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 the globalization and climate change turned city trees even more vulnerable to 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, both for disease prevention and control, especially in natural ecosystems, where the use of chemicals is strictly limited.

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ABSTRACT

Urban tree mortality is a serious worldwide problem. The stressful ecological situation created by the globalization and climate change turned city trees even more vulnerable to 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, both for disease prevention and control, especially in natural ecosystems, where the use of chemicals is strictly limited.

Keywords: phytopathogenic mycelial fungi, *Bacillus*, anifungal activity of bacteria.

I. INTRODUCTION

The death of urban trees and forests in general, is a serious environmental problem worldwide. Trees already been affected by pests and pathogens became especially sensitive and vulnerable to stresses in the era of globalization and climate change (Jentsch et al. 2007, Tubby & Webber 2010, Smith 2011, IPCC 2012).

Recently, biological control (biocontrol) is in 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, which would allow the survival of the host plant and, consequently, the entire population (Bale et al. 2008). Such an approach is often advantageous because in many cases biocontrol agents are found in the plant itself. They are endophytic microorganisms inhabiting plant tissues and are integral components of its microbiome (Jia et al. 2016). They can be represented by viruses as well as bacteria and fungi (Lenteren et al. 2018). Endophytes play an important role in plant development and resistance to adverse environmental conditions (Reid & Greene 2012).

Many plant diseases can be managed using antagonistic microorganisms (Shafi et al. 2017, Cook et al. 2002). 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 (Yánez-Mendizábal et al. 2011, Wang et al. 2015), increase of systemic resistance of plants (Lahlali et al. 2013), or competition with pathogens for nutrients and ecological niche (Kumar et al. 2011).

The majority of bacterial antagonists belong to the genus *Bacillus* (Soria et al. 2012, Wang et al. 2015, Zhang et al. 2015, Chen et al. 2016, Jha et al. 2016, Shafi et al. 2017). *B. subtilis* is one of the most popular biological agents for plant disease management (Wang et al. 2015, Tozlu et al. 2018, Yánez-Mendizábal et al.,2011, Chen et al. 2013, Li et al. 2013). *B. subtilis* QST 713 is widely used in biocontrol programs worldwide (Abbasi & Weselowski 2014).

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.

II.   MATERIAL AND METHODS

2.1 Isolation and identification of antagonistic bacteria

For the isolation of bacteria, antagonistic to mycelial fungi, those isolates that exhibited fungicidal activity on the primary inoculations of plant leaves and needles were transferred to nutrient agar. Sampling of the material from sick and externally healthy trees of Tbilisi and its surroundings and preparation of inocilations for phytopathological study was described previously (Danelia et al. 2021). 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 (Harley et al. 2005).

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

Bacteria with high fungicidal activity were identified by sequencing of corresponding sites of 16S rDNA at the University of Guelph (Canada) (Danelia et al. 2021).

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 as dominants in terms of prevalence and frequency among tested plants (Danelia et al. 2021). 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 (Agrios 2005), was studied as well, which had frequency less than 5% and was characterized by sporadic distribution.

The dual culture technique with a fungal block in the center of Petri dish was used to test the fungicidal activity of bacterial isolates (Soria1 et al. 2012). 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 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. 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. Inhibition of a fungus growth was calculated by the following formula:

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

x is the percentage of a fungus growth inhibition; a - diameter of a fungus in control variant; b - diameter of a fungus inhibited by a bacterium.
The mean values of two experimental results were used to analyze the fungicidal activity (Table 1).

III.   RESULTS AND DISCUSSION

Among the microbiota, isolated from plant samples, along with microscopic fungi, bacterial cultures with low frequency were found as well; most of them revealed clear antagonism to

phytopathogenic fungi. The primary inoculants of some of this specimen are shown in Figure 1.

Totally 40 pure cultures of endophytic bacteria were isolated; 38 out of them were identified as *Bacillus* according to their morphological characteristics (colony morphology, cell shape, size, Gram staining, and spore producing ability) (full list of bacteria is shown in Table 1). Two other isolates (conditional names - PL 201 and TS) had very weak antagonistic activity against studied fungi or did not reveal it at all (the data are not given).

