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Gene expression of lipopeptide biosynthesis in *Bacillus subtilis* analyzed by RT-qPCR using the method 2-ΔΔ CT

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***Abstract* — The genus *Bacillus* is a critical ally in combating phytopathogens such as *Alternaria* sp. and *Botrytis* sp., which cause losses to the agricultural sector in Ecuador. *Bacillus subtilis* generates important lipoproteins belonging to the fengicin and iturin families; therefore, this study analyzed the expression of genes involved in the synthesis of FEND and ITUDI lipoproteins. In addition, the antagonism of *Bacillus subtilis* against phytopathogenic microorganisms was analyzed through the PICR (percentage of radial growth). Using molecular techniques such as RT-qPCR, the expression levels of the aforementioned genes were quantified on three specific days: 1, 5 and 9 when *Bacillus subtilis* is in confrontation with phytopathogens. For this purpose, three treatments with the phytopathogens mentioned above were carried out. Total RNA was extracted, followed by retrotranscription, and finally, the cDNA was subjected to qPCR analysis to determine gene expression values. It was determined that the gene coding for phengicins is expressed seven times on the fifth day of treatment when *Bacillus subtilis* is in the presence of *Alternaria* sp. It was also determined that the ITUDI gene is expressed an average of 4.56 times more on the fifth day of treatment in the presence of *Alternaria* sp. Finally, the expression levels of FEND and ITUDI are expressed an average of 3.6 and 2.3 times, respectively, when it is in antagonism with *Botrytis* sp.**

***Keywords*: *Alternaria* sp., *Botrytis* sp.; plant-induced chemoresistance; ribonucleic acid; complementary DNA; Fengicins; Iturins; Phytopathogens.**

***Resumen*— El género *Bacillus* es un aliado fundamental en la lucha contra fitopatógenos como *Alternaria* sp. y *Botrytis* sp., los cuales ocasionan pérdidas en el sector agrícola del Ecuador. *Bacillus subtilis* produce importantes lipoproteínas pertenecientes a las familias de las fengicinas e iturinas; por ello, en este estudio se analizó la expresión de los genes involucrados en la síntesis de las lipoproteínas FEND e ITUDI. Además, se evaluó el antagonismo de *Bacillus subtilis* frente a microorganismos fitopatógenos mediante el PICR (porcentaje de inhibición del crecimiento radial). Utilizando técnicas moleculares como la RT-qPCR, se cuantificaron los niveles de expresión de los genes mencionados en tres días específicos: 1, 5 y 9, cuando *Bacillus subtilis* se encontraba en confrontación con los fitopatógenos. Para ello, se realizaron tres tratamientos con los fitopatógenos antes mencionados. Se extrajo el ARN total, seguido de la retrotranscripción y, finalmente, el ADNc fue sometido a análisis por qPCR para determinar los valores de expresión génica. Se determinó que el gen codificante de fengicinas se expresa siete veces más en el quinto día de tratamiento cuando *Bacillus subtilis* está en presencia de *Alternaria* sp. Asimismo, se estableció que el gen ITUDI se expresa en promedio 4,56 veces más en el quinto día de tratamiento en presencia de *Alternaria* sp. Finalmente, los niveles de expresión de FEND e ITUDI se incrementan en promedio 3,6 y 2,3 veces, respectivamente, cuando se encuentra en antagonismo con *Botrytis* sp.**

***Palabras clave*: *Alternaria* sp., *Botrytis* sp.; quimiorresistencia inducida en plantas; ácido ribonucleico; ADN complementario; Fengicinas; Iturinas; Fitopatógenos.**

I. INTRODUCTION

CONVENTIONAL agriculture has focused on the use of chemicals to solve the deficiencies of the agricultural field where continuous and indiscriminate use has been seen, which has caused damage to the environment and human health, as is the case of pesticides such as chlorobenzilate, ethylene dibromide, organochlorines, etc. [1]. 19% of Ecuador's territory is used for agriculture, where most crop losses are caused by serious phytopathogenic diseases such as those triggered by *Botrytis, Alternaria, Fusarium, Penicillium, Cercospora, Colletotrichum* [2]. In Ecuador, there is a great affection for crops of interest by fungi *Alternaria* sp. and *Botrytis* sp. For example, *Botrytis* cinerea can infect 235 species with the socalled "Gray Rot", where the most affected fruit is strawberries and roses. [3] [4] [5] [6] This fungus occurs in various environments, such as cold, temperate, and tropical climates. *Alternaria* sp. causes damage to cauliflower, beans, and tomato crops [7]. Therefore, options are being sought to control plant pathogens, where biological agents are an alternative since they will help eliminate or mitigate the damage caused by pathogenic

