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Original paper

***Halotolerant Bacillus amyloliquefaciens* 24.5 useful as a biological agent to control phyto-pathogenic fungi**

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Abstract

Phytopathogenic fungi are causal agents which determine significant economic losses during the pre- and post-harvest stages. Biological control of fungal diseases using antagonistic native bacteria is a promising alternative. In this study, we tested under *in vitro* conditions the antifungal potential of eighteen halotolerant bacterial strains against *Alternaria alternata, Aspergillus carbonarius, A. flavus, A. niger, Botrytis cinerea, Fusarium oxysporum, Penicillium digitatum, Rhizopus* sp. Among these strains, one has strongly inhibited the growth of the major fungal plant pathogens, except *Rhizopus* sp. This strain, coded as 24.5 was molecularly identified as *Bacillus amyloliquefaciens* by multiplex PCR. The presence of the antifungal genes involved in the biosynthesis of the lipopeptides such as surfactin (*srf/lch*), fengycin (*fen*), iturin D (*ituD*), iturin A (*ituA*) and bacillomycin (*bmyA*) were detected by PCR analysis in the targeted strain. For the strain conditioning, freeze drying process was taken into account; a high percentage of viability, ranging from 94 to 98%, was obtained by the use of different cryoprotective agents. Additionally, *in vivo* tests proved the effectiveness of the *Bacillus amyloquefaciens* 24.5 as a promising biocontrol agent to prevent the development of different phytopathogenic fungi on the surface of the infected lemons, tomatoes and corn.

Keywords *In-vitro* and *in-vivo* antifungal activity; *Bacillus amyloliquefaciens*; antifungal lipopeptide genes; freeze-drying conditioning.

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Introduction

Fruits, vegetables or cereals can be infected by different phytopathogenic fungi belonging to the genera *Aspergillus* sp., *Alternaria* sp., *Botrytis* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp. during the field growth, preharvest, postharvest, and storage, which causes significant economic losses (R. BARKAI-GOLAN & N. PASTER [1], M. BARTH & al [2], R.A. SAMSON & al [3], S. ROUSSEAUX & al [4], G. DOEHLEMANN & al [5], L. ESCRIVÁ & al [6]). Effective fungicides are available for fungal disease management. However, the fungicide residues in environment or on raw fruits and vegetables which can affect the human and animal health have attracted public concern (S. DROBY & al [7]). As a result, biological control using antagonistic microorganisms can be an alternative strategy to reduce the incidence of fungal diseases, in the context of the eco-friendly technologies applied for environmental and agricultural sustainability (S. DROBY & al [7], O. LASTOCHKINA & al [8], D. KOUR & al [9]).

Different species of the genus *Bacillus*, such as *B. amyloliquefaciens, B. subtilis, B. licheniformis* and *B. pumilus* have been already well represented commercially and widely used as biocontrol agents in agriculture. Previous studies mentioned *Bacillus* sp. to have a strong inhibition capacity against several vegetable fungal pathogens, including *Fusarium* sp. (O.A. SICUIA & al [10], A.I. GROSU & al [11], D. VITULLO & al [12], A. DE SENNA & A. LATHROP [13], T. LEE & al [14], M.-D. URSAN & al [15]), *Alternaria* sp. (T. MORITA & al [16]), *Botrytis cinerea* (C.F. DIGUȚĂ & al [17], H. CALVO & al [18]), *Aspergillus* sp. (T.C. EINLOFT & al [19], F. SIAH-MOSHTEH & al [20], M. ARFAOUI & al [21]), *Penicillium* sp. (H. CALVO & al [18], E. ARREBOLA & al [22],Y. WANG & al [23], Z. TIAN & al [24]).

Various mechanisms of biological control of *Bacillus* species against plant pathogens have been associated with the competition for nutrients and space (O.A. SICUIA & al [25], J. SHAFI & al [26], D. BEN ABDALLAH & al [27]), production of different antimicrobial metabolites (O.A. SICUIA & al [25], J. SHAFI & al [26], A. ARGUELLES-ARIAS & al [28], H. CAWOY & al [29], S.P. CHOWDHURY & al [30], S. CAULIER & al [31]), plant growth-promoting rhizobacteria (PGPR) (D. KOUR & al [9], O.A. SICUIA & al [25], J. SHAFI & al [26], D. BEN ABDALLAH & al [27], J.W. KLOEPPER & al [32], E. SANSINENEA [33]) and induction of systemically resistance in the plant (O.A. SICUIA & al [25], J. SHAFI & al [26], J.W. KLOEPPER & al [32]). Competition for space and nutrients is the primary mode of biocontrol activity. Despite the high diversity of bioactive metabolites produced by *Bacillus* spp, three main cyclic lipopeptides (iturins, fengycins, surfactins) play the dominant role in the antagonism to suppress the growth phytopathogenic fungi (ARGUELLES-ARIAS & al [28], H. CAWOY & al [29], S.P. CHOWDHURY & al [30], S. CAULIER & al [31], A.E. ARREBOLA & al [34], F. ALVAREZ & al [35], C.S. COMPAORÉ & al [36], D. FIRA & al [37], J. NANJUNDAN & al [38], H. CALVO & al [39]).

