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Original Article

Antagonistic Activity of Bacteria Isolated from Conifers of Tbilisi and its Surroundings against Phytopathogenic Fungi

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Abstract - *Urban tree mortality is a serious worldwide problem. the stressful ecological situation created by globalization and climate change turned city trees even more vulnerable to the impact of pests and pathogens. Many plant diseases can be managed using antagonistic microorganisms, and biological control allows the host plant to survive. Isolation and studying of the antagonistic activity of endophytic bacteria against those pathogenic fungi, which are likely to cause massive diseases and dying of coniferous plantations in Tbilisi and its surroundings was the aim of the presented work. Some strains of the genus Bacillus with high antagonistic activity against several phytopathogenic mycelial fungi (A. alternata, A.infectoria, E. nigrum, C. spicifera, C. inaequalis, D. gregaria, D. iberica, D. sapinea) have been revealed using the bicultural techniques. Further study of these strains may be promising as biocontrol agents for disease prevention and control, especially in natural ecosystems, where the use of chemicals is strictly limited.*

Keywords - *Antifungal activity of bacteria, Bacillus, Phytopathogenic mycelial fungi, Conifers.*

1. Introduction

The death of urban trees and forests, in general, is a serious environmental problem worldwide. Trees have already been affected by pests, and pathogens have become especially sensitive and vulnerable to stresses in the era of globalization and climate change [10, 12, 22, 26].

Recently, biological control (biocontrol) has been at the center of scientists' and practitioners' attention; it has become a constituent of an integrated approach to combating pests and diseases. the main purpose of biocontrol is to reduce the population of a pathogen or pests, allowing the host plant's survival and, consequently, the entire population [3]. Such an approach is often advantageous because, in many cases, biocontrol agents are found in the plant itself. Endophytic microorganisms inhabit plant tissues and are integral to their microbiome [13]. They can be represented by viruses, bacteria, and fungi [17]. Endophytes play an important role in plant development and resistance to adverse environmental conditions [20].

Plant diseases can be managed using antagonistic microorganisms [7, 21]. Some of them possess the ability to fight one particular phytopathogen (under certain conditions), while others can manage a wide range of plant pathogens. the mechanisms of growth inhibition of the phytopathogenic fungi may be different: production of biologically active substances [27, 28], an increase of systemic resistance of plants [16], or competition with pathogens for nutrients and ecological niche [14].

Most bacterial antagonists belong to the genus Bacillus [5, 21, 23, 24, 27, 29]. B. subtilis is one of the most popular biological agents for plant disease management [6, 18, 25, 27, 28]. B. subtilis QST 713 is widely used in biocontrol programs worldwide [1].

Isolation and studying of the antagonistic activity of endophytic bacteria against those pathogenic fungi, which are likely to cause massive diseases and dying of coniferous plantations in Tbilisi and its surroundings was the aim of the presented work.

2. Materials and Methods

2.1. Isolation and identification of antagonistic bacteria

To isolate bacteria antagonistic to mycelial fungi isolates that exhibited fungicidal activity on the primary inoculations of plant leaves and needles were transferred to nutrient agar. A sampling of the material from sick and externally healthy trees of Tbilisi and its surroundings and preparation of inocilations for the phytopathological study was described previously [8]. Bacterial isolates were incubated at 30 °C for 2 days. the Streak-plate and the Spread-plate techniques were used to obtain the pure cultures [9].

The primary identification of bacterial isolates was performed according to their morphological characters (colony morphology, cell shape, size, gram staining, and ability to form spores).

Bacteria with high fungicidal activity were identified by sequencing corresponding sites of 16S rDNA at the University of Guelph (Canada) [8].

2.2. Fungicidal activity test

The fungicidal activity of bacterial isolates was studied on microscopic fungi isolated from different plants growing in Tbilisi and its surroundings, which were considered dominants in terms of prevalence and frequency among tested plants [8]. Namely: *Alternaria alternata* (Fr.) Keissl., *Epicoccum nigrum* Link., *Curvularia spicifera* (Bainier) Boedijn, *Curvularia inaequalis* (Shear) Boedijn., *Dothiorella gregaria* Sacc., *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves., *Diplodia sapinea* (Desm.) Kickx. Another species of the genus *Alternaria* – *A. infectoria*, E.G., Simmons, also known for its high pathogenic properties [2], was studied as well, which had a frequency of less than 5% and was characterized by sporadic distribution.

