

ANTIBACTERIAL ACTIVITY OF SOME *BACILLUS* STRAINS IN DEPENDENCE ON THE CULTURE MEDIUM

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Abstract: *Biological control of plant pathogenic bacteria, which includes the use of antagonistic microorganisms, is an alternative method to the application of chemicals. Bacteria belonging to the Bacillus spp. have demonstrated antimicrobial activity against plant pathogenic microorganisms due to the produced secondary metabolites. Thus, it is of particular interest to study and to identify new microorganisms, in particular Bacillus spp., possessing pronounced antimicrobial activity against phytopathogens that can be used as biotechnological alternatives for agriculture, with the production of biologically active preparations based on the microorganism strains or metabolites. From 12 strains of Bacillus spp. three strains, grown on liquid media, were selected and characterized by high antimicrobial activity against pathogenic plant bacteria, both bacterial suspension and cell-free supernatant showed. The selected strains can be proposed as potential bioactive antagonists against phytopathogens, which can be used to obtain bioactive preparations useful for agriculture in the field of plant protection and cell-free supernatant may have prospects for use in the medical field to obtain bioactive preparations due to the ability to produce various secondary metabolites such as antibiotics.*

Keywords: *antibacterial activity, Bacillus, culture medium.*

INTRODUCTION

Fungal and bacterial phytopathogens are responsible for significant losses or decrease in the quality and safety of agricultural products by damaging crops. Among the plant pathogenic bacteria causing numerous crop plant diseases are those of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Xanthomonas* and so on and among pathogenic fungi the most common are from *Alternaria*, *Botrytis*, *Fusarium*, *Aspergillus* and *Penicillium* [1-3].

Chemical pesticides are mainly used to control diseases caused by plant pathogens. Besides the fact, that chemical pesticides have a number of side effects on both the environment and human health, pathogens develop resistance to them [1, 2]. Biological control of plant pathogenic bacteria, which includes the use of antagonistic microorganisms, is an alternative method to the application of chemicals, which can be achieved by destroying pathogens, and is a safe method

for an effective and integrated disease management system that is environmentally friendly and non-toxic to human health [1, 4].

Most species of the genus *Bacillus* are considered safe microorganisms and possess remarkable abilities to synthesize numerous substances that have been successfully used in agriculture and for industrial purposes [3, 5]. Bacteria of the genus *Bacillus* have demonstrated antimicrobial activity against phytopathogenic microorganisms due to the secondary metabolites produced by them through a variety of mechanisms of action, such as competition, induction of systemic resistance and antibiotic production [6-10]. *Bacillus* spp. strains are already adapted to the environment and can be applied for a biological control method, due to the large number of advantages such as the fact that most of them are isolated from the environment, grow rapidly in liquid media, form extremely resistant spores with high thermal tolerance, which allows them to be considered prioritizing candidate agents for the biological control of plant diseases [1, 3, 11].

All of the above points to the necessity of studying and identification of new microorganisms, especially *Bacillus* spp. possessing pronounced antimicrobial activity on phytopathogens that can be used as alternative biotechnological solutions for agriculture, with the production of biologically active antimicrobial preparations based on the strains of microorganisms or their metabolites.

Thus, the aim of this research focused on the identification of strains of *Bacillus* spp. possessing pronounced antibacterial activity against phytopathogenic bacteria, depending on the culture medium.

MATERIALS AND METHODS

The study included 12 bacterial strains from *Bacillus* spp.: *Bacillus subtilis* CNMN-BB-01, *Bacillus subtilis* CNMN-BB-06, *Bacillus subtilis* CNMN-BB-08, *Bacillus subtilis* CNMN-BB-09, *Bacillus subtilis* CNMN-BB-10, *Bacillus velezensis* CNMN-BB-12, *Bacillus velezensis* CNMN-BB-13, *Bacillus velezensis* CNMN-BB-14, *Bacillus velezensis* CNMN-BB-15, *Bacillus velezensis* CNMN-BB-16, *Bacillus velezensis* CNMN-BB-17 and *Bacillus velezensis* CNMN-BB-18, stored and deposited in the National Collection of Non-Pathogenic Microorganisms (NCNM) at the Institute of Microbiology and Biotechnology of Technical University of Moldova.

As growing media, served Nutrient Agar (NA) with the following composition (g/L): Meat Extract – 1.0; Yeast extract – 2.0; Peptone – 5.0; Sodium chloride – 5.0; Agar – 15.0 and Mueller Hinton Agar (MHA) with the following composition (g/L): Meat infusion – 2.0; Casein hydrolysate – 17.5; Starch – 1.5; Agar-agar – 13.0. The bacterial cultures were grown on the whole Petri dish surface, concentration was adjusted to 0.5 McFarland standard. Cultivation was carried out at a temperature of 35±1°C, 48 hours.

The bacterial cultures were also grown on liquid medium Nutrient Broth (NB) with the following composition (g/L): Peptic digest of animal tissue – 5.0; Sodium chloride – 5.0; Beef extract – 1.5; Yeast extract – 1.5. Experiments were carried out in 250 mL Erlenmeyer flasks with a working volume of 50 mL. The inoculum concentration was adjusted to 0.5 McFarland standard. Optimal cultivation conditions include a temperature of $30\pm 1^{\circ}\text{C}$, continuous illumination. Cultures were agitated continuously at 200 rpm for 48 hours.

