

SELECTED PLANT PROTECTION *Bacillus* STRAINS INCREASE FOOD SAFENESS BY INHIBITING HUMAN PATHOGENIC BACTERIA

Radu Cristian Toma¹, Oana-Alina Boiu-Sicuia^{1,2*}, Filofteia Camelia Diguță¹, Matilda Ciucă³, Florentina Matei¹, Călina Petruța Cornea¹

¹University of Agronomic Sciences and Veterinary Medicine of Bucharest, Mărăști Blvd, no. 59, 011464 Bucharest, Romania

²Research-Development Institute for Plant Protection, Ion Ionescu de la Brad Blvd, no. 8, 013813 Bucharest, Romania

³National Agricultural Research and Development Institute Fundulea, 915200 Fundulea, Călărași County, Romania

*Corresponding author. E-mail: sicuia_oana@yahoo.com

ABSTRACT

Food illnesses can occur due to the presence of human pathogen contaminants in fresh farm products. Herbs, vegetables and fruits, especially from organic agriculture, are highly exposed to animal and human pathogens. However, safe microbial antagonists, approved for plant protection, could be a solution to prevent this health risk to occur. The aim of this study is to reveal several beneficial bacterial strains reducing the prevalence of human and animal pathogens. Tested beneficial strains were previously described as promising biocontrol agents against soilborne pathogens of field crops and vegetables. Moreover, their endophyte adaptation, ensures an intimate relation with their plant hosts. Therefore, within this study we analyzed the inhibitory activity of seven biocontrol endophytes against 24 reference bacterial strains, of which 19 important human and animal pathogens. Some of the tested beneficial strains revealed antibacterial activity against a wide spectrum of pathogens, such as: *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria ivanovii*, *L. monocytogenes*, *Rhodococcus equi*, *Salmonella enterica*, *S. typhimurium*, *Staphylococcus aureus*, *S. epidermidis* and *Streptococcus pyogenes*. Due to their antagonistic activity, the beneficial strains were studied through molecular techniques to reveal their functional genes involved in antimicrobial compounds synthesis. Genes encoding for iturin A, surfactin, bacilysin, bacillomycin and bacillaene were found in these biocontrol strains. Therefore, we could consider such beneficial strains as promising candidates for plant protection and human safety.

Keywords: biocontrol endophytes, antagonism, pathogenic bacteria, functional genes.

INTRODUCTION

Today demand for low toxicity and eco-friendly agricultural inputs increased the trust in alternative control methods to chemicals (Brumă et al., 2021). Although for plant nutrition manure and organic composts are good to stimulate plant growth and development (Cirebea et al., 2020), as they increase soil fertility and improve soil structure, unfortunately sometime it could bring various pathogenic load. Studies have showed that using manure or compost, less than one-year old, increases 19 times the prevalence of *E. coli*, compared to older aged materials (Mukherjee et al., 2004).

Herbs and vegetables, as well as some fruits, especially from organic agriculture, are highly exposed to animal and human pathogens

contamination. For sure contamination can also occur during harvesting or while post-harvest handling (Alsanius et al., 2016). However, there are several cases explicitly linked to the consumption of organic plant products (Meerburg and Borgsteede, 2011). In 1992, a child lost his life due to an *E. coli* O157:H7 infection counteract while eating improper washed vegetables obtained from manure fertilized garden (Cieslak et al., 1993). In 1995, severe gastroenteritis and cases of haemolytic uraemic syndrome were attributed to *Citrobacter freundii* in a nursery school, were sandwiches with green butter, containing contaminated organic parsley, were given to the children (Tschäpe et al., 1995). Later on, in 2000, a comparative study was carried out in organic and conventionally produced lettuce and alfalfa sprouts, in order to quantify

the presence of important human pathogens. Higher load of *E. coli* (10^6 cfu/g) was found in organic lettuce spring mix (8 of 48 samples), compared to conventional spring mix (4 of 48 samples). *Salmonella* spp. was found in organic produced alfalfa sprouts in 7.7% cases (3 of 39 samples), while the conventional sprouts were free of pathogenic bacteria (Doyle, 2000).

There are also studies where there were no significant differences in the microbial load when analyzing different varieties of organic and conventional vegetables (Maffei et al., 2013). These showing that hygiene is very important in each production system.

Only a few studies were made to evaluate human pathogens presence in cereals. In Australia, Europe and United States, the prevalence of *B. cereus*, *E. coli*, and *Salmonella* spp. in wheat and flour is at low levels (Berghofer et al., 2003). However, in African countries, various cases of enteropathogenic *E. coli* were detected in maize and millet flours, in Côte d'Ivoire (Kouame N'zebo et al., 2017).

Unfortunately, important pathogens such as *Salmonella* spp., *L. monocytogenes* and *E. coli* could be present in organic fertilizers, such as manure (Johannessen et al., 2004). However, in the European Union, the new Regulation (EU) 2019/1009 on the fertilizing products available on the market provides safer organic fertilizers. Therefore, in certified organic agriculture, pathogens like *E. coli* or *Enterococcaceae* must not exceed 10^3 cfu in 1g or 1ml of organic fertilizer, while *Salmonella* spp. must be absent in 25 g or 25 ml of marketable product.