The reports regarding the *Bacillus* spp. conditioning/ formulation ready to be used against plant pathogens are rather limited (D.A. SCHISLER & al [40], Y. ZHAN & al [41], A. GOTOR-VILA & al [42], L. HAN & al [43], S. STAMENKOVIĆ STOJANOVIĆ & al [44]) and the undisclosed formulation procedure is owned by manufacturing companies (D.A. SCHISLER & al [40]). During the freeze-drying process different factors influence the bacterial cells survival / viability, such as targeted species, cryoprotectants, freeze-drying parameters, as well as rehydration conditions (D.A. SCHISLER & al [40], Y. ZHAN & al [41], A. GOTOR-VILA & al [42], L. HAN & al [43], S. STAMENKOVIĆ STOJANOVIĆ & al [44], G.R. NIREESHA & al [45]). The spore-forming Bacilli can be successfully formulated by freeze-drying process, because this form provides tolerance during stressful conditions (S. STAMENKOVIĆ STOJANOVIĆ & al [44]). Among the protective agents, saccharides are supposed to be preferable due to their relatively low-cost raw material and accessibility, chemically innocuous nature, high of survival rate of cells after freeze-drying and stability during storage (D.A. SCHISLER & al [40], Y. ZHAN & al [41], A. GOTOR-VILA & al [42], L. HAN & al [43], S. STAMENKOVIĆ STOJANOVIĆ & al [44], G.R. NIREESHA & al [45]).

The aims of this study were: (1) to select halotolerant bacterial strains with large-spectrum against phytopathogenic fungi; (2) to detect the presence of genes responsible for the fungal biocontrol ability; (3) to investigate the effectiveness of two cryoprotectant agents on the viability of 24.5 bacterial cells during freeze-drying process and (4) to study *in vivo* the efficiency of 24.5 isolate in the biological control of the fungal diseases of tomatoes, lemons and corn.

Materials and Methods

1. Biological materials

The vegetables (tomatoes), fruits (lemons), cereals (corn) at ripening stage were purchased from commercial market.

2. Microorganisms and growth conditions

Eighteen bacterial isolates originating from a hypersaline water in Lopătari, România (G.I. PROCA & al [46]) have been used in this study. The bacterial strains were grown and maintained on Nutrient Agar (Merck, Germany) for 24h-48h, at 37°C.

The fungi used in the antifungal assays were isolated, identified and stored in the microbial collection of the Faculty of Biotechnology/UASVM Bucharest and belong to the following species: *Alternaria alternata* M5 (isolated from hypersaline water in Lopătari), *Aspergillus carbonarius* MI-15 (isolated from grapes), *Aspergillus flavus* MI-24 (isolated from peanuts), *Aspergillus niger* M4 (isolated from hypersaline water in Lopătari), *Botrytis cinerea* MI-Aligote Husi (isolated from grapes), *Penicillium digitatum* MI-41 (isolated from lemons), *Fusarium oxysporum* MI-34

(isolated from wheat), and *Rhizopus* sp. MI-30 (isolated from grapes). All fungi were incubated at 27°C for 7 days in darkness and maintained on Potato Dextrose Agar (Vwr, USA) at 4°C. Spores and mycelium were recovered by scraping the surface of the plates with a sterile loop and transferred in sterile distilled water containing 0.2% (v/v) Tween 80. Spore concentration was adjusted to 10^5 spore/ml by counting using the Mallassez cell.

3. In vitro antifungal assay

In vitro screening of antifungal bacteria against phytopathogenic fungi was determined by dual cultural test, according to the method described by C.F. DIGUȚĂ & al [17]. Fresh mycelial plugs (5-mm-diameter) were transferred on the center of the PDA plates. Following this, fresh halotolerant bacterial strains were streaked in line at 2 cm apart from the centre of the PDA plate. The plates were incubated at 28°C for 7 days. The antifungal activity was evaluated by measuring in millimetres the inhibition zone (the distance between the mycelium and the bacterial colony).

