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Antifungal activity of *Bacillus* and *Pseudomonas* strains cultivated in the presence of iron, zinc and copper salts

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Abstract. Over 70–80% of plant diseases are caused by pathogenic fungi that have adverse effects on both crop growth and yield, becoming an important bottleneck for the development of sustainable agriculture. Some of the most common plant-pathogenic fungi belong to the genera *Alternaria, Fusarium, Botrytis*, etc. which attack a wide variety of plants causing black spots, gray mold, soft rot, responsible for causing considerable crop loss even post-harvest. The identification and selection of bacterial strains with the potential to increase the synthesis of bioactive compounds by enhancing antifungal activity on phytopathogens was focused on the need to develop innovative and sustainable biotechnological solutions for agriculture and the data obtained are presented in this article.

Keywords: antifungal activity, bacteria, micronutrients.

Activitatea antifungică a tulpinilor de bacterii din g. *Bacillus* și g. *Pseudomonas* cultivate în prezența sărurilor de fier, zinc și cupru

Rezumat. Peste 70–80% din bolile plantelor sunt cauzate de ciupercile patogene ce au efecte adverse atât asupra creșterii culturilor cât și randamentului acestora, devenind un blocaj important pentru dezvoltarea unei agriculturi durabile. Cei mai răspândiți fungi fitopatogeni sunt din g. *Alternaria*, *Fusarium, Botrytis* ș.a care atacă o gamă largă de plante provocând pete negre, mucegaiul cenușiu, putregaiul moale generând pierderi semnificative chiar și post-recoltare. Identificarea și selectarea unor tulpini de bacterii, cu potențial de sporire a sintezei substanțelor bioactive prin intensificarea activității antifungice asupra fitopatogenilor, s-a axat pe necesitatea elaborării unor soluții biotehnologice inovative și durabile pentru agricultură, iar datele obținute sunt prezentate în acest articol.

Cuvinte-cheie: activitate antifungică, bacterii, microelemente.

1. Introduction

Plant diseases result in an estimated annual loss of 10–15% of the world's major crops, with direct economic losses of up to hundreds of billions of dollars [5, 18]. Of

these diseases, 70–80% are caused by pathogenic fungi that have adverse effects on crop growth and yield, becoming an important bottleneck for the development of sustainable agriculture [13, 15, 18].

Phytopathogenic fungi synthesize various toxins that are non-enzymatic compounds, harmful to plants by even very low concentrations, and can destroy the normal physiological functions of plants [18, 23, 26]. Thus, phytopathogenic toxins are mostly low molecular weight secondary metabolites that can produce specific symptoms such as wilting, growth inhibition, chlorosis, necrosis, and leaf staining [18, 33]. The mechanism of action of toxins is complex, first affected by the cell membrane, mitochondria, and chloroplasts of the host plant, destroying the plant or interfering with its metabolism [18, 20].

Alternaria alternata is one of the most devastating phytopathogenic fungi. This microorganism infects various plant species of agronomical interest, such as potatoes, apples, pears, tomatoes, strawberries, and blueberries, causing black spots (necrosis) and generating significant post-harvest losses [4, 11, 25]. *Alternaria* produces a wide array of metabolites with phytotoxic, cytotoxic, and antimicrobial properties [4, 7, 9]. Like many phytopathogenic fungi, *Alternaria* can produce host-selective toxins, an ability that has been strictly associated with fungal pathogenesis [4, 9, 29].

Another fungal necrotrophic pathogens is *Botrytis cinerea*, which also attacks a wide range of plants. *Botrytis cinerea* is commonly known as gray mold [22, 31]. *B. cinerea* has a high ability to infect a wide range of plant species, similar to *Sclerotinia sclerotiorum*, causing mold diseases in hundreds of plants [10, 22]. This pathogen has a prolific reproductive capacity, remarkable survival mechanisms, and multiple infection strategies, being included among the top 10 most economically devastating phytopathogens [8, 22].

Fusarium is considered one of the most severe plant disease that cause a wide range of plant infections of many crops and are responsible for significant yield losses. In addition, some *Fusarium* spp. can produce mycotoxins that contaminate, in particular, infected grains and pose a threat to human and animal health. *F. oxysporum*, *F. solani*, *F. fujikuroi* and *F. graminearum* are representative species known as plant-pathogenic *Fusarium* [3, 30]. *F. oxysporum* infect crops such as tomatoes, eggplants, peppers, cucumbers, watermelons, etc., initially leading to wilting, yellowing and then to necrosis and death of plant tissue [3]. While *F. solani* is associated with root rot of the infected host plant of vegetables, fruits, and ornamental plants [24].