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fungi in crops [8] [9]. *Bacillus subtilis* is an optimal biocontroller since it can be foliar and root applied. In addition, it can colonize the rhizosphere (root-surrounding soil); it produces digestive enzymes that degrade and eliminate fungi and bacteria, while also exhibiting resistance to high temperatures, osmotic fluctuations, and other stressful environmental conditions. For this reason, it is a bacterium studied worldwide; it also produces peptide metabolites with antibiotic action (surfractins, iturins and fengicins) [10] [11]. Therefore, *Bacillus subtilis* is an alternative that must be considered to control various phytopathogenic fungi. Dual cultures are an excellent technique for studying the inhibitory capacity present in one microorganism against another [3]. This analysis consists of stressing a microorganism of interest, i.e., modifying certain conditions such as temperature, nutrients, and space so that it can generate metabolites that help to cope with adverse conditions, in addition it is subjected to a confrontation with another microorganism; with the main objective that they can compete for space and nutrients necessary to ensure their survival [12] [13] [14]. The metabolites generated from the dual culture can be quantified in two ways; the first is through physicochemical methods, e.g., chromatography, nuclear magnetic resonance, infrared transmission, and mass spectrophotometry [15]. The main advantage of these methods is that they identify different families of lipoproteins generated by *Bacillus* spp. The second way to quantify and identify lipoproteins is by molecular methods since they allow a more specific evaluation and an understanding of at what point there is a higher expression of these metabolites [13] [16]. The main molecular techniques for quantifying and identifying lipoproteins are RT-PCR and RT-qPCR [17] [18]. PCR is a tool widely used by molecular biology and, in conjunction with electrophoresis, allows the identification of the presence of genes encoding a particular lipoprotein, either surfactins, fengicins, or iturrins [16]. The second technique allows the researcher to quantify and identify in real time. RT-qPCR is a fast and efficient analysis where no other more laborious and expensive analysis is required. This analysis can be quantified using two methodologies, either absolute or relative. [19] [20]. Relative quantification is ideal for this type of analysis since it is more suitable for determining metabolic and physiological changes at the cellular level. According to [13] [21] [19]. To determine the levels above, the model $2\Delta\Delta Ct$ proposed by Livak and Schmittgen [22]. Where it consists of interpolating the data at a threshold point or cut-off cycle (CT) and determining the expression changes that can be generated by the microorganisms under study [16] [23]. This study aims to quantify the expression levels of the two principal genes involved in lipopeptide biosynthesis in *Bacillus sp.*, as a way to assess their potential contribution to the control of major phytopathogenic diseases affecting agriculture. Elevated expression of these biosynthetic genes would be expected to reflect a stronger biocontrol capacity against common crop phytopathogenic in Ecuador.

II. MATERIALS AND METHODS

A. Activation of microorganisms

The microorganisms involved in the molecular analysis, such as *B. subtilis, Alternaria* sp., and *Botrytis* sp., were cryopreserved at -80 °C. These microorganisms were provided by the Life Sciences Laboratories of the Universidad Politécnica Salesiana, Quito, Ecuador [24]. The reactivation of *Bacillus subtilis* was prepared in a TSB medium, where 30 gL^{-1} was used in 1 L of distilled water and then incubated at 35°C for 24 h. After this time, it was inoculated in a petri dish with nutrient agar and incubated under the abovementioned conditions. To reactivate the phytopathogenic microorganisms, the Papa Dextrose Agar (PDA) medium was used, and incubated at 25°C.

B. Morphological identification Bacillus subtilis.

Next, a morphological identification of the microorganism was performed to ensure the absence of contaminating agents that could compromise the assay. Colony characteristics of *Bacillus subtilis* were examined, revealing opaque colonies with a white to grayish coloration, medium-sized (approximately 3–4 mm in diameter), circular in shape, flat, and with a dry, matte appearance. Microscopic evaluation included Gram staining, in which *Bacillus subtilis* exhibited the typical morphology of Gram-positive bacilli. Endospore detection was conducted using endospore staining with malachite green, allowing clear visualization of spore-forming structures. For the phytopathogenic microorganisms (*Alternaria* sp. and *Botrytis* sp.), a monosporic culture was obtained prior to analysis, and their structures were confirmed via methylene blue staining to ensure the absence of foreign or contaminant elements.

C. DNA extraction

Previously, the microorganisms to be analyzed were seeded in TSB media cultivated for 24 hours at 35°C. After this time, the PureLink™ Microbiome DNA Purification Kit (Invitrogen, USA) was used, which was essential for the extraction of the genetic material of *Bacillus* sp., which was later quantified by Qubit™ 4 Fluorometer (Invitrogen, USA), and the dsDNA HS Assay Kit (Invitrogen, USA) was used. Subsequently, the 16S rRNA gene region (V4) was amplified using the universal primers 518F and 800R to verify the presence of the purified bacterial DNA template and corroborate that the primers of the housekeeping region were amplified correctly. This confirmed the sample viability for the subsequent amplification of the genes under study [25].

D. PCR

The genes involved in the analysis of lipoproteins were ITUDI and FEND, for which primers amplifying the regions of interest were used:

TABLE I
SEQUENCE OF PRIMERS USED FOR PCR AND QPCR AMPLIFICATION

	F 5’	R 5’
FEND	TTTGGCAGCAGGAGAAGTT	GCTGTCCGTTCTGCTTTTTC
	3’	3’
	F 5’	R 5’
ITUDI	GATGCGATCTCCTTGGATGT	ATCGTCATGTGCTGCTTGAG
	3’	3’

In addition, the 16S gene was used to calibrate the data to assess expression levels. Amplification to determine the presence of the FEND and ITDI genes was performed using the DreamTaq™ Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA). Following the manufacturer's instructions. The amplification protocol was then carried out, with an initial denaturation of 95°C for 1 minute, continued with 45 cycles that counted with denaturation of 95°C for 30 seconds, Annealing of 60°C for a time of 30 seconds; the protocol also counted with Extension of 72°C for 30 seconds. A final Extension of 72°C for 3 minutes was added [13]. A holding cycle of 4°C for an undetermined time was programmed to end the thermal phase. The thermal phase of the PCR was carried out in the Proflex TM PCR System Applied BiosystemsTM (Thermo Fisher Scientific, Waltham, MA, USA) thermal cycler ideal for conventional PCR reactions. Once the reaction was finished, electrophoresis was performed to observe the amplification of the PCR products.

