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Identification of lipopeptide synthesis genes by RT-qPCR in endophytic strains *Bacillus pumilus* and *Bacillus amyloliquefaciens*

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Abstract. Endophytic bacteria of the genus *Bacillus* are promising agents for biological plant protection due to their broad spectrum of antimicrobial metabolites, in particular cyclic lipopeptides (CLPs). The endophytic strains *Bacillus pumilus* E11 and *Bacillus amyloliquefaciens* E12, isolated from *Pinus sylvestris* L. seeds, exhibited antagonistic activity against phytopathogenic microorganisms; however, the molecular-genetic basis of this activity remained unestablished. The aim of the study was to characterise the transcriptional activity of CLPs synthesis genes (fengycin, surfactin, iturin A) in *B. pumilus* E11 and *B. amyloliquefaciens* E12 using RT-qPCR with specificity verification via melting curve analysis. Total RNA was isolated from pure cultures in the stationary growth phase using GuSCN buffer with DTT and proteinase K; DNA was eliminated by treatment with DNase I; reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit, and RT-qPCR analysis was carried out on a CFX96 Opus (Bio-Rad) amplifier with SYBR Green. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the 16S rRNA reference gene. During the study, the *fenD* gene of the fengycin operon was identified and its specificity was confirmed by a melting peak (74.0-74.5°C) in both strains studied. The mean C_q

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value for *fenD* amplification was 30.3 for *B. pumilus* E11 and 29.8 for *B. amyloliquefaciens* E12, and the mean C_q values for 16S rRNA were 21.8 and 23.8 respectively. The relative transcriptional activity of the fengycin biosynthesis gene in *B. amyloliquefaciens* E12 exceeds that of *B. pumilus* E11 by a factor of 5.7 ($\Delta\Delta C_t$ = -2.5). The surfactin (*srfA*) and iturin A (*ituA*) synthesis genes were not detected in any of the strains. Thus, fengycin proved to be the only transcriptionally confirmed antifungal CLP among the studied endophytic strains, confirming their activity against the microfungi *Fusarium oxysporum* and *Alternaria alternata*, as determined in previous studies. The high level of *fen* operon transcription in *B. amyloliquefaciens* E12 confirms the potential of this strain as a producer of antifungal metabolites for the biocontrol of plant pathogens

Keywords: microorganisms; fengycin; biosynthesis; operon; biocontrol; antifungal activity; *Pinus sylvestris*

INTRODUCTION

Microorganisms of the genus *Bacillus* are the most efficient producers of structurally diverse antibiotic compounds among soil prokaryotes: some strains produce between five and eight chemically unrelated classes of secondary metabolites simultaneously. A. Ortiz *et al.* (2025) identified a particularly pronounced antimicrobial metabolome in two species, *Bacillus pumilus* and *Bacillus amyloliquefaciens*. As noted by N. Vasilyeva *et al*. (2025), the *B. pumilus* genome contains up to 15 functional biosynthetic gene clusters (BGCs), three of which are responsible for the synthesis of bacteriocins. This genetic structure accounts for the specific multifunctionality of these microorganism species as effective producers of antimicrobial agents. Among the secondary metabolites of microorganisms of the genus *Bacillus*, cyclic lipopeptides (CLPs) play a key role in the biological defence of plants; these are amphiphilic compounds synthesised by multi-enzyme complexes of non-ribosomal peptide synthetases (NRPS), encoded by a gene cluster. Three main classes of CLPs are distinguished: surfactins (peptides that cyclise with a β-hydroxyfatty acid carbon chain containing 13 to 15 carbon atoms to form a lactone structure), iturins (cyclic heptapeptides that form a ring via an amide bond with a β-amino fatty acid carbon chain containing 14 to 17 carbon atoms) and fengycins, also known as plipastatins (cyclic lipopeptides with a complex structure in which the peptide moiety consists of 10 amino acid residues, 8 of which form an internal lactone ring between the amino acids). The biosynthesis of each class of CLP is controlled by a separate cluster of genes: surfactin – *srfAA*, *srfAB*, *srfAC* and *srfAD*; iturin – *ituA*, *ituB*, *ituC* and *ituD*; fengycin – *fenA*, *fenB*, *fenC*, *fenD* and *fenE*. Importantly, not all *Bacillus* strains carry all three operons in their genomes, and the composition and repertoire of BGCs vary significantly even within a single species (Dobrzynski *et al*., 2023; Markelova *et al*., 2025). G. Balleux *et al*. (2025) determined that *B. pumilus* and *B. licheniformis* typically produce only one type of CLP belonging to the surfactin class, whereas *B. subtilis* and *B. amyloliquefaciens* can synthesise two types of CLPs, and the plant-associated species *B. velezensis* and *B. siamensis* can produce all three classes. Fengycin is of particular practical interest as a lipodecapeptide containing a β-hydroxy fatty acid chain (C14-C18) incorporated into a cyclic structure through a lactone bond between tyrosine and isoleucine. The antifungal mechanism of action of fengycin involves the inhibition of ergosterol synthesis, damage to membrane structure and the induction of apoptosis in fungal cells. According to N. Markelova *et al*. (2025), fengycin exhibits lower haemolytic activity than iturins and surfactins, which is a significant advantage in the development of biological plant protection products. N. Markelova *et al*. (2025) and C.-Y. Cao *et al*. (2025) emphasised that the spectrum of fungicidal activity of fengycin covers a wide range of pathogenic filamentous fungi: *Fusarium* spp., *Sclerotinia* spp., *Alternaria solani*, *Magnaporthe grisea*, *Botrytis cinerea*, *Rhizoctonia solani* and others. According to S. Wang *et al*. (2022), the fengycin operon (*fenA-fenE*) and the iturin operon (*ituA-ituD*) encode fengycin synthase and iturin synthase, respectively. Transcription of these operons is activated by the regulators ComA, SigA, DegU, DegQ, and Spo0A, while deletion of the repressor gene abrB further increases fengycin and iturin yields.

