

Quantification of FEND and ITUDI Anti-fungal Lipopeptide Gene Expression in *Bacillus megaterium* using RT-qPCR

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Abstract

Phytopathogenic diseases are a major concern in modern agriculture, and for decades, pesticides have been used to prevent potential damage. *Bacillus megaterium* is proposed as a biological controlling agent, and gene expression of the lipopeptide genes FEND and ITUDI was assessed using RT-qPCR. Inhibition effects of *B. megaterium* on *Alternaria* sp. and *Botrytis* sp. were examined over a period of nine days, which confirmed the potential use of this bacterium to counteract these two pathogens. In addition, expression of FEND and ITUDI genes was assessed over nine days in the aforementioned dual cultures and inhibition tests. FEND expression in *B. megaterium* increased 20.16-fold in response to *Alternaria* sp., and ITUDI expression increased 3.20-fold in response to *Botrytis* sp. on day five of incubation. These results were corroborated by gene expression data obtained from *B. megaterium* during fermentation, where FEND and ITUDI gene expression increased 95.14- and 18.70-fold, respectively. In conclusion, *B. megaterium* can increase lipopeptide synthesis when exposed to these particular phytopathogens and can significantly increase the respective expression during fermentation.

Keywords: Gene expression, Antagonism, Antimicrobial genes, Plant pathogens

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INTRODUCTION

Agriculture is one of the most important economic fields. It is presented with serious problems, such as economic and production losses owing to diseases affecting numerous types of crops during growth and after harvest, most of which are caused by phytopathogenic fungi¹. A large number of pathogens that cause severe plant diseases in the most important staple crops belong to the fungal genera, *Penicillium*, *Botrytis*, *Diplodia*, *Mucor*, *Phomopsis*, *Fusarium*, *Rhizopus*, *Monilinia*, *Alternaria*, and *Colletotrichum*². In Ecuador, two of the genera that cause the greatest number of diseases in crops of great production and consumption value are *Botrytis* and *Alternaria*. For example, *Botrytis* can infect 596 vascular plants, of which strawberry plants are particularly affected. This fungus occurs in various geographical areas, ranging from tropical to temperate and cold zone environments^{3,4}. *Alternaria* sp. causes diseases in tomato plants and in plants of the genus *Brassica*⁵.

Damage caused by these pathogens is a severe concern for crop production; therefore, controlling their spread is an urgent matter. To control these pathogens, different techniques and compounds have been used, the most common of which are synthetic pesticides owing to their considerable efficiency; however, long-term use of such chemical agents has caused collateral damage to the environment and human health. Novel alternatives for the control of pathogens are therefore required, and biological agents that may help mitigate or eliminate fungal pathogen-induced damage of crop plants are a promising option⁶. *Bacillus megaterium* is a growth promoter in plants and also shows pathogen-controlling effects as it produces lipoproteins, antibiotics, and hydrolytic enzymes. *B. megaterium* may, therefore, be a promising and ecologically versatile alternative for controlling the spread and reproduction of various pathogens⁷.

Molecular methods may help elucidate certain mechanisms and factors, such as the optimal time point of applying biocontrolling agents, the optimal concentration, and the required conditions for optimal effects, all of which are crucial aspects and should be subsequently determined using *in vivo* tests. In this regard, quantification of gene expression allows us to

understand the underlying mechanisms and to design targeted studies on the efficiency of biological controllers⁸⁻¹⁰.

B. megaterium has been shown to contain antifungal genes, which can inhibit growth of *Alternaria* sp. and *Botrytis* sp.¹¹; however, it is important to examine the respective expression patterns and to assess how they change over time and under certain conditions so as to determine the factors that induce expression and thereby increase the pathogen-controlling potential. Therefore, using RT-qPCR, we aimed to assess expression of FEND and ITUDI antifungal lipopeptide genes in *B. megaterium* in response to *Alternaria* sp. and *Botrytis* sp. and during fermentation.

