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ANTIMICROBIAL ACTIVITY OF NEWLY ISOLATED *Bacillus* SP. AND *Pseudomonas* SP. STRAINS AND THEIR POTENTIAL USE AS BIOCONTROL AGENTS

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Abstract

Most of the plant diseases are caused by microorganisms. Among these, most often mentioned in the literature are bacteria and fungi. Diseases caused by phytopathogens like Erwinia carotovora and Xanthomonas campestris lead to lower production and quality, causing significant economic losses. In order to prevent the diseases can be used microbial antagonists which, besides protection capability, can stimulate plant growth by degrading the substrate and releasing of certain compounds needed for growth.
After a preliminary screening, from a total of 25 microorganisms isolated from plant materials, best antimicrobial activities were registered with bacteria B1 and Bm belonging to the genera Pseudomonas sp. respectively Bacillus sp.. Following biochemical tests in conjunction with microscopy studies and MALDI-TOF MS, selected bacteria were identified as Pseudomonas putida, respectively Bacillus mycoides. Their antimicrobial activity was comparable to that of microorganisms belonging to the same genera, from the collection of the National Institute for Chemical Pharmaceutical Research and Development-ICCF. When they were grown on agar media with different compositions, significant differences regarding antimicrobial activity have not been observed. However, substantial differences were recorded in terms of antagonistic ability between Pseudomonas putida and Bacillus mycoides, the last one making the biggest area of inhibition against both phytopathoges X. campestris ICCF 274 (40 mm) and E. carotovora ICCF 138 (20 mm).
These results suggest the possibility of using newly isolated antagonists to prevent diseases caused by Erwinia carotovora and Xanthomonas campestris.

Key words: *antagonists, antimicrobial activity, biocontrol, phytopathogens.*

INTRODUCTION

Diseases caused by phytopathogens from the genera *Erwinia* and *Xanthomonas* affects many species of plants, causing significant economic losses. Bacteria from *Xanthomonas* genus, for example, can affect over 300 species of plants. Moreover, even if some organisms may look less affected, the bacteria can locate at the seeds level and can be passed on to other susceptible plants.
Bacteria of the genus *Erwinia* have a rich enzymatic equipment of amylases, cellulase, xylanases, polygalacturanases and pectin-methyl esterases that cause maceration of the tissues affected (Opara and Asuquo, 2016). Species of *Erwinia caratovora* attack and infects a variety of vegetables and plants including carrots, potatoes, cucumbers, onions, tomatoes, lettuce and ornamental plants like iris (Wood, 1998). Some strains of the genus, like *Erwnia carotovora* are bacteriocine producers. These substances, released in the medium, have antibacterial action and enable the bacteria to compete with other microbial species that occupy the niche. These, together, are attributes that make those bacteria so adaptable and persistent in the environment.
Although researches in the field of chemical synthesis had resulted in discovering of new pesticides it is proved that the phytopathogens become resistant (McManus et al., 2002) to

them and harder to control. In addition, arises increasingly stronger, the problem of the environmental and crops pollution affecting people's health (Horrigan et al., 2002).

In this context, we need to find new solutions for combating the phytopathogens using beneficial microorganisms that can prevent plant illnesses through their own mechanisms such as: competition for occupying the niche, production of secondary metabolites (substances with bactericidal and fungicidal effect, enzymes), direct parasitism of the phytopathogens (hyperparasitism) etc.

Among the microorganisms with recognized activity in biocontrol are bacteria from the genera *Bacillus* and *Pseudomonas*. The most important mechanism of their action is to produce substances with bactericidal and fungicidal effect. *Bacillus* sp. produces and releases during sporulation or in stationary phase of growth, a number of lipopeptides with antibiotic role (Kalai-Grami et al., 2016; Ongena and Jacques, 2008) like iturins, fengycin, surfactin. These bacteria also produce lytic enzymes like cellulases, glucanases, proteases, chitinases, or volatile compounds like hydrogen cyanide. Many species of the genus have been utilized in agriculture and are considered as safe microbes (Fravel, 2005).

Besides the bioprotection role it seems that these microorganisms can stimulate plant growth by producing phytohormones and by increase the availability of mineral compounds with low solubility.

Moreover the secondary metabolites, like biosurfactants can have various industrial applications (Banat et al., 2010; Pathak and Keharia, 2014).

Many studies have demonstrate that *Pseudomonas* strains are in a close relationship with the suppressive soils (Raaijmakers et al., 1997). Some strains that produce the antimicrobial metabolite 2,4-diacetylphloroglucinol (Shanahan et al., 1992) were isolated from the soil, roots of various plants and even from different tissues of plant (Turner et al., 2013). Other antimicrobial substances produced by strains of *Pseudomonas* are phenazines, hydrogen cyanide, pyrrolnitrin (Nandi et al., 2015).