Iturin A is an effective antifungal agent whose mechanism of action involves the formation of ion

channels in the fungal cytoplasmic membrane, inducing apoptosis-like changes. J. Dobrzynski *et al*. (2023) and N. Markelova *et al*. (2025) argued that surfactin (the *srfAA-srfAD* cluster) plays a key role in biofilm formation and the induction of systemic plant resistance; its direct antifungal activity is lower than that of iturins and fengycins, yet it is critically important for effective colonisation of the root zone. Analysis of the transcriptional activity of genes involved in the biosynthesis of antimicrobial lipopeptides in the studied strains is a crucial step, facilitating the transition from the observed antimicrobial effect to the elucidation of its molecular basis and the scientifically grounded optimisation of the fermentation process. The increasing resistance of phytopathogenic fungi to chemical fungicides, linked to changes in ergosterol biosynthesis and the activity of transport systems, necessitates the search for new (alternative) means of biological control. Microorganisms of the genus *Bacillus*, by producing CLPs (surfactin, iturin A, fengycin), exhibit pronounced antifungal activity. As noted by C. Bakker *et al*. (2024), their action is associated with disruption of the integrity of the cell membrane, interaction with its lipid components, including ergosterol, as well as the induction of metabolic disturbances in fungal cells. The multifunctionality of CLPs is one of the factors that potentially reduces the development of resistance in phytopathogens compared to fungicides with a narrow-spectrum mechanism of action.

The endophytic microbiota of conifer seeds is an under-researched source of new strains capable of producing CLPs. In previous studies by V. Shopinskyi *et al*. (2025) and V. Shopinskyi & L. Butsenko (2026) the endophytic strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 were isolated from *Pinus sylvestris* L. seeds and identified using MALDI-TOF and VITEK® 2 Compact methods. For *B. pumilus* E11, antagonistic activity against phytopathogenic bacteria was detected: growth inhibition zones against *Pseudomonas syringae* UCM B-1027 were 35 ± 2 mm, against *Clavibacter michiganensis* subsp. *michiganensis* 10_2 – 33 ± 2 mm, against *Xanthomonas campestris* UCM B-1049 – 32 ± 2 mm, against *Pseudomonas fluorescens* 8573 – 25 ± 1 mm and against *Pectobacterium carotovorum* UCM B-1075 – 20 ± 1 mm (Shopinskyi *et al.*, 2025). Antagonistic activity against phytopathogenic bacteria was also detected for *B. amyloliquefaciens* E12: growth inhibition zones against *P. syringae* UCM B-1027 were 37 ± 2 mm, against *C. michiganensis* subsp. *michiganensis* 10_2 – 22 ± 1 mm, against *X. campestris* UCM B-1049 – 42 ± 2 mm, against *P. fluorescens* 8573 – 26 ± 1 mm and against *P. carotovorum* UCM B-1075 – 35 ± 2 mm. It was also determined that *B. amyloliquefaciens* E12 exhibits antagonistic activity against phytopathogenic microfungi: the strain under investigation inhibited the growth of *Fusarium oxysporum* GTF1 and *Alternaria alternata* GTA2 with a zone of inhibition of 15-18 mm (Shopinskyi & Butsenko, 2026). Given the detected biocontrol potential of the endophytic microorganisms *B. pumilus* E11 and *B. amyloliquefaciens* E12 against plant pathogens, the molecular mechanisms of their antagonistic activity, in particular the expression profile of genes responsible for the biosynthesis of lipopeptides, remain insufficiently studied. To characterise the antifungal phenotype and to develop targeted fermentation strategies, it is necessary to identify actively transcribed BGCs. The aim of this study was to establish the transcriptional activity profile of cyclic lipopeptide genes (*fenD*, *srfA*, *ituA*) in *B. pumilus* E11 and *B. amyloliquefaciens* E12 using RT-qPCR with SYBR Green detection and verification of specificity via melting curve analysis.

MATERIALS AND METHODS

The endophytic strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 under investigation were isolated from surface-sterilised seeds of *Pinus sylvestris* L. by serial dilution and identified based on morphological and cultural characteristics, MALDI-TOF mass spectrometry and the VITEK® 2 Compact analyser (Shopinskyi *et al.*, 2025). For gene expression analysis, pure cultures of both strains were grown in LB liquid nutrient medium (tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L) at 28°C with stirring at 240 rpm under aerobic conditions. Samples were collected during the stationary phase of growth after 48 hours of cultivation, as it is during this period that the maximum transcriptional activity of the genes encoding CLPs synthesis, under the regulation of the *Spo0A* and *DegU*/*ComA* cascades, is observed in most *Bacillus* strains. RNA was isolated from a pure bacterial culture. A bacterial colony was placed in a 1.5 mL sterile tube, 100 µL of RNase-free water was

added, and the sample was resuspended. Cell lysis was carried out sequentially: 10 µL of lysozyme (1 mg/mL) was added, followed by incubation for 10 minutes at 37°C, followed by 400 µL of 4 M GuSCN buffer with DTT and 10 µL of proteinase K; the samples were incubated for 10 minutes at room temperature, then centrifuged for 1 minute at 10,000 rpm. The supernatant was transferred to a new sterile microtube, 0.7× the volume of isopropanol was added, and the mixture was incubated at -20°C for 30 minutes to precipitate the RNA. After centrifugation at 12,000 rpm for 5 minutes, the pellet was washed with 70% ethanol followed by a similar centrifugation, dried for 5 minutes, and resuspended in 40 µL of RNase-free water. Residual genomic DNA was removed by treatment with DNase I (Thermo Scientific). The reaction mixture (20 µL) contained: 10 µL RNA, 1 U DNase I, 2 µL 10×$MgCl_2$ buffer and 7 µL DEPC-treated deionised water. The mixture was incubated for 30 minutes at 37°C; DNase I was inactivated by adding 2 µL of 0.5 M EDTA and heating at 65°C for 15 minutes. The concentration and purity of the RNA were determined on a Denovix 11FX spectrophotometer: based on the A_{260} (concentration) and the A_{260}/A_{280}, A_{260}/A_{230} ratios (purity). The absence of DNA contamination was verified by PCR without reverse transcription. Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Samples were incubated at 25°C for 5 minutes and at 42°C for 60 minutes. Enzyme inactivation was carried out at 70°C for 5 minutes. The composition of the reaction mixture is shown in Table 1.

Table 1. Composition of the reverse transcription reaction mixture

Component	Volume, µL
RNA (concentration 0.1 ng – 5 µg)	5
Random primer	1
5× reaction buffer	4
RNAse inhibitor	1
10 mm dTTP	2
Reverse transcriptase	1
Deionised water (DEPC-treated)	6
Total volume	20

Source: developed by the authors according to the manufacturer's instruction

cDNA samples were stored at -20°C for no longer than 1 week. For reverse transcription, 5 µL of the total RNA preparation, corresponding to 0.1 ng – 5 µg of RNA depending on the sample, was used as input in a 20 µL reaction; random hexamer primers were chosen to ensure unbiased, whole-transcriptome conversion independent of the position of the target genes within the lipopeptide operons. The reverse transcription was carried out in duplicate for each sample. The efficiency and integrity of the reverse transcription were assessed indirectly through the reproducible amplification of the 16S rRNA reference gene from the resulting cDNA: consistent low Cq values for 16S rRNA across replicates confirmed that intact, amplifiable cDNA was obtained, while the parallel no-reverse-transcriptase control excluded genomic DNA carry-over. Analysis of the transcriptional activity of CLP genes was performed using the RT-qPCR method on a CFX96 Opus amplifier (Bio-Rad), with data analysis carried out in Bio-Rad CFX Maestro. The iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad) was used. The composition of the reaction mixture is shown in Table 2.