METHODOLOGY

Inoculum preparation

Microbes that had been identified using molecular genetic means as *B. megaterium*, *Alternaria* sp. and *Botrytis* sp. were used in this study. The cultures, cryopreserved at -80°C, were obtained from the Laboratories of Life Sciences of the Universidad Politécnica Salesiana, Quito, Ecuador¹². First, a culture medium was prepared using 22 g of Nutritional Agar and 1 L distilled water, in which the bacteria were incubated at 36°C. For fungal cultures, potato dextrose agar (PDA) culture medium was prepared at the same concentration, which was then used for incubation at 26°C.

Dual culture assays to test antagonistic activity

In order to test inhibitory effects of *B. megaterium* on *Alternaria* sp. and *Botrytis* sp., a dual culture method was used, in which each pathogenic fungus was exposed to *B. megaterium* in PDA culture medium where the fungus was placed at the center of the box, and the bacteria was inoculated through a 3-mm striation between bacteria and fungus. Incubation was continued at 36°C for nine days^{13,14}. Genetic material and data for calculating the proportion of inhibition were obtained after this step. Data were collected on days 1, 5, and 9. Bacteria cultured without fungi were used as a control. The proportion of radial growth inhibition was calculated using the following equation¹⁵:

$$PICR = \frac{R_1 - R_2}{R_1} \times 100$$

Where R1 is the diameter of radial fungus growth and R2 is the diameter of radial growth of fungus exposed to *B. megaterium*.

Liquid Fermentation

Preparation of the inoculum and fermentation

Nutritional Agar medium was used to reactivate cryopreserved *B. megaterium* for incubation at 35°C for 24 h. Next, the bacterial concentration was recorded as a starting point. For this purpose, a suspension was prepared from normal saline that was set to a standard of 0.5 using spectrophotometry and the McFarland scale, thus representing a concentration of 10⁵ CFU mL⁻¹. After preparing the initial inocula, the fermentation process was initiated, starting with the preparation of 100 mL bacterial suspension (10⁵ CFU mL⁻¹) per Erlenmeyer flask using Triptocasa Soja Broth as the liquid culture medium. *B. megaterium* was incubated at 25°C under agitation at 100 rpm for 120 h.

RNA extraction

Extraction and quantification of *Bacillus megaterium* RNA from dual cultures

RNA was extracted from dual cultures of *Botrytis* sp. and *Alternaria* sp. exposed to *B. megaterium* on days 1, 5, and 9 using the PureLink

RNA Mini-Kit (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, RNA was quantified in µg/µL using a Qubit RNA BR Assay Kit (Thermo Fisher Scientific).

Extraction and quantification of RNA from *Bacillus megaterium* in fermentation

RNA was extracted from bacterial cultures fermented in broth for 24, 48, 72, and 120 h, as specified above.

Reverse transcription and quantification

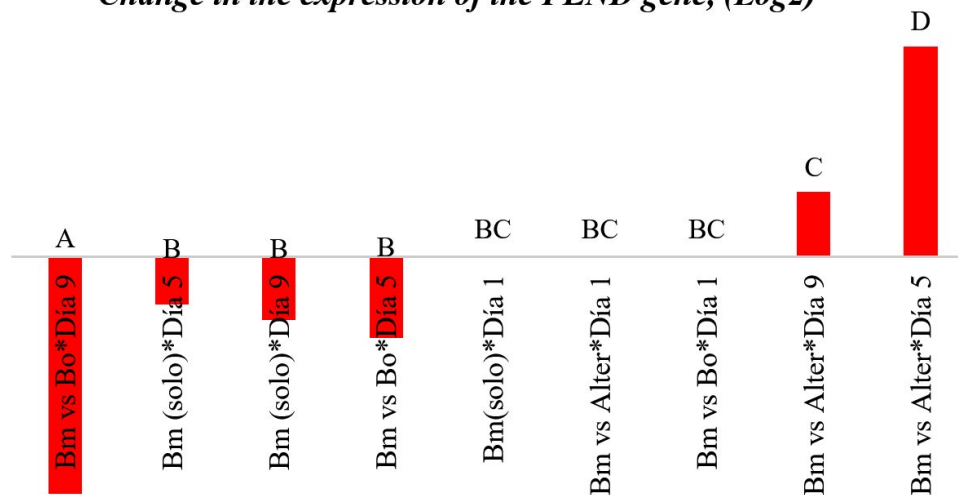
A Superscript® III First-Strand Synthesis SuperMix for RT-qPCR (Thermo Fisher Scientific) kit was used to obtain cDNA from the RNA templates, according to the manufacturer’s instructions. Briefly, 5 µg/µL RNA were used, and the reaction was incubated in a thermal cycler at 25°C for 10 min, 50°C for 30 min, and 85°C for 5 min before cooling to 4°C. Then, 1 µL *E. coli* RNase H was