In addition, many strains of *Bacillus* sp. and *Pseudomonas* sp. seem to have the capacity to induce systemic resistance in plants (Pieterse et al., 2001; Bargabus et al., 2002) by chemical elicitors like salicylic acid, siderophore, 2,3-butanediol, lipopolysaccharides.

The main objective of our research was to isolate and identify microorganisms capable of controlling the phytopathogens *Erwnia carotovora* and *Xanthomonas campestris*. Also antagonistic activity was examined in relation to the optimal growth medium for phytopathogens and respectively antagonists, and with inoculation moment.

MATERIALS AND METHODS

The microorganisms studied for antagonistic activity have been isolated from various plant materials (hay, beans).

The plant material from which microbial strains were isolated was collected from different areas of Romania (Vâlcea, Ilfov) and kept in a refrigerator in sterile containers until processing. Approximately 1g of plant material was inoculated onto liquid growth medium, specific to each type of microorganism as follows: IPS medium broth for bacteria, YMPG for yeasts and fungi strains. The IPS broth containing (% g/v) glucose 1.00%, corn extract 1.50%, KH_2PO_4 1.00%, NaCl 1.00%, $MgSO_4$ 0.05%, and YMPG containing (% g/v) yeast extract 3.00%, malt extract 3.00%, peptone 5.00%, glucose 10.00%, were sterilized at 115°C, for 20 minutes. The plant material was inoculated into these media in 500 ml shake flasks containing 100 ml medium and left to develop, in an incubator at 30±1°C and 220 rpm for 24 h. Serial dilution did follow and pour plated onto NA (nutrient agar), YMPG (Yeast Malt Peptone Glucose) and PDA (Potato Dextrose Agar). In order to obtain single colonies, streak plating technique was used.

A number of 25 microorganisms isolated in pure culture were grown and maintained on their specific media as follows: the bacteria (20 strains) on nutritive agar, the yeasts (3 strains) on YMPG and the fungus (2 strains) on PDA media.

For strains identification, microscopy studies in conjunction with biochemical tests and MALDI-TOF MS were done.

Morphological characterization was performed by microscopic examination using a Novex

microscope. Biochemical assays were done according to literature on diverse media for testing the capacity of the microbes to utilize or produce various compounds.
Microflex LT (MALDI-TOF mass-spectrometer manufactured by Bruker Daltonics Inc. - Billerica, MA, USA), equipped with nitrogen laser, was used to acquire the mass-spectra from fresh colonies. The equipment identify a microorganism by analyzing the expression of the most abundant ribosomal proteins from the acquired mass spectra and by comparing the specificity of his mass spectrum with a large number of reference patterns from its database (Tomulescu et al., 2015).
In order to establish their antagonistic capacity, newly isolated microorganisms were grown on agar medium along with the phytopathogenic strains *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274 by dual cultures method. During experiments were tested, in the same conditions, two other strains *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391. *Erwinia carotovora* ICCF 138, *Xanthomonas campestris* ICCF 274, *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391 belong from Culture Collection of Industrial Importance Microorganisms (CMII) of the National Institute for Chemical-Pharmaceutical Research and Development, Bucharest.
To ensure optimal conditions for development, the antagonists and the phyopathogens were grown on various types of agarized medium: NA, YMPG, PDA, M44. Medium M44 (containing (%g/v): yeast extract 1.00%, bacteriological peptone 1.00%, glycerol 5.00%, agar 2.00%) was the most appropriate for the majority of the strains and YMPG was the best for *X. campestris*. On these media were performed all subsequent experiments. For our research were used bacterial strains after 48 hours of development on their specific agaric medium. The broth medium for bacterial development has the same composition as mentioned above. After 24 hours at 30^{o}C and 220 rpm the culture was appropriate for pour plate inoculation. For carrying out the method of dual cultures, one ml of inoculum from the broth culture of phytopathogens was added by pipette to the center of the Petri dish, over the agar medium (cooled, but still molten) and rotated gently, to ensure that the culture and medium are thoroughly mixed. After solidification, 100µL of inoculum from the broth culture of antagonists was put in the middle of the same plate and allowed to be adsorbed in medium. After 48-72 hours of incubation at 30±1^{o}C the inhibition zones were checked.

RESULTS AND DISCUSSIONS

From a number of 25 microorganisms, were isolated in pure culture 20 strains of bacteria, 3 of yeasts and 2 of fungus. Two bacterial strains, who have registered the best antimicrobial action against *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274 when they were cultured together by dual cultures method, were further investigated for identification.
These strains noted B1 and Bm, were identified as *Pseudomonas putida* and respectively *Bacillus mycoides* through microscopy studies in conjunction with biochemical tests and MALDI-TOF MS (see figures and tables below).

