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Transcriptomic analysis of Bacillus licheniformis M2-7 in response to co-culture with Curvularia lunata, the causative agent of leaf spot disease in maize landrace

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Abstract

Curvularia lunata (Wakker) Boedijn is the main causal agent of corn leaf spot worldwide. Control of this disease is based on the use of chemical fungicides. Therefore, the use of biological control agents such as *Bacillus licheniformis* M2-7 is a sustainable measure for this disease in maize. However, there is limited information on the molecular mechanisms triggered in antagonistic bacteria against phytopathogenic fungi. The objective of this study was to investigate the molecular antifungal mechanisms of strain M2-7 against *C. lunata* using transcriptome analysis. Strain M2-7 caused the phytopathogenic fungus to swell, deform, rupture hyphae and reduce conidial germination from the second day of interaction. At this time, transcriptome analysis of the bacterium individually and in co-culture with the phytopathogenic fungus was performed, revealing that 35 genes were over-regulated and 2 genes were under-expressed. The over-regulated genes are genes associated with sporulation of the bacterium. The *coxA*, *liaL* and *sspB* genes were validated by using quantitative real-time PCR, showing 45.11-, 61.75- and 1.24-fold expressions, respectively, under interacting conditions. This study provides us with evidence that strain M2-7 would be exerting an antagonistic effect against *C. lunata* by spore action.

Introduction

Leaf spot disease, caused by *Curvularia lunata* (Wakker) Boedijn, is among the most serious fungal diseases worldwide. Particularly, this pathogen severely impacts sustainable maize production, as it can occur at all stages of plant development (Akonda et al. 2015; Zhang et al. 2019; Barupal et al. 2021). *C. lunata* can also infect maize cobs, especially if the incidence of this pathogen in the field has reached a critical point, hindering development and grain formation by reducing photosynthetic capacity (Gao et al. 2017). Furthermore, *Curvularia* produces the toxin M5HF2C upon infecting maize plants. This toxin gradually accumulates in the kernels, posing a serious health risk to humans (Gao et al. 2017). The management of this disease primarily involves the intensive use of chemical fungicides (propineb 0.2%, methyl thiophanate, mancozeb, and imazalil 800 ppm) and resistant varieties (Cristóbal-Alejo et al. 2013). However, prolonged and unreasonable use of fungicides can lead to pathogen resistance and adverse effects on human, animal, and environmental health (Rahman et al. 2018). Therefore, biocontrol strategies based on bacterial secondary metabolites have garnered increasing attention due to their efficacy and environmental friendliness from a food safety perspective (Mnif and Ghribi 2015).

Bacillus species are among the most researched biocontrol agents, contributing to the suppression of plant pathogens, particularly fungi (Mnif and Ghribi 2015; Fira et al. 2018). *Bacillus* species inhibit pathogen growth through several mechanisms, including competition for nutrients and space, production antibiotic compounds, hydrolytic enzymes, and siderophores, and/or induction of systemic resistance (Beneduzi et al. 2012; Miljaković et al. 2020). In recent decades, multiple secondary metabolites with antifungal properties have been identified in members of the *Bacillus* genus, including *Bacillus licheniformis* (Fira et al. 2018; Won et al. 2021). Several studies have reported on the promising biocontrol properties of *B. licheniformis* against a wide variety of plant diseases caused by fungi,