The European Union initiative, through the 'farm to fork' strategy, intend to facilitate the approval of beneficial microorganisms on the market, as plant protection products in order to reduce the dependence on synthetic chemicals. Therefore, starting from November 21st 2022 new regulations on the approval of microorganisms as active substances (Commission Regulation (EU) 2022/1438 amending Annex II to Regulation (EC) No 1107/2009), specific data requirements for such active substances (Commission Regulation (EU) 2022/1439, amending Regulation (EU)

No 283/2013) and commercial plant protection products (Commission Regulation (EU) 2022/1440, amending Regulation (EU) No 284/2013), as well as uniform principles for their evaluation and authorization [Commission Regulation (EU) 2022/1441, amending Regulation (EU) No 546/2011] are now applicable. However, several microbial based products are already available on the market (Abuhena et al., 2022), previously approved as plant protection products based on the same legislation as for synthetic pesticides. But new strains of microbial antagonists will be easier released on the market and used to control plant pathogens. Among plant beneficial microorganisms there could be found promising bio-fertilizing, bio-stimulant and biocontrol strains within *Bacillus subtilis* group (Siculia et al., 2015).

Bacillus spp. are promising microorganisms for agriculture. They are able to produce various growth promoting and biocontrol metabolites (Ek-Ramos et al., 2019; Boiu-Siculia and Cornea, 2020, 2021). Their enzymatic activity is useful not only in agriculture and environmental applications but also in several industries (Su et al., 2020). They are highly versatile and can adapt to various environmental conditions. Moreover, their sporulation ability makes them reliable for long term storage and extreme conditions, without affecting to much their viability (Kefi et al., 2015). An important aspect about *B. subtilis* is the fact that the European Food Safety Authority (EFSA) recognized it as qualified presumption of safety and accept it as zootechnical additive and plant protection product (Koutsoumanis et al., 2020; Spears et al., 2021). The use of *B. subtilis* and related bacteria as bio-based agro-inoculants is accepted also in organic farming (Ostroukhova et al., 2022). Strains of *Bacillus* sp., isolated from soil or fermented foods, demonstrated good antibacterial activity against human pathogens such as *B. cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *S. typhimurium* and *Staphylococcus aureus* (Avci et al., 2016).

Considering this, the aim of this study is to reveal several plant beneficial bacterial strains that can be used to promote plant

health and nutrition while reducing the prevalence of human and animal pathogens.

MATERIAL AND METHODS

Beneficial bacteria and growth conditions

Seven endophytic bacterial strains, isolated from various plant species grown in Romania, were used in this work (Table 1). These strains were previously selected for their plant beneficial characteristics (Boiu-Sicuia and Cornea, 2021), such as antagonism against various pathogenic fungi, or traits related to plant growth promotion.

Pathogenic bacteria and growth conditions

Various human and animal pathogenic bacteria were used in this study (Table 2). Both Gram positive and Gram negative, of 11

different genera, some of them antibiotic resistant strains. Beside pathogenic strains, commensals and non-pathogenic species, were also used as sensitive references for antibacterial tests. All strains used for bactericide testing were reference strains from the American Type Culture Collection (ATCC).

Endophytes were freshly grown on Luria Bertani (LB, Carl Roth GmbH + Co.KG) at 28°C. The submerged cultures were incubated under orbital shaking at 150 rpm. Cultures were stored in LB Broth with 25 % glycerol at -20°C.

These bacteria were stored in Tryptic Soy Broth (TSB, Oxoid™) with 20% glycerol at -20°C. Cultures were refreshed on Tryptic Soy Agar (TSA, Oxoid™) and routinely cultured on TSB at 37°C before each trial.

Table 1. Sources of plant beneficial bacteria

Endophytic Strain	Isolation source	
	Host plant	Plant part
LT MYM 1	<i>Lavandula angustifolia</i>	lavender stem
LFF MYM 5	<i>Lavandula angustifolia</i>	lavender leaves and flowers
St 1T2	<i>Solanum tuberosum</i>	potato tuber
E1Pv	<i>Phaseolus vulgaris</i>	bean roots
BPVs2	<i>Phaseolus vulgaris</i>	bean seeds
BAHs1	<i>Arachis hypogaea</i>	peanut seeds
BTA3	<i>Triticum aestivum</i> cv. Glosa	wheat kernels

Table 2. Pathogenic bacteria and other reference strains

Species	Type strain	Special characteristics
Gram positive bacteria		
<i>Bacillus cereus</i>	ATCC 11778	human and animal pathogen; it can cause foodborne illness and a wide range of opportunistic infections.
<i>B. subtilis</i>	ATCC 6633 ⁱ	non-pathogenic specie; antibiotic sensitive strain
<i>Enterococcus faecalis</i>	ATCC 29212	vancomycin-sensitive; could be found as opportunistic pathogen
<i>Ent. Faecium</i>	ATCC 6057 ⁱⁱ	commensal bacterium which gain prominence as a nosocomial pathogen
<i>Ent. Hirae</i>	ATCC 10541	zoonotic pathogen
<i>Listeria innocua</i>	ATCC 33090 ⁱ	non-pathogenic specie
<i>L. ivanovii</i>	ATCC 19119	infect ruminants, and is rarely reported as human pathogen
<i>L. monocytogenes</i>	ATCC 7644	human and animal pathogen responsible for food borne illness.
	ATCC 13932	enteric and infectious disease
<i>Rhodococcus equi</i>	ATCC 6939	responsible for infections in multiple-hosts, animals and humans
	ATCC 6538	human pathogenic specie
<i>Staphylococcus aureus</i> subsp. <i>aureus</i>	ATCC 25923	human pathogenic specie
	ATCC 33592	gentamicin and methicillin resistant strain; responsible for a wide spectrum of clinical infections
	ATCC 43300	methicillin and oxacillin resistant strain; responsible for a wide spectrum of clinical infections
<i>S. epidermidis</i>	ATCC 12228	vancomycin sensitive strain; important human opportunistic pathogen of nosocomial infections
	ATCC 51625	methicillin-resistant strain; important human opportunistic pathogen of nosocomial infections
<i>Streptococcus pyogenes</i>	ATCC 19615	human pathogenic specie
Gram negative bacteria		
<i>Citrobacter freundii</i>	ATCC 43864	responsible for nosocomial infections and diarrheal infections; it become a multidrug resistant specie
<i>Escherichia coli</i>	ATCC 8739	faecal strain; known as a human and animal pathogen
	ATCC 25922	human and animal pathogen
<i>Proteus hauseri</i>	ATCC13315 ⁱⁱ	commensal of the normal flora of human gastrointestinal tract, that can switch in opportunistic pathogen
<i>Pseudomonas aeruginosa</i>	ATCC 9027 ⁱ	can cause animals and humans diseases; non-virulent strain
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Enteritidis</i>	ATCC13076	an enteric foodborne pathogen
<i>S. typhimurium</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i>	ATCC 14028	an enteric foodborne pathogen; multiple antibiotic resistances.