Table 1. Percentage of Radial Growth Inhibition

Growth	Day	RPI	
<i>B. megateirum</i> vs.	1	4,55 ± 5,25	a
<i>Botrytis</i> sp.	5	10,71 ± 0,00	a
	9	30,00 ± 0,00	ab
<i>B. megateirum</i> vs.	1	43,33 ± 5,62	b
<i>Alternaria</i> sp.	5	76,45 ± 5,22	c
	9	98,48 ± 2,14	c

Note: RPI: Percentage of Radial Growth Inhibition.

Change in the expression of the FEND gene, (Log2)



Note: Alter: *Alternaria* sp.; Bo: *Botrytis* sp.; Bm: *Bacillus megaterium*.

Fig. 1. Change in FEND gene expression (logarithmic scale, base 2).

added, and the reaction mixture was incubated at 37°C for 20 min. cDNA was quantified using a Qubit ssDNA Assay Kit (Thermo Fisher Scientific). RT-qPCR

The ITUDI fragment was amplified using primers F: 5'-GATGCGATCTCCTGGATGT-3' and R: 5'-ATCGTCATGGCTGAG-3', and the FEND fragment was amplified using primers F: 5'-TTTGGCAGCAGGAGAAGTT-3' and R: 5'-GCTGTCCGTGCTTT-3' at a concentration of 10 µM. The 16S rRNA housekeeping gene was used for data normalization.

A LightCycler 2.0 (Roche, Basel, Switzerland) and 2 µg/µL cDNA were used to perform RT-qPCR with primers and Fast Sybr Green Master Mix (Thermo Fisher Scientific) at a final volume of 20 µL, according to the manufacturer's instructions. Thermocycling was performed as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s, and 72°C for 30 s, after which a melting curve was generated at 95°C for 0 s, 65°C for 1 min, and 95°C for 0 s. The reaction was stopped by cooling to 4°C. To calculate expression of FEND and ITUDI, the initial concentration of

gene copies (N₀) was required, including the respective efficiencies (E) of each reaction, which was computed using LinRegPCR version 11.0, 16 which uses the 2^{-ΔΔct} method^{17,18}. Data were standardized with the 16S housekeeping gene as a reference, according to the following equation¹⁹:

$$N_0 \text{ (normalizado)} = \frac{N_0 \text{ gen problema}}{N_0 \text{ gen normalizador}}$$

Gene expression was calculated from standardized data by comparing all treatments with the control, which in this case was cultivation of *B. megaterium* without fungi, using the following equation¹⁹:

$$\text{Relative expression level} = \frac{N_0 \text{ gene day } n}{N_0 \text{ gene day } 1}$$

Statistical analyses

FEND and ITUDI gene expression in *B. megaterium* were considered the response variables, and presence of the phytopathogenic

