, solubilization of phosphorus, siderophore production, metabolite biosynthesis in root cells and gene transcription with direct influence on root physiology.

MATERIAL AND METHOD

Microbial consortia in this study contain strains isolated from different soil types (Haplic Chernozem, Albic Luvisol, Fluvisol as per WRB-SR - 1998) and vermicompost selected based on their ability to increased excreting in Czapek growth medium of soluble secondary metabolites.

Bacterial and fungal isolates (22 isolated) belonged to the genera *Bacillus, Pseudomonas, Lactobacillus, Streptomyces, Fusarium, Aspergillus, Botrytis*, and were grouped into four consortia based on the growth rate of the isolates and their capability in biosynthesis of the enzymes, exopolysaccharides and pigments.

Four microbial consortia (C1, C2, C3, C4) were analyzed in order to determine the optimal composition in the relations of co-culture isolates of the mixture and assessment of synergism in microbial biosynthesis of organic complexes consisting solely of exometabolites, after 5 days at 27^{o}C temperature.

Paper chromatography techniques evidenced qualitative contributions of the exometabolite accumulations in time and released in the growth culture medium by the presence of 2 or 4 isolates of *Fusarium culmorum* inoculated in Czapek broth media with a density of 3x10^{3} cfu g^{-1}, at a temperature of 30^{o}C for 5 days.

The paper chromatograms of exometabolites distribution from the C1-C4 consortia were used for qualitative comparing with distribution of the humus compounds from haplic chernozem isolated according with IHSS method.

Cellulase activity influenced by the type of microbial consortia C1-C4 was qualitative assayed on CMC media, in agar wells (diffusion method). The influence of secondary metabolites from broth culture on the activity of cellulase was quantitatively assayed using DNS method. The results were statistically analyzed, according to the Student test.

The influence of the C1-C4 consortia metabolites on laccase activity was estimated according with bromophenol blue protocol (Tekere et al. 2001).

Production of exopolysaccharides by the C1-C4 consortia was tested qualitatively by inoculating culture on the sterile filter paper disc and the total amount of exopolysaccharides produced was determined by calculating the weight difference between the dry weight of the paper with the polymer and the initial weight of filter paper. The results are the average of three replicates of each consortium and comparing the quantities of exopolysaccharides was performed by the statistical analysis using Student's

test (Read et al., 1987)

The antimicrobial activity of exometabolites from the C1-C4 consortia was tested in Petri against spots of *Aspergillus ochraceus* from pure culture (spot-on-lawn method). The influence of exometabolites on the fungus development was assayed after 7 days at 25^{o}C temperature.

The influence of sterile filtrate culture media of the C1-C4 consortia on the cucumber root development was assayed in Petri dish with sterile filter paper on the bottom of the plate. The cucumber seeds surface was disinfected with chloramine and washed with distilled water. For each variant were made three replicates. The root branching and length was assayed after 7 days at 27^{o}C.

RESULTS AND DISCUSSIONS

For an evaluation of individual contribution in exometabolites excreted in culture media we used the pure cultures of *Fusariu*m isolates from different soil types. Erlenmeyer vials with Czapek broth media non-inoculate and inoculate with 2 and 4 fungal isolates were used for assessing the qualitative contribution in microbial exometabolites accumulations. The Figure 1 presents the fungal isolates inoculated in culture media after 5 days growth at 30^{o}C temperature. The image presents the accumulations and distribution of exometabolites in circular chromatograms, as a reflection of complexity molecular growth of the compounds biosynthesized by fungal isolates. The secondary metabolites accumulations were more intense when 4 isolates of *Fusarium* were inoculated in broth media as against 2 isolates or non-inoculate.