Table 2. Composition of the RT-qPCR reaction mixture

Component	Volume, µL
iTaq™ Universal SYBR® Green mix	10
Forward primer (300 nM final concentration)	0.5
Reverse primer (300 nM final concentration)	0.5
cDNA (100 ng)	2

Table 2. Continued

Component	Volume, μL
Deionised water	7
Total volume	20

Source: compiled by the authors according to the manufacturer's instruction

A separate reaction mixture was prepared for each sample and gene studied. The reaction was carried out in triplicate. Amplification temperature profile: initial denaturation for 3 minutes at 95°C; 40 cycles: 20 s at 95°C and 30 s at 58°C (fluorescence signal detection was performed on the FAM/SYBR channel); melting curve analysis: 10 s at 65-95°C with a temperature increase of 0.5°C per cycle. The primers for the fengycin (*fenD*), surfactin (*srfA*) and iturin A (*ituA*) genes were designed specifically for this study in Primer-BLAST (NCBI) on the basis of the corresponding biosynthetic operon sequences of reference strains *Bacillus amyloliquefaciens* FZB42 (GenBank CP000560) and *Bacillus velezensis* (reference lipopeptide synthetase genes), with the target amplicon length constrained to 180-220 bp and the melting temperature of the primers set to 60°C to ensure uniform annealing under a single thermal profile. Primer specificity was verified in silico against the NCBI nucleotide database, and only primer pairs yielding a single predicted product for the intended target were retained. The reference-gene primers (16S rRNA) were taken from H. Younas *et al*. (2023). The nucleotide sequences of the primers are given in Table 3.

Table 3. Primers used for RT-qPCR analysis of cyclic lipopeptide synthesis genes

Lipopeptide	Gene	Primers (5′→3′)	Expected amplification product, bp
Fengycin	*fenD*	F: ATGGGAAATGTTCGGAGCGT R: CTGTAAAGCTTCTCCGCCGA	220
Surfactin	*srfA*	F: CCGCACCAAAAGAAGAACGG R: GCTCGCCCTTCCTAGACTTC	182
Iturin A	*ituA*	F: AAGACGCCATTGCCACTGTA R: CGAGTTCCTCCGCTCTGATC	187

Source: compiled by the authors based on H. Younas *et al*. (2023)

Amplification specificity was confirmed by three independent criteria: a single, narrow peak in the melting-curve analysis; the correspondence of the melting temperature to the value predicted for the target amplicon; and the absence of any signal in the negative control (NTC). Since the study addressed the qualitative detection of transcripts and their relative quantification by the $2^{-\Delta\Delta Ct}$ method, in which the target and reference genes are amplified from the same cDNA under identical conditions, amplification efficiency was taken as equivalent for the compared genes; the amplicons were kept short (180-220 bp) precisely to keep efficiency close to the theoretical maximum, as recommended for SYBR Green assays (Bustin *et al*., 2009). A limitation of the present design is that no external standard dilution series was run to determine the exact PCR efficiency and the coefficient of determination (R^2) of a standard curve, and no positive control template was available for *srfA* and *ituA*; consequently, the negative results for these two genes are interpreted as the absence of a detectable transcript under the conditions studied rather than as definitive proof of the absence of the corresponding gene clusters, which will be verified by whole-genome sequencing. The relative level of transcriptional activity of CLP genes was calculated using the $2^{-\Delta\Delta Ct}$ method described by K.J. Livak & T.D. Schmittgen (2001). 16S rRNA was used as the reference gene, as its stable transcription has been confirmed under various cultivation conditions for strains of the genus *Bacillus* (Younas *et al*., 2023). ΔC_t was defined as: $\Delta C_t = C_q$ (target gene) – C_q (16S rRNA). The $\Delta\Delta C_t$ value was calculated relative to the calibrator strain *B. pumilus* E11: $\Delta\Delta C_t$ (*B. amyloliquefaciens* E12) = ΔC_t (*B. amyloliquefaciens* E12) – ΔC_t (*B. pumilus* E11). The relative expression level was calculated as $2^{-\Delta\Delta Ct}$ (Livak *et al*., 2001). Statistical analysis of the results was performed using CFX Maestro (Bio-Rad) and Microsoft Excel software. Each C_q measurement was performed in three

technical replicates for each sample studied. Reproducibility between replicates was assessed by the difference in C_q values between duplicates: a difference of $\Delta C_q \leq 0.5$ between technical replicates was considered acceptable. For the 16S rRNA reference gene and the genes of the lipopeptides under investigation, the mean C_q value was calculated as the arithmetic mean of the three technical replicates (Bustin *et al.*, 2009). The $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of the target gene's expression level, using the *B. pumilus* E11 strain as the primary calibrator ($2^{-\Delta\Delta Ct} = 1.0$). Dispersion was assessed via the standard deviation of technical replicates. The absence of non-specific amplification products was confirmed by the correspondence of melting peak temperatures for each target gene and the absence of a signal in the NTC. Genes were considered absent or as having undetectable transcripts in the absence of an amplification signal ($C_q = 0$ or failure to reach the threshold) after 40 cycles and in the absence of a melting peak in all technical replicates.

RESULTS AND DISCUSSION

The total RNA preparations obtained from strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 were of sufficient quality for RT-qPCR analysis, and the synthesised cDNA enabled reproducible amplification of the 16S rRNA reference gene in all samples studied. The amplification curves in Figure 1 provide the primary evidence for the transcriptional status of each target gene.