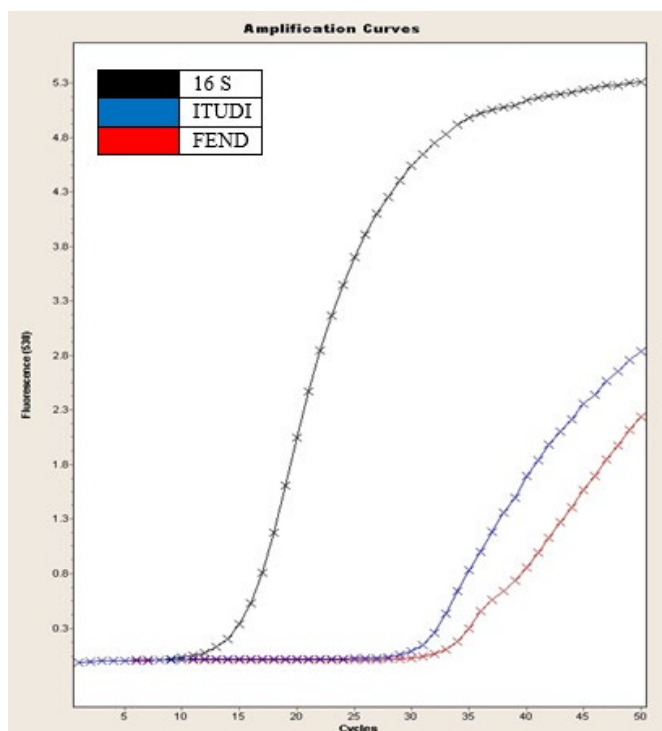


Fig. 2. 16S gene amplification curve, FEND, ITUDI of *Bacillus megaterium* vs *Alternaria* sp. at day 5 (LightCycler®, Roche).

fungi *Botrytis* sp. and *Alternaria* sp. and incubation time (1, 5, or 9 days) were used as independent variables. A completely randomized design was used for a 3 X 3 factorial analysis. The 27 experimental units were categorized according to incubation time and growth using three replicates of exposure of bacteria to each pathogen, plus the respective controls. Gene expression data obtained from dual cultures were compared with data collected at 24, 48, 72, and 120 h of bacterial fermentation under optimal growth conditions. For this, an analysis of variance (ANOVA) was performed, followed by Tukey's post hoc test at 5%, using the statistical software, Infostat (2018).

RESULTS AND DISCUSSION

Radial growth inhibition

ANOVA showed a highly significant difference for both growth and incubation time ($p < 0.0001$), and the interaction between these variables was significant ($p = 0.0395$). Therefore, radial growth inhibition differed between the two fungi and depended on incubation time. Tukey's test (Fig. 1) showed three ranges of significance, A, B, and C, with range C representing antagonism of *Alternaria* sp. on day 5 of incubation with an average inhibition of 76.45% and day 9, with inhibition averaging 98.48%, cultures that presented the highest inhibition, which indicated

Table 2. Change in relative gene expression (RGE) of the FEND gene in *Bacillus megaterium* alone and against pathogens

Growth	Incubation days	Average EGR change (Linear)	Average EGR change (Log2)
<i>B. megaterium</i>	1	1,00 ± 0,00	0,00 ± 0,00
	5	0,56 ± 0,29	-0,97 ± 0,78
	9	0,53 ± 0,35	-1,31 ± 1,49
<i>B. megaterium</i> vs. <i>Botrytis</i> sp.	1	1,00 ± 0,00	0,00 ± 0,00
	5	0,32 ± 0,06	-1,66 ± 0,27
	9	0,04 ± 0,01	-4,89 ± 0,60
<i>B. megaterium</i> vs. <i>Alternaria</i> sp.	1	1,00 ± 0,00	0,00 ± 0,00
	5	20,16 ± 0,18	4,33 ± 0,02
	9	2,55 ± 0,47	1,34 ± 0,27

Change in the relative gene expression of ITUDI, (Log2)