E. Antagonistic test: Dual culture

Dual culture was used to microbiologically detect the regulatory effect of B. *subtilis* against *Alternaria* sp. and *Botrytis* sp. 22 g of PDA were prepared in one liter of distilled water to reduce the necessary nutrients, forcing the microorganisms to fight for their survival. For this purpose, a disc of 5 mm diameter was inoculated with the fungus in the middle of the Petri dish; then, a groove was made with a separation of 3 mm [13]. The microorganisms were incubated at 25°C and were evaluated on days 1, 5, and 9 according to the methodology proposed by [16] [26]. These days, it is necessary to determine the percentage of radial growth inhibitors (PIRC) and to obtain the genetic material needed for the analysis by qPCR. To obtain the PICR, the formula proposed by Acurio [27]. Eq. 1:

$$PICR = \frac{R1–R2*100}{R1} \quad (1)$$

R1 represents the radius of the fungus without biocontroller and R2 is the radius of the fungus with the controller.

F. RNA extraction

The genetic material necessary for the qPCR analysis was extracted from the dual cultures from days 1,5 and 9, to which B. *subtilis* was subjected to the phytopathogenic microorganisms. The Purelink RNA Mini-Kit extraction kit (Ambion, Life Technologies, USA) was used to obtain the material. The RNA obtained from the extraction was then quantified in $\mu g(\mu L)^{-1}$ using the Qubit™ 4 Fluorometer system (Invitrogen, USA), and the RNA HS Assay Kit (Invitrogen, USA) was applied. Additionally, to ensure optimal subsequent amplification, consider that high-quality samples correspond to integrity values of RIN ≥ 7–8, which are generally required for reliable amplification and cDNA-based analyses that provide the best performance.

G. Retrotranscription

Retrotranscription was carried out using the Superscrit® III First-Strand Synthesis SuperMIX for RT-qPCR kit (Invitrogen) to obtain cDNA, and an RNA concentration of at least one $\mu g(\mu L)^{-1}$ [26] was required. The reaction was then incubated at 25 °C for an estimated 10 min, 50 °C for 30 min, 85 °C for 5 min only. The reaction was cooled to 4 °C to add 1 μL of *E. coli* RNase H at 37 °C for 20 min to eliminate any interfering RNA template. The product obtained was quantified using the Qubit ssDNA Assay Kit (Invitrogen).

H. qPCR

The qPCR was carried out in the QuantStudio ® 5 kit (Thermo Fisher Scientific, Waltham, MA, USA). The reaction was prepared from 10 μL of Fast Sybr Green Master Mix (Thermo Fisher Scientific) following the manufacturer's recommendations, 1 μL of Forward and Reverse primers was added, and 5 μL of cDNA, whose minimum concentration is 2 $\mu g(\mu L)^{-1}$ was added. [16] completed with nuclease-free water until a final volume of 20 μL was obtained. *Bacillus megaterium* was included as a positive control, given its previous characterization under the same experimental protocol. The negative control consisted of a reaction with PCR-grade water, excluding the microorganism's DNA/RNA template to confirm the absence of contamination. It was then taken to the thermal cycler, where it was set up with the following temperatures:

TABLE II
REAL TIME AMPLIFICATION PROTOCOL

Amplification program

Amplification phase	Temperature/Time-Cycle
Initial denaturation	95°*C*/ 1 min-1 cycle
Denaturation	95°*C*/ 30 seg-45 cycles
Annealing	60°*C*/ 30 seg-45 cycles
Extension	72°*C*/ 30 seg-45 cycles
Final Extension	72°*C*/ 3 min -1 cycle
Melting curve	95°*C*, 65°*C*, 95°*C*/(15 seg-1min-1 seg)
Cooling	4°*C*

The gene expression levels were calculated using the method $2 - \Delta\Delta CT$ [22] [19] To perform expression calculations, obtaining a calibrator group CT helps to minimize the error between samples. For this, a CT is set for both the gene of interest and the reference gene, that is Eq. 2:

$$\Delta CTCalibrador = CT\ gen\ de\ interés - CT\ gen\ referencia \quad (2)$$

The $\Delta CTCalibrador$ will be used to make the comparison between the ΔCT of the samples of the genes of interest to be evaluated in Eq. 3:

$$\Delta\Delta CT = \Delta CTCalibrador - \Delta CT\ gen\ de\ interés \quad (3)$$

Finally, we determine the expression levels of the $\Delta\Delta CT$ obtained by Morales [23]. Eq. 4:

$$Expression\ levels = 2 - \Delta\Delta CT \quad (4)$$

I. Statistical analysis

A 3 * 3 factorial design was used, taking the independent variable raised about the development of *Bacillus subtilis* aga-

inst phytopathogenic microorganisms on days 1, 5, and 9, the dependent variable raised will be the levels of gene expression in lipopeptides, in addition to three replicates per treatment were raised, resulting in 27 experimental units where it will be related to the time that *B. subtilis* is in contact with phytopathogenic organisms, also if the time implies that there is a change at the level of gene expression. For this, ANOVA analysis was used to understand statistically how the results obtained behaved. Subsequently, Tukey's Post Hoc test was used with a significance level of 5%, using the INFOSTAT statistical program.