Pesticides are used to control fungi that harm plants, but these chemicals can pose risks to the environment. Research on the use of microorganisms for biological control can lead to the development of novel approaches and effective strategies for the development of

sustainable agriculture and environmental protection. It is well-known that *Bacillus* and *Pseudomonas* strains have the ability to synthesize exometabolites, including siderophores that have antimicrobial activity and are widely used in the field of protection of plant growth [12, 27].

Modification of the composition of the medium, used for bacterial growth, to which salts of various micronutrients or macronutrients Fe, Zn, Cu, Cd have been added, leads to the production of synthesized exometabolites [6, 17] and can enhance their properties.

Zinc and iron are essential micronutrients necessary for plant metabolic pathways and involved in chlorophyll biosynthesis, photosynthesis, redox reactions, and other physiological activities [1, 14, 16, 19, 21, 32], but also copper, which is important for organic agriculture for plant protection [28]. Therefore, our research was initiated for the identification and selection of some bacterial strains of *Bacillus* and *Pseudomonas* cultivated in the presence of inorganic salts of iron, zinc and copper, with the potential to synthesize bioactive substances to stimulate biosynthetic processes such as antifungal activity in phytopathogens.

2. Materials and Methods

This study aimed to identify of 8 *Bacillus* strains (*B. subtilis var. mesentericus* CNMN-BB-02, *B. cereus var. fluorescens* CNMN-BB-07, *B. velezensis* CNMN-BB-12, *B. velezensis* CNMN-BB-13, *B. velezensis* CNMN-BB-14, *B. velezensis* CNMN-BB-15, *B. safensis* CNMN-BB-26, *B. velezensis* CNMN-BB-31) and 4 *Pseudomonas* strains (*P. fluorescens* CNMN-PsB-01, *P. fluorescens* CNMN-PsB-02, *P. fluorescens* CNMN-PsB-11, *P. fluorescens* CNMN-PsB-12) with high potential for application in agriculture and stored in the National Collection of Non-pathogenic Microorganisms within the Institute of Microbiology and Biotechnology of the Technical University of Moldova.

Antifungal activity of *Bacillus* and *Pseudomonas* strains was determined against phytopathogenic fungi also stored in the NCNM: *Alternaria alternata* CNMN-FF-09, *Botrytis cinerea* CNMN-FF-10, *Fusarium oxysporum* CNMN-FF-06 and *Fusarium solani* CNMN-FF-07, which have ability for causing severe plant diseases and yield losses.

Bacillus strains were cultivated on nutrient agar and *Pseudomonas* strains on King B medium, both supplemented with 10 mg/L of iron, zinc, and copper salts ($FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $ZnCl_2$, $CuSO_4 \cdot 7H_2O$) for 48 h at 36 ± 1°C [34].

Assessment of antifungal activity was determined using the disc diffusion method and the well plate method [2]. The test fungi were cultured on malt agar. After 3 to 5 days of incubation at 29 ± 1°C, the diameters of the growth inhibition zones were measured.

Statistical analysis of data was performed using Microsoft Excel 2010, antifungal activity was performed in triplicate. The statistical significance threshold was $p \leq 0.05$.

3. Results and Discussions

The result of screening for antifungal activity of tested bacteria indicated that from 8 *Bacillus* strains five showed inhibition zones against four phytopathogens, the inhibition zones varying between 17 and 34 mm, depending on the strain (Table 1).

Table 1. Antifungal activity of *Bacillus* and *Pseudomonas* bacterial strains against phytopathogenic fungi, zone of inhibition, mm (Note: $p \leq 0.05$, Ni – no inhibition).

Bacterial strains	**Phytopathogenic strains. zone of inhibition. mm**			
	Alternaria alternata CNMN-FF-09	*Botrytis cinerea* CNMN-FF-10	*Fusarium oxysporum* CNMN-FF-06	*Fusarium solani* CNMN-FF-07
Bacillus subtilis var. mesentericus CNMN-BB-02	15.3±1.3	Ni	Ni	19.3±1.3
Bacillus cereus var. fluorescens CNMN-BB-07	24.7±0.7	33.0±1.1	24.3±1.3	22.3±1.7
Bacillus velezensis CNMN-BB-12	22.7±0.7	27.7±1.3	23.7±1.3	Ni
Bacillus velezensis CNMN-BB-13	26.7±1.3	29.3±1.3	19.7±0.7	20.3±1.7
Bacillus velezensis CNMN-BB-14	24.0±2.3	34.0±1.1	23.0±2.3	19.0±1.1
Bacillus velezensis CNMN-BB-15	24.0±2.0	26.3±1.7	17.7±0.7	17.0±1.1
Bacillus safensis CNMN-BB-26	Ni	Ni	Ni	Ni
Bacillus velezensis CNMN-BB-31	26.3±2.4	33.3±1.7	25.0±1.1	23.0±1.1
Pseudomonas fluorescens CNMN-PsB-01	Ni	12.7±1.7	10.7±0.7	Ni
Pseudomonas fluorescens CNMN-PsB-02	Ni	12.3±1.7	12.3±0.7	Ni
Pseudomonas fluorescens CNMN-PsB-11	10.3±0.7	Ni	Ni	Ni
Pseudomonas fluorescens CNMN-PsB-12	13.0±1.1	Ni	Ni	Ni