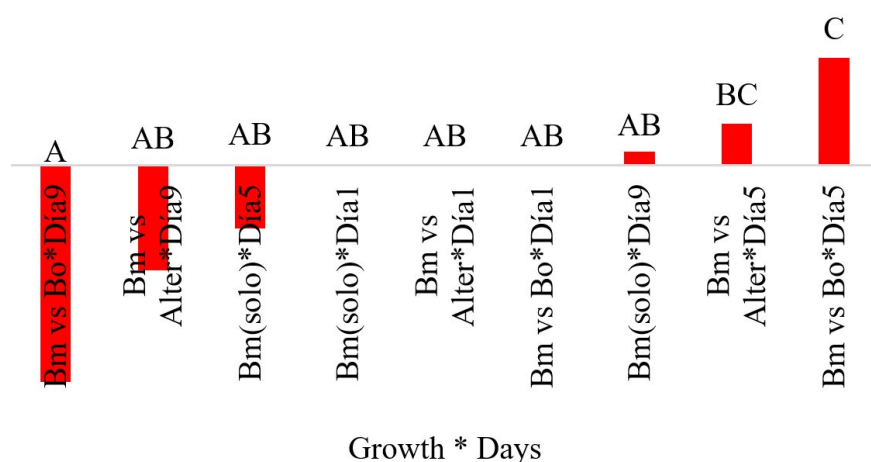


Fig. 3. Change in the expression of the ITUDI gene (logarithmic base scale 2).

that *B. megaterium* inhibits *Alternaria* sp. growth more effectively, with inhibition levels of almost 99%. In previous studies, *Bacillus* sp. produced inhibition effects on *Alternaria* sp. ranging from 11% to 31% on day 5, 20, 11, which are comparable to the results of the current study. Moreover, *B. subtilis* was previously reported to show a 60% inhibition of *Botrytis* sp. and 80% inhibition of *Alternaria* sp. 21, which is consistent with our results.

Kurniawan *et al.* (2018)²⁰ and García (2016)²² established that several species of the genus *Bacillus*, such as *B. megaterium*, can potentiate their inhibitory effects if they are grown under exposure to a pathogen. Moreover, *B. megaterium*, *A. alternata* on biopreparations when applied to broccoli crops infected with *A. japonica* kept values below 15.56 of severity²³.

FEND gene expression in *B. megaterium*

When *B. megaterium* was not challenged with pathogenic fungi, no increase in relative expression of FEND by more than 2 was observed, which was not considered significant. Lengyel (2018)²⁴ reports that molecules involved in gene expression are an inevitable source of chance because they are found under certain conditions

in the cells, which causes proteins to be produced randomly and at minimal quantities, which is termed as the “noise” of expression. However, when *B. megaterium* was exposed to *Alternaria* sp., a significant increase in gene expression was evident on day 5 of incubation. ANOVA showed a highly significant difference in growth between pathogens and incubation times, and the interaction between the two variable was also significant ($p < 0.0001$, each); thus, relative expression of FEND depended on the fungus and time of incubation.

In Fig. 2, according to Tukey’s test, four ranges of significance are presented: A, B, C, and D; within range A, the test presented the lowest expression, and range D includes the effect of *Alternaria* sp. on *B. megaterium* on day 5, which showed the greatest increase in FEND expression since day 1. FEND expression was down-regulated over time in the control. A similar phenomenon was observed when *B. megaterium* was exposed to *Botrytis* sp.

FEND expression was upregulated in *B. megaterium* exposed to *Alternaria* sp. with a 20-fold increase until day 5. On day 9, FEND expression decreased, which is in line with the results of