III. RESULTS AND DISCUSSIONS

A. Radial growth inhibition

The ANOVA analysis showed a p-value < 0.0001 for the variables day, growth, and their interaction, indicating a significant difference among treatments and confirming that the inhibition percentage produced by *Bacillus subtilis* depends on the incubation time. Tukey's test identified four significance groups (A, B, C, and D), with the highest inhibition values occurring in group D (*B. subtilis* vs. *Alternaria* sp. on day 9, PICR = 86.57%) and group C (*B. subtilis* vs. *Alternaria* sp. on day 5, PICR = 47.16%). These values represent the strongest inhibitory effects observed in this study, demonstrating that *B. subtilis* can suppress the growth of *Alternaria* sp. by nearly 90%. It is important to emphasize that inhibition reference ranges reported in the literature are relative, as inhibition levels vary according to antagonism time and the target phytopathogen; studies have described inhibition values as low as 4.75%–25% under certain conditions, while higher ranges (33.26%–98.48%) are considered the most effective. Comparable research has shown that *Bacillus* sp. often exhibit stronger inhibition from day 5 onward, reaching 40%–60% against *Alternaria*, consistent with the patterns observed here. Additionally, references [26] and [27] indicate that several *Bacillus* species, including *B. subtilis*, are effective antagonists against multiple phytopathogens.

TABLE III
INHIBITION PERCENTAGES

Growth	Day	PICR(%)
B. subtilis against	1	18,19
Alternaria sp.	5	47,1
	9	86,57
B. subtilis against	1	13,33
Botrytis sp.	5	24,7
	9	33,26

B. Gene expression of the FEND gene

In this study, *Bacillus subtilis* was shown to inhibit the growth of the phytopathogenic fungi to which it was subjected. The qPCR analysis helped to determine the gene involved in synthesizing phengicins, one of the main families of lipoproteins [28]. The study monitored the expression levels on the proposed evaluation days, which were days 1, 5, and 9. Low expression levels on the first evaluation day, as shown in Figure 1. On day 5 of the assessment, it can be found that the expression levels present higher levels of abundance of FEND, indicating a greater inhibition against phytopathogenic organisms on this day. This coincides with the findings of Chiluisa-Utreras, Medrano, et al. [16], which concluded that the day of greatest performance of the FEND gene is the fifth day of evaluation. On day 9 of the review, the expression levels are low, mainly due to [13], the highest FEN expression observed on day 5 suggests that fengycin biosynthesis occurs early in the growth kinetics. This behavior indicates that *Bacillus* sp. activates the fengycin production pathway before entering the stationary phase, likely in response to competition signals or early stress associated with the late exponential phase. This identifies day 5 as the optimal point for detecting or harnessing maximum fengycin production, which is consistent with the fact that *Bacillus subtilis* later enters a dormancy phase, making the production of this gene impossible at that stage.

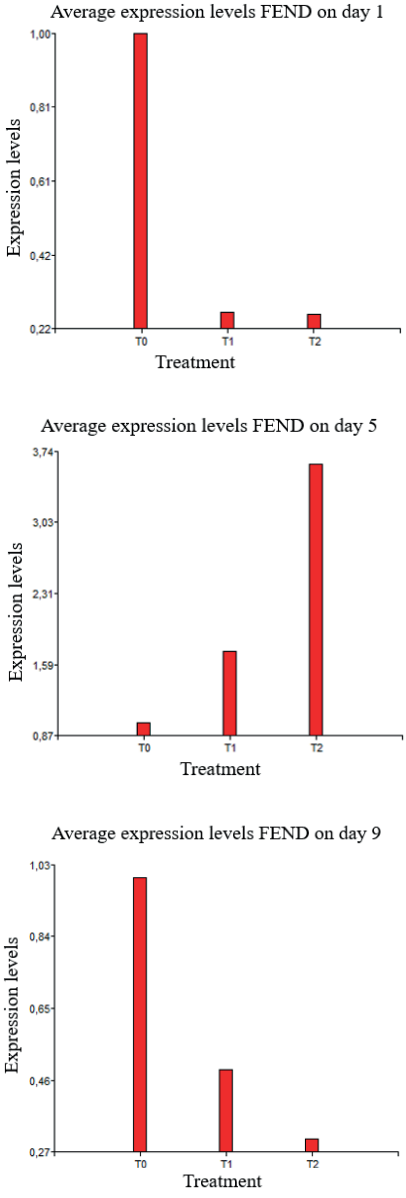


Fig. 1. Average expression levels of the FEND gene on days 1, 5, and 9 of the trial.