The dual culture technique with a fungal block in the center of the Petri dish was used to test the fungicidal activity of bacterial isolates [23]. 5 mm agar blocks of 5-day-old fungal test cultures, grown on a beer wort agar nutrient medium at 25 °C, were placed in the center of a 90 mm diameter Petri dish with beer wort agar. 2.5 cm-s were measured from the center crosswise in four directions at each dish, and one loopfool of 24-hour culture suspensions of four different bacteria were inoculated. Each fungal culture was inoculated in the same manner without bacterial cultures as control. the Petri dishes were incubated at 25 °C for 5 days. the test was performed twice on each fungus and bacterium. After 5 days, the diameters of fungal cultures grown on both control and test variants were measured. the following formula calculated the inhibition of a fungus growth:

$$x = \frac{a - b}{a} \times 100\%$$

x is the percentage of a fungus growth inhibition; a - the diameter of a fungus in the control variant; b - the diameter of a fungus inhibited by a bacterium.

The mean values of three experimental results were used to analyze the fungicidal activity. Obtained results and their standard deviations are given in table 1.

3. Results and Discussion

Among the microbiota isolated from plant samples and microscopic fungi, bacterial cultures with low frequency were also found; most of them revealed clear antagonism to phytopathogenic fungi. the primary inoculants of some of these specimens are shown in **Figure 1**.

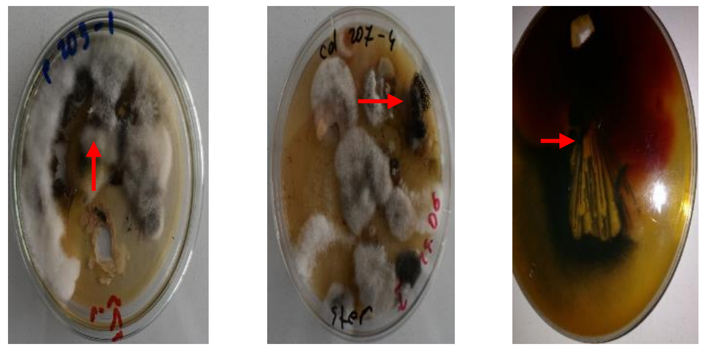

Fig. 1 Primary inoculants of needles in which the fungicidal activity of bacteria was detected (indicated by an arrow)

40 pure cultures of endophytic bacteria were isolated; 38 were identified as *Bacillus* according to their morphological characteristics (colony morphology, cell shape, size, Gram staining, and spore-producing ability). All of them revealed fungicidal activity against at least several tested microscopic fungi. Two bacterial isolates had weak antagonistic activity against studied fungi or did not reveal it. (the data are not given).

Table 1 is demonstrated the results of bacteria that showed the maximal antifungal activity by dual culture technique towards at least one micromycete. It is evident that presented bacterial isolates inhibited the growth of all studied fungi to various degrees; this is acceptable to the antagonistic activity against different strains of the same fungal species.

The highest antagonistic susceptibility towards all three *Epicoccum nigrum* (F5, P4-1-1, F8) was found for bacterial isolates with the conditional names T347 and CD461; However, the growth of *E. nigrum* F5 was more inhibited by the bacterial strain T 347 (41.2%), *E. nigrum* P4-1-1 was sensitive to strain CD 293 (50%), which demonstrated high effectivness against *E. nigrum* F8 as well (54.5%). Five other strains (CD 207, TI 286, CAN 334, CP 452, and CD 461) have revealed a similar growth inhibition rate (54.5%). Since the genus *Alternaria* is a powerful plant pathogen, which causes great harm to agricultural products [2, 15], and was detected in 91.5% of the tested plants [8], the antagonistic activity of bacteria against its four isolates - *A.*

alternata K1, *A. alternata* K12, *A. alternata* P314-5-1, *A. infectoria* P362-1-1, was investigated as well.

Bacteria with the conditional names T347 and CD461 showed the highest activity in growth inhibition of all four strains of *Alternaria*. T347 was especially effective against *A. alternata* K12 and *A. infectoria* P362-1-1 (53.1% and 62%, respectively); while CD461 revealed the better results against *A. alternata* K1 and *A. alternata* P314-5-1 (51.7% and 55%, respectively); similar inhibitory effect (62%) on *A. infectoria* P362-1-1 demonstrated the bacterial isolate AC 427, whereas B 462 – was effective against *A. alternata* P314-5-1 (55%).