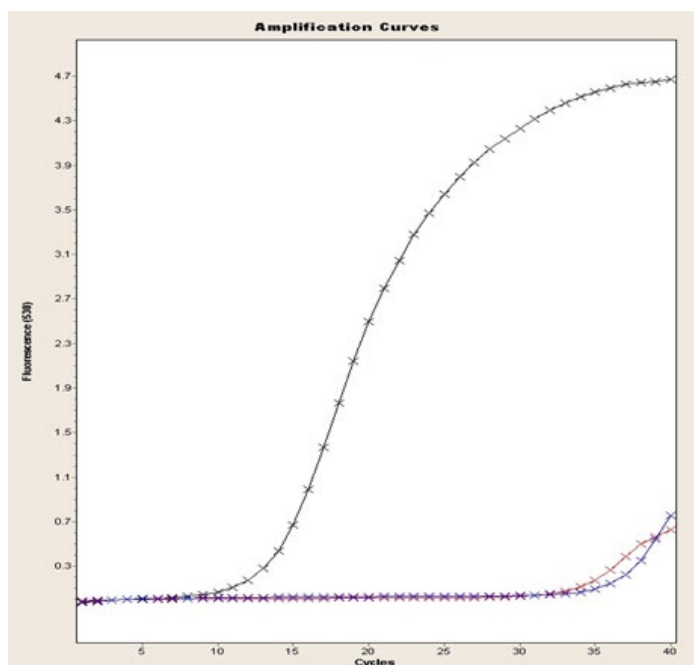


Fig. 4. 16S gene amplification curve, FEND, ITUDI of *Bacillus megaterium* versus *Botrytis* sp., at day 5 (LightCycler®, Roche).

Dragovoz *et al.* (2016), who reported that different strains of *Bacillus* exposed to *A. alternata* showed a 20- to 100-fold increased lipopeptide gene expression from day 1, with FEND as the highest expressed gene. They confirmed that fengicins are lipopeptides that are produced in large quantities. A different study found that fengicines are specific inhibitors of fungi²⁵; however, surfactins more specifically inhibit bacteria.

The FEND gene is largely expressed as a defense against specific pathogens that are considered targets. Velho *et al.* (2015)¹⁰ suggested that biocontrollers increase the production of specific and effective antimicrobial proteins as a defense against certain microorganisms. The results of the current study showed that *B. megaterium* may be a promising biocontroller of *Alternaria* sp. as it showed upregulation of FEND expression when challenged with this phytopathogenic fungus.

Knight *et al.* (2018) reported that *B. subtilis*, a species very similar to *B. megaterium*, is an effective controller of the phytopathogens,

Alternaria sp. and *Fusarium* sp., with upregulated FEND expression on day 3 of incubation. A different study using in vitro assays found that *B. subtilis* inhibited the growth of *Alternaria* sp. and *Fusarium* sp., and on day 4 of incubation confirmed the presence of genes such as *ituA*, *bamC*, and *sfp* which encode iturine A, bacilomycin D, and surfactin, respectively²⁶.

ITUDI gene expression in *B. megaterium*

No significant changes in ITUDI expression over time were observed; however, *B. megaterium* exposed to *Botrytis* sp. and *Alternaria* sp. showed ITUDI upregulation on day 5. Exposure to *Botrytis* sp. led to a 3-fold increased expression, compared to that on day 1 (Table 3).

Only moderate changes in ITUDI expression were observed; however, the strength of inhibitory effects depends on several metabolites, including other lipopeptides (fengicins, surfactins, bacilliomycins, etc.), suggesting that each metabolite is required. Thus, both fengicins and surfactins depend on the expression of iturins, as they play an important role for effective inhibitory activity^{27,28}.

Table 3. Change in relative gene expression (RGE) of ITUDI in *Bacillus megaterium* alone and against pathogens

Growth	Incubation days	Average EGR change (Linear)	Average EGR change (Log2)
<i>B. megaterium</i>	1	1,00 ± 0,00	0,00 ± 0,00
	5	0,81 ± 0,94	-0,97 ± 1,65
	9	1,22 ± 0,61	-0,17 ± 0,71
<i>B. megaterium</i> vs. <i>Alternaria</i> sp.	1	1,00 ± 0,00	0,00 ± 0,00
	5	1,73 ± 1,02	0,64 ± 0,78
	9	0,33 ± 0,08	-1,61 ± 0,36
<i>B. megaterium</i> vs. <i>Botrytis</i> sp.	1	1,00 ± 0,00	0,00 ± 0,00
	5	3,20 ± 0,64	1,66 ± 0,31
	9	0,10 ± 0,01	-3,31 ± 0,15

Table 4. 16S gene amplification curve, FEND, ITUDI of *Bacillus megaterium* versus *Botrytis* sp., at day 5 (LightCycler®, Roche)