4. Bacterial identification

Colonies' descriptors (color, shape and size) and morphological characteristics (Gram staining procedure, cells shape, endospore-forming) were examined after 24 h incubation at 37ºC. On specie-level, the identification of 24.5 isolate was confirmed by molecular tool. Genomic DNA extraction was performed by the use of ZR Fungal/Bacterial MiniPrep™ Kit (Zymo Research, USA), according to the manufacturer's instructions. The 24.5 isolate was identified in this study by analyzing unique nucleotide sequences of the *rpo*A, *gyr*A and 16S rDNA genes by multiplex PCR, as described by F. CAO & al [47]. PCR reactions were performed in 50 µl of 10X DreamTaq Green Buffer (contains 20 mM $MgCl_2$), 0.2 mM dNTPs, 0.5 mM of each primer, 0.025 U of DreamTaq DNA Polymerase (Thermo Scientific, USA) and 10 ng of bacterial DNA. PCR protocol was performed according to F. CAO & al [47]. The PCR products were detected by agarose gel electrophoresis (2% (w/v) agarose, 90 V, 60 min) followed by ethidium bromide staining. The gels were visualized with an ultraviolet transilluminator.

5. Detection of antifungal lypopeptides genes presence

Sequences of the gene-specific primer pairs used in this study are listed in Table 1. PCR amplifications were carried out in MultiGene Thermalcycler (Labnet International, Inc., Cambridge, United Kingdom) with the following conditions: 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 43°C (As1 F/Ts2 R), at 45°C (Af2 F/Tf1 R), at 55°C (ITUD F1/ITUD R1, ITUD1 F/IITUD1 R, bmyA F/bmyA R) for 30 s, and extension at 72°C for 75 s. Final elongation was at 72°C for 7 min. The band of the appropriate size was approximated using the DNA ladder (GeneRuler 100bp Plus DNA Ladder, Thermo Scientific, USA).

Table 1. Primer sequences

Lypopeptides	Genes	Primers	Primers sequence 5`-3`	Expected PCR product size (bp)	References
Fengycin	*fen*	Af2 F	GAATAYMTCGGMCGTMTKGA	443, 452	A. TAPI et al [48]
		Tf1 R	GCTTTWADKGAATSBCCGCC		
Iturin	*ituD*	ITUD F1	TTGAAYGTCAGYGCSCCTTT	482	S. CHUNG et al [49]
		ITUD R1	TGCGMAAATAATGGSGTCGT		
Iturin A	*ituA*	ITUD1 F	GATGCGATCTCCTTGGATGT	647	SARANGI et al [50]
		ITUD1 R	ATCGTCATGTGCTGCTTGAG		
Surfactin	*srf/lch*	As1 F	CGCGGMTACCGVATYGAGC	419-431	A. TAPI et al [48]
		Ts2 R	ATBCCTTTBTWDGAATGTCCGCC		
Bacillomycin	*bmyA*	bmyA F	CTCATTGCTGCCGCTCAATC	853	C.S. COMPAORÉ et al [36]
		bmyA R	CCG AAT CTA CGA GGG GAA CG		

6. Freeze-drying conditions

The halotolerant isolate 24.5 was grown in Nutrient Broth (Merck, Germany) at 37°C for 36 h (stationary phase). Cells were centrifuged (5 000 x *g*, 10 min, 4°C). The cell pellets were washed twice with distilled water and resuspended in 2 ml of solutions with the following cryoprotectant in four concentrations: maltodextrin noted as M (5%, 10%, 20%, 30% (*w*/*v*)) and sucrose noted as S (5%, 10%, 20%, 30% (*w*/*v*)). Solutions of the cryoprotectants were prepared with distilled water and were sterilized with 0.22 µm filter membrane (Merck, Germany). The bacterial suspension was frozen in sterile vials at –20°C, overnight. The frozen samples were conditioned in a chamber type freeze-drier (FreeZone6, LABCONCO, 6 Liter Benchtop Freeze Dry System, USA) for 4 h at –52°C and 0.04 mbar, conditions which may lead to products with <5% residual moisture.. Dried cells were stored at 4°C.

7. Viable cells estimation

Freeze-dried samples were rehydrated to the original volume with 2 ml sterile distilled water for 30 minutes at room temperature, and appropriate dilutions were seeded in Nutrient Agar. Plates were incubated at 37°C for 48 h; the number of viable cells per mililiter (CFU/ml) was counted from samples taken before and after freeze-drying. The survival rate was calculated by the following formula:

$$\text{Viability (\%)} = \frac{\text{Log CFU/ml after freeze drying}}{\text{Log CFU/ml before freezedrying}} \times 100$$