At the end of the cultivation cycle, the bacterial biomass was separated from the culture liquid by centrifugation to obtain the cell-free supernatant (CFS), then the dry mass of the supernatant was determined, which was on average 10 mg/mL.

The antimicrobial activity was tested against *Agrobacterium tumefaciens* CNMN-AB-01, *Corynebacterium michiganense* CNMN-CrB-01, *Erwinia carotovora* CNMN-BE-01 and *Xanthomonas campestris* CNMN-BX-01 – bacteria that cause various crop plant diseases. The assessment of antibacterial activity was determined by disc diffusion method and well plate method [12]. For disc diffusion method, antibacterial activity was defined by using agar blocks. For well diffusion method, a quantity of 100 μL (bacterial suspension (BS) or cell-free supernatant (CFS)) obtained after cultivation in liquid media was dosed into wells of 8.0 mm diameter in Petri plates with medium already containing seeded test-bacterial strains. Plates were left at room temperature for 1 hour to allow diffusion, then incubated at $+35\pm 1^{\circ}\text{C}$. Inhibition zone diameters were measured after 24 hours of incubation.

Statistical analysis of data was performed using Microsoft Excel 2016, there were three replications for each test and the biocidal assessment was performed twice. The statistical significance threshold was $p\leq 0.05$.

RESULTS AND DISCUSSIONS

The results obtained on the antibacterial activity of *Bacillus* spp. strains, cultivated on agarized media, demonstrated that out of five investigated strains of *B. subtilis*, only *B. subtilis* CNMN-BB-10 showed activity against the four strains of phytopathogenic bacteria, regardless of the cultivation medium NA or MHA, the inhibition zones varying between 14.7–27.0 mm and 14.3–25.7 mm, respectively (Table 1).

In contrast, the *B. velezensis* strains all had antibacterial activity against phytopathogens, the zones of inhibition being dependent on the strain or medium tested. The most sensitive was the *A. tumefaciens* CNMN-AB-01 strain, with zones of inhibition of growth and development ranging from 19.7–27.3 mm, depending on the growth medium used.

Table 1. Antibacterial activity of some *Bacillus* strains in dependence on the culture medium, by disc diffusion method, zone of inhibition, mm

	<i>A. tumefaciens</i> CNMN-AB-01		<i>C. michiganense</i> CNMN-CrB-01		<i>E. carotovora</i> CNMN-BE-01		<i>X. campestris</i> CNMN-BX-01	
	NA	MHA	NA	MHA	NA	MHA	NA	MHA
<i>B. subtilis</i> CNMN-BB-01	Ni	Ni	Ni	Ni	Ni	10.7±0.7	Ni	10.3±0.7
<i>B. subtilis</i> CNMN-BB-06	Ni	Ni	Ni	Ni	Ni	Ni	Ni	11.7±1.7
<i>B. subtilis</i> CNMN-BB-08	Ni	Ni	Ni	Ni	Ni	Ni	Ni	Ni
<i>B. subtilis</i> CNMN-BB-09	Ni	Ni	Ni	Ni	Ni	Ni	Ni	12.0±1.1
<i>B. subtilis</i> CNMN-BB-10	23.3±1.3	24.0±1.1	27.0±1.1	25.7±1.7	17.0±1.1	14.7±0.7	14.7±0.7	14.3±0.7
<i>B. velezensis</i> CNMN-BB-12	21.7±0.7	27.3±0.7	15.0±1.1	20.7±2.6	16.3±0.7	23.3±1.3	19.3±1.3	23.7±0.7
<i>B. velezensis</i> CNMN-BB-13	24.0±1.1	20.3±1.7	27.7±0.7	25.7±0.7	16.3±0.7	16.3±0.7	15.7±0.7	15.7±0.7
<i>B. velezensis</i> CNMN-BB-14	20.7±0.7	24.3±0.7	22.0±1.1	24.7±0.7	14.7±0.7	14.0±1.1	14.3±0.7	14.3±0.7
<i>B. velezensis</i> CNMN-BB-15	19.7±0.7	21.3±0.7	12.3±0.7	12.7±0.7	19.7±2.4	22.3±0.7	17.0±1.1	23.7±0.7
<i>B. velezensis</i> CNMN-BB-16	26.3±0.7	27.0±1.1	14.3±0.7	13.3±0.7	22.3±0.7	24.7±0.7	22.0±1.1	23.3±0.7
<i>B. velezensis</i> CNMN-BB-17	21.0±1.1	25.3±0.7	15.3±0.7	13.7±0.7	18.7±1.7	22.0±2.0	18.7±1.3	21.7±1.7
<i>B. velezensis</i> CNMN-BB-18	20.3±0.7	25.0±2.0	13.3±0.7	13.7±0.7	22.3±0.7	23.7±0.7	18.3±0.7	21.7±0.7

Note: NA – Nutrient Agar, MHA – Mueller Hinton Agar, Ni – no inhibition, $p \leq 0.05$.