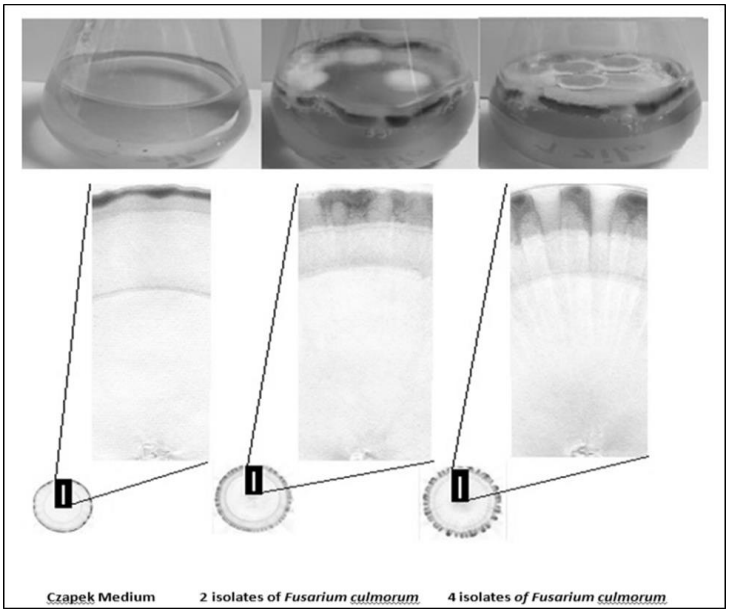

Figure 1 Circular chromatograms of broth media 5 days after inoculation with 2 and 4 isolates of Fusarium culmorum

The assay of exometabolites composition of the mix of isolates from the C1-C4 consortia aimed to characterize each consortium from metabolic point of view and if these were the result of a possible synergistic interactions between selected isolates. Also, it was assayed the production, the diversity and the complexity of exometabolites taking in account that the result of an optimum combination of isolates could determine to obtain compounds with a composition similar to the soil humic acids.

Ascendant chromatograms were used to compare the exometabolites of the C1-C4 consortia which were extracted from the broth culture media with the humic acids fraction isolated from Haplic Chernozem. In the image of chromatograms, the metabolomic

differences were observed in the exometabolites distribution in the C1-C4 consortia and to the control. Then they were qualitatively compared with distribution of the soil compounds of the humic fraction to estimate the metabolic capabilities of each mix of isolates (Figure 2).

Exometabolites of the C4 consortium had a diversity and complexity of compounds biosynthesized in the culture media nearest to control. The level of organization of the organic compounds in the C1 consortium is higher, their diversity enables progress towards the formation of more complex compounds. In the C3 and C2 consortia it highlights areas of accumulation with different molecular structures, no trends to complexing, due to their inability to biosynthesis of intermediate compounds by the microbial consortia or due to the incompatibility between microbial isolates which subsequent induce the biochemical processes evolution in the broth culture media.

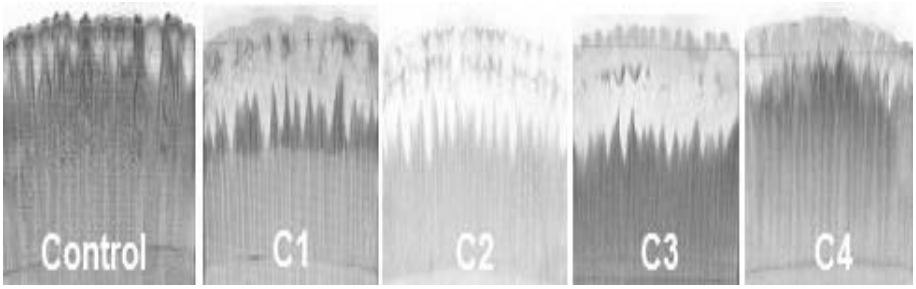

Figure 2 The chromatograms of exometabolites distribution in C1-C4 consortia

By using microbial consortia C1-C4, it was obtained a wide variety of the cellulase complexes with different potential in the breakdown of methylcellulose or of similar substrates with differing degrees of polymerization, and of polydispersity. In our tests, the methylcellulose was used as substrate for the qualitative assay of the cellulolytic activity of consortia. The cellulolytic activity of the C4 consortium was the most intense and that of the C2 consortium with the lowest intensity. The diameter of reaction zone after the supernatant diffusion was of 27mm for agar-wells with the C4 mix culture and 17mm for agar-wells with the C2 supernatant (Figure 3). The consortia C2 and C3 had intermediate values, 20mm and respectively 24mm diameter.