oomycetes, bacteria, viruses, and nematodes (Bhai et al. 2019; Abdelkhalek et al. 2020; Panichikkal et al. 2021; Upadhyay and Mohan 2021; Bahena-Oregón et al. 2022). Particularly, *B. licheniformis* M2-7 is known to produce secondary metabolites such as amylases, proteases, esterases, biosurfactants, and volatile organic compounds, which can act as biocontrol agents (Bolaños-Dircio et al. 2021). *In vitro* tests have revealed that *B. licheniformis* M2-7 inhibits *C. lunata* SY17 mycelial growth and germination of conidia by 73.75% and 78.66%, respectively (Bolaños-Dircio et al. 2024). *In vivo* tests revealed that concentrations of 1×10^3 and 1×10^6 CFU of strain M2-7 significantly decreased disease severity relative to the control (Bolaños-Dircio et al. 2024). However, little is known regarding the molecular mechanisms through which bacteria respond to phytopathogenic fungi to establish antagonistic activity and Next-generation sequencing technologies (NGS) provide an effective means for whole-genome sequencing and investigating specific biological control genes (Dunlap et al. 2015). Complete genome sequencing of several *Bacillus* strains and other biological control agents has revealed functional genes involved in disease control (Lefort et al. 2014). Genomic analysis offers a broad overview of the potential mechanisms involved in controlling plant pathogenic fungi, whereas massive RNA sequencing (RNA-seq) allows for the analysis of specific molecular mechanisms through the quantification of gene expression levels under known experimental conditions (Wilhelm and Landry 2009). Therefore, this approach enables the characterization of genes involved in the interaction between antagonistic and phytopathogenic organisms (Zhang et al. 2015). For example, Jiang et al. (2019) identified nearly one thousand differentially expressed genes that were related to the maturation process of *Bacillus velezensis* F21, triggering induced systemic resistance (ISR) in the presence of *Fusarium oxysporum* f. sp. *niveum* in watermelon plants. These changes in gene expression patterns were accompanied by an increase in the expression of transcription factors (TF), plant disease resistance genes, and the activities of defense enzymes such as CAT, PDO, and SOD. Similarly, transcriptome analyses conducted by Zhang et al. (2022a) revealed that that up to 304 genes of *Alternaria solani* were differentially expressed upon treatment with fengycins produced by *Bacillus subtilis*, with cell wall and cell membrane metabolism being among the main processes affected by fengycin stress. Zhang et al. (2022b) conducted transcriptome analyses of *Fusarium pseudograminearum* in the presence of *Bacillus velezensis* YB-185, revealing 5,086 differentially expressed genes (DEGs) compared to the control. These DEGs included genes for glucan synthesis, fatty acids, mechanosensitive ion channels, peroxide dismutase, thioredoxin, and plant cell wall-degrading enzymes. However, although the aforementioned studies provide valuable insights into the underlying mechanisms of plant-pathogen interactions, the molecular mechanisms that regulate biocontrol processes have remained largely unexplored.

Therefore, our study sought to identify changes in gene expression patterns during the interaction between *Bacillus licheniformis* M2-7 and *C. lunata* SY17 through RNA-seq analyses. We expect that our findings will not only provide valuable insights into the interactions between beneficial and pathogenic microorganisms, but may also serve as a basis for the development of biofungicides with antimicrobial substances as their main components.

Materials and methods

Biological materials and culture conditions

All experiments were conducted using the *B. licheniformis* M2-7 strain and the phytopathogenic fungus *C. lunata* SY17, both belonging to the collection of the Laboratorio de Microbiología Molecular y Biotecnología Ambiental (Facultad Académica de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero). Strain M2-7 was grown in Luria Bertani (LB) liquid medium containing 10.0 g/L casein peptone, 5.0 g/L wash extract, and 10.0 g/L NaCl (pH 7.3). The cells were incubated at 180 rpm for 24 hours at 37°C. The phytopathogenic fungus was activated using potato dextrose agar (PDA) medium containing 4.0 g/L potato extract, 20.0 g/L dextrose, and 15.0 g/L bacteriological agar, with a final pH of 5.5 ± 0.2. The plates were incubated at 30°C for 7 days.

Preparation of the pathogen inoculum

The pathogen inoculum was prepared using a one-week-old fungal culture grown on PDA at 30°C. *C. lunata* SY17 conidia were diluted in 2 mL of sterile 0.9% saline (Corrales-Ramirez et al. 2012), after which 500 µL of this resuspension was used for inoculation in downstream experiments.

Preparation of *B. licheniformis* M2-7 inoculum

Bacterial cells were recovered from a 24-hour incubation culture at 37°C in liquid LB medium by centrifugation at 0.29 g for 10 min. The cell pellet was washed twice with sterile 0.9% saline and resuspended in 2 mL of 0.9% saline. The initial concentration was adjusted to 0.05 cells/mL by an optical density of OD_{600} (Márquez et al. 2003).