8. In vivo testing of the antifungal activity

The freeze-dried *Bacillus* 24.5 cells was assessed *in vivo* for its antifungal activity against molds on artificially infected tomatoes, lemons and corn, according to the method described by C.F. DIGUȚĂ & al [17]. Healthy samples were disinfected with 1% (v/v) sodium hypochlorite for 5 min, then rinsed three times with sterile distilled water and air-dried. The tomatoes and lemons were wounded (approximately 3 mm wide and 3 mm deep) using a sterile needle. The rehydrated bacterial suspension (10^7CFU/ml) was sprayed on each sample. Following this, 10 µl of mold spores suspension of (10^5 spores/ml) were inoculated into the wounds and 100 µl sprayed on the surface of the corn, respectively. The spore suspension of phytopathogenic fungi was used separately under the same conditions, as a positive control. Negative control was treated with the rehydrated bacterial suspension (107CFU/ml), under the same conditions. Then, the infected samples were placed in covered sterile plastic boxes to maintain high humidity conditions by adding 5 ml sterile water in each box and incubated at 28°C for 7 days. After the incubation period, the samples were examined and the percentage of fungal inhibition was determined as follows: Inhibition (%) = [(DP-DB)/DP] x 100 (DP is the disease incidence in trial treated by phytopathogenic fungi alone, DB is the disease incidence in trial co-inoculated with *Bacillus* 24.5 and phytopathogenic fungi. A reduction in disease incidence ranged 50%-70%, compared to the control treatment was adopted as criteria of selection of the best biocontrol bacteria C.F. DIGUȚĂ & al [17].

9. Statistical Analysis

All experiments were performed in triplicate. Data represented as mean ± standard deviation. Differences between groups were analysed by one-way ANOVA. *In vivo* testing of the antifungal activity was analysed by LSD post hoc test and Wilcoxon signed ranks test. $P < 0.05$ was considered statistically significant and all tests used were two-sided. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY).

Results

1. Screening of halotolerant bacterial strains with antifungal activity against a broad spectrum of phytopathogenic fungi

In this study, we have screened among eighteen halotolerant bacterial isolates, originating from a hypersaline water streams located in Lopatari, Buzău County, România, for their inhibitory activity on different species of phytopathogenic fungi, respectively *Alternaria alternata*, *Aspergillus carbonarius*, *A. flavus*, *A. niger*, *Botrytis cinerea*, *Penicillium digitatum, Fusarium oxysporum* and *Rhizopus* sp., under dual culture assay (Figure 1).

From the total of eighteen bacterial isolates, fifteen haven't proved any inhibitory activity on the fungal growth (Table 2). The isolate 47.5 inhibited the growth of a limited number of alteration fungi, respectively only on *Aspergillus carbonarius* and *Botrytis cinerea*. In the case of the isolate 11.5 positive results have been obtained on five molds, like *Alternaria alternata, Aspergillus carbonarius, A. niger, Botrytis cinerea* and *Fusarium oxysporum* (Table 2). For all tested bacteria, no inhibition has been noticed against *Rhizopus* spp.

Among the tested bacteria, the isolate 24.5 (Table 2, line bolded) exerted a broad-spectrum antifungal activity against seven phytopathogenic fungi, respectively on *Alternaria alternata, Aspergillus carbonarius, A. flavus, A. niger, Botrytis cinerea, Fusarium oxysporum, Penicillium digitatum*. As a consequence, the isolate 24.5 was selected for further investigations.

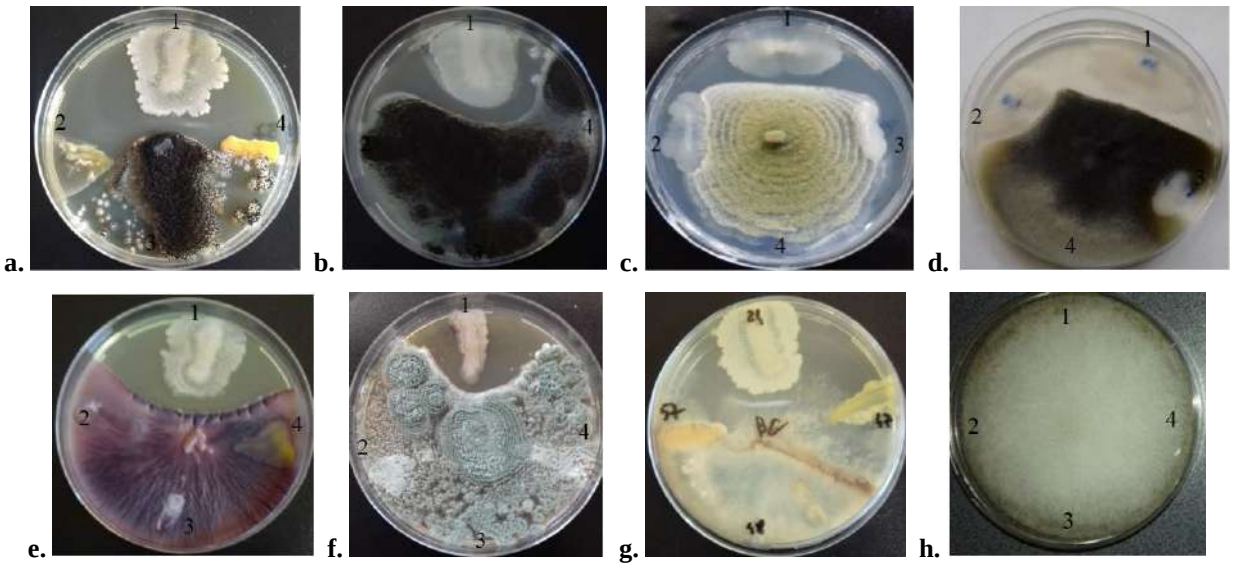

Figure 1. Aspects of antifungal activity screening of several halotolerant bacterial isolates:
1: 24.5, **2:** 47.5, **3:** 48.5, **4:** 57.5 against phytopathogenic fungi: **a.** *Aspergillus carbonarius*, **b.** *Aspergillus niger*, **c.** *Aspergillus flavus*, **d.** *Alternaria alternate*, **e.** *Fusarium oxysporum*, **f.** *Penicillium digitatum*, **g.** *Botryris cinerea* and **h.** *Rhizopus* sp.