Genes	Fermenting days	Average EGR change (Linear)	Average EGR change (Log2)
FEND	1	1,00 ± 0,00	0,00 ± 0,00
	2	0,85 ± 0,05	-0,24 ± 0,08
	3	1,16 ± 0,04	0,21 ± 0,05
	5	95,14 ± 6,59	6,57 ± 0,10
ITUDI	1	1,00 ± 0,00	0,00 ± 0,00
	2	0,42 ± 0,10	-1,27 ± 0,37
	3	12,96 ± 1,02	3,96 ± 0,12
	5	18,70 ± 0,38	4,23 ± 0,03

Even so, gene expression differed between incubation conditions. For this purpose, an ANOVA was carried out considering each variable of interest separately, as well as the interactions between them. No significant

difference was found regarding growth, whereas the effect of incubation time showed 1.91-fold increased expression until day 5, compared to that on day 1. In Fig. 4, data are shown as a logarithm to the base 2, and in Fig. 5, amplification curves until

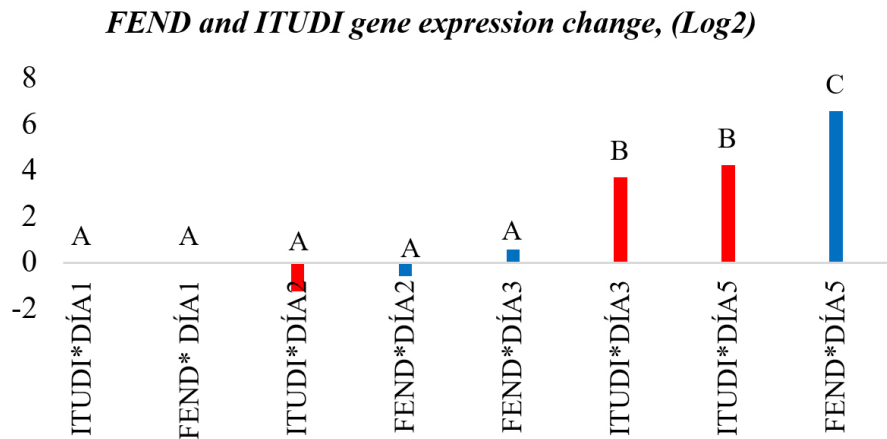


Fig. 5. Representation of relative gene expression change in logarithmic scale base 2 of FEND and ITUDI in *Bacillus megaterium* under fermentation.

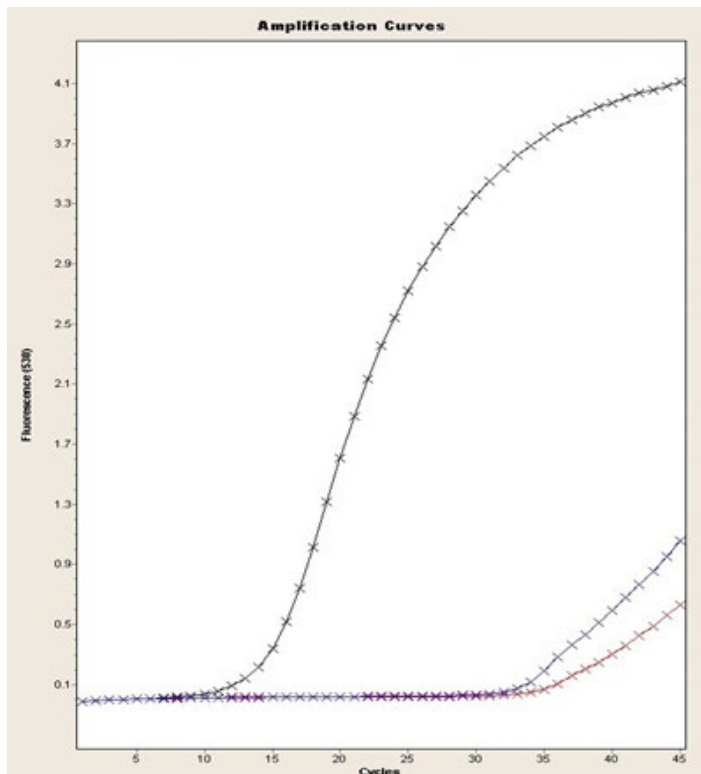


Fig. 6. 16S gene amplification curve, FEND, ITUDI of *Bacillus megaterium* versus *Botrytis* sp., at day 5 (LightCycler®, Roche).