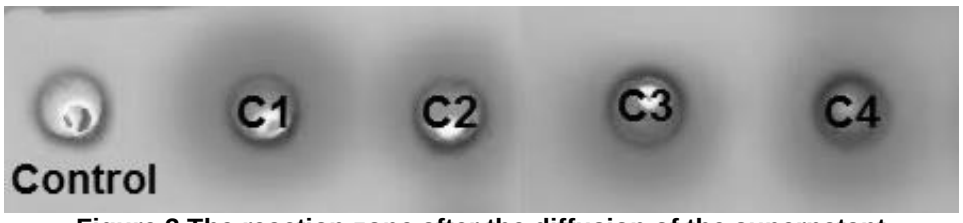

Figure 3 The reaction zone after the diffusion of the supernatant from C1-C4 mix culture in CMC agar media

The influence of the exometabolites from broth culture media on the cellulase activity was quantitatively assayed for the C1-C4 consortia. A equal volume of co-culture supernatant from each consortium was mixed with equal volume of citrate solution in test tubes with CMC substrate. The mixture was incubated and the DNS reagent was added to the reaction mixture. The C4 consortium had a cellulase activity estimated to 269mgL^{-1} D-glucose and 244mgL^{-1} D-glucose concentration for C1 consortium, both values were statistic significant in comparing to the C3 and C2 consortia, respectively with values of 166 and 148mgL^{-1} D-glucose concentration (Figure 4).

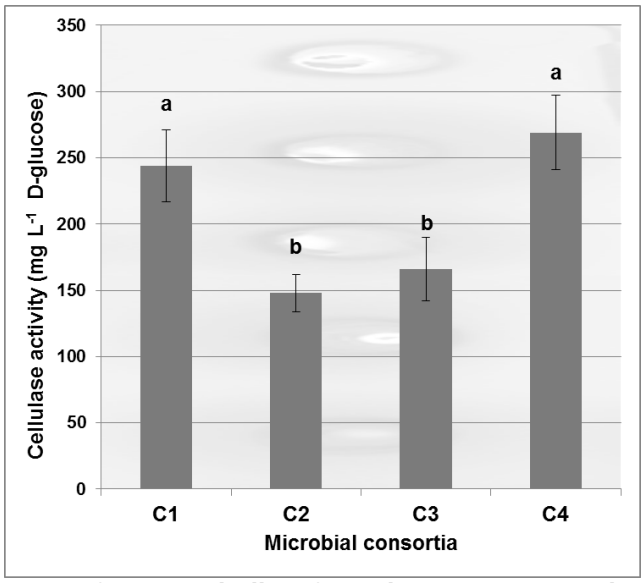

Figure 4 The influence of exometabolites from the C1-C4 consortia on cellulase activity. The values followed by the same letter are not significantly different for p <0.05 (Student test)

The microbial consortia C1-C4 can biosynthesize laccase, polyphenolic enzymes, which play a role in the morphogenesis, the lignin biodegradation, the pigments formation in fungal mycelia, the cell-to-cell adhesion, in the formation of the rhizomorphs and of the polyphenolic glue by that binds the fungal hyphae. Recent studies postulated their involving in various cellular and microbial activities, in the physiological functions such as the cell wall biosynthesis, phytopathogenesis, the degradation and the humification, melanization, and the melanin related virulence for humans (Thurston 1994; Xu 1999). These enzymes are able to overcome the immune response of the host plant to pathogens, facilitating the detoxification via the oxidation of antifungal phenols or deactivation of phytoalexins, also.

For qualitative assay of laccase activity of the C1-C4 consortia, the culture media incubated in wet chamber with supernatant from all the cultures in agar-wells revealed the emergence of clear halos around the wells (Figure 5).