The results of fungicidal activity of some bacterial isolates by dual culture technique are demonstrated in Table 1 and Fig. 2.
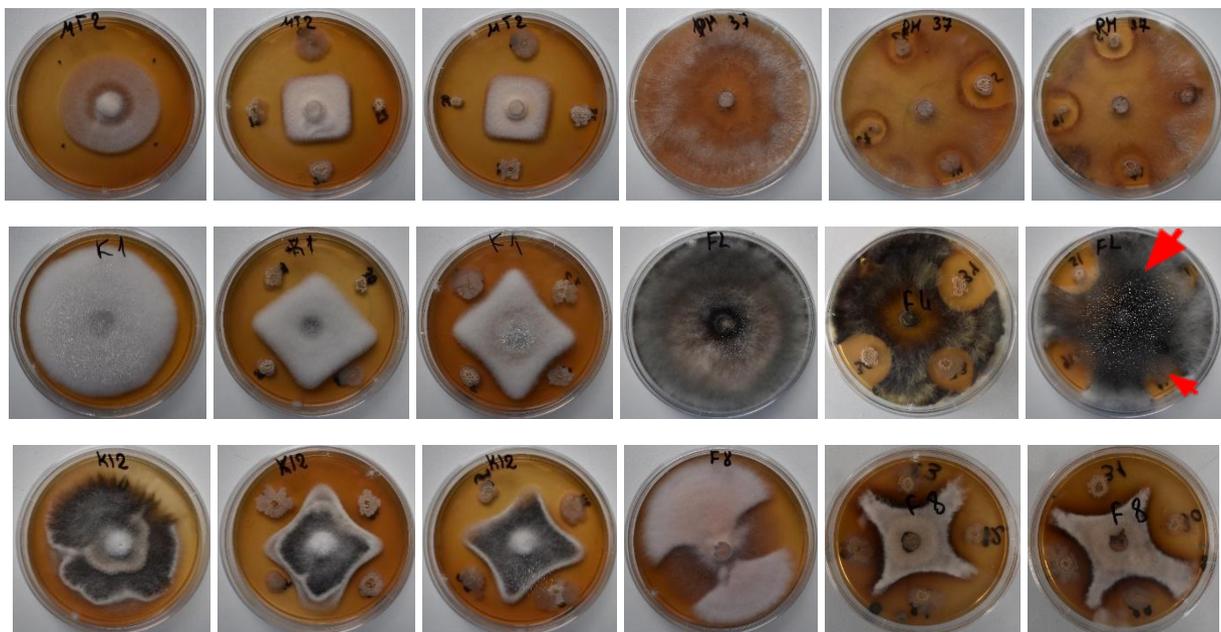

Fig. 2: Antagonistic relation of some bacterial isolates towards different micromycetes

From Table 1 it is clear that 38 isolates of bacteria inhibited the growth of all studied fungi at various degrees; this is acceptable to the antagonistic activity against different strains of the same fungal species as well.

The highest antagonistic susceptibility towards all three strains of *Epicoccum nigrum* (F5, P4-1-1, F8) was found for bacterial isolates with the conditional names T347 and CD461; although 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 rate of growth inhibition (54.5%).