A represents the average levels on the first day of the evaluation, whereas Treatment 2 (T2) is *Bacillus subtilis* vs *Alternaria* sp., which shows low expression levels. On the fifth day, the expression levels for T2 represent an increase compared to day 1. On the ninth day, the expression levels were low. The ANOVA analysis indicates a p-value of 0.0205; significant differences are evident in the day variable, which shows that the highest production of this gene is found on the fifth day (Figure 2). This study found no differences in the treatments, indicating that although phengicins are present, their yield will depend on time. Chowdhury et al. [29] argue that other *Bacillus* genera will have greater control against certain microorganisms. Chiluisa-Utreras, Medrano, et al. [16] determined that the yield of FEND in *B. megaterium* was 20 times more than that of B. *subtilis*, which manages to inhibit both the variable days and treatments, demonstrating that it is capable of exerting biocontrol of *Alternaria* sp. and *Botrytis* sp. In addition, *B. subtilis* has good efficacy in inhibiting *Alternaria* sp. In addition, Pedraza et al. [30] and Ley-lópez et al. [28] found that *B. amyloliquefaciens*, when stimulated by *Collectotrichum* spp. generates an increased production of fengycins capable of neutralizing its competitors.

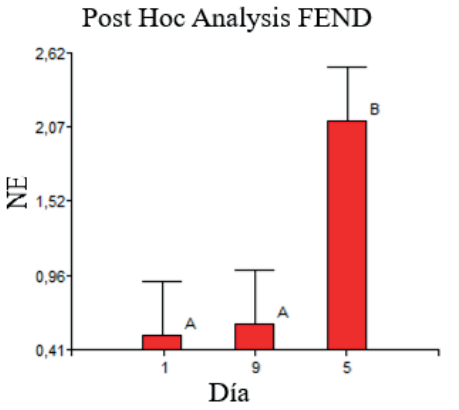


Fig. 2. The post hoc analysis and the Tukey test indicated that the fifth day of the trial showed higher expressions levels.

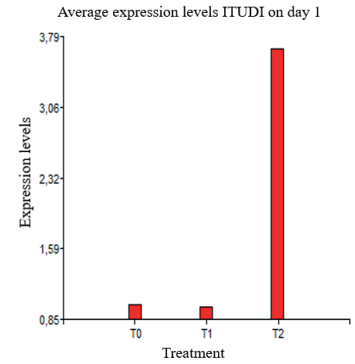
TABLE IV
ANOVA ANALYSIS FEND

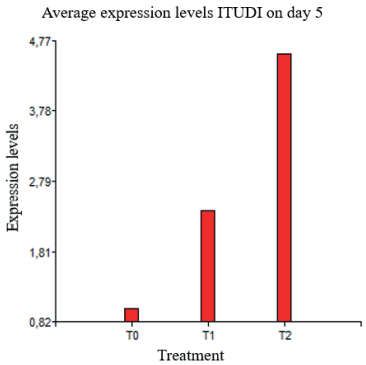
F.V.	GL	SC	CM	F	P-VALOR
TREATMENT	2	1,51	0,76	0,5	0,6136
DAY	2	14,64	7,32	4,86	0,0205
TREATMENT*DAY	4	11,28	2,82	1,87	0,1589
ERROR	18	27,09	1,5		
TOTAL	27				

C. Gene expression of the ITUDI gene

Fengicins represent one of the main families of lipoproteins, however, analyzing the expression of genes coding for iturrins is essential, since the expression levels of this lipoprotein act in synergy with the other families of lipoproteins increasing the levels of inhibition [31]. The expression levels of the ITUDI gene show that on the first day of the evaluation, average ITUDI production was from 0.98 to 3.66 for both *Botrytis* sp. and *Alternaria* sp., respectively. By the fifth day of the assessment, the averages ranged from 2.39 to 4.58, indicating an increase in metabolite production. By the last day of the evaluation, the expression levels dropped from 1.8 to 1.6, as shown in Figure 3. The production levels of this metabolite show low levels of expression; [32] state that the production range is from 0 to 3, but its presence also indicates that other lipoproteins such as surfactins may develop. Cadena & Medrano [13] mentioned that iturins stimulate the production of fengycins and surfactins. In the present study, the gene expression levels of the ITUDI gene are not high, but their presence helps stimulate better antagonistic activity.

A represents the average levels on the first day of the evaluation, whereas Treatment 2 (T2) is *Bacillus subtilis* vs *Alternaria* sp., which shows considerable expression levels. On the fifth day, the expression levels for T2 represent an increase compared to day 1. On the ninth day, the expression levels present low levels in contrast to the previous evaluation days. The ANOVA analysis does not indicate a significant p-value for the variable days. However, it does show differences for the variable treatments, which suggests that *B. subtilis* is an excellent biocontroller for treating *Alternaria* sp.; B. *subtilis* presented low levels of expression of this gene, but Dang et al. [33] found that this *Bacillus* genus tends to develop better inhibition when they are in the presence of *Alternaria* sp. Thanks to this, we were able to see evidence that there is better control microbiologically.