Thus, the laccase activity of the C4 consortium was the most intense followed by the C1 consortium. The diameter of reaction zone after diffusion was of 21mm for C4 under the influence of supernatant with laccase and 19mm for agar-wells with the C1 culture supernatant. The values of the halos diameter varied from 18mm for C3 to 15mm for C2. For all the consortia, the clear halos around the wells with the C1-C4 supernatant indicate positive and different influences induced by the presence of the laccases.

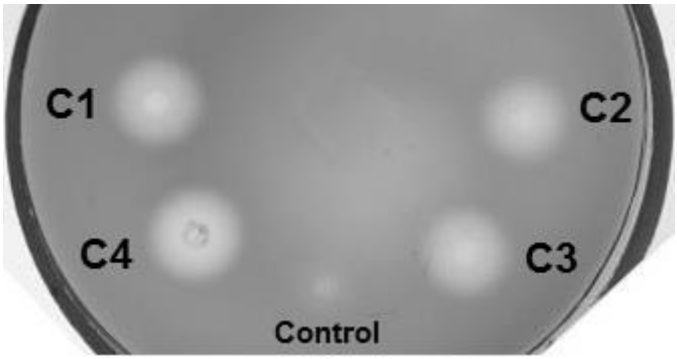

Figure 5 The emergence of the clear halos around the wells with laccase biosynthesized by the C1-C4 microbial consortia

Production of exopolysaccaharides by the C1-C4 consortia was qualitatively assayed by inoculating the sterile discs of filter paper and their presence was recognized after the formation of colonies mucoid in appearance around the disks as a consequence of exopolysaccharides associated to microbial production of the consortia. All the consortia, exceptin the C2 consortium, produced similar quantities of mucoid exopolysaccaharides around the paper disks (Figure 6).

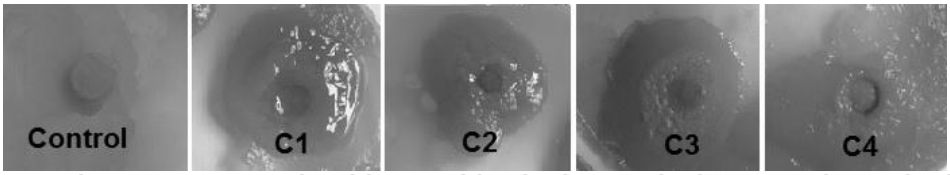

Figure 6 The C1-C4 consortia with mucoid colonies producing exopolysaccharides

Exometabolites, like exopolysaccharides from broth culture media were quantitative assayed from the C1-C4 consortia. The precipitate of exopolysaccharides was collected and the total amount produced from each consortium was determined through the dry weight of the polymers. The C4 consortium produced 87mgL^{-1} exopolysaccharides, the C3 and C1 consortia produced 76mgL^{-1} and 74mgL^{-1}. All results statistical assayed for the C4, C3, C1 microbial consortia were significantly higher as compared with the C2 consortium with a production of 58mgL^{-1} exopolysaccharides (Figure 7).

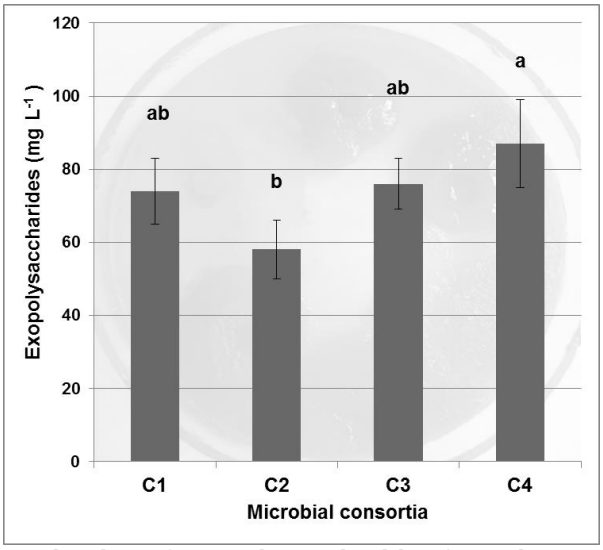

Figure 7 The production of exopolysaccharides from the C1-C4 consortia.
The values followed by the same letter are not significantly different for p <0.05 (Student test)

