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Identification of antimicrobial peptide biosynthetic genes of *Bacillus pumilus* in suppression of *Phytophthora spp.*

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Abstract. *Phytophthora* species adversely impact citrus growth and health, leading to significant reductions in quality and yield in commercial orchards. *Bacillus pumilus*, a natural inhabitant of soil, shows potential as a biological control agent for managing *Phytophthora*. PCR analysis for antimicrobial peptide genes demonstrated that *B. pumilus* strains possessed up to four distinct antibiotic biosynthesis genes: *BacA*, *BmyB*, *spaS*, and *ituC*, which contributed to the production of antibiotics including bacilysin, bacillomycin, subtilin, and iturin. This study underscores *Bacillus pumilus* isolates as effective bacterial biocontrol agents against *Phytophthora*. Moreover, applying bacterial suspensions of *B. pumilus* strains shows promise in alleviating root rot in citrus seedlings, with observed plant survival rates of 100% and maximal growth promotion particularly notable in plants treated with *B. pumilus* (VN-K13).

Keywords: lipopeptides, biosynthetic genes, bacillus, antimicrobial peptides, bacillomycin, surfactin, biological control

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Научная статья

Идентификация генов биосинтеза антимикробных пептидов *Bacillus pumilus* в подавлении *Phytophthora spp.*

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Аннотация. Виды *Phytophthora* негативно влияют на рост и здоровье цитрусовых, что приводит к значительному снижению качества и урожайности в коммерческих садах. *Bacillus pumilus*, естественный обитатель почвы, показывает потенциал как биологический агент контроля за *Phytophthora*. Анализ методом ПЦР генов антимикробных пептидов продемонстрировал, что штаммы *B. pumilus* обладают четырьмя различными генами биосинтеза антибиотиков: *BacA, BmyB, spaS* и *ituC*, которые способствуют производству антибиотиков, включая бацилизин, бацилломицин, субтилин и итурин. Это исследование показало, что штаммы *Bacillus pumilus* – эффективные бактериальные биологические агенты контроля за *Phytophthora*. Кроме того, применение бактериальных суспензий штаммов *B. pumilus* показывает многообещающие результаты в борьбе с корневой гнилью у саженцев цитрусовых. При этом наблюдаются 100-процентная выживаемость растений и максимальное стимулирование роста; особенно заметное у растений, обработанных *B. pumilus* (VN-K13).

Ключевые слова: липопептиды, биосинтетические гены, бактерии, антимикробные пептиды, бацилломицин, сурфактин, биологический контроль

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Introduction. Citrus (*Citrus spp.*) stands as one of Vietnam's most economically significant fruit crops. Nevertheless, diseases like root rot, stem rot, gummosis, and brown fruit rot, primarily caused by *Phytophthora*, are prevalent and highly damaging, notably impacting orange and pomelo varieties [1, 2]. *Bacillus pumilus*, a Gram-positive, spore-forming bacterium, is ubiquitous across diverse habitats, including marine environments, deep-sea sediments, and soils [3]. Notably resilient, it withstands adverse conditions such as nutrient scarcity, desiccation, irradiation, hydrogen peroxide, and chemical disinfection [4]. The ecological significance of *B. pumilus* is underscored by its ability to produce compounds antagonistic to fungal and bacterial pathogens, including the production of lipopeptides and hydrolytic enzymes [5]. Lipopeptides such as fengycin, iturin, bacillomycin, and surfactin are known for their broad antimicrobial spectrum and potent surfactant activities [5].

B. pumilus MTCC7615, isolated from a rice field, has been identified as an antagonist against *Rhizoctonia solani* under in vitro conditions [6]. Meanwhile, strain *Bacillus pumilus* PTB185 has demonstrated secretion of lipopeptides from the surfactin, iturin, and fengycin families against *Botrytis cinerea* [2]. Additionally, surfactin produced by *B. pumilus* strains HR10 may play a role in inhibiting the growth of *Rhizoctonia solani* in *Pinus massoniana* seedlings [7].