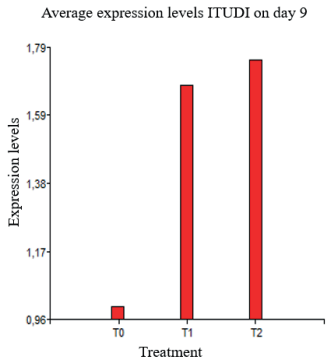


Fig. 3. Average expression levels of the ITUDI gene on days 1, 5, and 9 of the trial.

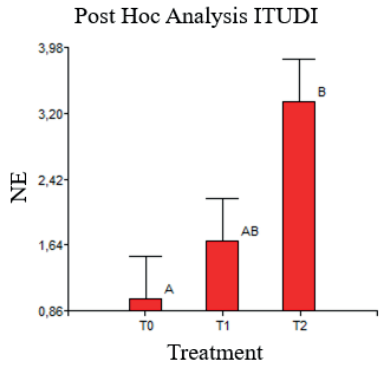


Fig. 4. Post hoc analysis and Tukey test indicated that the variable trial treatments showed higher expressions levels at T2.

TABLE V
ANOVA ANALYSIS ITUDI

F.V.	GL	SC	CM	F	P-VALOR
TREATMENT	2	25,92	12,96	5,68	0,0122
DAY	2	6,47	3,24	1,42	0,2679
TREATMENT-DAY	4	8,98	2,24	0,98	0,4410
ERROR	18	41,06	2,28		
TOTAL	26	82,43			

The greater inhibitory activity observed, despite the low ITUDI levels in *B. subtilis*, is consistent with the findings of Dang et al. [33], who found that this genus develops better inhibition in the presence of *Alternaria* sp. The expression of the ITUDI genes will depend on several factors, one of which is the species of *Bacillus*, since each one will develop optimal expression levels for inhibition [31] [34]. Furthermore, the increase in fungicidal capacity is due to synergistic factors: the presence of other types of lipopeptides enhances the great antagonistic capacity. Corrales [31] and Yánez-Mendizábal et al. [34] state that the presence of other lipopeptides, especially surfactins, is necessary, as their action in concert potentiates antifungal activity, which is crucial for the inhibition of filamentous fungi like those analyzed in the present study.

IV. CONCLUSIONS

The results obtained in the study showed that the FEND gene is stimulated in the presence of an external agent, i.e., *Alternaria* sp. stimulates the presence of this gene, and on the fifth day of the trial, the expression levels tend to be higher than on other days. The study did not find data of relevance when *B. subtilis* is confronted with *Botrytis* sp. Still, inhibition can be evidenced, suggesting a synergy between other families of lipoproteins that tend to inhibit *Botrytis* sp. Therefore, It is concluded that the fengycins generated by *B. subtilis* have an inhibitory capacity against *Alternaria* sp. and can be used as a biocontroller to combat this phytopathogen. However, this family of proteins is a fundamental precursor for the development of other families of lipoproteins, such as surfactins, which, together with fengycins, enhance the inhibitory development essential to combat phytopathogenic microorganisms such as *Alternaria* sp., *Botrytis* sp., *Collectotrichum* sp., among others, these characteristics highlight the potential of *B. subtilis* to contribute to more sustainable phytopathogen-management strategies through the natural production of bioactive molecules with broad inhibitory capacity, as *B. subtilis*–based formulations could be developed as sustainable alternatives to chemical fungicides, contributing to environmentally friendly crop protection and enabling future applications such as optimized microbial consortia or enhanced fermentation strategies to maximize lipopeptide production.

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CONFLICT OF INTEREST

The authors declare the following conflict of interest: statements Universidad Politécnica Salesiana, Research Group BIOARN and Master's degree in molecular biology

ARTIFICIAL INTELLIGENCE STATEMENT

The authors declare that generative artificial intelligence tools were used for the following purposes: Translation of abstract. The tool(s) used include: Google translator. The authors take full responsibility for the content of the manuscript

REFERENCES

[1] FAOSTAT, "Food and agriculture organitation of the united nations," https://www.fao.org/faostat/en/#data, 2017.

[2] Instituto Nacional de Estadística y Censos - INEC, "Información ambiental en la agricultura 2016," https://www.ecuadorencifras.gob.ec/documentos/web-inec/Encuestas_Ambientales/Informacion_ambiental_en_la_agricultura/2016/PRESENTACION_AGRO_AMBIENTE_2016.pdf, 2016.

[3] C. Espinoza-Ahumada, G. Gallegos-Morales, Y. Ochoa-Fuentes, F. Hernández-Castillo, R. Méndez-Aguilar, and R. Rodríguez-Guerra, "Antagonistas microbianos para biocontrol de la marchitez y su efecto promotor en el rendimiento de chile serrano," *Revista Mexicana de Ciencias Agrícolas*, vol. 10, no. spe23, pp. 187–197, 2019. [Online]. Available: https://doi.org/10.29312/remexca.v0i23.2020

[4] S. Jha, S. Joshi, and S. Geetha, "Lipopeptide production by bacillus subtilis r1 and its possible applications," *Brazilian Journal of Microbiology*, vol. 47, no. 4, pp. 955–964, 2016. [Online]. Available: https://doi.org/10.1016/j.bjm.2016.07.006

[5] M. Lisboa, "Efectividad de bacillus subtilis y de una cepa nativa de trichoderma harzianum sobre la incidencia y severidad de pudrición gris (botrytis cinerea) en vid," http://dspace.utalca.cl/handle/1950/919, 2003.