Since the genus *Alternaria* is a powerful plant pathogen, which causes a great harm to agricultural products (Lagopodi & Thanassoulopoulos 1998, Agrios 2005), and was detected in 91.5% of the tested plants (Danelia et al. 2021), 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 terms of 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 nigrum			Curvularia		Diplodia		Dothyorella		Alternaria			
	F5	F8	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	41.5	21	40.7	32	36.0	**60**	27.8	19	44.1	42.0	53	48
AB 206	30.4	48.0	35	44.4	32	54.6	58	25.0	14	48.0	44.9	50	48
CD 207	35.8	**54.5**	39	40.7	41	**58.1**	58	38.9	5	50.3	48.4	47	51
CP 216	2.7	43.6	32	ND	41	0	53[1]	27.7	ND	0	0	53	34
PH 269	29.0	49.1	39	44.4	64	45.3	56	33.3	23	48.0	46.1	50	45
PH 269	31.6	49.1	39	44.4	64	47.7	56	31.5	14	23.6	46.1	56	45
TJ 270	33.0	51.7	43	40.7	0	48.8	58	24.1	5	41.5	40.6	40	38
TI 286	30.2	**54.5**	39	40.7	64	45.3	51[1]	35.2	14	47.3	40.6	35	48
CD 293	33.0	**54.5**	**50**	44.4	73	46.5	58	37.0	23	48.9	44.7	50	48
PC 296	31.8	46.7	43	44.4	73	47.7	58[1]	31.5	23	40.3	41.8	53	45
CPT	33.0	44.1	39	**51.8**	68	50.0	53[1]	37.9	5	45.5	23.4	56	41
CTA 315	34.4	50.6	25	44.4	77	46.5	56	37.5	5	48.9	41.2	53	48
CP 325	38.5	51.7	43	44.4	68	47.6	56	41.4	19	45.0	36.0	56	48
CAN 334	34.4	**54.5**	46	48.1	68	53.5	58	35.6	23	45.7	47.3	59	48
T 347	**41.2**	53.2	46	48.1	77	56.9	58	45.6	14	50.3	**53.1**	**62**	48
AC 427	26.2	44.1	29	44.4	**77.5**	43.0	53[1]	36.2	14	44.3	45.8	**62**	45
JUN 442	31.6	49.3	39	33.3	64	44.2	53[1]	32.5	14	29.9	38.3	53	48
CP450-5Y	2.7	46.1	ND	ND	ND	37.8	ND	13.0	ND	0	51.1	ND	ND
CP 450-5	31.6	46.6	39	44.4	68	57.3	53	38.1	ND	45.5	48.1	50	51
CP 452	31.6	**54.5**	43	48.1	73	**58.1**	56	39.3	ND	45.7	48.4	56	45
P 456	39.8	51.9	39	48.1	68	48.8	58	41.2	ND	45.5	44.7	56	45
CD 457	28.8	47.9	ND	44.4	ND	51.1	ND	39.6	ND	42.9	46.7	ND	ND
P 460	35.7	50.6	29	40.7	64	52.3	56[1]	43.0	45	48.3	48.7	56	48
CD 461	39.9	**54.5**	**50**	48.1	73	53.5	58	**48.2**	42	**51.7**	48.7	56	**55**
B 462	28.8	50.4	39	48.1	73	50.0	**60**	42.4	**63**	44.3	44.7	53	**55**
CD 463	33.1	51.8	39	44.4	73	50.0	**60**	44.4	0	46.9	47.3	53	48
PC 464	39.8	53.2	29	48.1	73.5	47.6	**60**	38.5	50	46.6	47.0	43	48
P 475	34.4	46.6	ND	40.7	68	48.8	56	41.4	58	41.5	42.1	50	51
CD 477	34.4	50.6	43	40.7	64	51.1	58	41.2	42	45.0	43.5	50	48
P 503	39.8	50.4	43	40.7	68	47.7	58[1]	41.4	50	41.7	47.5	53	45
CP 513	33.0	44.1	25	44.4	68	45.3	**60**	37.5	50	42.9	47.3	53	45
PC 515	37.2	45.4	32	40.7	68	48.8	**60**	39.3	42	42.9	46.3	53	51[1]
PC 515-3	34.4	51.8	35	44.4	64	52.3	58	42.1	42	44.1	47.9	50	51
CP 521	26.2	50.6	35	44.4	73	44.1	60[1]	39.8	54	40.3	46.1	53	48
CD 534	34.4	53.0	43	44.4	36	52.3	**62**	43.5	ND	46.6	48.4	50	ND
CD 539	28.9	45.5	39	33.3	36	41.8	56[1]	33.3	50	40.3	40.0	59	ND
TB	26.1	53.1	35	33.3	70	45.3	56	39.9	42	44.1	47.0	47	45
P 526	**41.6**	47.0	29	40.7	43	48.8	60	37.0	50	38.8	48.5	47	45

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 a particular fingi 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 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 additionaly 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.

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 aforementioned *Epicocum*, *Alternaria*, and *Curvularia*, are dangerous plant pathogens (Phillips et al. 2005, Ivanová et al. 2016, Bußkamp et al. 2020) and were distinguished with high frequency of occurrence among the studied plants of Tbilisi and its surroundings (Danelia et al. 2021).

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

IV. 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 towards tested pathogens.

Tree death mostly is 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).

ACKNOWLEDGMENTS

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