day 5 are shown. These results indicated that ITUDI expression by *B. megaterium* exposed to *Botrytis* sp. increased by 3.20-fold until day 5, compared to that on day 1. Such a response was expected as *B. megaterium* can reach the exponential growth phase after 48 h; thus on day 9, the bacteria would have already entered the dying phase²⁹. In addition, production of lipopeptides by several species of the genus *Bacillus* to which inhibitory effects of *Botrytis* sp. are attributed suggest the presence of iturines, among other lipopeptides. Stable production occurs from 120 h to 168 h. Thus, on day 5, it increases by up to three fold³⁰. Moreover, fengicines³¹ can also be produced during the stationary phase which occurs from 48–72 hours of incubation; however, inhibitory effects of *B. megaterium* are attributed to the synthesis of lipopeptides, as observed previously in microbiological test including exposure to *Alternaria* sp. and *Botrytis* sp.³².

FEND and ITUDI gene expression in fermenting *B. megaterium*

FEND and ITUDI expression during fermentation of *B. megaterium* was evaluated using RT-qPCR on days 1, 3, 5, and 9 (Table 4), and we observed that expression of both genes significantly increased until day 5 of fermentation; however, it was necessary to perform an ANOVA. Tukey's post hoc test showed a significant difference in gene expression between days of incubation. Gene expression of both lipopeptides increased 56.93-fold until day 5. Regarding differences in expression between genes, the results showed stronger upregulation of fengicines with a 95.14-fold increase, and an 18.71-fold increase in iturines. This indicates that lipopeptide synthesis increases during fermentation. Fig. 6 shows data of logarithmic phase 2 and Fig. 7 shows the amplification curve during fermentation.

The fermentation environment is more appropriate for bacterial growth³³. In addition, with respect to time, the production of iturines is associated with the early stationary phase, and fengicines accumulate during the later stationary phase, which should occur after 72 hours, as reflected in the observed changes in gene expression³⁴. During fermentation, the results were more promising, regardless of whether liquid or solid culture media are used³⁵. Large commercial producers use this route of

production because of its strong performance and high yield of metabolites such as antibiotics, bioregulators, and toxins for use in different fields including agriculture. *B. subtilis*, which is closely related to *B. megaterium*, showed considerable production of iturines and fengicines from days 2–5 of fermentation in our study, and similar results have been obtained previously³⁶. Therefore, *B. megaterium* apparently increases the expression of both lipopeptides independently when exposed to pathogens and during fermentation.

CONCLUSIONS

We conclude that *B. megaterium* inhibits *Alternaria* sp. growth by 98.48% during 9 days of incubation. FEND gene expression increased 20.16-fold until day 5 of incubation when *B. megaterium* was exposed to *Alternaria* sp., which was the maximum of upregulation, suggesting that *B. megaterium* is a promising controlling agent to counteract specifically *Alternaria* sp. Regarding iturines, gene expression in *B. megaterium* increased three times more when exposed to *Botrytis* sp., and incubation time is a key factor for the synthesis of this lipopeptide, as evidenced by our results. Fermentation was associated with a significant increase in expression of both genes as under optimal growth conditions, this bacterium could produce metabolites at higher yields. Both genes were upregulated rapidly, but upregulation of the FEND gene was stronger. *B. megaterium* can be considered an promising candidate for agricultural use as it produced excellent results in in vitro microbiological and molecular tests; specifically, it may be used as a biological controlling agent to counteract *Alternaria* sp. and *Botrytis* sp.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

VC authorship and direction of the project, management of resources for research

and publication, review and translation of the manuscript.

KM execution of laboratory tests, analysis, processing of results and writing of the manuscript.

AC execution of laboratory tests, analysis, processing of results and writing of the manuscript.

RA donation of microbiological material, support in statistical analysis and review of the manuscript.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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