Legend: ⁱNon-pathogenic bacteria or non-virulent strain; ⁱⁱCommensal bacteria.

Bacterial identification procedure

Endophytic bacteria were identified based on Biolog technique, using the semi-automated GEN III system, according to the manufacturer guidelines. Therefore, fresh cultures were prepared on Biolog Universal Growth (BUG) media. From the fresh cultures, single colonies were suspended in Biolog type B inoculation fluid up to 97% turbidity, in 590 nm light. Obtained bacterial suspension was inoculated in Biolog GEN III microplate, using 100 µl/well. Plates were incubated at 33°C. The microbial identification was made after 24 and 48 h of incubation using the semi-automat Biolog MicroStation Plate Reader coupled to the

Microbial Identification Databases for Biolog Systems. The GEN III redox chemistry reveals the physiological prophyll of the analyzed strain and compares it to other 1568 taxa. The microbial preferences to metabolize certain carbon sources and tolerance certain chemicals, reveals the unique pattern of the strain and enables the identification to genus or species level if there is a proper correlation with the systems' database.

Plate screening of bacterial antagonism

The spot diffusion assay was used to evaluate the antibacterial activity of endophytic strains against mentioned human and animal pathogenic

bacteria. Tests were performed in vitro, on TSA medium. Overnight grown pathogens were inoculated in the melted agar in 1:10 (v/v) ratio, using up to 0.3 OD cell suspension quantified at 600 nm. On top of the solidified pathogenic cultures tested endophytes were inoculated in spots, using 5 µl of 24 h old plant beneficial bacteria. Control plates were also prepared, were instead of the pathogen, antibiotic sensitive strains were used. Tests were performed in triplicate and incubated at 30°C. Plates were analyzed after 24 to 48 h of incubation and the antibacterial activity was evaluated by measuring the inhibitory halo revealed around the endophytic strains. Pathogens were considered sensitive to the endophytes if the inhibitory halo were completely clear, while tolerant pathogens were considered those which revealed a tern growth in the presence of the antagonistic strain.

Antibacterial functional genes and PCR conditions

The presence of certain functional genes involved in antibacterial compounds synthesis was carried out by polymerase chain reaction (PCR).

Certain primers pairs were used in this study to reveal genes encoding for antimicrobial compounds (Table 3). The PCR mix was performed in 25 µl reaction volume containing 1X Buffer, 2 mM MgCl₂, 0.2 mM dNTPs (ThermoScientific LSG), 0.5 µM of each primer (Alpha Scientific Solutions), 0.25U of MangoTaq DNA Polymerase (BioLine) and 20 ng of template DNA. The DNA was purified using the ZR Fungal/Bacterial MiniPrep kit (Zymo Research, USA) according to Zaharia et al. (2022) protocol.

Table 3. Primers used in this study

Antibacterial compound	Gene	Primers	Primer sequence 5' - 3'	Amplification product (bp)	Annealing temperature	Reference
Iturin A	<i>ituA</i>	ITUD1 f	GATGCGATCTCCTTGGATGT	647	55°C	Sarangi et al., 2017
		ITUD1 r	ATCGTCATGTGCTGCTTGAG			
Surfactin	<i>urfA</i>	SrfA F1	AGAGCACATTGAGCGTTACAAA	626	55°C	Chung et al., 2008
		SrfA R1	CAGCATCTCGTTCAACTTTCAC			
Bacilysin	<i>Bac A/B</i>	bacA/B F	TGCTCTGTTATAGCGCGGAG	910	55°C	Compaoré et al., 2013
		bacA/B R	GTCATCGTATCCCACCCGTC			
Bacillomycin	<i>bmyA</i>	bmyA F	CTCATTGCTGCCGCTCAATC	853	55°C	Compaoré et al., 2013
		bmyA R	CCGAATCTACGAGGGGAACG			
Bacillaene	<i>baeA</i>	BaeR F	ATGTCAGCTCAGTTTCCGCA	688	55°C	Compaoré et al., 2013
		BaeR R	GATCGCCGTCTTCAATTGCC			

The PCR reaction involved one step of 4 minutes at 94°C for initial denaturation, followed by 30 cycles in three steps, one of 30 seconds at 94°C for denaturation, a second step of 30 seconds at 55°C for primers' annealing and the third step of 75 seconds at 72°C for elongation, with a final elongation step of 7 minutes at 72°C.

The amplified products (7 µl PCR product) were analyzed by 1.2 % (w/v) agarose gel electrophoresis in TBE buffer (Tris 84 mM; boric acid 89 mM; EDTA 2mM, pH 8-8.5) containing 1 µg/ml (w/v) of ethidium bromide. The migration was performed at 100 V for 1 h, while the analysis was made under UV-light exposure, using the BioDoc-It

Imaging System. Molecular weight of the bands was estimated by co-migration and band comparison with a 1 Kbp DNA ladder (ThermoScientific LSG). The PCR was scored positive when amplicons of appropriate size were detected.