Notably, several strains of *Bacillus* have linked the biocontrol of plant pathogens to the presence of antimicrobial peptide biosynthetic genes, such as *bmyB, fenD, ituC, srfAA,* and *srfAB* [8]. The simultaneous production of various antimicrobial peptides (AMPs) is crucial for efficient disease control and underlies the broad antagonistic activity observed in *Bacillus*. For instance, the production of mixtures of bacillomycin, fengycin, and iturin A by *B. subtilis* has been associated with the control of *Podosphaera fusca* in cucurbits [9]. Similarly, the production of bacilysin, iturin, and mersacidin in *B. subtilis* ME488 has been linked to the suppression of *Fusarium* wilt in cucumber and *Phytophthora* blight in pepper [10]. Consequently, *Bacillus* strains positive for all aforementioned AMP biosynthetic genes exhibit greater efficacy in inhibiting fungal growth compared to other isolates lacking one or more of these markers [11].

In a separate investigation, we discovered four *Bacillus pumilus* strains (VN-H5, VN-H8, VN-F8, VN-K13) to be highly effective against *Phytophthora* (unpublished data). Therefore, to assess their broad-spectrum antifungal activity, we conducted *in vivo* studies to evaluate the bioefficacy of these strains against *Phytophthora parvispora*. Additionally, we identified secondary metabolite biosynthetic genes using PCR-based molecular characterization. To the best of our knowledge, this study represents the first examination of the biocontrol ability of *Bacillus pumilus* strains with distinct lipopeptide signatures against *Phytophthora* in citrus through greenhouse assays.

Materials and methods. *Culture of B. pumilus and P. parvispora.* The microbial cultures *B. pumilus* strain H5, H8, F8 and K13 were collected from the microbial culture collection of Van Tran at the Department of Plant Pathology, Faculty of Agronomy, Vietnam National University of Agriculture.

P. parvispora – VN-Oo10 causing root rot and gummosis in citrus in northern Viet Nam was employed in this study [1, 2]. This isolate was cultured on Potato Dextrose Agar (PDA) medium at 25 °C for 5 days before further use.

Detection of biosynthetic genes from Bacillus species. DNA isolated from bacterial colonies was dissolved in a 50 µl volume of TE buffer (Tris + EDTA) at pH 8.0 within 1.5-ml Eppendorf tubes. The bacterial aliquot was then heat-treated at 100 °C for 10 minutes and utilized as the PCR template. To detect the presence of biosynthetic genes responsible for the production of bioactive compounds of the well-known antagonistic

bacterial genus *Bacillus*, six housekeeping genes were amplified employing specific primers as described in Table 1.

The PCR reaction was conducted with a total volume of 25 µl, comprising 12.5 µl of 2× MytagMM, 0.4 µl of each primer, 0.5 µl of DNA, and 11.2 µl of H_2O.

The PCR procedure was executed in a thermal cycler following these conditions: a single cycle of denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 20 seconds, annealing for 15 seconds, extension at 72 °C for 1 minute, and a final extension step at 72 °C for 5 minutes. The annealing temperature was set to 58 °C for for *fenD, ituC, srfAA, bacA* and *spaS*, to 55 °C for *bmyB*. Subsequently, the reaction was halted and allowed to cool to room temperature. The PCR products were visualized by electrophoresis on an agarose gel.

Evaluation of Bacillus spp. against Phytophthora under Protected Cultivation.

Preparation of plant materials. Orange (*Citrus sinensis* cv. Sanh) seeds were subjected to surface sterilization by rinsing with 95% ethanol for 30 seconds, followed by immersion in a 2.5% sodium hypochlorite (NaClO) solution for 10 minutes under constant gentle shaking. The seeds were then thoroughly rinsed with sterile distilled water ten times to remove any residual sodium hypochlorite.