Interaction system

To evaluate the morphological changes of *C. lunata* SY17 in the presence of *B. licheniformis* M2-7, a direct interaction assay was conducted in liquid LB. A monoculture of the phytopathogenic fungus was used as a control. This assay was performed in triplicate. To visualize the interaction, 5 µL of the samples were taken at 2, 4, 6, and 8 days and placed on a slide. Each sample was then stained with cotton blue, covered with a coverslip, and visualized under an optical microscope (Olympus).

Total RNA extraction

Triplicate samples of *B. licheniformis* M2-7 were taken from liquid LB cultures after two days of interaction. The control group consisted of *B. licheniformis* M2-7 only (B1_S20, B2_S21 and B4_S9), whereas the experimental group consisted of *B. licheniformis* M2-7 in the presence of *C. lunata* SY17 (BC1_S23, BC2_S24 and BC4_S7 and BC5_S8). Cells were collected and resuspended in lysis buffer (15 mg/mL lysozyme, 30 mM Tris-HCl, and 1 mM EDTA), incubated at 37°C for 30 min, and vortexed to break the cell walls. RNA was then extracted using the TRIzol® reagent (Thermo Fisher Scientific, Cat. No. 15596-026, Waltham, MA, USA) according to the manufacturer's instructions. High-throughput RNA sequencing was performed at the Unidad Universitaria de Secuenciación Masiva Service (UUSMB), Instituto de Biotecnología, Universidad Nacional Autónoma de México. Subsequently, the residual DNA

using DNase I (Thermo Scientific) was removed. Total RNA was chemically fragmented into 150–200 bp fragments. Complementary DNA (cDNA) was synthesized using a random hexamer primer and reverse transcriptase. The final products were subsequently ligated to adapters and purified to obtain the final cDNA library. The libraries were sequenced on an Illumina Genome Analyzer IIx.

Quality control, alignment sequences and Mapping in the genome of reference

RNA-seq sequence quality analysis was performed using the Fastp v0.22.0 data ultraprocessing tool. This algorithm allowed us to perform quality control and remove chimeras and adapters that may have remained after sequencing. To assemble the sequences, the bioinformatics program FLASH (v1.2.11) was used, once the overlapping sequences were obtained, the next step consisted of a de *novo assembly* of the transcripts using the RnaSPAdes software (v3.13.1). The “contigs” were then used to perform a BLAST-NCBI. The BWA and SAMtools packages were used to map the transcripts. BWA (v.0.7.17-r1188) is a software package used to map the sequences with the reference genome through alignments. This software's BWA-MEM function was used to map the reads resulting from quality control. SAMtools (v1.13) was also used to transform short-read DNA sequence alignments from SAM to BAM formats. Both programs allow the creation of a series of data related to alignments, classification, indexing, data extraction, and format conversion. These sequences were used to perform a BLAST analysis on the NCBI database, which yielded 100% coverage with the reference genome of *Bacillus licheniformis* strain P8_B2, whose accession number (CP045814.1).

Differential gene expression analysis

The analysis was performed using two programs: Cuffdiff v2.2.1 (Trapnell et al. 2013) and DESeq2 v1.40.2 (Love et al. 2014). Both programs estimated the relative abundance of transcripts based on the mapped reads and provided an estimate of differential expression. The functional annotation of differentially expressed genes, regarding the affected pathways, was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000), using our own R’s scripts. The RNA-seq transcriptome data were deposited in the NCBI Gene Expression Omnibus database under accession number (GSE330209).

Nf-core/differentialabundance (v.1.2.0).

This platform is a bioinformatics pipeline used to analyze RNAseq data, comparing a group of observations to generate differential statistics for subsequent analysis. The pipeline is created using Nextflow v23.04.2.5870, a workflow tool used to run tasks on multiple computing infrastructures. It uses different containers such as Docker v20.10.25, making installation simple and the results highly reproducible. The results report generated is presented in two forms: the first is an HTML file created from R Markdown, generating an interactive index with tables and graphs, and the second is the generation of an interactive analysis from Shiny.