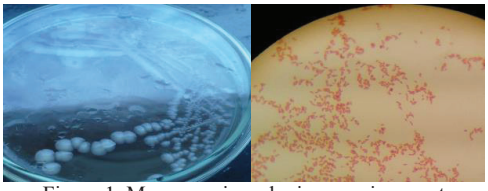

Figure 1. Macroscopic and microscopic aspect of B1 strain

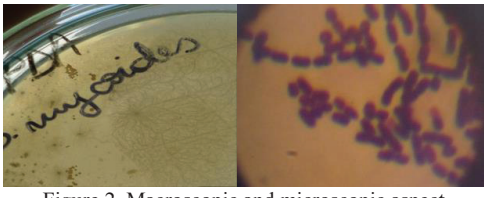

Figure 2. Macroscopic and microscopic aspect of Bm strain

Regarding microbiological and biochemical characteristics (shown in Table 1), the results were similar to those reported for *Pseudomonas putida* and *Bacillus mycoides* in ABIS online Encyclopedia and in the works of other researchers (Borah et al., 2002; Egamberdiyeva, 2005).

Table 1. Microbiological and biochemical characteristics for B1 and Bm strains

MICROBIOLOGICAL CHARACTERISTICS	RESULTS	
	B1	Bm
Gram staining	-ve	+ve
Rod shaped	+ve	+ve
Spore forming	-ve	+ve
Motility	+ve	-ve
BIOCHEMICAL TESTS		
Citrate utilization	+ve	-ve
H_2S production	-ve	-ve
Gas production	-ve	-ve
Gelatin utilization	-ve	+ve
Catalase production	+ve	+ve
Urea hydrolysis	+ve	-ve
Starch hydrolysis	+ve	+ve
Glucose fermentation	+ve	+ve
Glycerol utilization	+ve	+ve

Note: +ve indicates positive and -ve indicates negative results

By MALDI-TOF MS analysis the strain noted as B1 was identified as *Pseudomonas putida* (data not shown).

For growth and development of the microorganisms tested, were investigated some culture media: NA, YMPG, PDA, M44. From these, medium M44 was the most appropriate for the majority of the strains involved in this study and YMPG was the best for *X. campestris*.

As it shows in figures below, Bm presented the best action against *X. campestris* ICCF 274 (a 40 mm inhibition area) and *E. carotovora* ICCF 138 (a 20 mm inhibition area). It was followed, in descending order, B1 (20 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138), *B. subtilis* ICCF 84 (20 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138) and *P. putida* ICCF 391 (16 mm against *X. campestris* ICCF 274 and 8 mm against *E. carotovora* ICCF 138).

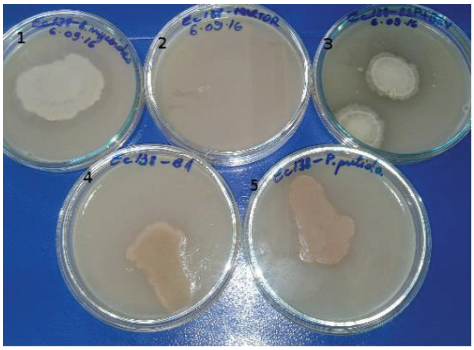

Figure 3. Dual cultures method - front view: 1) Bm, 2) Control, 3) *B. subtilis*, 4) B1, 5) *P. putida* -against *Erwinia carotovora* ICCF 138

Figure 4. Dual cultures method - reverse view: 1) Bm, 2) Control, 3) *B. subtilis*, 4) B1, 5) *P. putida* - against *Erwinia carotovora* ICCF 138

Figure 5. Dual cultures method - front view: 1) B1, 2) Control, 3) *P. putida*, 4) *B. subtilis*, 5) Bm - against *Xanthomonas campestris* ICCF 274

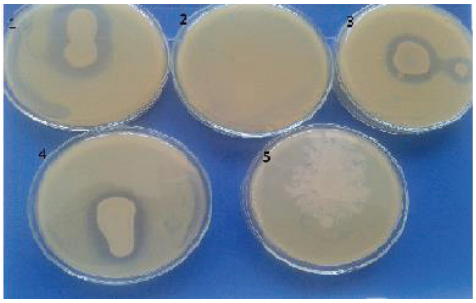

Figure 6. Dual cultures method - reverse view: 1) B1, 2) Control, 3) *P. putida*, 4) *B. subtilis*, 5) Bm - against *Xanthomonas campestris* ICCF 274

CONCLUSIONS

Microscopy studies in conjunction with biochemical tests and MALDI-TOF MS led to the conclusion that the strains noted B1 and Bm are *Pseudomonas putida* and respectively *Bacillus mycoides*.

Optimal conditions for development of the phyopathogens were obtained with *X. campestris* on medium YMPG and *E. carotovora* on M44. Medium M44 was best for most bacteria used in this research.

Among the microorganisms isolated, Bm and B1 recorded the best antimicrobial activity against phytopathogens *Erwinia carotovora* ICCF 138 and *Xanthomonas campestris* ICCF 274. It was followed *Bacillus subtilis* ICCF 84 and *Pseudomonas putida* ICCF 391. These strains will be used in further studies for obtaining microbial origin products for plant protection.

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