RESULTS AND DISCUSSION

Bacterial phenotypic identification

The Biolog GEN III analysis revealed the biochemical profile of the analyzed endophytic strains. The phenotypic fingerprint of each studied bacterial strain was compared with the pattern of all references from the Biolog database, using the MicroLog3 software.

Based on their similarities, the studied strains were identified at specie level. All studied endophytic bacteria were closely related and belong to *Bacillus subtilis* group (LT MYM 1, LFF MYM 5, BPVs2, E1Pv, BAHs1 and BTAs3). The St 1T2 strain was identified as *B. pumilus*, which is also included in *Bacillus* group.

Antibacterial activity

The diffusion assay was used to determine antibacterial activity against the 24 ATCC

strains, of which 19 important human and animal pathogens. When no growth developed from the pathogenic strains around the biocontrol spots, the pathogens were considered sensitive to antibacterial compounds released by the beneficial bacteria (Figure 1a). However, some pathogens could develop at reduced density in the presence of the biocontrol endophytes. In such cases pathogens were considered tolerant to the antimicrobial compounds or their dose (Figure 1b).

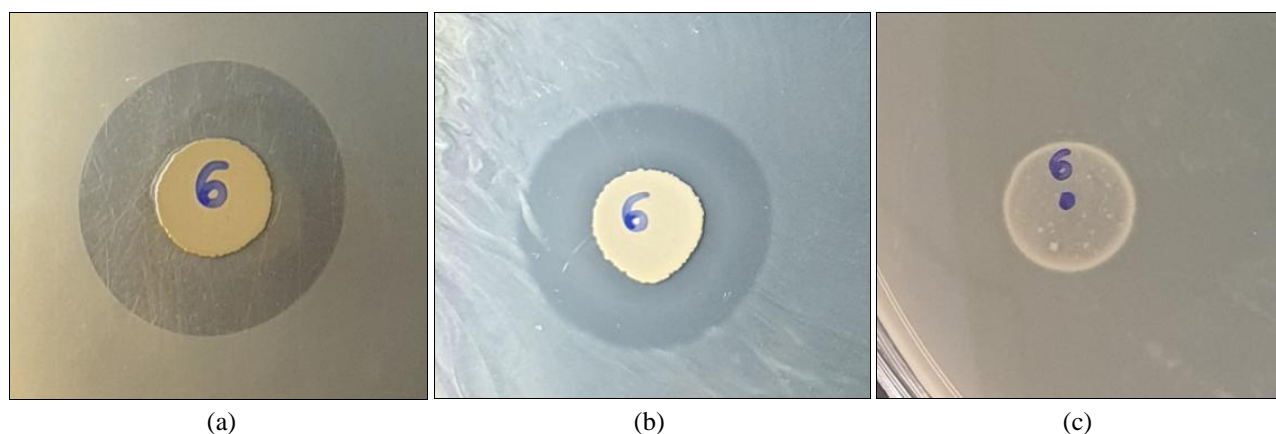


Figure 1. BAHs1 endophytic strain against different bacterial pathogens.

- a) Complete inhibition of *L. monocytogenes* ATCC7644; b) reduced pathogenic growth of *R. equi* ATCC6939; c) no inhibition of *P. aeruginosa* ATCC 9027 growth

Some of the studied strains revealed antibacterial activity against a wide spectrum of pathogens, such as: *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria ivanovii*, *L. monocytogenes*, *Rhodococcus equi*, *Salmonella enterica*, *S. typhimurium*,

Staphylococcus aureus, *S. epidermidis* and *Streptococcus pyogenes* (Table 4). However, an antibacterial activity should be considered if inhibition zone has maintained for at least 1 mm in the first 48 h of co-cultivation.

RADU CRISTIAN TOMA ET AL.: SELECTED PLANT PROTECTION *Bacillus* STRAINS INCREASE FOOD SAFENESS BY INHIBITING HUMAN PATHOGENIC BACTERIA

Table 4. Antibacterial activity of plant beneficial endophytes used in this study