Aspergillus ochraceus as a pathogenic fungus is adapted to varied ecological niches. Traditionally, a soil fungus can contaminate agricultural products at different stages and, as opportunistic pathogen, is encountered as storage mould. The mycotoxins (aflatoxins and ochratoxin A) have been identified in foods contaminated and the aflatoxins (B1, B2, G1, G2) are the most toxic and carcinogenic mycotoxins naturally occurring.

The exometabolites from the C1-C4 consortia diffused enough in the culture medium (after 7 days of microbial growth in darkness and at 25^{o}C) to inhibit the growth of fungal mycelia on the surface of the agar plate. The antimicrobial properties of exometabolites were present and relatively equal for all the consortia (C1-C4) against *Aspergillus ochraceus* (Figure 8).

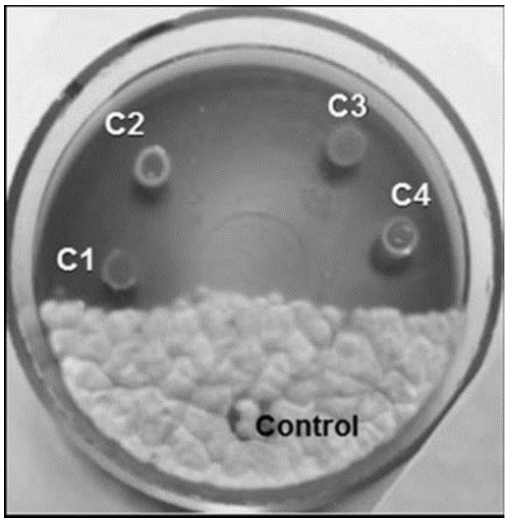

Figure 8 The antimicrobial properties of exometabolites from the C1-C4 consortia against Aspergillus ochraceus

The microbial exometabolites can induce changes in root enzymes activity or production of the root metabolites (phenazines which enhance the total efflux of amino acids in plant) as shown for exometabolites from *Chryseobacterium* or *Azospirillum* which can influences the exudation of the flavonoids, informations important in design of the mix of inoculants (Heulin et al., 1987; Shaw et al., 2006; Dardanelli et al., 2010).

In the present experiments, the roots of cucumber plantlets were measured (length and the branching) to estimate their evolution after the treatment with sterile filtrate from culture medium of the C1-C4 consortia. The C4 sterile filtrate influenced the growth of roots length to the 97mm in comparing with the control at which the length was only to 43mm. The sterile filtrates of the C2, C1 and C3 consortia influenced the growth of roots length to values of 88mm, 69mm and 54mm, respectively. No one of sterile filtrates influenced the branching of the roots in analyzed period (Figure 9).

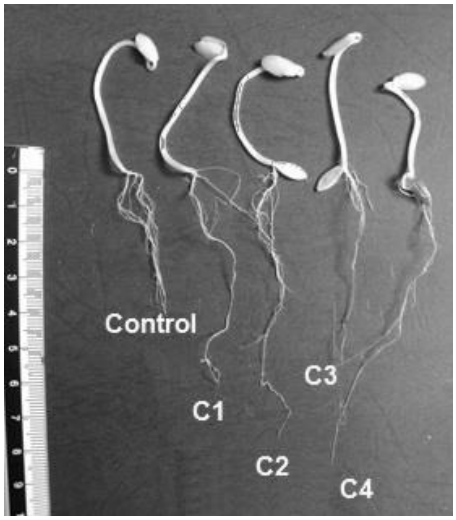

Figure 9 The length and the branching evolution of the cucumber plantlets roots under treatment with sterile filtrates from culture medium of the C1-C4 consortia.