Table 1. Inhibition of microscopic fungi growth by bacterial isolates (%)

Conditional name of a bacterial isolate	***Epicoccum***			***Curvularia***		***Diplodia***		***Dothyorella***		***Alternaria***			
	***nigrum* F5**	***nigrum* F8**	***nigrum* P4-1-1**	***inaequalis* FE**	***spicifera* 456-2-1**	***sp.* FL**	***sapinea* P361-1-1**	***gregaria* MT2**	***iberica* CP450-5-2-2**	***alternata* K1**	***alternata* K12**	***infectoria* P362-1-1**	***alternata* P314-5-1**
P 203	33.0 ±1.65	41.5±2.1	21±1.1	40.7±2.0	32±1.5	36.0±1.8	**60**±3.0	27.8±1.4	19±0.9	44.1±2.2	42.0±2.1	53±2.6	48±2.4
AB 206	30.4 ± 1.5	48.0±2.4	35±1.7	44.4 ± 2.2	32±1.5	54.6 ± 2.7	58±2.9	25.0±1.3	14±0.7	48.0±2.4	44.9±2.2	50±2.5	48±2.4
CD 207	35.8 ± 1.8	**54.5**±2.7	39±1.9	40.7 ± 2.0	41±2.0	**58.1** ± 2.9	58±2.9	38.9 ± 1.9	5±0.2	50.3±2.5	48.4±2.4	47±2.4	51±2.5
TI 286	30.2 ± 1.5	**54.5**±2.7	39±1.9	40.7 ± 2.0	64±3.2	45.3 ± 2.3	51[1] ±2.5	35.2 ± 1.8	14±0.7	47.±2.4	40.6±2.9	35±1.7	48±2.4
CD 293	33.0± 1.7	**54.5**±2.7	**50**±2.5	44.4 ± 2.2	73±3.6	46.5 ± 2.3	58±2.9	37.0 ± 1.9	23±1.1	48.9±2.5	44.7±2.2	50±2.5	48±2.4
CPT	33.0 ± 1.7	44.1±2.2	39±1.9	**51.8** ± 2.6	68±3.4	50.0 ± 2.5	53[1] ±2.6	37.9 ± 1.0	5±0.2	45.5±2.3	23.4±2.3	56±2.8	41±2.0
CTA 315	34.4 ± 1.7	50.6±2.5	25±1.2	44.4 ± 2.2	**77**±3.8	46.5 ± 2.3	56 ±	37.5±1.9	5±0.2	48.9±2.5	41.2±2.1	53±2.6	48±2.4
CAN 334	34.4 ± 1.7	**54.5**±2.7	46±2.3	48.1 ± 2.4	68±3.4	53.5 ± 2.7	58±2.9	35.6±1.8	23±1.1	45.7±2.3	47.3±2.4	59±2.9	48±2.4
T 347	**41.2**±2.1	53.2±2.7	46±2.3	48.1 ± 2.4	**77**±3.8	56.9±2.8	58±2.9	45.6±2.3	14±0.7	50.3±2.5	**53.1**±2.7	**62**±3.1	48±2.4
AC 427	26.2 ± 1.3	44.1±2.2	29±1.4	44.4 ± 2.2	**77.5**±3.9	43.0 ± 2.1	53[1] ±2.6	36.2± 1.8	14±0.7	44.3±2.2	45.8±2.3	**62**±3.1	45±2.2
CP 452	31.6 ± 1.6	**54.5**±2.7	43±2.1	48.1 ± 2.4	73±3.6	**58.1** ± 2.9	56 ±2.8	39.3 ±2.0	ND	45.7±2.3	48.4±2.4	56±2.8	45±2.2
CD 461	39.9 ± 2.0	**54.5**±2.7	**50**±2.5	48.1 ± 2.4	73±3.6	53.5 ± 2.7	58±2.9	**48.2**±2.4	42±2.8	**51.7**±2.6	48.7±2.4	56±2.8	**55**±2.7
B 462	28.8 ± 1.4	50.4±2.5	39±1.9	48.1 ± 2.4	73±3.6	50.0 ± 2.5	**60**±3.0	42.4±2.1	**63**±3.1	44.3±2.2	44.7±2.2	53±2.6	**55**±2.7
CD 463	33.1±1.7	51.8±2.6	39±1.8	44.4±2.2	73±3.6	50.0±2.5	**60**±3.0	44.4±2.2	0	46.9±2.3	47.3±2.4	53±2.6	48±2.4
PC 464	39.8 ± 2.0	53.2±2.7	29±1.4	48.1 ± 2.4	73.5±3.7	47.6 ± 2.4	**60**±3.0	38.5±1.0	50±2.5	46.6±2.3	47.0±2.4	43±2.1	48±2.4
P 475	34.4 ± 1.7	46.6±2.3	ND	40.7 ± 2.0	68±3.4	48.8 ± 2.4	56 ±2.8	41.4±2.1	58±2.9	41.5±2.1	42.1±2.1	50±2.5	51±2.5
CP 513	33.0±1.7	44.1±2.2	25±1.2	44.4±2.2	68±3.4	45.3±2.3	**60**±3.0	37.5±1.9	50±2.5	42.9±2.1	47.3±2.4	53±2.6	45±2.2
PC 515	37.2±1.8	45.4±2.3	32±1.5	40.7±2.0	68±3.4	48.8±2.4	**60**±3.0	39.3±2.0	42±2.1	42.9±2.1	46.3±2.3	53±2.6	51±2.5
CD 534	34.4 ± 1.7	53.0±2.7	43±2.1	44.4 ± 2.2	36±1.8	52.3 ± 2.6	**62** ±3.1	43.5 ± 2.2	ND	46.6±2.3	48.4±2.4	50±2.5	ND
P 526	**41.6** ±2.1	47.0±2.3	29±1.4	40.7±2.0	43±2.1	48.8±2.4	60±3.0	37.0±1.8	50±2.5	38.8±1.9	48.5±2.4	47±2.3	45±2.2