Next, the seeds were treated with gibberellic acid (GA3) at a concentration of 80 ppm and incubated at 25 °C for 12 hours. These treated seeds were then planted in plastic pots (7×9 cm) filled with sterile sand. The pots were placed in an environment with temperature fluctuations ranging between 15–30 °C and a relative humidity of 60–80%. The seedlings were irrigated three times daily, and a nutrient solution was supplied weekly. After a 3-month period, orange seedlings with 3–4 true leaves were successfully grown. The pots were flooded several times with water to remove excess nutrient salts that could potentially affect zoospores before inoculation.

Preparation of biocontrol agents. Bacillus pumilus strains H5, H8, F8, and K13 were cultured on nutrient agar (NA) medium supplemented with yeast extract (3 g), peptone (5 g), NaCl (5 g), agar (15 g), 1,000 mL of H_2O. A loop of 24-hr-old culture of individual strains were inoculated into liquid Nutrient Broth (NB) medium, which consists of peptone (5 g/L), yeast extract (3 g/L), NaCl (5 g/L), 1,000 ml of water.

The flasks were placed on a shaker and incubated for 48 hours at 28 °C in darkness with shaking at a rate of 200 rpm. This suspension was then adjusted 1×10^6 colony forming units (CFU)/ml and $1x10^8$ CFU/ml, respectively for the use in study.

In vivo biological control of Phytophthora sp. on citrus. Five mycelial

Table 1 – Characteristic of specific primers

Gene	Produce name	Primer name	Sequence (5’ to 3’)	Size, bp
spaS	Subtilin	SPASF	GGTTTGTTGGATGGAGCTGT	375
		SPASR	GCAAGGAGTCAGAGCAAGGT	
fenD	Fengycin	FENDF	GGCCCGTTCTCTAAATCCAT	269
		FENDR	GTCATGCTGACGAGAGCAAA	
bmyB	Bacyllomicin	BMYBF	GAATCCCGTTGTTCTCCAAA	370
		BMYBR	GCGGGTATTGAATGCTTGTT	
bacA	Bacylisin	BACF	CAGCTCATGGGAATGCTTTT	498
		BACR	CTCGGTCCTGAAGGGACAAG	
ituC	Iturin	ITUCF	GGCTGCTGCAGATGCTTTAT	423
		ITUCR	TCGCAGATAATCGCAGTGAG	
srfAA	Surfactin	SRFAF	TCGGGACAGGAAGACATCAT	201
		SRFAR	CCACTCAAACGGATAATCCTGA	

plugs from the representative *P. Parvispora* (VN-Oo10) isolate were applied to the base region of the seedlings. Two days after inoculation with *Phytophthora*, 3 ml aliquots of the supernatant solution derived from four *Bacillus* bacterial strains, at two distinct concentrations (1×106 and 1×108), were administered to each seedling. The experimental setup followed a completely randomized block design, with each treatment group comprising 12 seedlings. Notably, positive controls (inoculated with *P. parvispora*) and negative controls (untreated controls) were included.