Our results are supported by data from literature reporting that the microbial exometabolites can influence the elicitation of isoflavone, alkaloids, terpenoids, benzoxazinoids, starch content, phenolic compounds or total soluble sugars accumulation in plant roots, sugars known to be involved in low-temperature tolerance (Ait Barka et al., 2006; Fernandez et al., 2012; Chamam et al., 2013).

CONCLUSIONS

The accumulations of exometabolites from secondary metabolism of *Fusarium culmorum* were more intense when 4 isolates were inoculated in broth culture media.

Exometabolites biosynthesized by the C4 consortium had a diversity and complexity of compounds in the culture media nearest to control. In the C3 and C2 consortia were seen slow trends to complexing, due to their inability in the biosynthesis of intermediate compounds or an incompatibility between microbial isolates.

In agar-wells, the quality of cellulase activity was influenced most intensely by the C4 consortium exometabolites, followed by C1 and the lowest influence was determinated by the C2 consortium.

The activity of cellulase was estimated under the influence of the exometabolites from the C4 consortium to 269mgL^{-1} D-glucose and to 148mgL^{-1} D-glucose from the C2 consortium.

The influence of the exometabolites from the consortia on the laccase activity was the highest of the C4 consortium (21mm) followed by the C1 consortium. For all, the clear halos around the wells indicate positive and different influence on laccase enzymes.

All consortia produced similar quantities of mucoid exopolysaccaharides around the paper disks, excepting C2.

Exometabolites, like exopolysaccharides from broth culture were quantitatively estimated in C4 consortium to 87mgL^{-1} exopolysaccharides and 58mgL^{-1} exopolysaccharides at the C2 consortium

Extracelullar compounds from the C1-C4 consortia had an individual and cumulative inhibitory effect on the pathogenic soil fungus *Aspergillus ochraceus*.

Microbial metabolites from all the C1-C4 consortia stimulated the growth of the roots from cucumber plantlets, as compared with control.

ACKNOWLEDGEMENTS

This paper was published under the frame of PNCDI II Research Programme, Project PNII C68/2012 (acronym FRIENDLYHORT) and PN 16 07 01 11/2016.

BIBLIOGRAPHY

1. **Ait Barka, E., Nowak, J., Clement, C.,** 2006 - *Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN*, Appl. Environ. Microbiol., no.72, p. 7246–7252

2. **Carmichael, W.W.,** 1992 - *Cyanobacteria secondary metabolites – the cyanotoxins,* J. Appl. Bacteriol., no.72, p. 445–459.

3. **Chamam, A., Sanguin H., Bellvert F., Meiffren G., Comte G., Wisniewski-Dyé F.,** 2013 - *Plant secondary metabolite profiling evidences strain-dependent effect in the Azospirillum-Oryza sativa association*, Phytochem., no.87, p.65–77.

4. **Dardanelli, M. S., Manyani, H., Gonzalez-Barroso, S., Rodriguez-Carvajal, M. A., Gil-Serrano A. M., Espuny M. R.,** 2010 - *Effect of the presence of the plant growth promoting rhizobacterium (PGPR) Chryseobacterium balustinum Aur9 and salt stress in the pattern of flavonoids exuded by soybean roots*, Plant. Soil, no.328, p.483–493.

5. **DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F.**, 1956 - *Colorimetric Method for Determination of Sugars and Related Substances*, *Anal.Chem.*,vol.28, no.3, p. 350–356.

6. **Fernandez, O., Theocharis A., Bordiec, S., Feil, R., Jacquens L., Clement, C.,** 2012 - *Burkholderia phytofirmans PsJN acclimates grapevine to cold by modulating carbohydrate metabolism,* Mol. Plant Microbe. Interact., no.25, p.496–504.