Note: *ND - No data available; [1] - Incomplete inhibition of air mycelium development; [2] - the air mycelium of the fungus "covered" the bacteria (see Fig. 2; (indicated by an arrow); the best results of fungicidal activity of bacterial isolates towards particular fungi are given in red)*

The fungicidal activity of tested bacteria was studied against two species of the genus *Curvularia*: *C. inaequalis* FE and *C. spicifera* 456-2-1. the strain with the conditional name CPT revealed the highest activity against *C. inaequalis* FE (51.8%), while strains CTA 315, T 347, and AC 427 were active against *C. spicifera* 456-2-1 (77% - 77.5%). It should be noted that additionally, four other bacterial isolates: CP 452, CD 461, B 462, and PC 464, demonstrated close to the best fungicidal activity against both strains of *Curvularia*: their inhibitory effect was 48.1% against *C. inaequalis* FE and 73-73.5% - against *C. spicifera* 456-2-1.

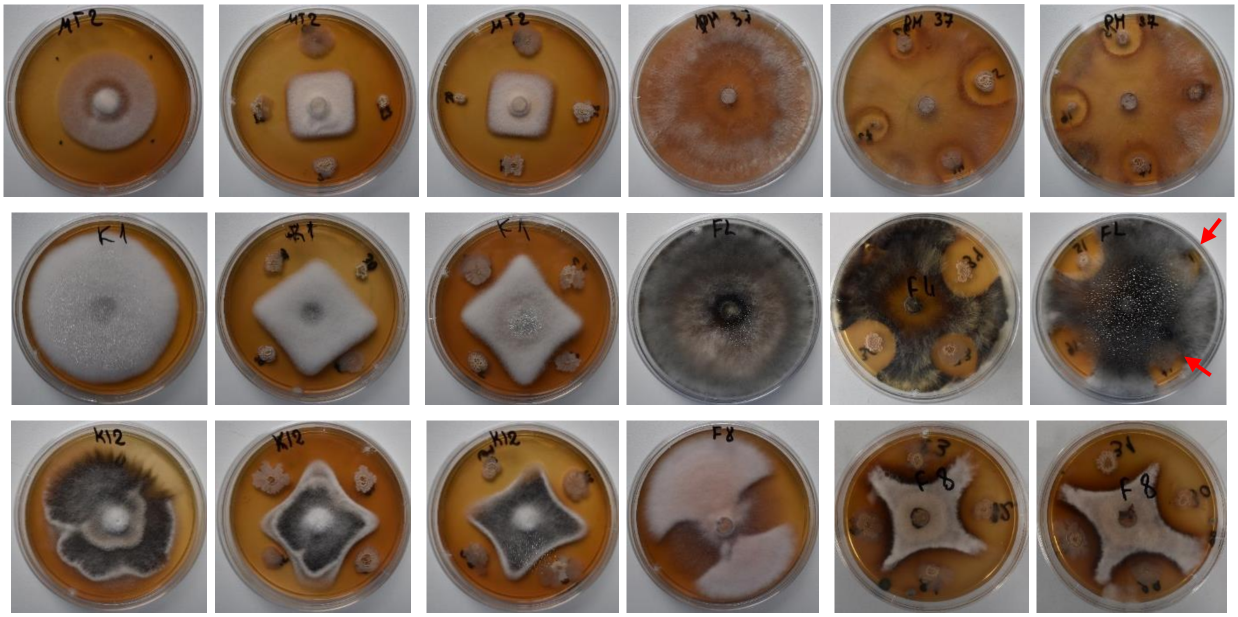

Fig. 2 Antagonistic relation of some bacterial isolates towards different micromycetes