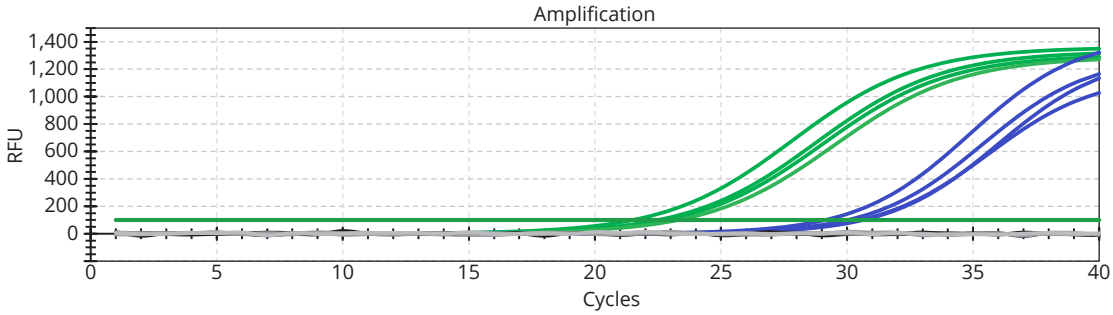

Figure 1. Summary amplification curves (obtained by RT-qPCR) of the target genes for strains *B. pumilus* E11 and *B. amyloliquefaciens* E12
Note: RFU – fluorescence units; green – 16S rRNA (reference gene); blue – fengycin (*fenD*); black – surfactin (*srfA*); grey – iturin A (*ituA*)
Source: compiled by the authors

The sigmoidal profile of the *fenD* and 16S rRNA curves, with a clearly defined exponential phase, indicates efficient template amplification, whereas the flat, sub-threshold traces of *srfA* and *ituA* over the full 40 cycles reflect the absence of the corresponding transcripts rather than reaction inhibition, since the reference gene amplified normally from the same cDNA. The earlier exponential rise of the *fenD* curve in *B. amyloliquefaciens* E12 relative to *B. pumilus* E11 already anticipates the quantitative difference in transcriptional activity established below, and the flat NTC baseline across all channels excludes genomic DNA carry-over and primer-dimer artefacts. The negative control did not produce an amplification signal for any of the analysed genes, confirming the absence of reagent contamination. To verify the specificity of the amplification, melting curve analysis was performed separately for each gene under investigation. For the 16S rRNA reference gene, a single symmetrical melting peak at 83.0-83.5°C, reproduced in both strains with comparable peak heights, confirmed that amplification yielded one homogeneous product without secondary peaks or shoulders (Fig. 2).

The narrow width of the derivative peak and its stable position between strains confirm both the specificity of the reference primers and the uniform quality of the cDNA preparations, which is a prerequisite for valid normalisation in the $2^{-\Delta\Delta Ct}$ calculation. For the surfactin biosynthesis gene *srfA*, the amplification traces of both technical replicates for each strain remained at baseline and did not cross the fluorescence threshold

within 40 cycles, while the corresponding melting-curve derivatives showed no peaks across the 65-95°C range, and no peak was observed in the no-template control (NTC) (Fig. 3).

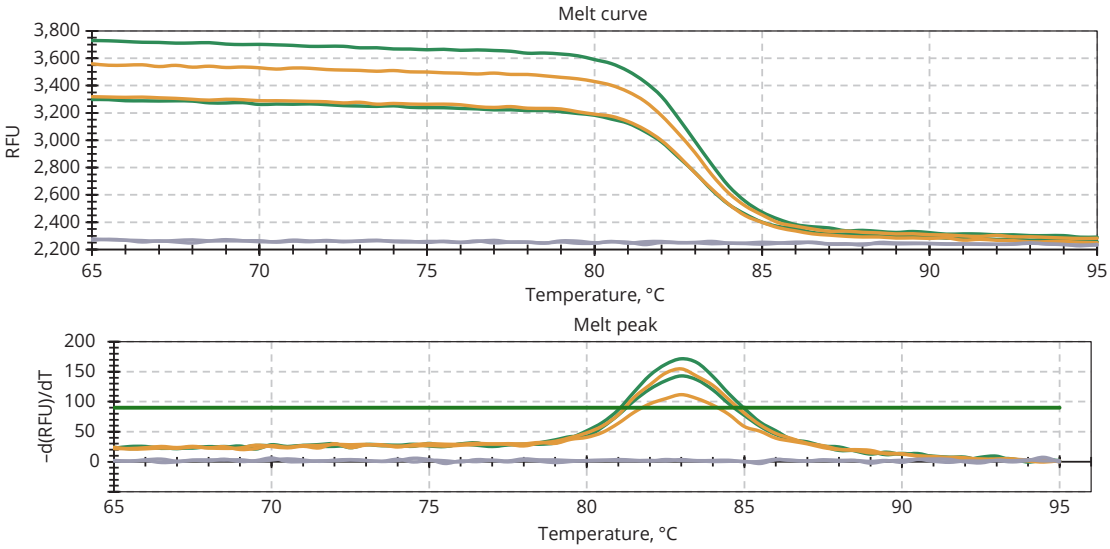

Figure 2. Melting curves and their derivatives for the 16S rRNA reference gene
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

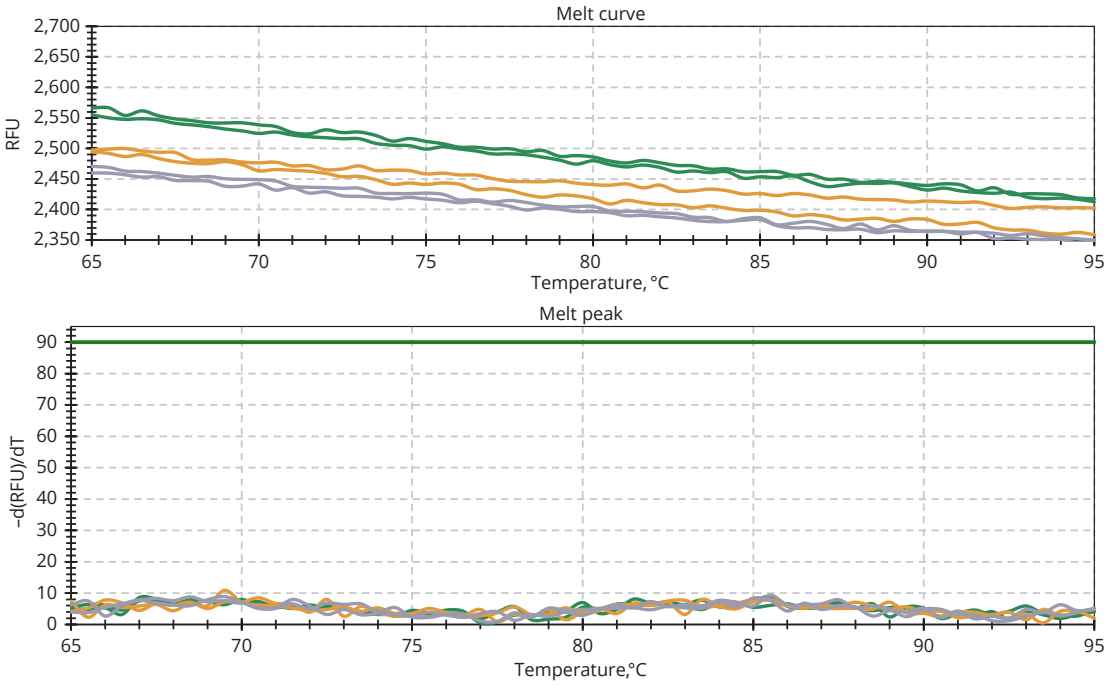

Figure 3. Melting curves and their derivatives for the surfactin biosynthesis gene (*srfA*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