Table 2. *In vitro* antifungal activity of halotolerant bacterial isolates (in mm of inhibition area)

Bacterial isolates code	*Aspergillus carbonarius*	*Aspergillus niger*	*Aspergillus flavus*	*Alternaria alternata*	*Botrytis cinerea*	*Fusarium oxysporum*	*Penicillium digitatum*	*Rhizopus* sp.
10.5	-[a]	-	-	-	-	-	-	-
11.5	7.50 ± 0.70*	5.25±1.26	-	3.00±0.00	9.50 ± 0.70	6.50 ± 0.70	-	-
13.5	-		-	-	-	-	-	-
14.5	-	-	-	-	-	-	-	-
24.5	**10.00 ± 1.41***	**6.50 ± 0.70**	**3.75 ± 0.35**	**3.50 ± 0.70**	**16.50 ± 2.12**	**9.00 ± 1.41**	**7.50 ± 0.70**	-
45.5	-	-	-	-	-	-	-	-
46.5	-	-	-	-	-	-	-	-
47.5	4.00 ± 0.00*	-	-	-	10.50 ±2.12	-	-	-
48.5	-	-	-	-	-	-	-	-
50.5	-	-	-	-	-	-	-	-
51.5	-	-		-	-	-	-	-
54.5	-	-	-	-	-	-	-	-
55.5	-	-	-	-		-	-	-
57.5	-	-	-	-	-	-	-	-
58.5	-	-	-	-	-	-	-	-
59.5	-	-	-	-	-	-	-	-
60.5	-	-	-	-	-	-	-	-
62.5	-	-	-	-	-	-	-	-

[a]- : absence of antifungal activity
The data are expressed as the mean ± SD.
Values followed by asterisk symbol (*) indicates significant differences ($P < 0.05$) among halotolerant bacterial isolates, according to ANOVA test.

2. Bacterial identification

In a first step, classical, morphological identification has been performed. The bacterial isolate 24.5 is forming flat or raised colonies, of cream colour and sometimes the margin of the colony became folded on Nutrient Agar. The macroscopic results have been completed by microscopic observations (rod-shaped cells, Gram positive, endospore-forming). Based on the preliminary phenotypic identification, the 24.5 isolate was identified as belonging to the genus *Bacillus*. Also, the other two halotolerant isolates (11.5 and 47.5) showing antifungal activity were identified as belonging to *Bacillus* group due to their morphological characteristics.

For additional confirmation at specie level, the isolate 24.5 was further identified using molecular methods. In a previous study, F. CAO & al [47] has chosen the more conserved *B. amyloliquefaciens* gyrase subunit A (*gyrA*) gene to discriminate from *B. subtilis*, *B. licheniformis* and *B. pumilus* by multiplex PCR. In our study, by multiplex PCR has amplified a PCR fragment estimated to be approximately at 730 bp, which corresponded to *B. amyloliquefaciens* profile (Fig. 2), as described by F. CAO & al [47].

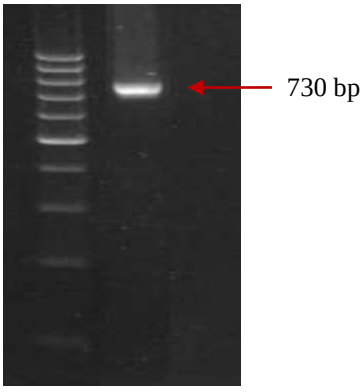

Figure 2. Identification of bacterial isolate 24.5 by multiplex PCR presented of the conserved gyrase subunit A (*gyrA*). The corresponding lanes are as follows: **M:** 100-bp DNA ladder, **1:** 24.5 and **2:** non-template control.

3. PCR detection of the genes involved in antimicrobial lipopeptide synthesis

The antifungal activity of *B. amyloliquefaciens* 24.5 strain could be due to the competition among space and or nutrition, but also could be correlated with the biosynthesis potential of antimicrobial compounds. Among the antimicrobial compounds synthesized by the different microorganisms, iturins, fengycin, surfactins are intensively studied for their strong inhibitory activity against plant phytopathogens and also for their mechanism to induce systemic resistance in plant.