The antagonistic activity of experimental bacteria was studied against *Diplodia,* and *Dothyorella* strains as well (in particular: *Diplodia sp*. FL, *Diplodia sapinea* P361-1-1, *Dothyorella gregaria* MT2, *Dothyorella iberica* CP450-5-2-2). These genera, like the Epicocum mentioned earlier, *Alternaria*, and *Curvularia*, are dangerous plant pathogens [4, 11, 19] and were distinguished by the high frequency of occurrence among the studied plants of Tbilisi and its surroundings [8].

The highest fungicidal activity against *Diplodia sp*. FL was observed in bacterial strains - CD 207, CP 452 (58.1%), while against *Diplodia sapinea* P361-1-1 strain CD 534 was effective (62%); bacterial strain CD 461 showed positive results towards *Dothyorella gregaria* MT2 (48.2%); against *Dothyorella iberica* CP450-5-2-2 the strain B 462 (63%) was effective.

Almost the maximal antagonistic activity against *Diplodia* and *Dothyorella* strains was revealed among several tested bacterial isolates: in the case of *Diplodia sp*. FL and *Diplodia sapinea* P361-1-1 were active strains AB 206, CD 207, and T 347 against *Dothyorella gregaria* MT2 and *Dothyorella iberica* CP450-5-2-2 – strain P 475 (41.4% and 58%, respectively).

Summarizing the experimental results, it is clear that several bacterial strains revealed high antagonistic activity against different species of various genera of microscopic fungi. Especially two strains have demonstrated the maximal or near-maximal antagonistic activity against almost all studied fungal cultures: isolates CD 461 (out of 13 tested fungal strains was active against 11 ones, with maximal activity against 5 ones) and T 347 (was active against 11 fungal strains, with maximal fungicidal activity against 4 ones).

Nine bacterial strains with the best fungicidal activity were identified by sequencing corresponding sites of 16S rDNA at the University of Guelph (Canada); their 95% similarity to some species of the genus *Bacillus* was revealed (**Table 2**).

Table 2. Results of the identification of bacterial cultures.

Conditional name of isolate	Coefficient of similarity detected by sequence	Similarity Coefficient was Identified with the following species
P 203	>99.6%	*B. subtilis, B. mojavensis, B. halotolerans, B. tequilensis,* and *B. vallismortis*
CD 207	>99.5%	*B. velezensis, B. amyloliquefaciens, B. subtilis, B. vallismortis,* and *B. siamensis*
TI 286	>99.5%	*B. velezensis, B. amyloliquefaciens, B. subtilis, B. vallismortis,* and *B. siamensis*
CTA 315	>99.6%	*B. subtilis, B. mojavensis, B. halotolerans, B. tequilensis,* and *B. vallismortis*
CAN 334	>99.5%	*B. velezensis, B. amyloliquefaciens, B. subtilis, B. vallismortis,* and *B. siamensis*
P 460	>99.5%	*B. velezensis, B. amyloliquefaciens, B. subtilis, B. vallismortis,* and *B. siamensis*
CD 452	>99.6%	*B. amyloliquefaciens, B. vallismortis, B. nematocida, B. velezensis,* and *B. subtilis*
T 347	>99.7%	*B. subtilis, B. tequilensis, B. mojavensis, B. halotolerans,* and *B. vallismortis*
CP 461	>99.5%	*B. velezensis, B. amyloliquefaciens, B. subtilis, B. vallismortis,* and *B. siamensis*

4. Conclusion

Some strains of the genus *Bacillus* characterized by the high antagonistic activity against different genera of phytopathogenic mycelial fungi have been revealed, so the selected bacterial cultures may be promising biocontrol agents for tested pathogens.Tree death is mostly caused by several pathogenic fungi simultaneously. Therefore, further study of the fungicidal potential of revealed individual bacterial strains against various phytopathogenic fungi would be of great importance for their bioprotective use in natural ecosystems, where chemical control is strictly limited; It must be taken into consideration that all tested phytopathogenic fungi belong to the same class; moreover, pairs *Diplodia-Dotiorella* and *Curvularia-Alternaria* - to the same family (*Botryosphaeriaceae* and *Pleosporaceae*, respectively).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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