In SYBR Green chemistry a genuine transcript, even at a high C_q, would generate a defined melting peak; its complete absence in every replicate therefore indicates that no *srfA* template was reverse-transcribed and amplified, and the concordance between the two replicates confirms th e reproducibility of this negative result. For *B. pumilus* E11 the outcome is consistent with the species-level restriction of its surfactin-family repertoire to pumilacidin, whose variant operon is not necessarily recognised by primers designed against the *srfA* sequence. For the iturin A biosynthesis gene *ituA*, the flat amplification traces that remained below the fluorescence threshold and the absence of a derivative melting peak confirmed that no specific amplicon was generated in either strain, thereby excluding the presence of a detectable iturin A transcript (Fig. 4).

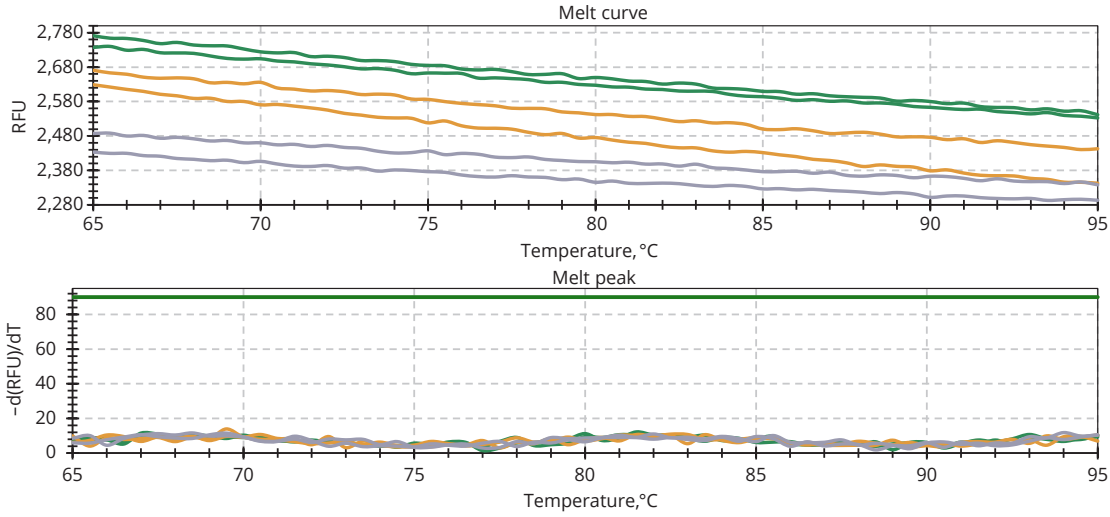

Figure 4. Melting curves and their derivatives for the iturin A biosynthesis gene (*ituA*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

The result is particularly informative for *B. amyloliquefaciens* E12, in which the iturin operon is generally present, and therefore points to a regulatory cause, namely insufficient activation of the *itu* promoter by the Spo0A-DegQ cascade on nutrient-rich LB medium in the absence of a fungal inducer, rather than to the absence of the gene cluster itself. For the fengycin biosynthesis gene *fenD*, both strains produced a single clear, sharp melting peak within the narrow range of 74.0-74.5°C, with no additional peaks, shoulders, or low-temperature signals indicative of non-specific amplification or primer-dimer formation, while no peak was detected in the no-template control (NTC) (Fig. 5). The near-identical peak position in *B. pumilus* E11 and *B. amyloliquefaciens* E12 confirms that the same specific product was generated regardless of the strain of origin, while the greater peak height recorded for E12 is consistent with a larger amount of accumulated amplicon and, by extension, with a higher initial transcript abundance, in agreement with its lower C_q value. The mean values of amplification C_q and melting temperatures obtained during RT-qPCR analysis for the target genes and strains under investigation are presented in Table 4.

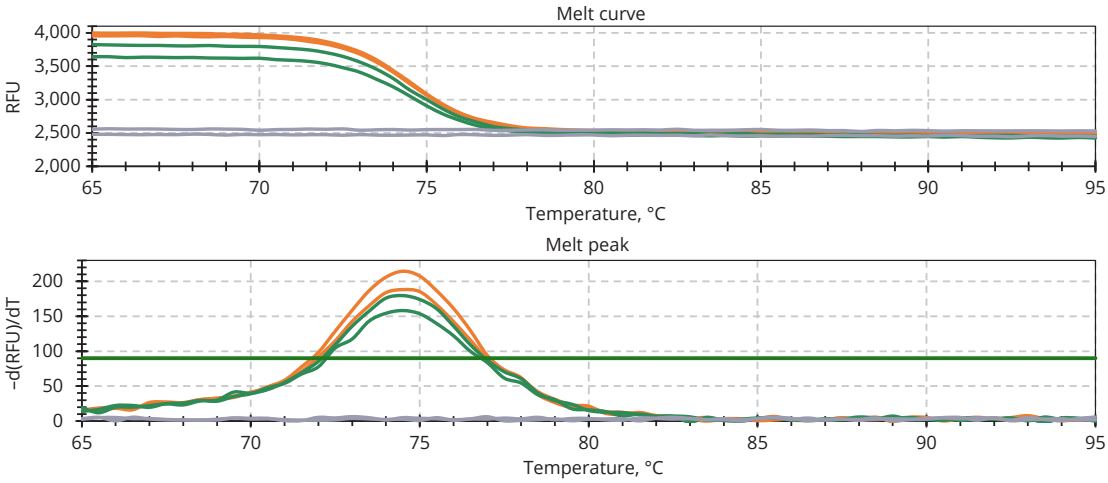

Figure 5. Melting curves and their derivatives for the fengycin biosynthesis gene (*fenD*)
Note: green – *B. pumilus* E11; orange – *B. amyloliquefaciens* E12; grey – NTC
Source: compiled by the authors

Table 4. Results of RT-qPCR analysis:
Cq values and melting temperatures for cyclic lipopeptide synthesis genes

Target gene	B. pumilus E11, C_q	B. amyloliquefaciens E12, C_q	Melting temperature (°C)
16S rRNA (reference)	21.8	23.8	83.0-83.5
Fengycin (*fenD*)	30.3	29.8	74.0-74.5
Surfactin (*srfA*)	N/D	N/D	No peak
Iturin A (*ituA*)	N/D	N/D	No peak
NTC (negative control)	N/D	N/D	No peak