Quantitative real-time PCR (RT-qPCR)

To validate the accuracy of our RNA-seq analyses, three DEGs (*coxA*, *lialI* and *sspB*) were selected and verified through RT-qPCR. Total RNA was extracted as described by Damm et al. (2015), with some modifications. First-strand cDNA was synthesized from 1 µg of total RNA using the RevertAid H Minus First Strand cDNA kit (Thermo Scientific) and reverse oligonucleotides for each treatment and control gene. The *rpoB* gene was used as a reference gene. Each sample was analyzed in triplicate in a final reaction volume of 20 µL, consisting of 1 µl cDNA (5 ng), 10 µL Master Mix 2x (Light Cycler 480 SYBR Green I; Roche), and 1 µL oligonucleotides (2.5 mM). Amplification was carried out with the following temperature profiles: denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and elongation at 72°C for 40 s. All oligonucleotide sequences were designed using Primer3Plus (https://primer3plus.com). Relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (Prism 7; GraphPad Software Inc., San Diego, USA). Cuffdiff is a model for reading counts based on a negative β-binomial (BN) distribution and a t-test to identify genes that have undergone changes, allowing the analysis of differential genetic expression between treatment and control based on a paired analysis with and without replicates. DESeq is a program that applies a false discovery rate (FDR) adjusted $P < 0.05$ cutoff to identify differentially expressed genes. The program requires a minimum of three replicates per sample. To relative expression of marker genes Unpaired Student´s *t* was performed to determine statical significance ($P < 0.05$)

Results

B. licheniformis M2-7 affects the structure of *C. lunata* SY17

The co-culture of *B. licheniformis* M2-7 and *C. lunata* SY17 was observed under an optical microscope to analyze their interaction at 2, 4, 6, and 8 days (Fig. 1). In parallel, a fungal monoculture was examined as a control. The growth of strain SY17 in monoculture was normal (Fig. 1a). In contrast, strain SY17 exhibited swelling, hyphal deformation, hyphal breakage, and conidial deformities in the presence of *B. licheniformis* M2-7 (Fig. 1b). These results allowed us to determine when the bacterium activates its antagonism mechanisms, which informed the timing of the transcriptomic response analysis.

Interaction with *C. lunata* SY17 activates genes associated with spore wall and spore germination in *B. licheniformis* M2-7

To gain insights into the molecular responses of *B. licheniformis* M2-7 to *C. lunata* SY17, comparative analysis of the transcriptome of the bacterial strain cells was conducted in the presence and absence of the phytopathogenic fungus. The Q30 values of the transcript sequences were between 94.31% and 95.14%, indicating high-quality reads with few errors and ambiguities (Table 1).

Table 1
Summary of sequencing quality metrics obtained after quality control and filtering using the fastp program.

Sample	Gross reading	Clean bases	Q30 (%)*
B1_S20	15196322	1104043	94.88
B2_S21	11776932	856841455	94.31
B4_S9	20242822	1458811	95.14
BC1_S23	14366210	1040516	94.99
BC2_S24	14517662	1053282	95.13
BC4_S7	16828802	1224084	94.84
BC5_S8	16897651	1243180	94.10

*Q30: the percentage of bases with of bases with a Phred quality score of ≥ 30, equivalent to a read accuracy of 99.9%. The GC content corresponds to the percentage of guanine and cytosine present in the cleaned sequences obtained after quality control using the fastp program.

The interaction of *B. licheniformis* M2-7 with *C. lunata* SY17 resulted in 37 DEGs. Among these genes, 35 genes were significantly upregulated, whereas 2 genes were significantly downregulated (Fig. 2). Interestingly, the upregulated genes were associated with spore wall and spore germination (Table 2).

Table 2

Bacillus licheniformis M2-7 genes with significant differential expression during direct interaction with *Curvularia lunata* SY17.