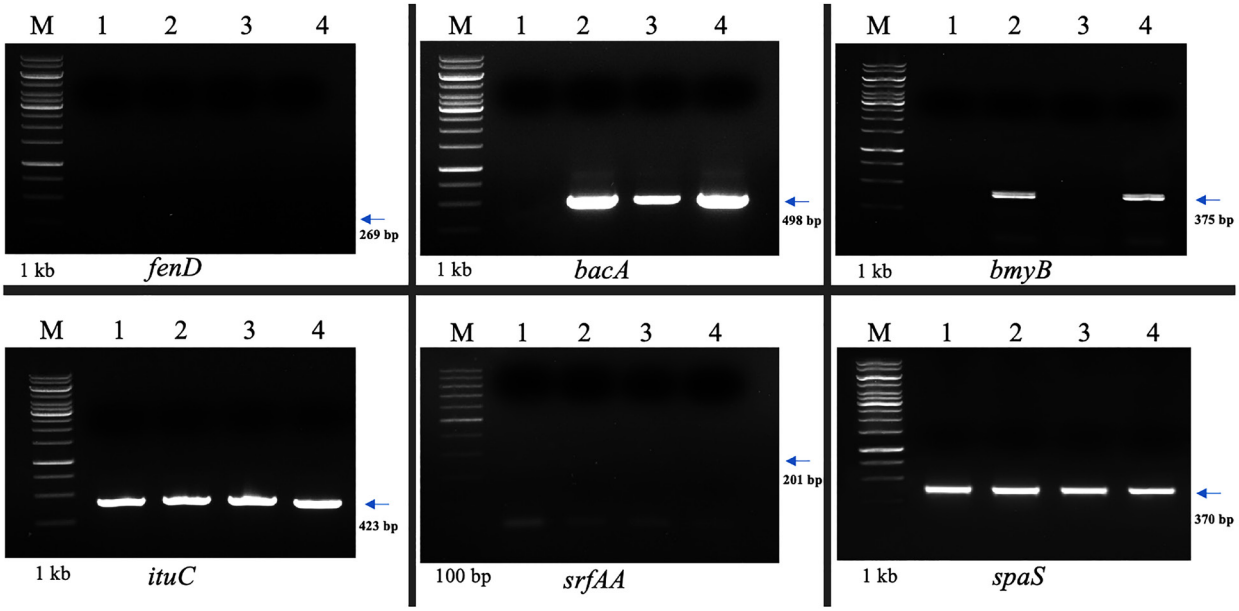
Four months after transplantation, the seedlings were carefully removed from the pots, and their roots underwent a thorough washing procedure to eliminate any residual sand particles. Subsequently, the roots and shoots of the seedlings were separated and subjected to a drying process at 80 °C for 72 hours before subsequent weighing.

Statistical analysis. All experiments were performed in triplicate. Significant differences between treatments were analysed in the SPSS statistics v. 26 software by one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference test (P <0.05).

Results and discussion. *Bacillus pumilus harbors various biosynthetic genes encoding antifungal compounds.* The production of antifungal compounds plays a pivotal role in the defense mechanisms employed by Biological Control Agents (BCAs) derived from *Bacillus* species against pathogens. These antifungal compounds can directly combat pathogenic microorganisms.

Investigating antibacterial resistance involved conducting Polymerase Chain Reaction (PCR) amplifications utilizing specific primer pairs. Six primer pairs were employed to amplify genes encoding antibacterial properties in four bacterial isolates. Positive amplifications were identified using the *bacA* primer pair, linked to bacylisin production, in three isolates VN-H8, VN-F8, and VN-K13.

Concurrently, all four strains exhibited positive PCR results with the *ituC* and *spaS* primer pair, correlated with iturin and subtilin productions. Concerning the *bmyB* primer pair for bacyllomicin production, positive amplification occurred exclusively in two isolates, VN-H8 and VN-K13. However, the other two targeted genes, *fenD* and *srfAA* associated with fengycin and surfactin productions, did not yield any PCR products (Fig. 1).



M: 1 kb marker (*bmyB, fenD, ituC, bacA*, and *spaS*). M: 100 bp marker (*srfAA*)
Lane 1: VN-H5. Lane 2: VN-H8. Lane 3: VN-F8. Lane 4: VN-K13

Figure 1 – The presence of the six Antimicrobial peptides genes was determined in the corresponding *Bacillus* isolates

In vivo biological control of phytophthora sp. on Orange (cv. Sanh) trees. After a precisely controlled 16-week inoculation period, the results indicated a significant reduction in disease severity for all four bacterial isolates – VN-H5, VN-H8, VN-F8, and VN-K13 (Table 1, Fig. 2).

However, no significant difference was observed at both dosages (1×106 and 1×108 colony-forming units (CFU)/ml). This decrease in disease severity, attributed to antagonistic bacteria, is presumed to be associated with biological control mechanisms and their adaptation to the host plant environment.