Note: N/D – not detected
Source: compiled by the authors

The fengycin operon gene (*fenD*) was detected and confirmed in both strains studied. For *B. pumilus* E11, the mean C_q value across three technical replicates was 30.3; for *B. amyloliquefaciens* E12, it was 29.8. Stable amplification was also observed for the 16S rRNA reference gene: for *B. pumilus* E11, the mean C_q value across three technical replicates was 21.8, and for *B. amyloliquefaciens* E12, it was 23.8. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected (Cq = 0) in any of the samples studied. The specificity of the amplification was confirmed by a single melting peak at (74.0-74.5°C), corresponding to the *fenD* gene. No melting peak was detected in the NTC. The data in Table 4 show a consistent gap of approximately 8-9 cycles between the reference gene 16S rRNA (mean C_q 21.8 and 23.8 for E11 and E12) and the target gene *fenD* (mean Cq 30.3 and 29.8). Since each cycle corresponds to an approximate doubling of template, this gap indicates that *fenD* transcripts are present at a substantially lower abundance than the constitutively expressed 16S rRNA, which is expected for a growth-phase-regulated secondary-metabolite operon rather than a housekeeping gene. It is important that the higher reference C_q of *B. amyloliquefaciens* E12 does not by itself imply lower target expression, because in the $2^{-\Delta\Delta Ct}$ method each target is normalised to its own reference; after normalisation the balance shifts in favour of E12, as shown in Table 5. The uniform absence of amplification and melting peaks for *srfA*, *ituA* and the NTC delimits the fengycin-restricted transcriptional profile common to both strains. The values of the relative transcriptional activity of the fengycin biosynthesis gene, calculated using the $2^{-\Delta\Delta Ct}$ method, are presented in Table 5.

Table 5. Calculation of the relative level of transcriptional activity of the fengycin biosynthesis gene using the $2^{-\Delta\Delta Ct}$ method

Strain	C_q		ΔC_t	$2^{-\Delta\Delta Ct}$ (relative expression)
	fenD	**16S rRNA**		
B. pumilus E11	30.3	21.8	8.5	1.0 (calibrator)
B. amyloliquefaciens E12	29.8	23.8	6.0	5.7

Source: compiled by the authors

The value of ΔC_t for *B. pumilus* E11: 30.3 – 21.8 = 8.5. The value of ΔC_t for *B. amyloliquefaciens* E12: 29.8 – 23.8 = 6.0. The value of $\Delta\Delta C_t$ for *B. amyloliquefaciens* E12 relative to *B. pumilus* E11: 6.0 – 8.5 = -2.5. Accordingly, $2^{-(-2.5)} \approx 5.7$. Thus, the relative transcriptional activity of the fengycin operon in *B. amyloliquefaciens* E12 is 5.7 times higher than in *B. pumilus* E11. This 5.7-fold difference should be interpreted at the level of relative transcript abundance rather than as a direct measure of secreted fengycin, since transcription and final peptide yield may diverge because of post-transcriptional and translational regulation. Nevertheless, a positive relationship between *fenD/fenA* transcription and fengycin output has been reported for engineered *B. amyloliquefaciens* strains, in which increased *fen*-operon transcription was accompanied by higher fengycin accumulation (Zhao *et al*., 2016). On this basis, the markedly higher *fenD* transcription in *B. amyloliquefaciens* E12 predicts a correspondingly greater fengycin-producing capacity and provides a quantitative, molecular rationale for prioritising this strain in subsequent fermentation-optimisation and LC-MS verification experiments, while *B. pumilus* E11, with a confirmed but lower level of *fenD* transcription, remains a relevant complementary producer. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected in any of the strains in three technical replicates (no C_q values were obtained, and no melting peaks were detected).

G. Balleux *et al*. (2025) found that microorganisms belonging to different Bacillus species possess different sets of CLP operons. It has been established that *B. pumilus* and *B. licheniformis* are typically capable of synthesising only one class of CLP – from the surfactin family; *B. subtilis* and *B. amyloliquefaciens* produce two types of CLPs, whilst *B. velezensis* and *B. siamensis* produce three types of CLPs simultaneously. These data provide a fundamentally important context for interpreting the results obtained: the absence of the *srfA* transcript in *B. pumilus* E11 can be explained either by the absence of the *srf* cluster itself or by species-specific features of its regulation. Furthermore, the characteristic lipopeptide of *B. pumilus* is pumilacidin, a structural analogue of surfactin synthesised by an *srf*-like operon with a modified amino acid composition. As emphasised by A. Saggese *et al*. (2018) and J. Dobrzynski *et al*. (2023), primers for *srfA*, developed primarily for strains of *B. subtilis* and *B. amyloliquefaciens*, may not recognise the variant pumilacidin operon. For *B. pumilus* E11, only the fengycin operon has been identified, despite the fact that the synthesis of surfactin-like cyclic lipopeptides is more characteristic of this species. However, J. Dobrzynski *et al*. (2023) reported the presence of fengycin biosynthesis genes in certain strains of *B. pumilus* and found that a mixture of *B. pumilus* SS-10.7 and *B. amyloliquefaciens* SS-12.6 and SS-38.4 produced fengycin A and inhibited the growth of *Pseudomonas syringae* pv. *aptata* on sugar beet. S. Wang *et al*. (2022) and D. Zhang *et al*. (2025) reported that the iturin operon is present in most described strains of *B. amyloliquefaciens*; therefore, the absence of the *ituA* transcript in strain E12 may be considered atypical. This may be due to the specific features of the regulation of the expression of the corresponding gene cluster. In particular, transcription of the *itu* cluster is controlled by the level of the phosphorylated regulator *Spo0A* and the involvement of *DegQ*; under conditions of cultivation on enriched LB medium, the level of *Spo0A*~P may be insufficient to activate the *itu* promoter (Wang *et al*., 2022). G. Balleux *et al*. (2025) also found that, in some endophytic strains, iturin synthesis is induced only through interaction with phytopathogenic fungi or their volatile metabolites, indicating the co-stimulated production of lipopeptides. D.J. Hazarika *et al*. (2019) found that the *fenA* gene may be present in the genome of the endophytic microorganism *B. subtilis*, although the corresponding product was not detected by LC-ESI-MS

under standard cultivation conditions. This indicates the conditionally specific nature of fengycin biosynthesis and a possible mismatch between the presence of the biosynthetic cluster and the production of the final metabolite. The data obtained are consistent with the fact that the presence of BGCs in the genome is not necessarily accompanied by their expression under standard conditions, and the use of RT-qPCR analysis allows the actual transcriptional activity of the corresponding genes to be assessed and expands the possibilities of genomic research.