Identifier	Gene	Protein	Fold change value
gene-GII88_00310	*sspF*	SspF	3.972
gene-GII88_04415	-	-*	1.475
gene-GII88_04775	*yfhD*	YfhD	1.719
gene-GII88_04805	*ypzG*	YpzG	2.178
gene-GII88_04810	*sspK*	Acid soluble spore protein	1.822
gene-GII88_04860	*sspE*	Acid soluble spore protein	4.828
gene-GII88_05280	*yhcV*	YvcV	1.736
gene-GII88_05610	*sspL*	Acid soluble spore protein B	2.598
gene-GII88_05650	BLi01058	-	1.038
gene-GII88_05865	BL05233	Spore coat protein	3.072
gene-GII88_06400	*yizC*	YizC	1.492
gene-GII88_06755	*cotY*	Spore coat protein Y	1.655
gene-GII88_06760	*cotX*	Spore coat protein X	1.387
gene-GII88_06765	*cotW*	Spore coat protein W	1.872
gene-GII88_06790	*spoVIF*	Sporulation-specific transcription factor	1.132
gene-GII88_08195	*ykzV*	Membrane protein	1.213
gene-GII88_08845	*yloV*	Glycerone kinase protein	1.024
gene-GII88_11030	BL01387	Spore coat protein	1.337
gene-GII88_11205	*gerT*	Spore Germination Protein	1.864
gene-GII88_14165	BL03704	-	1.997
gene-GII88_15085	*yrzI*	YrzI	1.288
gene-GII88_15185	*yrzQ*	YrzQ	1.292
gene-GII88_15190	*yrzR*	YrzR	2.842
gene-GII88_15195	*yrrD*	YrrD	2.844
gene-GII88_15375	*coxA*	Sporulation Cortex Protein CoxA	3.022

-*Protein with unknown function.

Identifier	Gene	Protein	Fold change value
gene-GII88_15770	*gerE*	Spore germination protein	1.063
gene-GII88_16320	*sspB*	Acid soluble spore protein	5.379
gene-GII88_18105	BL05329	Spore coat protein	1.160
gene-GII88_18225	*liaS*	LiaS	1.509
gene-GII88_18230	*liaF*	LiaF	1.575
gene-GII88_18235	*liaG*	LiaG	2.857
gene-GII88_18240	*liaH*	LiaH	5.070
gene-GII88_18245	*liaI*	LiaI	5.091
gene-GII88_18485	*mntA*	Protein of manganese transport system	1.657
gene-GII88_20050	*ssb*	-	1.301
gene-GII88_03505	*tatA*	TatA	-1.675
gene-GII88_03510	*tatC*	TatC	-1.185
-*Protein with unknown function.			

Validation of Gene Expression Profiles by RT-qPCR

To validate gene expression profiles obtained through RNA-seq analyses, three unigenes (*coxA*, *liaL*, and *sspB*) of *B. licheniformis* M2-7 were evaluated via RT-qPCR (Fig. 3). The expression levels of *coxA* and *liaL* were significantly increased by 45.11- and 61.75-fold (Fig. 3a and 3b) in the interaction system with *C. lunata* SY17, respectively, whereas the *sspB* gene exhibited a 1.24-fold increase (Fig. 3c).

Discussion

B. licheniformis M2-7 has been previously shown to inhibit the growth of *C. lunata* SY17 (Bolaños-Dircio et al. 2024), demonstrating its potential as a promising biocontrol candidate. Our results revealed that *B. licheniformis* M2-7 caused severe morphological damage to the hyphae, conidia, and spores of *C. lunata* SY17 (Fig. 1). The antifungal effect of *Bacillus* species is often associated with the secretion of metabolites that damage the plasma membrane and cell wall of various plant pathogenic fungi (Blake et al. 2021; Soni and Kaheria 2021; Zhang et al. 2022a). In a previous study, our findings revealed that *B. licheniformis* M2-7 produces lytic enzymes, siderophores, biosurfactants, and volatile organic compounds (VOCs). Therefore, the antifungal capacity of this strain is likely attributable to some of these compounds (Bolaños-Dircio et al. 2021). Species of the genus *Bacillus* can sporulate in environments with low nutrient availability (Galperin 2013; Toukabri et al. 2023). However, spores are capable of sensing their environment and quickly resume vegetative growth when exposed to nutrients, a process known as germination (Galperin et al. 2022). During the interaction between *B. licheniformis* M2-