Human pathogens	Plant beneficial endophytes						
	LT MYM 1	LFF MYM 5	St 1T2	E1Pv	BPVs2	BAHs1	BTAs3
<i>Bacillus cereus</i> ATCC 11778	0.5 S	1.0 S	1.0 S	0.5 S+2.0 T	2.0 S	1.0 S	1.0 S
<i>B. subtilis</i> ATCC 6633	0	0	0	0.5 T	0.5 S + 3.0 T	0.5 S	0,5 S
<i>Ent. Faecalis</i> ATCC 29212	0	2.0 T	2.0 T	3.0 T	1.0 T	1.5 T	1.5 T
<i>Ent. Faecium</i> ATCC 6057	1.0 T	1.0 T	0	1.5 T	1.0 T	1.0 T	2.0 T
<i>Ent. Hirae</i> ATCC 10541	2.0 S	1.5 S	0	0	0.5 T	0.5 T	1.0 T
<i>L. innocua</i> ATCC 33090	2.0 T	2.0 T	1.5 T	3.0 T	2.0 S+1.0 T	2.0 T+1.0 S	3.0 T
<i>L. ivanovii</i> ATCC 19119	3.0 S+1.0 T	1.0 S+3.0 T	3.0 S	4.5 S	5.0 S	5.0 S	5.0 S
<i>L. monocytogenes</i> ATCC 7644	1.5 S	2.0 S+3.0 T	2.0 S+1.0 T	4.0 T	5.0 S	3.0 S	4.0 S
<i>L. monocytogenes</i> ATCC 13932	2.0 T	2.0 T	1.5 T	1.0 T	3.0 T	2.0 T	2.5 T
<i>R. equi</i> ATCC 6939	2.0 S	3.0 S+2.0 T	2.0 S+3.0 T	3.0 T	5.0 S	3.0 S+2.0 T	4.0 S+2.0 T
<i>S. aureus</i> ATCC 6538	1.5 T	1.0 T	3.5 T	0.5 T	1.0 T	2.5 T	2.5 T
<i>S. aureus</i> ATCC 25923	2.0 T	2.5 T	0.5 T	2.0 T	2.0 T	1.5 T	2.5 T
<i>S. aureus</i> ATCC 33592	2.0 T	1.5 S	3.0 T	1.5 T	1.0 T	0.5T	1.0 T
<i>S. aureus</i> ATCC 43300	2.0 T	1.0 T	0.5 T	0.5 T	1.0 T	2.0 T	3.0 T
<i>S. epidermidis</i> ATCC 12228	2.0 T	3.0 T	3.0 T	4.0 T	2.5 T	3.0 T	4.0 T
<i>S. epidermidis</i> ATCC 51625	1.5 T	3.0 T	5.0 T	2.0 T	3.0 T	3.0 T	4.0 T
<i>Streptococcus pyogenes</i> ATCC 19615	0	1.0 T	3.0 T	0	3.0 T	2.0 T	4.0 T
<i>Citrobacter freundii</i> ATCC 43864	0.5 T	1.0 T	0	0	1.0 T	1.0 T	0.5 T
<i>Escherichia coli</i> ATCC 8739	1.0 T	1.0 T	0	0.5 T	1.0	1.0 T	0.5 T
<i>E. Coli</i> <i>F.</i> ATCC 25922	2.0 T	2.0 T	2.0 T	0	2.0 S	1.0 T	2.0 T
<i>Proteus hauseri</i> ATCC 13315	2.5 T	2.0 T	0	1.5 T	1.5 T	2.0 T	2.0 T
<i>Pseudomonas aeruginosa</i> ATCC 9027	0	0	0	0	0	0	0
<i>Sal. Enterica</i> ATCC 13076	2.0 T	3.0 T	1.5 T	4.0 T	2.0 T	2.0 T	3.0 T
<i>Sal. Typhimurium</i> ATCC 14028	1.0 T	1.0 T	0	1.0 T	1.0T	1.0 T	1.0 T

Legend: S = sensitive, no pathogenic growth was developed; T = tolerant, the pathogenic growth was reduced; 0 - resistance, the pathogenic growth was not disturbed.

The endophytic, plant beneficial strains were more efficient in suppressing Gram positive pathogenic bacteria. Against *B. cereus*, *L. ivanovii*, *L. monocytogenes* and *R. equi* the effect was bactericidal. The pathogenic growth was completely suppressed for up to 5 mm radius around the endophyte spots, depending on the tested strains. In all other cases of antibacterial activity, the pathogens were not completely suppressed, but only delayed, as they could developed at reduced density. Among the tested strains, best results

were obtained with the lavender and seeds endophytes.

Molecular detection of antibacterial functional genes

The endophytic bacterial strains were analyzed through molecular techniques in order to detect five functional genes (Table 5), each encoding for a different antimicrobial compound (iturin A, surfactin, bacilysin, bacillomycin and bacillaene).

Table 5. Molecular detection of the genes encoding antimicrobial compounds

Endophytic strain	Functional gene				
	<i>ituA</i>	<i>urfA</i>	<i>bacA/B</i>	<i>bmyA</i>	<i>baeA</i>
LT MYM 1	+	-	+	+	+
LFF MYM 5	+	-	+	+	+
St 1T2	-	-	-	-	-
E1Pv	-	+	-	-	+
BPVs2	+	-	+	+	+
BAHs1	+	-	+	+	+
BTAs3	+	-	+	+	+

Legend: present (+) / absent (-) functional gene.

Regarding endophytes potential to produce antimicrobial compounds, best results were obtained with those strains isolated from the lavender and seeds (LT MYM 1, LFF MYM 5, BPVs2, BAHs1 and BTAs3). They are able to produce iturin A (Figure 2a), surfactin,

bacilysin, bacillomycin and bacillaene. These results explaining the higher antibacterial activity of this strains. The gene encoding for surfactin synthesis was detected only in E1Pv strain (Figure 2b).