The use of disc diffusion method is a rapid method, useful both for screening of strains that possess antimicrobial activity and for obtaining of biologically active preparations as a result of the cultivation of microorganisms of biotechnological interest for various use. It is more efficient to grow them in a liquid medium, which offers wider possibilities of manipulation and obtaining biopreparations from microbial biomass and the resulting culture liquid due to the synthesis of different secondary metabolites with various actions.

Thus, as a result of the screening of *Bacillus* strains, were selected three most active strains *B. subtilis* CNMN-BB-10, *B. velezensis* CNMN-BB-12 and *B. velezensis* CNMN-BB-16, cultured on liquid Nutrient Broth medium. The antibacterial activity of both the bacterial suspension and the cell-free supernatant, following centrifugation and separation from the microbial biomass was determined after 48 hours of cultivation. The results have demonstrated that the selected *Bacillus* spp., *B. subtilis* CNMN-BB-10, *B. velezensis* CNMN-BB-12 and *B. velezensis* CNMN-BB-16 strains, maintained their antibacterial activity against phytopathogens (Table 2).

Table 2. Antibacterial activity of some *Bacillus* strains cultivated on Nutrient Broth medium, by well plate method, zone of inhibition, mm

	<i>A. tumefaciens</i> CNMN-AB-01		<i>C. michiganense</i> CNMN-CrB-01		<i>E. carotovora</i> CNMN-BE-01		<i>X. campestris</i> CNMN-BX-01	
	BS	CFS	BS	CFS	BS	CFS	BS	CFS
<i>B. subtilis</i> CNMN-BB-10	24.4±0.7	22.1±0.9	25.0±1.1	22.5±1.6	13.8±0.7	11.7±0.4	14.7±0.7	11.5±0.6
<i>B. velezensis</i> CNMN-BB-12	25.4±0.7	23.7±0.7	14.7±0.7	12.7±0.7	20.0±1.0	13.0±0.4	20.8±0.5	12.8±0.7
<i>B. velezensis</i> CNMN-BB-16	25.0±0.5	19.9±0.9	25.3±1.4	24.3±1.1	15.7±0.4	13.9±0.5	19.7±1.3	Ni

Note: BS – Bacterial Suspension, CFS – Cell-free supernatant, Ni – no inhibition, $p \leq 0.05$.

Analysing the obtained data, both the BS and CFS show inhibitory action on the growth and development of phytopathogenic bacteria, with growth inhibition values ranging from 13.8 – 25.4 mm and 11.5 – 24.3 mm, respectively. The exception is only the CFS obtained from the cultivation of *B. velezensis* CNMN-BB-16, which lost its activity against *X. campestris* CNMN-BX-01.

As in the case of cultivation on agarized media, the *A. tumefaciens* CNMN-AB-01 strain proved to be the most sensitive strain, growth being inhibited over areas between 24.4 – 25.4 mm when using bacterial suspension and between 19.9 – 23.7 mm when using CFS.

Therefore, following the analysis of the obtained data, it was found that the exometabolites synthesized in the medium by *B. subtilis* CNMN-BB-10, *B. velezensis* CNMN-BB-12 and *B. velezensis* CNMN-BB-16 have antibacterial action on plant pathogenic bacteria, and the given strains can be proposed as potential bioactive antagonists against phytopathogens, which can be used to obtain bioactive preparations useful for agriculture in the field of plant protection.

The obtained results on the antimicrobial activity of the CFS obtained from the cultivation of *Bacillus* spp. bacteria may have prospects for use in the medical field to obtain bioactive preparations based on it. According to literature data, it is known that *Bacillus* spp. strains are capable of producing in the medium various secondary metabolites, diverse in function and structure, such as peptide antibiotics as: gramicidins, bacitracin and thyrocidins that possess pronounced antimicrobial activity [13-16].

CONCLUSIONS

Results obtained on the antibacterial activity of bacterial strains of *Bacillus* spp., cultivated on agarized media, showed that out of five *B. subtilis* strains investigated only *B. subtilis* CNMN-BB-10 showed activity against the four phytopathogenic bacterial strains, while those of *B. velezensis*, all seven had

activity, regardless of the culture medium Nutrient Agar or Mueller Hinton Agar, zones of inhibition ranging from 13.3-27.7 mm and 12.7-27.3 mm, respectively.

Cultivation of *B. subtilis* CNMN-BB-10, *B. velezensis* CNMN-BB-12 and *B. velezensis* CNMN-BB-16 strains on liquid Nutrient Broth medium demonstrated that both the bacterial suspension and the cell-free supernatant possessed quite high antibacterial activity on test cultures of phytopathogenic bacteria, in some cases the zones of inhibition being larger than those grown on agarized media.

The selected strains can be proposed as potential bioactive antagonists against phytopathogens, which can be used to obtain bioactive preparations useful for agriculture in the field of plant protection and cell-free supernatant may have prospects for use in the medical field to obtain bioactive preparations due to the ability to produce various secondary metabolites such as antibiotics.

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