Analyzing tree height parameters, the three bacterial strains VN-H8, VN-F8, and VN-13 demonstrated dimensions comparable to the uninoculated control. Only the VN-H5 bacterial strain displayed a lower height than the uninoculated control but a height higher than that of the inoculated control.

Shifting focus to tree diameter parameters, only the strain VN-K13 exhibited a diameter surpassing that of the uninoculated control. The remaining bacterial strains showed measurements nearly equivalent to the uninoculated control and surpassed those of the inoculated control.

Regarding fresh weight parameters of shoots, the VN-H5 bacterial strain presented a lower weight than the uninoculated control. In contrast, the other three bacterial strains demonstrated weights higher than the uninoculated control, with VN-K13 exhibiting superiority, followed by VN-F8 and VN-H8. This trend persisted when considering shoot dry weight parameters.

Upon scrutinizing fresh weight parameters of roots, only the VN-K13 bacterial strain had a lower weight than the uninoculated control, while the VN-F8 bacterial strain had a weight higher than equivalence to the uninoculated control. In contrast, both VN-H5 and VN-H8 strains displayed weights lower than the uninoculated control.

Concerning root dry weight parameters, only the strain VN-K13 had a weight higher than the uninoculated control. In contrast, the remaining three strains VN-H5, VN-H8, and VN-F8 demonstrated weights lower than the uninoculated control but higher than the inoculated controls. The survival rate of seedlings subjected to treatment with *B. pumilus* bacterial strains was higher than that of the inoculated control, highlighting the potential efficacy of these bacterial strains in enhancing plant resilience against phytopathogens.

Discussion. To predict and to some extent elucidate the antifungal compounds

Table 2 – *In vivo* evaluation of biological activity of biological control agents on the control of *Phytophthora* root rot of Orange (cv. Sanh) seedlings

Treatment	Plant height, cm	Plant diameter, cm	Shoot fresh weight, g	Shoot dry weight, g	Root fresh weight, g	Root dry weight, g	Percentage survival, %
Uninoculated	17.58 c[1]	4.55 cd	57.2 d	19.73 d	40.86 f	12.04 g	100
Inoculated	11.83 a	3.39 a	38.24 a	12.18 a	19.12 a	6.62 a	75
K13 (a)	17.55 c	4.61 cd	64.71 f	21.21 f	49.78 g	14.23 h	100
K13 (b)	17.61 c	4.66 d	66.67 g	22.22 g	51.28 h	14.66 i	100
H5 (a)	16.19 b	4.11 b	44.64 b	14.88 b	27.90 b	7.65 b	91.67
H5 (b)	16.18 b	4.18 b	48.51 c	16.17 c	30.32 c	8.30 c	83.33
F8 (a)	17.48 c	4.47 c	61.71 e	20.57 ef	39.81 f	11.07 f	100
F8 (b)	17.52 c	4.46 c	61.63 e	20.54 ef	39.76 f	11.03 f	100
H8 (a)	17.46 c	4.48 c	58.84 d	19.61 d	36.78 d	10.21 d	100
H8 (b)	17.53 c	4.51 cd	60.97 e	20.32 de	38.11 e	10.59 e	91.67

Bacillus isolates (K13 (a), H5 (a), F8 (a), H8 (a) and K13 (b), H5 (b), F8 (b), H8 (b)) were drenched at 1×10^6 or 1×10^8 CFU/ ml respectively.

[1] Means (n=12) in both columns and rows followed by the same letters are not significantly different ($p < .05$, Tukey's HSD test).