7 and *C. lunata* SY17, DEGs related to bacterial wall and spore germination were identified (Table 2). Three genes that were upregulated during this interaction encode for the CotY, CotX and CotW proteins, which are spore coat proteins related to stages IV and V of sporulation and are among the last proteins to be morphologically expressed (McKenney et al. 2010; Tu et al. 2021). The *yfhD* and *coxA* genes, which were differentially expressed during the interaction, are associated with low-temperature stress and in the developing forespore respectively (Ahmad et al. 2017; Flores et al. 2023). The transcription factor *spoVIF* is involved in spore coat assembly and favors the development of resistant spores in *Bacillus* species. Other differentially expressed genes include *sspF*, *sspK*, *sspE*, and *sspB*, which encode small acid-soluble spore proteins (SASP). These proteins are found exclusively in the spore nucleus, where they saturate spore DNA. Protein binding drastically alters DNA structure and properties, playing a crucial role in spore resistance to heat and various chemicals (Tu et al. 2021). Additionally, the *gerE* and *gerT* genes, involved in spore germination in bacterial strains such as *B. subtilis*, were differentially expressed during the interaction. The YrzI, YrzQ, and YrzR proteins have also been identified in spores, although their functions remain unknown. The *lia*H and *lia*I genes reached fold change values of 5.070 and 5.091, respectively. Under stress conditions that compromise cell membrane integrity, the liaH protein forms higher-order oligomeric structures that bind peripherally to affected membrane regions to mitigate the potentially lethal effects of phospholipid bilayer perturbations (Thurotte et al. 2017). In other words, this protein stabilizes the membrane by binding to highly compromised areas in the form of high-order oligomeric structures. Domínguez-Escobar et al. (2014) demonstrated that membrane stabilization by *liaH* requires interaction with its membrane anchor (*liaI*) at static points. The *liaS* gene encodes for a histidine kinase with two membrane-crossing regions and is involved in sensing cell envelope perturbations (Jani et al. 2020). Previous studies have indicated that the *mntA* gene is linked to the presence of Mn^{2+}. Zhang et al. (2022b) reported that bacillomycin D (BD) promotes spore formation in *B. amyloliquefaciens* fmbJ and exhibited synergistic effects with certain concentrations of Mn^{2+}. Finally, five DEGs encoding for hypothetical proteins (*liaG*, *yloV*, *ypzG*, *yizC*, and *yrrD*) and two with unknown functions (*liaF* and *yhcV*) were also identified. Liang et al. (2023) reported that the co-culture of *Bacillus velezensis* E68 with *Fusarium graminearum* DAOMC 180378 resulted in overexpression of genes related to stages II, III, IV, and V of sporulation. The upregulated genes encode for membrane proteins, sigma factors, regulatory proteins, membrane engulfment-related proteins, and spore cortex proteins, suggesting that the presence of the phytopathogenic fungus promotes *B. velezensis* sporulation in response to stress. The spores of some *Bacillus* species have been found to be efficient in controlling insect pests (Álvarez-Lagazzi et al. 2021; Dubovskiy et al. 2021). However, very few studies have characterized the relationship between the sporulation of *Bacillus* species and the antagonistic effect of their spores against phytopathogenic fungi. Feio et al. (2004) reported that the sporulation of *Bacillus subtilis* is related to the production of active metabolites. These metabolites exhibited antifungal effects on different taxonomic groups contaminating wood surfaces (e.g., *Cladosporium cucumerium*, *Botrytis cinerea*, *Fusarium oxysporum*, *Aspergillus niger*, and *Penicillium expansum*). Ben et al. (2012) reported that the supernatant of *Bacillus subtilis* L194 spore culture exhibited twice as much antifungal activity as vegetative cells against the phytopathogenic fungus *Phoma medicaginis*. Furthermore, *in vivo* experiments revealed that L194 spores were highly protective against the ascomycete fungus *P.*

medicaginis, reducing disease symptoms in *Medicago truncatula* seedlings. Similarly, we recently reported that *Azotobacter vinelandii* cysts exert antifungal effects against *Fusarium brachygibbosum*, *Aspergillus niger*, and *Colletotrichum gloeosporioides* (Bolaños-Dircio et al. 2022). A strain unable to synthesize alkylresorcinols lost its ability to inhibit the growth of phytopathogenic fungi, indicating that these lipids play a crucial role in the antifungal properties of cysts (Bolaños-Dircio et al. 2022). Similar to spores, cysts are formed under adverse conditions to ensure survival (Segura et al. 2020). The main components of cysts are alkylresorcinols, phenolic lipids located in the intestine and exine layers (Romero et al. 2016). Therefore, spores might also contain compounds or proteins with antifungal effects. To the best of our knowledge, no previous reports had explored the effects of *C. lunata* co-culture on the molecular and genetic systems of strain M2-7. Collectively, our findings provide key insights into the molecular mechanisms of bacterial and fungal interaction. However, additional studies are needed to explore the link between the production of antifungal compounds and sporulation.