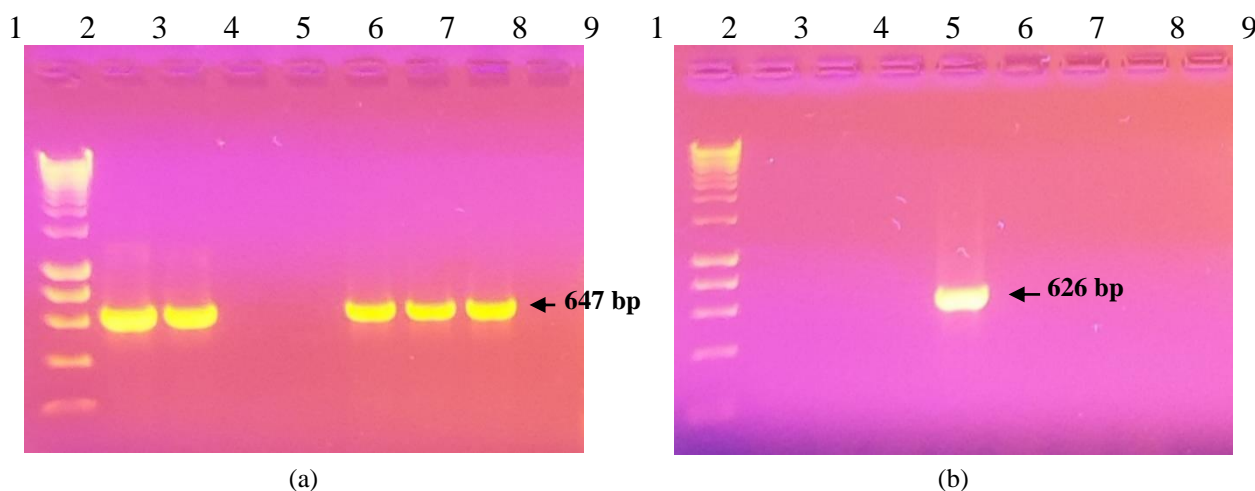


Figure 2. Example of electrophoretic profiles of the PCR products revealing *ituA* (a) and *urfA* (b) genes
Lines: 1 – 1 Kbp DNA marker, 2 – LT MYM 1 strain, 3 – LFF MYM 1 strain, 4 – St 1T2 strain,
5 – E1Pv strain, 6 – BPVs2 strain, 7 – BAHs1 strain, 8 – BTAs3 strain, 9 – Negative control.

In the present study, the seven endophytic bacteria were identified based on the phenotypic/biochemical profiles obtained in the Biolog GEN III MicroPlate assay. Thus, it was revealed they belong to the *Bacillus subtilis* group and *B. pumilus*. Identifying endophytic bacteria from *Bacillus subtilis* group is a common aspect revealed in many studies (Xia et al., 2013; Lopes et al., 2015; Jasim et al., 2016; Bolivar-Anillo et al., 2021), some of them highly reliable, as their genome was complete or partial sequenced (Deng et al., 2011; Jeong et al., 2014; Sun et al., 2015; Cai et al., 2016). *Bacillus* spp. are ubiquitous bacteria; they could be found in various environments (Schultz et al., 2017), including as endophytes (Gond et al., 2015; Shahzad et al., 2016; Boiu-Sicuia and Cornea, 2019; Cheng et al., 2020).

Although *Bacillus* spp. are known as promising biocontrol microorganisms (Etesami and Alikhani, 2018). Several studies mention them also to have the ability to inhibit human and animal pathogens, such as *Bacillus cereus*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *S. typhimurium* and *Staphylococcus aureus* (Avci et al., 2016). However, the *Bacillus* strains used in this study were not able to inhibit *Pseudomonas aeruginosa* growth. Our strains were more efficient against Gram-positive pathogenic bacteria, some strains having bactericidal activity against *B. cereus*, *L. ivanovii*, *L. monocytogenes*, and *Rhodococcus equi*.

Most studies revealing beneficial bacteria inhibiting or suppressing human and animal pathogens are those focused in lactic acid bacteria (Vătuui and Popa, 2015). Although these bacteria are generally recognized as safe (de Lacerda et al., 2016), their use as agro-inoculants is limited. Still, *Bacillus* are having multiple qualities, they are having wide ecological plasticity, are spore forming bacteria (Kefi et al., 2015), and can produce a wide variety of enzymes (Su et al., 2020) and biologic active compounds (Ek-Ramos et al., 2019).

An important aspect for which they are used as biocontrol agents is their ability to

produce various antimicrobial compounds with antifungal and antibacterial activity. Among the important metabolites of *Bacillus* spp. that demonstrated antibacterial activity are bacteriocines and certain classes of lipopeptides, such as iturins and surfactins, as well as bacilysin and bacitracin A and F (Caulier et al., 2019).

In this study, the lavender and seeds endophytes (LT MYM 1, LFF MYM 5, BPVs2, BAHs1 and BTAs3) revealed to possess genes involved in iturin A, bacilysin, bacillomycin and bacillaene antimicrobial compounds synthesis. Such compounds are mentioned as antibacterial compounds in various studies (Fira et al., 2018; Patel et al., 1995), thus sustaining the results.

CONCLUSIONS

Selected endophytic *Bacillus* strains could be considered promising biocontrol agents due to their antimicrobial activity. The selected lavender and seeds endophytes (LT MYM 1, LFF MYM 5, BPVs2, BAHs1 and BTAs3) could be used in both conventional and organic farming as they are generally recognized as safe microbial inoculants. They revealed antagonistic activity against important multidrug resistant and highly virulent human and animal pathogens. Moreover, their antibiotic compounds are of different nature than those of pharmaceutical use, thus they have no restrictions to be used in agriculture.

ACKNOWLEDGEMENTS

This research was funded by the University of Agronomic Sciences and Veterinary Medicine of Bucharest, project number 2021-0005/13.07.2021 - Developing a microbial bio-fertilizer technology.

REFERENCES

- Abuhena, M., Al-Rashid, J., Azim, M.F., Khan, M.N.M., Kabir, M.G., Barman, N.C., Rasul, N.M., Akter, S., Huq, M.A., 2022. Optimization of industrial (3000 L) production of *Bacillus subtilis* CW-S and its novel application for minituber and industrial-grade potato cultivation. Scientific Reports, 12: 11153.