In this study, the total DNA of the *Bacillus amyloliquefaciens* 24.5 has amplified five genes of interest *srf/lch*, *fen*, *ituD*, *ituA* and *bmyA* (Figure 3), coding the following lipoproteins: surfactin, fengycin, iturins and bacillomycin. This finding is coming to support our initial screening results for antifungal activity, knowing that these encoding genes were reported to be responsible for the suppression of wide range of phytopathogenic fungi (H. CAWOY & al [29], J. NANJUNDAN & al [38], H. CALVO & al [39], A.L. MOYNE & al [51]).

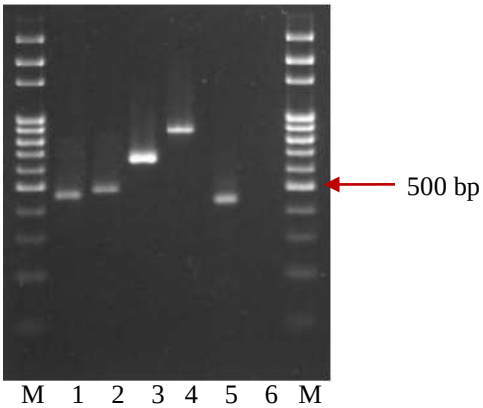

Figure 3. PCR amplification of various antimicrobial lipoprotein genes in *Bacillus amyloliquefaciens* 24.5. The corresponding lanes are as follows: **M:** 100-bp DNA ladder, **1:** *fen* (450bp), **2:** *ituD* (480bp), **3:** *ituA* (650bp), **4:** *bmyA* (850 bp), **5:** *srf/lch* (420bp) and **6:** non-template control.

4. Effect of cryoprotective agents on viability of freeze-dried Bacillus amyloliquefaciens 24.5

For the freeze-dried process, polysaccharides have been reported to ensure a good bacterial stability against freeze drying injuries, to have a low cost, to have a good matrix for rehydration, to do not affect the antifungal properties (D.A. SCHISLER & al [40], Y. ZHAN & al [41], A. GOTOR-VILA & al [42], L. HAN & al [43], S. STAMENKOVIĆ STOJANOVIĆ & al [44], G.R. NIREESHA & al [45]). In this study, the protective ability of two polysaccharides such as sucrose and maltodextrin (in four concentrations) was evaluated and compared against cells formulated without cryoprotectant (control). As procedure, have been used bacterial cells in the stationary phase because it was demonstrated that the cells in this phase are more resistant to a variety of chemical and physical stresses. The cells' viability was determined as described in methodological section and the results are presented in Table 3. No significant difference was observed between the tested protective agents. When *Bacillus amyloliquefaciens* 24.5 was suspended in sucrose and maltodextrin, the viability of cells after freeze drying ranged between 94-98%.

5. In vivo testing of the antifungal activity of freeze-dried Bacillus amyloliquefaciens 24.5

In our study, artificially infected tomatoes, lemons and corn were used to test the antifungal activity of freeze-dried *B. amyloliquefaciens* 24.5 cells (10^7 CFU/ml) with 5% sucrose (selected for *in vivo* testing) by co-inoculation technique. The growth of all fungal strains tested was significantly inhibited (Table 4).

The efficacy of *Bacillus amyloliquefaciens* 24.5 against *Alternaria alternata* and *Botrytis cinerea* was evaluated *in vivo* on wounded tomatoes (Table 4) and determined the reduction of fungal infections by *A. alternata* with 73.3% and *B. cinerea* with 100%, respectively.

B. amyloliquefaciens 24.5 inhibited the growth of *Penicillium digitatum* on lemons with almost 90% (Table 4).

Meanwhile, the tests proved that *B. amyloliquefaciens* 24.5 can effectively reduce the incidence of *F. oxysporum* to 99% on corn cops; (Table 4); also, can inhibit the growth of *A. flavus* to 84% and *A niger* to 75% (Table 4).

Table 3. Effect of cryoptrotectant agents on viability of *Bacillus amyloliquefaciens* 24.5 after freeze-drying

Parameters	**Control**	**Sucrose**				**Maltodextrin**			
		S5%	S10%	S20%	S30%	M5%	M10%	M20%	M30%
Log CFU/mL	5.9±0.14	7.3±0.07*	7.2±0.25*	7.1±0.35*	7.1±0.34*	7.3±0.08*	7.3±0.21*	7.3±0.31*	7.3±0.04*
% viability	**77.6**	**98.0**	**96.2**	**94.3**	**96.6**	**97.9**	**98.0**	**97.1**	**96.9**

The data are expressed as the mean ± SD.
Values followed by asterisk symbol (*) were significantly different from the control ($P < 0.05$) between treatments with cryoprotectants *versus* treatments without cryoprotectants, according to ANOVA test.