The results obtained, in particular the detection of transcription of the fengycin cluster in the absence of transcriptional activity of the surfactin and iturin biosynthesis genes, differ from the typical profile described in the literature for most endophytic strains of *B. amyloliquefaciens*, in which all three classes of CLPs are detected simultaneously. S. Ma *et al*. (2025) used PCR to analyse the profile of genes involved in lipopeptide biosynthesis in three endophytic strains isolated from cereal crops – *B. velezensis* ZH179 and *B. amyloliquefaciens* ZH409 and ZH99 – which actively inhibited *Aspergillus flavus*. In all three strains, the genes *srfAA*, *srfAD*, *fenA*, *fenB*, *ituA*, *ituB*, *ituD*, *bmyA*, *bmyB*, and *bmyC* were simultaneously detected; LC-MS analysis confirmed the production of surfactin, fengycin, and iturin. Thus, *B. amyloliquefaciens* strains ZH409 and ZH99 are effective co-producers of all three classes of CLPs, whereas *B. amyloliquefaciens* E12 in the current study exhibited only a fengycin transcript. This reflects strain-specific variability in BGC expression, which is consistent with the concept of condition-specific lipopeptide synthesis (Balleux *et al*., 2025). As noted by X.H. Chen *et al*. (2007), such interspecies variability has a genomic basis: the reference strain *B. amyloliquefaciens* FZB42 allocates 8.5% of its genetic capacity to the non-ribosomal synthesis of secondary metabolites, which exceeds the corresponding value for *Streptomyces avermitilis* (6.4%), and the number and composition of biosynthetic gene clusters differ markedly between strains of the same species. T. Ahmad *et al*. (2023) investigated the lipopeptide profile of the soil-isolated *B. subtilis* Y17B against *Alternaria alternata*. PCR screening identified the genes involved in the biosynthesis of fengycin, surfactin and iturin; UPLC Q-TOF mass spectrometry confirmed the production of surfactin (m/z 994.64; 1,022.68; 1,026.62), iturin (m/z 1,043.56) and fengycin (m/z 1,491.85) in the culture extract. The presence of fengycin in *B. subtilis* Y17B correlated with the inhibition of *A. alternata* growth, which, in terms of mechanism of action, is consistent with the results obtained: the detection of the fengycin transcript *fenD* in *B. amyloliquefaciens* E12 correlates with the endophyte's antagonistic activity against *A. alternata* GTA2 (Shopinskyi & Butsenko, 2026).

However, unlike *B. subtilis* Y17B, the studied strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 showed no transcriptional activity of the surfactin and iturin synthesis genes. K. Yang *et al*. (2024) conducted a genomic analysis of the endophytic strain *B. amyloliquefaciens* MR4, which was isolated from the roots of wild medicinal plants. Using PCR, 8 of the 9 antimicrobial agent synthesis genes under investigation were detected, including *fenA*, *ituA* and *srfAA*. Compared to *B. amyloliquefaciens* MR4, the *B. amyloliquefaciens* E12 exhibited a more restricted transcriptional profile under standard cultivation conditions. The absence of the *ituA* transcript may be related to the specifics of expression regulation, in particular insufficient promoter activation in LB medium, or to the absence of the corresponding cluster or mutations in regulatory regions, which requires further verification by whole-genome sequencing. In addition, J. Zhao *et al*. (2016) used RT-PCR to investigate the transcription level of the *fenA* gene in *B. amyloliquefaciens* ES-2-4 under various conditions. Increased fengycin production (an 8.30-fold increase) in the recombinant strain *B. amyloliquefaciens* FMB72 was accompanied by a 12.77-fold increase in the *fenA* transcription level compared to the wild-type *B. amyloliquefaciens* ES-2-4. This confirms that the quantitative level of *fenA* transcription directly correlates with the efficiency of fengycin biosynthesis. Therefore, the 5.7-fold difference in relative transcriptional activity between *B. amyloliquefaciens* E12 and *B. pumilus* E11, which was identified in this study, may predict a significant difference in the actual concentrations of fengycin in the culture media of the strains under investigation, which can be confirmed by chemical methods (HPLC or mass spectrometry). D.J. Hazarika *et al*. (2019) found that the presence of the fenA gene in the B. subtilis genome was not always accompanied by fengycin production under

standard cultivation conditions, emphasising the dependence of biosynthetic gene cluster expression on environmental conditions. In this study, the use of RT-qPCR analysis allows the assessment of the actual transcriptional activity of the relevant genes and provides functional verification of the results of genomic analysis. The data obtained indicate that cultivation in the stationary phase on LB medium is sufficient to activate the fengycin cluster in the studied strains *B. pumilus* E11 and *B. amyloliquefaciens* E12.

The detected transcriptional activity of the fengycin biosynthesis gene in both strains provides direct molecular-genetic confirmation of the antifungal activity reported by V. Shopinskyi & L. Butsenko (2026), who assessed the strains using the agar diffusion method. *B. amyloliquefaciens* E12, which exhibits 5.7-fold higher transcriptional activity of the fengycin synthesis gene compared to *B. pumilus* E11, formed zones of growth inhibition against *F. oxysporum* GTF1 and *A. alternata* GTA2 with a diameter of 15-18 mm. This correlation between the level of *fenD* transcription and the antifungal phenotype is consistent with the results of other scientific studies. Using *B. subtilis* NCD-2 as a model, Q. Guo *et al*. (2014) demonstrated that fengycin is the principal antifungal agent against *Rhizoctonia solani* associated with soil-borne cotton diseases, as inactivation of the fengycin biosynthesis gene *fenC* resulted in a complete loss of antifungal activity. H. Fan *et al*. (2017) investigated the biocontrol potential of *B. subtilis* 9407 against *Botryosphaeria dothidea* and found that inactivation of the *ppsB* gene, an orthologue of *fenA*, completely suppressed the antifungal activity of the strain against this phytopathogen. These results confirm the important functional role of fengycin as the primary antifungal agent, particularly under conditions where the synthesis of iturin and surfactin is absent or limited. D. Romero *et al*. (2007) determined that iturin, surfactin and fengycin are important factors in the antagonism of *B. subtilis* against *Podosphaera fusca*: the strains studied, which produced only fengycin (without iturin), exhibited characteristic, albeit somewhat reduced, antifungal activity. Thus, the fengycin-dominated profile identified for *B. pumilus* E11 and *B. amyloliquefaciens* E12 is functionally sufficient to provide an antifungal effect.