- Alsanius, B., Dorais, M., Doyle, O., Oancea, F., Spadaro, D., 2016. *Potential food hazards from organic greenhouse horticulture*. Bio GreenHouse, doi: 10.18174/375668.
- Avci, A., Üzmez, S., Alkan, F.B., Bagana, İ., Nurçeli, E., Çiftçi, E., 2016. *Antimicrobial activity spectrums of some Bacillus strains from various sources*. GIDA, 41(5): 323-328. doi: 10.15237/gida.GD16036
- Berghofer, L.K., Hocking, A.D., Miskelly, D., Jansson, E., 2003. *Microbiology of wheat and flour milling in Australia*. International Journal of Food Microbiology, 85: 137-149.
- Boiu-Sicuia, O.A., and Cornea, C.P., 2019. *Isolation procedures for endophytes harvesting*. AgroLife Scientific Journal, 8(1): 43-52.
- Boiu-Sicuia, O.A., and Cornea, C.P., 2020. *Bacterial endophytes improving plant growth*. AgroLife Scientific Journal, 9(2): 56-70.
- Boiu-Sicuia, O.A., and Cornea, C.P., 2021. *Bacterial strains involved in soilborne phytopathogens inhibition*. Scientific Papers, Series A, Agronomy, LXIV(1): 641-646.
- Bolivar-Anillo, H.J., González-Rodríguez, V.E., Cantoral, J.M., García-Sánchez, D., Collado, I.G., Garrido, C., 2021. *Endophytic bacteria Bacillus subtilis, isolated from Zea mays, as potential biocontrol agent against Botrytis cinerea*. Biology, 10: 492.
- Brumă, I.S., Rodino, S., Petcu, V., Micu, M.M., 2021. *An overview of organic sunflower production in Romania*. Rom. Agric. Res., 38: 495-504.
- Cai, X., Kang, X., Xi, H., Liu, C., Xue, Y., 2016. *Complete genome sequence of the endophytic biocontrol strain Bacillus velezensis CC09*. Genome Announcements, 4: e01048-16.
- Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., Mahillon, J., 2019. *Overview of the antimicrobial compounds produced by members of the Bacillus subtilis group*. Frontiers in Microbiology, 10: 302.
- Cheng, T., Yao, X.Z., Wu, C.Y., Zhang, W., He, W., Dai, C.C., 2020. *Endophytic Bacillus megaterium triggers salicylic acid-dependent resistance and improves the rhizosphere bacterial community to mitigate rice spikelet rot disease*. Applied Soil Ecology, 156: 103710.
- Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S.D., 2008. *Isolation and partial characterization of Bacillus subtilis ME488 for suppression of soilborne pathogens of cucumber and pepper*. Applied Microbiology and Biotechnology, 80(1): 115-123.
- Cieslak, P.R., Barrett, T.J., Griffin, P.M., Gensheimer, K.F., Beckett, G., Buffington, J., Smith, M.G., 1993. *Escherichia coli O157:H7 infection from a manured garden*. The Lancet, 342(8867): 367.
- Cirebea, M., Rotar, I., Vidican, R., Pleșa, A., Morea, A., Ranta, O., 2020. *Impact of organo-mineral fertilization upon phytocoenosis and feed quality of the grasslands in the region of Transylvania*. Rom. Agric. Res., 37: 179-188.
- Compaoré, C.S., Nielsen, D.S., Sawadogo-Lingani, H., Berner, T.S., Nielsen, K.F., Adimpong, D.B., Diawara, B., Ouédraog, G.A., Jakobsen, M., Thorsen, L., 2013. *Bacillus amyloliquefaciens ssp. plantarum strains as potential protective starter cultures for the production of Bikalga, an alkaline fermented food*. Journal of Applied Microbiology, 115: 133-146.
- de Lacerda, J.R.M., da Silva, T.F., Vollú, R.E., Marques, J.M., Seldin, L., 2016. *Generally recognized as safe (GRAS) Lactococcus lactis strains associated with Lippia sidoides Cham. are able to solubilize/mineralize phosphate*. SpringerPlus, 5(1): 828.
- Deng, Y., Zhu, Y., Wang, P., Zhu, L., Zheng, J., Li, R., Ruan, L., Peng, D., Sun, M., 2011. *Complete genome sequence of Bacillus subtilis BSn5, an endophytic bacterium of Amorphophallus konjac with antimicrobial activity for the plant pathogen Erwinia carotovora subsp. carotovora*. Journal of Bacteriology, 193: 2070-2071.
- Doyle, M.P., 2000. *Reducing foodborne disease: what are the priorities?* Nutrition, 16: 647-649.
- Ek-Ramos, M.J., Gomez-Flores, R., Orozco-Flores, A.A., Rodríguez-Padilla, C., González-Ochoa, G., Tamez-Guerra, P., Tamez-Guerra, P., 2019. *Bioactive products from plant-endophytic Gram-positive bacteria*. Frontiers in Microbiology, 10: 1-12.
- Etesami, H., Alikhani, H.A., 2018. *Bacillus species as the most promising bacterial biocontrol agents in rhizosphere and endorhiza of plants grown in rotation with each other*. European Journal of Plant Pathology, 150: 497-506.
- Fira, D., Dimkić, I., Berić, T., Lozo, J., Stanković, S., 2018. *Biological control of plant pathogens by Bacillus species*. Journal of Biotechnology, 285: 44-55.
- Gond, S.K., Bergen, M.S., Torres, M.S., 2015. *Endophytic Bacillus spp. produce antifungal lipopeptides and induce host defence gene expression in maize*. Microbiological Research, 172: 79-87.
- Jasim, B., Sreelakshmi, K.S., Mathew, J., Radhakrishnan, E.K., 2016. *Surfactin, iturin, and fengycin biosynthesis by endophytic Bacillus sp. from Bacopa monnieri*. Microbial Ecology, 72: 106-119.
- Jeong, H., Choi, S.K., Kloepper, J.W., Ryu, C.M., 2014. *Genome sequence of the plant endophyte Bacillus pumilus INR7, triggering induced systemic resistance in field crops*. Genome Announcements, 2: e01093-14.
- Johannessen, G.S., Froseth, R.B., Solemdal, L., Jarp, J., Wasteson, Y., Rorvik, L.M., 2004. *Influence of bovine manure as fertilizer on the bacteriological quality of organic Iceberg lettuce*. Journal of Applied Microbiology, 96(4): 787-794.
- Kefi, A., Slimene, I.B., Karkouch, I., Rihouey, C., Azaeiz, S., Bejaoui, M., Belaid, R., Cosette, P., Jouenne, T., Limam, F., 2015. *Characterization of endophytic Bacillus strains from tomato plants (Lycopersicon esculentum) displaying antifungal activity against Botrytis cinerea Pers.* World