Analysis of the sensitivity of phytopathogens revealed that *B. cinerea* CNMN-FF-10 showed the highest sensitivity on exometabolites produced by *Bacillus* strains, inhibition growth zones varied between 26.3 and 34.0 mm, followed by *A. alternata* CNMN-FF-09 with a diameter of growth inhibition up to 26.7 mm.

Pseudomonas strains showed lower antifungal activity, where *P. fluorescens* CNMN-PsB-01 and *P. fluorescens* CNMN-PsB-02 inhibited growth of only *B. cinerea* CNMN-FF-10 and *F. oxysporum* CNMN-FF-06 inhibiting its growth on an area with a diameter between 10.7 and 12.7 mm, while *P. fluorescens* CNMN-PsB-11 and *P. fluorescens* CNMN-PsB-12 strains were characterized by antifungal activity only against *A. alternata* CNMN-FF-09.

Thus, following the screening of the antifungal activity, the strains *B. cereus var. fluorescens* CNMN-BB-07, *B. velezensis* CNMN-BB-13, *B. velezensis* CNMN-BB-31, *P. fluorescens* CNMN-PsB-01, *P. fluorescens* CNMN-PsB-02 and *P. fluorescens* CNMN-PsB-12 were selected as candidates for stimulation of biosynthetic activity, being cultivated in the previously described media with the addition of 10 mg/L of inorganic iron, zinc and copper salts.

The chemical stimulators in the bacterial culture medium modulated the antifungal properties of the selected strains by altering the synthesis of bioactive antifungal substances, with effects depending on the strain and salt type.

Thus, among the three *Bacillus* strains it was observed that the biosynthetic activity of *B. cereus var. fluorescens* CNMN-BB-07 cultivated in the presence of 10 mg/L of salts mostly decreases and leads to a reduction or complete disappearance of antifungal activity, with the exception of iron and copper salts that stimulated antifungal activity against the *F. solani* CNMN-FF-07 strain by 19.40–37.31% compared to the control sample (Table 2).

The addition of iron, copper or zinc in the cultivation medium for *B. velezensis* CNMN-BB-31 increased sensitivity of *F. solani* CNMN-FF-07 by 15.94–44.93% compared to the control samples, with results that varied according to the compound applied, the most pronounced effect was observed with copper (II) sulfate.

B. velezensis CNMN-BB-13 cultivated in the presence of 10 mg/L iron, zinc and copper salts significantly increased antifungal activity against all phytopathogenic strains, except for *A. alternata* CNMN-FF-09, whose activity varied within the control limit. Thus, antifungal activity against the *B. cinerea* CNMN-FF-10 strain increased by up to 30.68%, against *F. oxysporum* CNMN-FF-06 by 98.31% and against *F. solani* CNMN-FF-07 by up to 62.30%, depending on the compound used.

Table 2. Antifungal activity of *Bacillus* strains cultivated in the presence of iron, zinc and copper salts against phytopathogenic fungi, zone of inhibition, mm (Note: p≤0.05, Ni – no inhibition, % C – % Control).