Table 4. Inhibitory activity of freeze-dried *Bacillus amyloliquefaciens* 24.5 against the growth of phytopathogenic fungi

Phytopathogenic fungi	**Biologic material**	**Growth inhibition (%) ***
Alternaria alternata	Tomatoes	73.3 ± 9.4
Aspergillus flavus	Corn	84.0 ± 5.7
Aspergillus niger	Corn	75.0 ± 7.1
Botrytis cinerea	Tomatoes	100.0 ± 0.0
Fusarium oxysporum	Corn	99.0 ± 1.4
Penicillium digitatum	Lemons	90.0 ± 14.1

The data are expressed as the mean ± SD.
Values followed by asterisk symbol (*) were significantly different from the control ($P < 0.05$) according to Wilcoxon Signed Ranks Test between treatments with co-inoculated with *Bacillus* 24.5 and phytopathogenic fungi *versus* treatments with phytopathogenic fungi alone.

Discussions

Fruits, vegetables and cereals are the main components of food and feed. These are exposed frequently to the attacks of pathogenic fungi (*Alternaria, Aspergillus, Botrytis, Fusarium, Penicillium* etc.) during the field growth, preharvest, postharvest, and storage, which causes significant economic losses (R. BARKAI-GOLAN & N. PASTER [1], M. BARTH & al [2], R.A. SAMSON & al [3], S. ROUSSEAUX & al [4], G. DOEHLEMANN & al [5], L. ESCRIVÁ & al [6]). *Alternaria alternata* is the most common specie in nature and causes the appearance of brown rot disease causing serious losses to tomatoes on both fruits and leaves (L. ESCRIVÁ & al [6]). *Botrytis cinerea* is responsible for the production of gray rot, causing serious losses to various plants such as: vegetables (tomatoes, cucumbers), and fruits (strawberries, apples, grapes) (M. BARTH & al [2]). Green mold caused by *Penicillium digitatum* is main postharvest pathogen of citrus fruit (R. BARKAI-GOLAN & N. PASTER [1]).

Fusarium sp. (*F. graminearum, F. culmorum, F. moniliforme, F. oxysporum* etc.) are the main fungi infecting cereals during the growing season, while fungi belonging to the genera *Aspergillus* (*A. flavus, A. ochraceus, A. niger* etc.) and *Penicillium* (*P. verrucosum, P. citrinum* etc.) are more associated with storage of crops (R.A. SAMSON & al [3], G. DOEHLEMANN & al [5]). Some species in these genera produce toxic secondary metabolites, called mycotoxins which can represent a risk to human and animal health (R. BARKAI-GOLAN & N. PASTER [1], S. ROUSSEAUX & al [4], L. ESCRIVÁ & al [6]). In the last years, special attention is paid to the biological control of the phytopathogenic fungi using antagonistic native bacteria, in the context of the eco-friendly alternative approaches applied for agricultural and environmental sustainability (S. DROBY & al [7], O. LASTOCHKINA & al [8], D. KOUR & al [9]).

In this study, eighteen halotolerant bacterial isolates, originating from a hypersaline water streams located in Lopatari, Buzău County, România were used as a starting point for *in vitro* antifungal screening against *Alternaria alternata, Aspergillus carbonarius, A. flavus, A. niger, Botrytis cinerea, Fusarium oxysporum, Penicillium digitatum, Rhizopus* sp. Strain 24.5 proved to have strongly inhibitory effects against the growth of the major fungal plant pathogens. No inhibitory activity of strain 24.5 against of *Rhizopus* spp. was found, which is not in accordance with A.T. CALDEIRA & al [52] who isolated *B. amyloliquefaciens* strain CCMI 1051 with strong inhibitory activity against *Rhizopus* sp. L-122. The isolate 24.5 was molecularly identified as *Bacillus amyloliquefaciens* by multiplex PCR developed F. CAO & al [47]. Also, A.I. GROSU & al [11] successfully used this multiplex PCR to identify *B. amyloliquefaciens* and *B. licheniformis* isolated from composted tea.