From left to right: Control without inoculated, inoculated with *P. parvispora* and *B. pumilus* K13, inoculated with *P. parvispora* and (B) root, respectively; Symptom was pictured at 4 months after inoculation

Figure 2 – Response of the leaves and roots of orange seedlings to treatment with *Phytophthora parvispora* isolate VN-Oo10 and *Bacillus pumillus* isolate VN-K13

potentially synthesized by the *B. pumilus* strains, the study investigated the presence of genes responsible for the biosynthesis of specific antimicrobial antibiotics like bacillomicin, iturin, bacylisin, subtilin, Fengycin, and surfactin using PCR. PCR-based detection of bacteria producing these specific antibiotics is preferred over screening and random isolation methods due to its efficiency and reduced time requirements.

Result, our VN-K13 strain was found to harbor genes, including *bmyB, ituC, bacA*, and *spaS*, which encode well-known antifungal compounds such as bacillomicin, iturin, bacylisin, and subtilin. These findings suggest that these *B. pumilus* strains have the potential to produce a range of antibiotics. This paves the way for an investigation into whether the combined action of these antibiotics plays a role in disease suppression, or if it's the result of the individual antibiotics, and whether this serves as a competitive strategy against other microorganisms.

The existence of biosynthetic genes responsible for antibiotic production may provide a plausible rationale for the antifungal properties observed in these *B. pumilus* strains. These molecules are capable of decreasing pathogen growth [12].

Hence, our investigation furnishes compelling evidence affirming the pivotal role of genes encoding lipopeptides in combating *Phytophthora*. The bacterial capacity to synthesize lipopeptides is critical for assessing its potential as a biological control agent (BCA) against plant pathogens [13].

Plant growth-promoting rhizobacteria (PGPR) produce various antibiotic compounds, among which lipopeptides are significant contributors to the antifungal activity of *Bacillus* species [14]. Cao et al. (2018) demonstrated that iturin and fengycin, secreted by *B. velezensis*, are responsible for its antimicrobial properties, while surfactin is implicated in biofilm formation and cell motility, crucial for successful rhizosphere colonization [14]. *Bacillus velezensis* exhibits versatility in producing antibiotic compounds, including surfactin, iturin, fengycin, ericin, and others [15].

Zalila-Kolsi et al. (2016) highlighted the broad-spectrum antifungal activity of *B. amyloliquefaciens* and *B. subtilis*, producing iturin and surfactin, and surfactin

and fengycin, respectively, against various phytopathogenic fungi [16].

Gong et al. (2015) reported that both iturin A and plipastatin (fengycine). A display fungicidal activity, with iturin A being more potent at lower concentrations than plipastatin A [17]. Furthermore, treatment with these molecules induces deformities and damages in hyphal morphology [17].

Toral et al. (2018) demonstrated that the biocontrol of *Botrytis cinerea* by *Bacillus* XT1 is facilitated by lipopeptides, suggesting that the mycelial structure of *B. cinerea* is likely degraded by these compounds [18]. Importantly, this study marks the first instance of the identification of VN-K13 and VN-F8 strains as positive for the presence of the *Bacyllomicin B* gene.

Nonetheless, additional research is imperative as this observation could be linked to a malfunction in the transfer of 4'-phosphopantetheine from coenzyme A to peptidyl transport protein, potentially induced by mutations in the *sfp* gene [19], resulting in the aforementioned strains being incapable of producing any lipopeptide.

Our results underlined an increase in the plant growth of Orange (cv. Sanh) seedlings treated with antagonist bacteria (bacterization) in comparison with untreated controls. Previous studies documented the existence of multiple biocontrol mechanisms among the studied bacteria that explains their potential as successful biocontrol [20].

In the current study, we have investigated a biocontrol strain that could be used as an alternative agent for controlling Phytophthora disease in citrus trees. Our experimental results have significantly enhanced our comprehensive understanding of the potential antifungal mechanisms of B. pumilus VN-K13. It has the potential for development as a biocontrol agent and biofertilizer due to the presence of genes encoding antimicrobial antibiotics such as bacillomicin, iturin, bacylisin, subtilin. To the best of our knowledge, this is the first study focused on citrus biocontrol using the antagonistic bacterium B. pumilus.

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