RADU CRISTIAN TOMA ET AL.: SELECTED PLANT PROTECTION *Bacillus* STRAINS INCREASE FOOD SAFENESS BY INHIBITING HUMAN PATHOGENIC BACTERIA

- Journal of Microbiology and Biotechnology, 31: 1967-1976.
- Kouame N'zebo, D., Dadie, A., Anin-Atchibri, O.L., Kouassi, N., Dje K.M., 2017. *Prevalence of Enteropathogenic Escherichia coli in Maize (Zea mays) or Millet (Pennisetum glaucum) Flours and Porridges*. International Journal of Current Microbiology and Applied Sciences, 6(1): 819-833.
- Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Koutsoumanis, K., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., 2020. *Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 12: suitability of taxonomic units notified to EFSA until March 2020*. EFSA Journal, 18(7): 6174.
- Lopes, R.B.M., Costa, L.E., Vanetti, M.C., de Araujo, E.F., de Queiroz, M.V., 2015. *Endophytic bacteria isolated from common bean (Phaseolus vulgaris) exhibiting high variability showed antimicrobial activity and quorum sensing inhibition*. Current Microbiology, 71: 509-516.
- Maffei, D.F., Ferraz de Arruda Silveira, N., Mortatti Catanozi, M.P.L., 2013. *Microbiological quality of organic and conventional vegetables sold in Brazil*. Food Control, 29(1): 226-230.
- Meerburg, B.G., and Borgsteede, F.H., 2011. *Organic agriculture and its contribution to zoonotic pathogens*. In: Krause, D.O., Hendrick, S. (eds.), *Zoonotic Pathogens in the Food Chain*. CAB International: Wallingford, UK: 167-181.
- Mukherjee, A., Speh, D., Dyck, E., Diez-Gonzalez, F., 2004. *Preharvest evaluation of coliforms, Escherichia coli, Salmonella, and Escherichia coli O157:H7 in organic and conventional produce grown by Minnesota farmers*. Journal of Food Protection, 67: 894-900.
- Ostroukhova, E., Peskova, I., Levchenko, S., Vyugina, M., Belash, D., Shadura, N., 2022. *The use of a microbiological preparation based on Bacillus subtilis in organic viticulture*. BIO Web of Conferences, April 6, 48, 02006.
- Patel, P.S., Huang, S., Fisher, S., Pirmik, D., Aklonis, C., Dean, L., Meyers, E., Fernandes, P., Mayerl, F., 1995. *Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by Bacillus subtilis: Production, taxonomy, isolation, physico-chemical characterization and biological activity*. Journal of Antibiotics, 48: 9.
- Sarangi, T., Ramakrishnan, S., Nakkeeran, S., 2017. *Antimicrobial peptide genes present in indigenous isolates of Bacillus spp. exhibiting antimicrobial properties*. International Journal of Current Microbiology and Applied Sciences, 6(8): 1361-1369.
- Schultz, M., Burton, J.P., Chanyi, R.M., 2017. *Use of Bacillus in human intestinal probiotic applications*. In: Floch, M.H., Ringel, Y., Walker, W.A. (eds.), *The microbiota in gastroin-testinal pathophysiology*. Academic Press: 119-123.
- Shahzad, R., Waqas, M., Khan, A.L., Asaf, S., Khan, M.A., Kang, S.M., Yun, B.W., Lee, I.J., 2016. *Seed-borne endophytic Bacillus amyloliquefaciens RWL-1 produces gibberellins and regulates endogenous phytohormones of Oryza sativa*. Plant Physiology and Biochemistry, 106: 236-243.
- Sicuia, O.A., Constantinescu, F., Cornea, C.P., 2015. *Biodiversity of Bacillus subtilis group and beneficial traits of Bacillus species useful in plant protection - A Review*. Romanian Biotechnological Letters, 20(5): 10737-10750.
- Spears, J.L., Kramer, R., Nikiforov, A.I., Rihner, M.O., Lambert, E.A., 2021. *Safety assessment of Bacillus subtilis MB40 for use in foods and dietary supplements*. Nutrients, 13: 733.
- Su, Y., Liu, C., Fang, H., Zhang, D., 2020. *Bacillus subtilis: a universal cell factory for industry, agriculture, biomaterials and medicine*. Microbial Cell Factories, 19: 173.
- Sun, Z., Hsiang, T., Zhou, Y., Zhou, J., 2015. *Draft genome sequence of Bacillus amyloliquefaciens XK-4-1, a plant growth-promoting endophyte with antifungal activity*. Genome Announcements, 3: e01306-15.
- Tschäpe, H., Prager, R., Streckel, W., Fruth, A., Tietze, E., Böhme, G., 1995. *Verotoxinogenic Citrobacter freundii associated with severe gastroenteritis and cases of haemolytic uraemic syndrome in a nursery school: green butter as the infection source*. Epidemiology and Infection, 114: 441-450.
- Vătuțiu, D.S.E., and Popa, M.E., 2015. *Lactic acid bacteria inhibitory activity on the pathogens Salmonella and Listeria monocytogenes*. Scientific Bulletin, Series F, Biotechnologies, XIX: 337-344.
- Xia, Y., DeBolt, S., Dreyer, J., Scott, D., Williams, M.A., 2013. *Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices*. Frontiers in Plant Science, 6: 490.
- Zaharia, R., Petrișor, C., Cornea, P., Diguță, C., Cristea, S., Ștefan, S., 2022. *Isolation and molecular identification of fungal isolates from stored cereals using PCR-RFLP method*. Rom. Agric. Res., 39: 13-22.