Numerous *in vitro* and *in vivo* antifungal tests were performed using *Bacillus amyloliquefaciens* cells, endospores, cells free supernatant (CFS) or purified lypopeptides on postharvest fruits and vegetables; the results have demonstrated the bioprotective abilities against a wide range of phytopathogenic fungi such as *Aspergillus, Alternaria, Botryosphaeria, Botrytis, Monilinia, Colletotrichum, Fusarium, Penicillium, Rhizoctonia, Rhizopus* genera (O.A. SICUIA & al [10], A.I. GROSU & al [11] D. VITULLO & al [12], A. DE SENNA & A. LATHROP [13], T. LEE & al [14], M.-D. URSAN & al [15], H. CALVO & al [18], M. ARFAOUI, & al [21], E. ARREBOLA & al [22], J. NANJUNDAN & al [38], H. CALVO & al [39], D. PRETORIUS & al [53], X. CHEN & al [54]). It has been reported that the application of cell-free supernatant (CFS) obtained from *B. amyloliquefaciens* inhibited the growth of *B. cinerea* (D. PRETORIUS & al [53]); CFS from *B. amyloliquefaciens* DH-4 (K. CHEN & al [55]) or *Bacillus* sp. w176 inhibited citrus green mold (*Penicillium digitatum*) (Z. TIAN & al [24]) and can provide new strategies to control phytopathogens. H. CALVO & al [18] demonstrated the potential of cells, endospores and CFS from *Bacillus amyloliquefaciens* BUZ-14 to inhibit the growth of *Botrytis cinerea, Monilinia fructicola, Monilinia laxa, Penicillium digitatum, Penicillium expansum,* and *Penicillium italicum* on postharvest fruit. *B. amyloliquefaciens* and *B. pumilus* were reported as effective biocontrol agents for controlling *Fusarium* wilt on tomatoes with beneficial effect on plant growth (N. HEIDARZADEH & al [56]), ochratoxigenic *Aspergillus* spp. (*A. ochraceus* and *A. carbonarius*) on grapes (M. ARFAOUI & al [21]). *Bacillus pumilus* TM-R suppressed the mycelial growth of five fungi (*Alternaria alternata, Cladosporium cladosporioides, Curvularia lunata, F. oxysporum,* and *P. italicum*) but promoted the growth of *Aspergillus niger* (T. MORITA & al [16]). In the study of I. DIMKIĆ & al [57], *B. amyloliquefaciens* strain SS-12.6, efficient producer of iturin and surfactin compounds, exhibited a strong antagonistic activity to control many fungal species such as *Alternaria alternata, Aspergillus flavus, Botryosphaeria obtusa, Colletotrichum acutatum, C. gloeosporioides, Fusarium oxysporum, F. solani, Monilinia fructigena, Mucor sp.* and *Penicillium expansum*.

The antifungal activity of *B. amyloliquefaciens* 24.5 strain could be due to the competition among space and or nutrition, but also could be correlated with the biosynthesis potential of antimicrobial compounds. The genes responsible for the biosynthesis of the four major lipopeptides (iturin, surfactin, fengycin and bacyllomicin) have been detected. Previous studies have reported that different *B. amyloliquefaciens* strains represent a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens, including the non-ribosomally synthesized cyclic lipopetides of the surfactin, iturin and fengycin families (D. VITULLO & al [12], A. ARGUELLES-ARIAS & al [28], H. CAWOY & al [29], S.P. CHOWDHURY & al [30], D. FIRA & al [37], J. NANJUNDAN & al [38], A. TAPI & al [48], A.L. MOYNE & al [51]).

Freeze drying has been reported by different authors as gentle and suitable technology for the production of dried bacterial cells formulations (D.A. SCHISLER & al [40], Y. ZHAN & al [41], A. GOTOR-VILA & al [42], L. HAN & al [43], S. STAMENKOVIĆ STOJANOVIĆ & al [44], G.R. NIREESHA & al [45]). The high survival potential of *Bacillus amyloliquefaciens* 24.5 (94-98%) during freeze drying process could be explained due its endospore production that provides tolerance under stressful conditions; meanwhile, should be taken into account the origin of the strain (salt lake) which is a stressful environment for the microorganisms due the high osmotic pressure; such living conditions may lead to different resistance mechanisms to physical stresses. According to G.I. PROCA & al [46], the isolate 24.5 showed tolerance up to 7.5% NaCl concentration. These results demonstrated that freeze drying process have been used successfully to obtain good formulated *Bacillus amyloliquefaciens* 24.5 products. L. HAN & al [43]

evaluated different cryoprotectants and centrifugal conditions to improve the survival of strain *Bacillus amyloliquefaciens* B1408, during freeze-drying. According to the authors, the survival rate of cells after freeze-drying was founded to be 91.24%, when used the suitable centrifugation conditions (5000 r/min, 10 min) and the optimized concentrations of cryoprotectants (glucose 1.00%, trehalose 4.74% and xylitol 1.45%). In the case of our strain, *Bacillus amyloliquefaciens* 24.5, the survival rate was even higher (98%).

Conclusions

In this study, we identified and characterised a halotolerant bacterial isolate, coded 24.5, as *Bacillus amyloliquefaciens* with high antifungal activity against a broad-spectrum phytopathogenic fungi. Strain 24.5 showed high survival rate when using two cryoprotective agents during freeze-drying process, showing a viability percentage ranging between 94-98%. The freeze dried cells maintained their antifungal ability. Further research will be focused to investigate the mechanism of the antifungal activity and to explore the *Bacillus amyloliquefaciens* 24.5 as ecofriendly biocontrol agent to a wider range of pathogens during the pre- and postharvest management.

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Conflict of interest

The authors declare that they have no conflict of interest.

Equal contribution: IRINEL GABRIEL PROCA, CAMELIA FILOFTEIA DIGUȚĂ

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