[6] J. Méndez-Úbeda, M. Flores, and P.-A. López, "Aislamiento e identificación de bacillus subtilis y evaluación del antagonismo in vitro frente hongos fitopatógenos," *Nexo*, vol. 30, no. 02, pp. 96–110, 2017. [Online]. Available: http://dx.doi.org/10.5377/nexo.v30i2.5530

[7] A. Albán, "Alternativas para la reducción de fusarium sp en el cultivo de alelí (matthiola incana), utilizando técnicas de control fitosanitario establecidas en la finca gemmolles s.a., cotopaxi," http://repositorio.utc.edu.ec/handle/27000/7616, 2021.

[8] Y. Yaseen, F. Gancel, M. Béchet, and D. Drider, "Study of the correlation between fengycin promoter expression and its production by bacillus subtilis under different culture conditions and the impact on surfactin production," *Archives of Microbiology*, vol. 199, no. 10, pp. 1371–1382, 2017. [Online]. Available: http://dx.doi.org/10.1007/s00203-017-1406-x

[9] D. Zhang, R. Qiang, Z. Zhou, Y. Pan, S. Yu, and W. Yuan, "Biocontrol and action mechanism of bacillus subtilis lipopeptides' fengycins against alternaria solani in potato as assessed by a transcriptome analysis," *Frontiers in Microbiology*, vol. 13, pp. 1–18, 2022. [Online]. Available: http://dx.doi.org/10.3389/fmicb.2022.861113

[10] R. Plascencia-Tenorio, V. Olalde-Portugal, H. Mena-Violante, L. Ceja-Torres, J. Venegas-Gonzáles, G. Oyoque-Salcedo, and V. Angoa-Pérez, "Antagonismo in vitro de aislados bacterianos de fresa comercial y silvestre vs. botrytis cinerea y rhizopus stolonifer," *Ra Ximhai*, vol. 8, pp. 103–110, 2012.

[11] F. Sánchez, "Importance of bacillus subtilis lipopeptides in the biological control of diseases in crops of high economic value," *Bionatura*, vol. 1, no. 3, pp. 135–138, 2016. [Online]. Available: http://dx.doi.org/10.21931/RB/2016.01.03.7

[12] L. Acosta, D. Azania, and R. Azania, "Cultivo dual in vitro de cepas nativas de trichoderma spp. frente a botrytis sp. patógeno de passiflora ligularis juss," *Revista de investigación Agropecuaria Science and Biotechnology*, vol. 1, no. 4, pp. 43–55, 2021. [Online]. Available: https://doi.org/10.25127/riagrop.20214.720

[13] A. Cadena and K. Medrano, "Evaluación de la expresión de los genes involucrados en la biosíntesis de lipopéptidos antimicrobianos en bacillus megaterium mediante rt-qpcr," http://dspace.ups.edu.ec/handle/123456789/18503, 2020.

[14] Y. Duarte-Leal, L. Pozo-Martínez, and B. Martínez-Coca, "Antagonismo in vitro de cepas de trichoderma asperellum samuels, lieckfeldt & nirenbergfrente a aislados de fusarium spp," *Revista de Protección Vegetal*, vol. 33, no. 1, pp. 00–00, 2018.

[15] V. Valenzuela, G. Gálvez, E. Villa, F. Parra, G. Santoyo, and S. De los Santos-Villalobos, "Lipopéptidos producidos por agentes de control biológico del género bacillus: revisión de herramientas analíticas utilizadas para su estudio," *Revista Mexicana de Ciencias Agrícolas*, vol. 11, no. 2, pp. 419–432, 2020. [Online]. Available: http://dx.doi.org/10.29312/remexca.v11i2.2191

[16] V. Chiluisa-Utreras, K. Medrano, A. Cadena, and R. D. Acurio, "Quantification of fend and itudi anti-fungal lipopeptide gene expression in bacillus megaterium using rt-qpcr," *Journal of Pure and Applied Microbiology*, vol. 14, no. 4, pp. 2339–2349, 2020. [Online]. Available: https://doi.org/10.22207/JPAM.14.4.12

[17] A. Campaña, "Identificación microbiológica y molecular mediante pcr en tiempo real de dos bacterias del género bacillus, de interés agro-biotecnológico," http://dspace.ups.edu.ec/bitstream/123456789/5081/1/UPS-CYT00109.pdf, 2018.

[18] V. Chiluisa-Utreras, M. Campaña, and R. Acurio, "Microbiological and molecular determination by real-time pcr of two bacteria of the genus bacillus of agro biotechnological interest," *Bionatura*, vol. 5, no. 2, pp. 1106–1110, 2020. [Online]. Available: https://doi.org/10.21931/RB/2020.05.02.4

[19] J. Morales and V. Chiluisa-Utreras, "Mejoramiento biotecnológico de plantas y modificación," http://142.93.18.15:8080/jspui/bitstream/123456789/794/1/GENNBIO_JMorales_Libro_2022%20(1).pdf, 2022.

[20] A. Salazar, A. Sandoval, and J. Armendáriz, "Fundamentos y aplicaciones en las ciencias de la salud," http://dipsa.com/ClanDunant/Textos/TUM-BiologiamolecularFundamentosyaplicacionesenlascienciasdelasalud-Salazar.pdf, 2013.