M. Ongena *et al*. (2005) and S. Breen *et al*. (2015) also identified fengycin as a key antifungal component produced by *B. subtilis* M4, reporting that the protection of bean plants against *Pythium ultimum*, apples against *Botrytis cinerea*, cucumbers against *Colletotrichum lagenarium*, and tomatoes against *Pythium aphanidermatum* was associated with the production of lipodecapeptides, particularly fengycin. The results obtained are of significant comparative importance, as *B. cinerea* and *Alternaria* spp. are characterised by similar mechanisms of pathogenesis. Therefore, fengycin synthesised by *B. amyloliquefaciens* E12 has the potential to exhibit similar efficacy against a broad spectrum of phytopathogenic fungi. Thus, the fengycin-dominant transcriptional profile of strains *B. pumilus* E11 and *B. amyloliquefaciens* E12 is molecularly confirmed and ensures the expression of antifungal biocontrol activity. The results obtained indicate the advisability of optimising the conditions for fengycin biosynthesis (selection of the C:N ratio, regulation via the *ComA/DegU* system and induction in the presence of phytopathogenic fungi), whilst the induction of iturin or surfactin synthesis is not a priority. Strain *B. amyloliquefaciens* E12, characterised by a higher level of transcription of the *fen* cluster genes, can be considered a priority agent for the development of a biopesticide. In contrast, for *B. pumilus* E11, whole-genome sequencing is advisable to determine the presence or absence of a complete set of CLP-cluster genes and to verify the structure and functional characteristics of the fengycin operon.

CONCLUSIONS

Using the RT-qPCR method with SYBR Green detection and verification of specificity via analysis of melting curves (peak 74.0-74.5°C), the transcriptional activity of the fengycin operon gene (*fenD*) was detected and confirmed in the endophytic microorganisms *Bacillus pumilus* E11 and *Bacillus amyloliquefaciens* E12, isolated from the seeds *of Pinus sylvestris* L. Analysis of the results obtained using the $2^{-\Delta\Delta Ct}$ method revealed that the relative transcriptional activity of the *fen* cluster in *B. amyloliquefaciens* E12 was 5.7 times higher than the corresponding value in *B. pumilus* E11 ($\Delta\Delta C_t = -2.5$). This finding confirms the effective antifungal activity of the *B. amyloliquefaciens* E12 strain against *F. oxysporum* GTF1 and *A. alternata*

GTA2, as determined in previous studies using the agar diffusion method. The surfactin (*srfA*) and iturin A (*ituA*) biosynthesis genes were not detected in any of the strains in all technical replicates under stationary-phase growth conditions on LB medium. The absence of the *srfA* transcript in *B. pumilus* E11 corresponds to the specific limitation of the set of cyclic lipopeptides in this species, where the characteristic lipopeptide is pumilacidin rather than surfactin. The absence of *ituA* in *B. amyloliquefaciens* E12 indicates the conditionally specific nature of *itu* operon transcription under the cultivation conditions studied, or the absence of this BGC in the strain's genome, which requires verification by whole-genome sequencing followed by antiSMASH analysis. Thus, fengycin proved to be the only transcriptionally confirmed antifungal CLP for both endophytic strains under the conditions studied, providing the necessary functional efficacy for the realisation of antifungal activity, which is consistent with similar results for other fengycin-producing biocontrol strains of *Bacillus* spp. The *B. amyloliquefaciens* E12 strain, which exhibits a higher level of *fen*-operon transcription compared to the *B. pumilus* E11 strain, has been identified as a promising candidate for further optimisation of fengycin biosynthesis conditions and the development of biocontrol agents for plant protection against phytopathogens. Further studies should include whole-genome sequencing and antiSMASH analysis of the BGC of both strains under investigation, LC-MS verification of fengycin production in culture filtrates, and optimisation of fermentation conditions.

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CONFLICT OF INTEREST

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Виявлення генів синтезу ліпопептидів методом RT-qPCR у ендофітних штамів *Bacillus pumilus* та *Bacillus amyloliquefaciens*

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Анотація. Ендофітні бактерії роду *Bacillus* є перспективними агентами біологічного захисту рослин завдяки широкому спектру антимікробних метаболітів, зокрема циклічних ліпопептидів (ЦЛП). Ендофітні штами *Bacillus pumilus* E11 і *Bacillus amyloliquefaciens* E12, виділені з насіння *Pinus sylvestris* L., виявляли антагоністичну активність щодо фітопатогенних мікроорганізмів, однак молекулярно-генетичні засади цієї активності залишалися невстановленими. Метою дослідження було охарактеризувати транскрипційну активність генів синтезу ЦЛП – фенгіцину, сурфактину та ітурину А – у *B. pumilus* E11 і *B. amyloliquefaciens* E12 за допомогою RT-qPCR із перевіркою специфічності методом аналізу кривих плавлення. Тотальну РНК виділяли з чистих культур у стаціонарній фазі росту із застосуванням буфера GuSCN, що містив DTT і протеїназу K; ДНК усували шляхом оброблення DNase I; зворотну транскрипцію проводили з використанням набору RevertAid First Strand cDNA Synthesis Kit, а RT-qPCR-аналіз здійснювали на ампліфікаторі CFX96 Opus (Bio-Rad) із застосуванням SYBR Green. Відносні рівні експресії

розраховували методом $2^{-\Delta\Delta Ct}$, використовуючи 16S rRNA як референсний ген. У ході дослідження в обох досліджуваних штамах було виявлено ген *fenD* оперону фенгіцину, а його специфічність підтверджено піком плавлення в діапазоні 74,0-74,5 °C. Середнє значення Cq для ампліфікації *fenD* становило 30,3 для *B. pumilus* E11 і 29,8 для *B. amyloliquefaciens* E12, тоді як середні значення Cq для 16S rRNA дорівнювали 21,8 і 23,8 відповідно. Відносна транскрипційна активність гена біосинтезу фенгіцину у *B. amyloliquefaciens* E12 у 5,7 раза перевищувала відповідний показник у *B. pumilus* E11 ($\Delta\Delta Ct = -2,5$). Гени синтезу сурфактину (*srfA*) та ітурину А (*ituA*) не було виявлено в жодному зі штамів. Отже, серед досліджених ендофітних штамів фенгіцин виявився єдиним протигрибковим ЦЛП, транскрипційну активність якого було підтверджено, що узгоджувалося з установленою в попередніх дослідженнях активністю цих штамів щодо мікроміцетів *Fusarium oxysporum* і *Alternaria alternata*. Високий рівень транскрипції оперону *fen* у *B. amyloliquefaciens* E12 підтвердив потенціал цього штаму як продуцента протигрибкових метаболітів для біоконтролю патогенів рослин

Ключові слова: мікроорганізми; фенгіцин; біосинтез; оперон; біоконтроль; протигрибкова активність; *Pinus sylvestris*