Bacterial strains		**Phytopathogenic fungal strains**							
		Alternaria alternata CNMN-FF-09		*Botrytis cinerea* CNMN-FF-10		*Fusarium oxysporum* CNMN-FF-06		*Fusarium solani* CNMN-FF-07	
		mm	% C	mm	% C	mm	% C	mm	% C
Bacillus cereus var. fluorescens CNMN-BB-07	Control	24.7±0.7	100	33.0±1.1	100	24.3±1.3	100	22.3±1.7	100
	$FeSO_4 \cdot 7H_2O$	24.3±0.7	98.65	32.0±3.9	96.97	17.7±0.7	72.60	26.7±1.7	119.40
	$FeCl_3 \cdot 6H_2O$	26.3±1.3	106.76	34.0±2.3	103.03	19.0±1.1	78.08	27.7±2.8	123.88
	$ZnSO_4 \cdot 7H_2O$	19.7±0.7	79.73	15.0±1.1	45.45	Ni	Ni	16.0±1.1	71.64
	$ZnCl_2$	21.0±1.1	85.14	Ni	Ni	Ni	Ni	13.7±0.7	61.19
	$CuSO_4 \cdot 7H_2O$	29.0±1.1	117.57	30.3±0.7	91.92	20.7±1.3	84.93	30.7±1.3	137.31
Bacillus velezensis CNMN-BB-13	Control	26.7±1.3	100	29.3±1.3	100	19.7±0.7	100	20.3±1.7	100
	$FeSO_4 \cdot 7H_2O$	24.3±1.7	91.25	38.3±3.6	130.68	18.0±1.1	91.53	27.7±2.4	136.07
	$FeCl_3 \cdot 6H_2O$	26.7±2.8	100.00	38.0±2.3	129.55	21.7±1.3	110.17	27.3±1.7	134.43
	$ZnSO_4 \cdot 7H_2O$	24.3±0.7	91.25	36.3±1.3	123.86	39.0±2.0	198.31	30.7±0.7	150.82
	$ZnCl_2$	23.3±0.7	87.50	35.7±1.3	121.59	23.0±2.3	116.95	28.3±0.7	139.34
	$CuSO_4 \cdot 7H_2O$	30.7±1.3	115.00	30.7±1.3	104.55	25.7±1.3	130.51	33.0±1.1	162.30
Bacillus velezensis CNMN-BB-31	Control	26.3±2.4	100	33.3±1.7	100	25.0±1.1	100	23.0±1.1	100
	$FeSO_4 \cdot 7H_2O$	23.0±1.1	87.34	34.0±2.0	102.00	17.0±1.1	68.00	30.3±0.7	131.88
	$FeCl_3 \cdot 6H_2O$	22.7±2.4	86.08	31.7±3.3	95.00	18.0±1.1	72.00	28.0±2.3	121.74
	$ZnSO_4 \cdot 7H_2O$	25.3±1.7	96.20	34.7±0.7	104.00	18.7±1.7	74.67	28.3±3.3	123.19
	$ZnCl_2$	21.3±1.3	81.01	31.0±4.1	93.00	15.0±1.1	60.00	26.7±0.7	115.94
	$CuSO_4 \cdot 7H_2O$	29.3±1.3	111.39	31.3±2.6	94.00	26.3±4.0	105.33	33.3±3.3	144.93

By testing *Pseudomonas* strains cultivated on King B medium with the addition of 10 mg/L of iron and zinc salts, no antifungal activity was registered against any phytopathogen, the initial activity disappeared. Although the growth of *Pseudomonas* strains on media supplemented with 10 mg/L copper(II) sulfate has caused a significant increase in antifungal activity in all phytopathogenic fungi tested (Table 3).

Table 3. Antifungal activity of *Pseudomonas* strains cultivated in the presence of 10 mg/L of $CuSO_4 \cdot 7H_2O$, zone of inhibition, mm (Note: $p \leq 0.05$).

Bacterial strains	**Phytopathogenic fungal strains**			
	Alternaria alternata CNMN-FF-09	*Botrytis cinerea* CNMN-FF-10	*Fusarium oxysporum* CNMN-FF-06	*Fusarium solani* CNMN-FF-07
P. fluorescens CNMN-PsB-01	26.0±1.1	34.0±2.0	27.0±3.0	30.7±1.3
P. fluorescens CNMN-PsB-02	30.0±2.3	24.0±2.3	26.7±3.3	31.3±1.7
P. fluorescens CNMN-PsB-12	27.0±3.0	31.7±2.3	25.7±1.3	30.7±3.3

Therefore, *P. fluorescens* CNMN-PsB-01 exhibited increased antifungal activity against phytopathogenic fungal strains, with inhibition zones ranging from 26.0 to 34.0 mm. *P. fluorescens* CNMN-PsB-02 inhibited the growth of the tested strains with inhibition zones between 24.0 and 31.3 mm, while the *P. fluorescens* CNMN-PsB-12 strain showed inhibition zones ranging from 25.7 to 31.7 mm. It should be noted that *F. solani* CNMN-FF-07 and *B. cinerea* CNMN-FF-10 exhibited the highest sensitivity to the exometabolites synthesized by *Pseudomonas* grown in the presence of copper(II) sulfate, with the diameter of the growth inhibition zones exceeding 30.7 mm.

4. Conclusions

The results of this study showed that the cultivation of *Bacillus* strains, particularly *B. velezensis* CNMN-BB-13, in the presence of 10 mg/L iron, zinc, and copper salts led mainly to increased antifungal activity against phytopathogenic fungi. The antifungal activity of *Pseudomonas* strains was significantly enhanced by the addition of copper salt to the nutrient medium.

Based on the results obtained, new biotechnological procedures can be developed for the cultivation of *Bacillus* and *Pseudomonas* strains with enhanced antifungal activity against phytopathogens such as *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Fusarium solani*. The integration of these results through the development of microbial preparations for the control of fungal diseases can serve as an alternative to the chemical pesticides widely used today in plant protection systems.

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