[21] V. Carpena, "Diseño de un ensayo de qpcr para la detección y cuantificación de bacillus subtilis en cultivo mixto con fusarium oxysporum en medio con polietileno como única fuente de carbono," http://hdl.handle.net/10835/13723, 2021.

[22] K. Livak and T. Schmittgen, "Analysis of relative gene expression data using realtime quantitative pcr and the 2ˆ−ΔΔ*Ct* method," *Methods*, vol. 408, pp. 402–408, 2001. [Online]. Available: https://doi.org/10.1006/meth.2001.1262

[23] J. Morales, "Expresión génica de flavanona 3-hidroxilasa y p-cumaril ester 3-hidroxilasa en el fruto de solanum caripense dunal mediante rt-pcr y rt-qpcr," https://hdl.handle.net/20.500.12996/4152, 2019.

[24] A. Vinueza and R. Nuñez, "Bacterias del género bacillus como agentes de control biológico in vitro del hongo fitopatógeno alternaria sp." http: //dspace.ups.edu.ec/handle/123456789/25311, 2023.

[25] F. Rodríguez, M. Ríos, and C. Muñoz, "Aislamiento y evaluación de agentes de biocontrol contra patógenos presentes en cultivos agrícolas," *Scientia Agropecuaria*, vol. 14, no. 2, pp. 159–170, 2023. [Online]. Available: https://revistas.unitru.edu.pe/index.php/scientiaagrop/article/view/6273/6944

[26] J. Morales, R. Blas, V. Chiluisa-Utreras, J. Flores, and G. Ortega, "Gene expression of flavanone 3-hydroxylase (f3h), anthocyanidin synthase (ans), and p-coumaroyl ester 3-hydroxylase (c3h) in tzimbalo fruit," *International Journal on Advanced Science, Engineering and Information Technology*, vol. 11, no. 2, pp. 805–813, 2021. [Online]. Available: http://dx.doi.org/10.18517/ijaseit.11.2.13681

[27] R. Acurio, C. Nacato, and M. Valencia, "Cepas autóctonas de bacillus subtilis como agente de biocontrol in vitro de alternaria spp. en brassica oleracea var. italica," *Bionatura*, vol. 3, no. 2, pp. 607–611, 2018. [Online]. Available: https://doi.org/10.21931/RB/2018.03.02.8

[28] N. Ley-lópez, J. Basilio, C. Martín-Hernández, J. R. Ibarra-Rodríguez, M. Angulo-Escalante, and S. García-Estrada, "Biosíntesis inducida de fengicina y surfactina en una cepa de bacillus amyloliquefaciens con actividad oomiceticida sobre zoosporas de phytophthora capsica," *Revista Argentina de Microbiología*, vol. 54, pp. 91–100, 2022. [Online]. Available: https://doi.org/10.1016/j.ram.2022.03.002

[29] S. Chowdhury, A. Hartmann, X. Gao, and R. Borriss, "Biocontrol mechanism by amyloliquefaciens fzb42 – a review," *Frontiers in Microbiology*, vol. 6, pp. 1–11, July 2015. [Online]. Available: https://doi.org/10.3389/fmicb.2015.00780

[30] L. A. Pedraza, C. E. López, and D. Uribe-vélez, "Ecología mecanismos de acción de bacillus spp . (bacillaceae) contra microorganismos fitopatógenos durante su interacción con plantas," *Acta Biológica Colombiana*, vol. 25, no. 1, pp. 112–125, 2020. [Online]. Available: http://dx.doi.org/10.15446/abc.v25n1.75045

[31] M. Corrales, "Evaluación del efecto de lipopéptidos antifúngicos producidos por bacillus subtilis ctpx s2-1, en la inducción de expresión de genes de crecimiento y resistencia de lupinus mutabilis," http://dspace.udla.edu.ec/handle/33000/10259, 2018, [Universidad de las Américas].

[32] G. Farace, O. Fernandez, L. Jacquens, F. Coutte, F. Krier, P. Jacques, C. Clément, E. A. I. T. Barka, C. Jacquard, and S. Dorey, "Cyclic lipopeptides from bacillus subtilis activate distinct patterns of defence responses in grapevine," *Molecular Plant Pathology*, vol. 16, no. 2, pp. 177–187, 2015. [Online]. Available: https://doi.org/10.1111/mpp.12170

[33] Y. Dang, F. Zhao, X. Liu, X. Fan, R. Huang, W. Gao, and S. Wang, "Enhanced production of antifungal lipopeptide iturin a by bacillus amyloliquefaciens ll3 through metabolic engineering and culture conditions optimization," *Microbial Cell Factories*, vol. 18, no. 68, pp. 1–14, 2019. [Online]. Available: https://doi.org/10.1186/s12934-019-1121-1

[34] V. Yánez-Mendizábal, H. Zeriouh, and I. Viñas, "Biological control of peach brown rot (monilinia spp.) by bacillus subtilis cpa-8 is based on production of fengycin-like lipopeptides," *European Journal of Plant Pathology*, vol. 132, pp. 609–619, 2012. [Online]. Available: http://dx.doi.org/10.1007/s10658-011-9905-0