7. **Grata, K., and Nabrdalik, M.,** 2014 - *Effect of Bacillus subtilis exometabolites on the growth rate of Rhizoctonia solani*, Proceedings of ECOpole, vol.8, no.1, p.37-41.

8. **Heulin, T., Guckert, A., Balandreau, J.**, 1987 - *Stimulation of root exudation of rice seedlings by Azospirillum strains – carbon budget under gnotobiotic conditions*, Biol. Fertil. Soil no.4, p.9–14.

9. **Jaki, B., Orjala, J., Heilmann, J., Linden, A., Vogler, B., Sticher, O.,** 2000 - *Novel extracellular diterpenoids with biological activity from the cyanobacterium Nostoc commune,* J. Nat. Prod., no.63, p.339–343.

10. **Keneni, A. and Gupta, V.K.,** 2011 - *Characterization of a Red bacterium strain isolated from root nodule of a Faba Bean (Vicia faba. L.) for Growth and Pigment production,*J. J.Adv.Lab.Res.Biol., vol.2, no3, p.138-146.

11. **Mahendran, S., Vijayabaskar, P., Saravanan, S., Anandapandian, K.T.K., Shankar, T.,** 2013 - *Structural characterization and biological activity of exopolysaccharide from Lysinibacillus fusiformis*, Afr. J. Microbiol.Res., vol. 7, no. 38, p. 4666-4676.

12. **Meenupriya, J., Thangaraj, M.,** 2011 - *Analytical characterization and structure elucidation of metabolites from Aspergillus ochraceus MP2 fungi*, Asian Pac J Trop Biomed, vol.1, no 5, p.376–80.

13. **Nagaraju, M., Narasimha, G., Rangaswamy, V.,** 2009 - *Impact of sugar industry effluents on soil cellulase activity*, Int.Biodeter. Biodegr. no.63, p.1088-1092.

14. **Rainer-B, V., Furkert, H. F.,** 2006 - *Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth*, Microbiological Research, vol.161, no2, p.180–186.

15. **Read, R.R., Costerton, J.W.,** 1987 - *Purification and characterization of adhesive exopolysaccharides from Pseudomonas putida and Pseudomonas fluorescens*, Can J Microbiol no.33, p.1080–1090.

16. **Schmidt, M. W.** 2011 - *Persistence of soil organic matter as an ecosystem property*, Nature, no.478,p. 49–56.

17. **Shaw, L. J., Morris, P., Hooker, J. E.,** 2006 - *Perception and modification of plant flavonoid signals by rhizosphere microorganisms*, Environ. Microbiol., no.8, p.1867–1880.

18. **Smith, G.D. and Doan, N.T.,** 1999 - *Cyanobacterial metabolites with bioactivity against photosynthesis in cyanobacteria, algae and higher plants*,J.Appl.Phycol., no.11, p. 337–344.

19. **Swift, R.S.,** 1996 - *Organic matter characterization (In D.L. Sparks et al. (eds) Methods of soil analysis. Part 3. Chemical methods*. Soil Sci. Soc. Am. Book Series: 5. Soil Sci. Soc. Am. Madison, WI, *chap 35, p.1018-1020.*

20. **Tekere et al**., 2001- *Growth, dye degradation and ligninolytic activity studies on Zimbabwean white rot fungi,* Enzyme and Microbial Technology., no 28, p. 420–426.

21. **Thurston, C. F.,** 1994 - *The structure and function of fungal laccases,* Microbiology, no.140, p.19-26.

22. **Timokhina, T.Kh.,** 2009 - *Daily dynamics of particular pathogenicity factors and persistence of Staphylococcus aureus under the influence of exametabolites of associative microflora*, Zh. Mikrobiol., no 4, p.15-18.

23. **Wallace, G.I., and Neave, S.I.,** 1927 - *The nitrite tests applied to bacterial cultures,* Jour. Bact., no.14, p.377-384.

24. **Xu, F.,**1999 - *Laccase*, In Flickinger, M. C. and Drew, S. W. (eds.), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, Bioseparation,New York, p.1545-1554.