Conclusion

B. licheniformis M2-7 causes morphological changes in mycelia and conidia of *C. lunata* and transcriptomic results under this interaction condition showed that sporulation-related genes are overexpressed in strain M2-7. Consequently, this investigation offers critical information that will enable future research to analyze the potential for spores to be associated with a mechanism of action in the biological regulation of plant-pathogenic fungi.

Declarations

Ethical Approval:

This is not applicable.

Consent to Participate:

This is not applicable.

Consent to Publish:

This is not applicable.

Competing Interests:

There is no conflict of interest

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Author Contribution

Authors' Contributions: Investigation: Alejandro Bolaños Dircio, Data Curation: Reyes-Prieto Mariana and Rojas-Aparicio Augusto, Formal analysis: Rodríguez-Barrera Miguel Ángel, Methodology and Validation: Toribio-Jiménez Jeiry and Ortuño-Pineda Carlos and Supervision, Writing - Review & Editing: Romero-Ramírez Yanet.

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Data Availability Statement:

The datasets generated and/or analyzed during the current study are available in the [Gene Expression Omnibus] repository, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE330209. Accession number: GSE330209.

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Figures

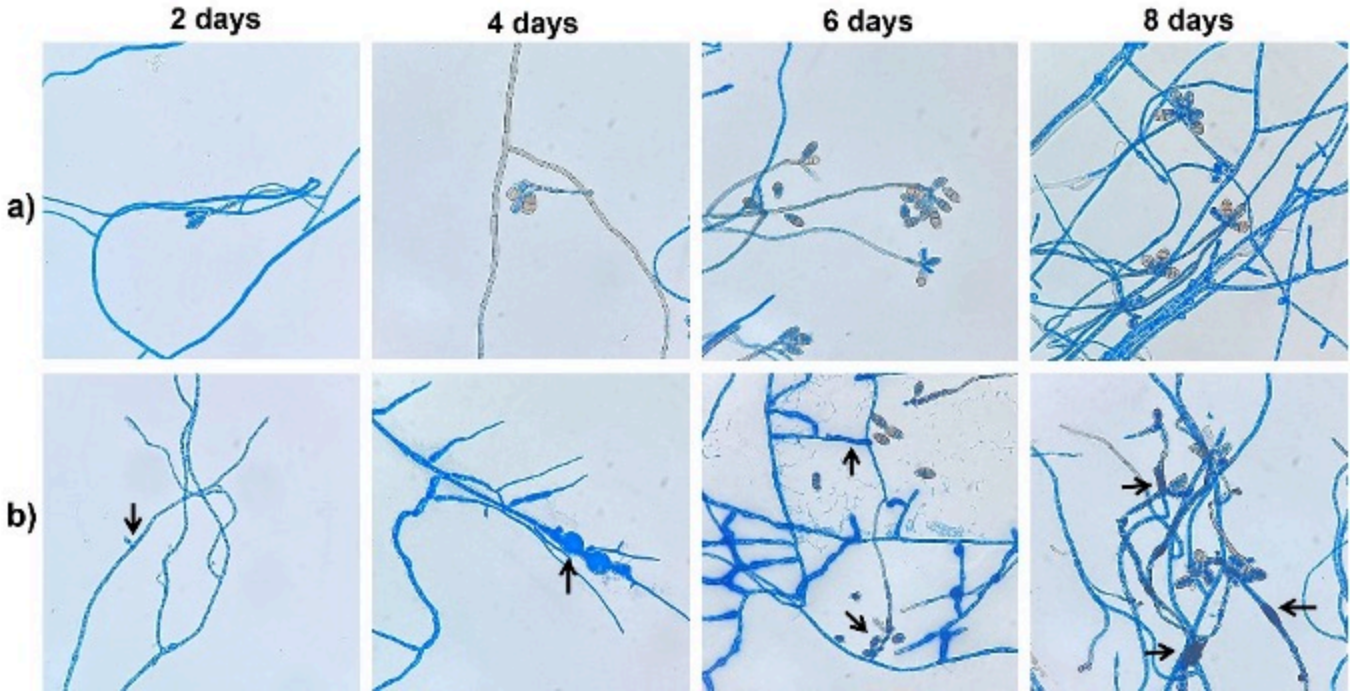


Figure 1

Direct interaction of *B. licheniformis* M2-7 and *C. lunata* SY17. a) Monoculture of *C. lunata* SY17 showing normal growth in liquid LB medium during the days of observation. b) Direct interaction of *C. lunata* SY17 and *B. licheniformis* M2-7 in liquid LB medium. The photographs were taken with the 40X objective of an Olympus light microscope. The arrows indicate swelling, deformities, hyphal breakage, conidial deformities, and spore reduction.

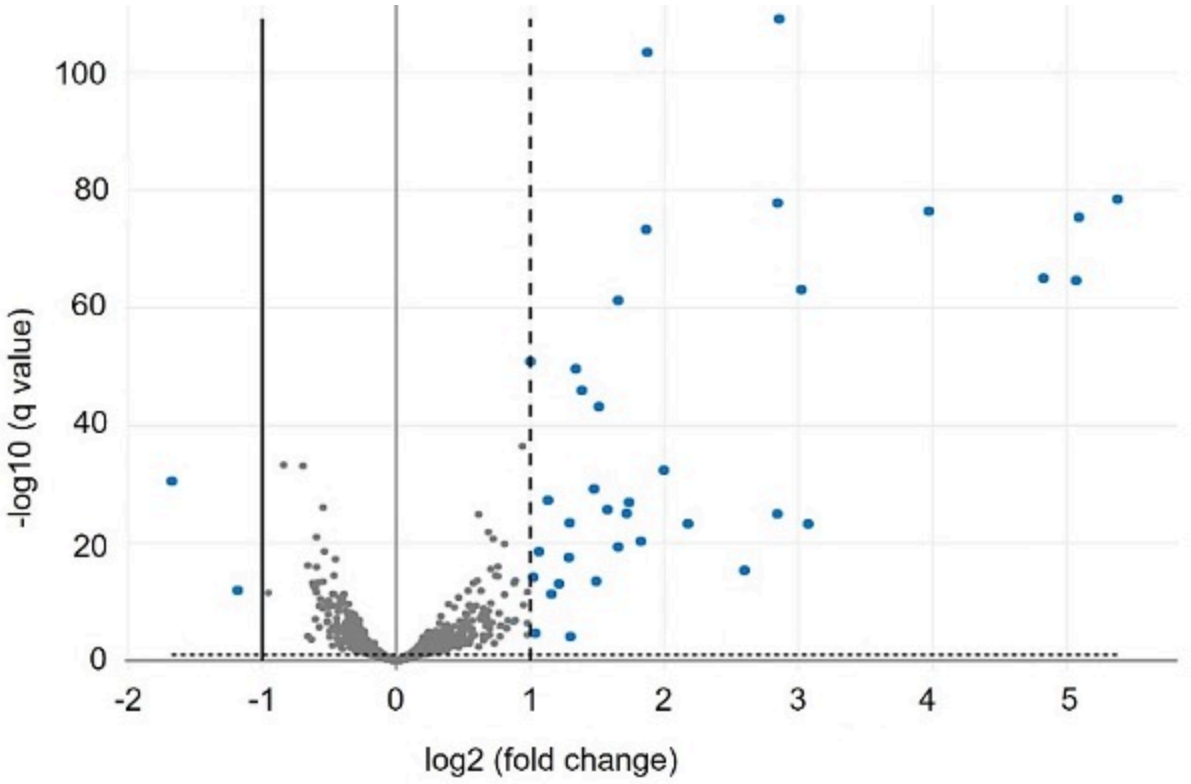


Figure 2

Volcano plot of DEG pattern in *B. licheniformis* M2-7 in the presence of *C. lunata* SY17. The dots from left to right represent downregulated genes, genes with no difference in expression, and upregulated genes, respectively. A gene was considered differentially expressed if it exhibited a Log2 fold change >1 and a false discovery rate (FDR) adjusted p-values ≤ 0.05.

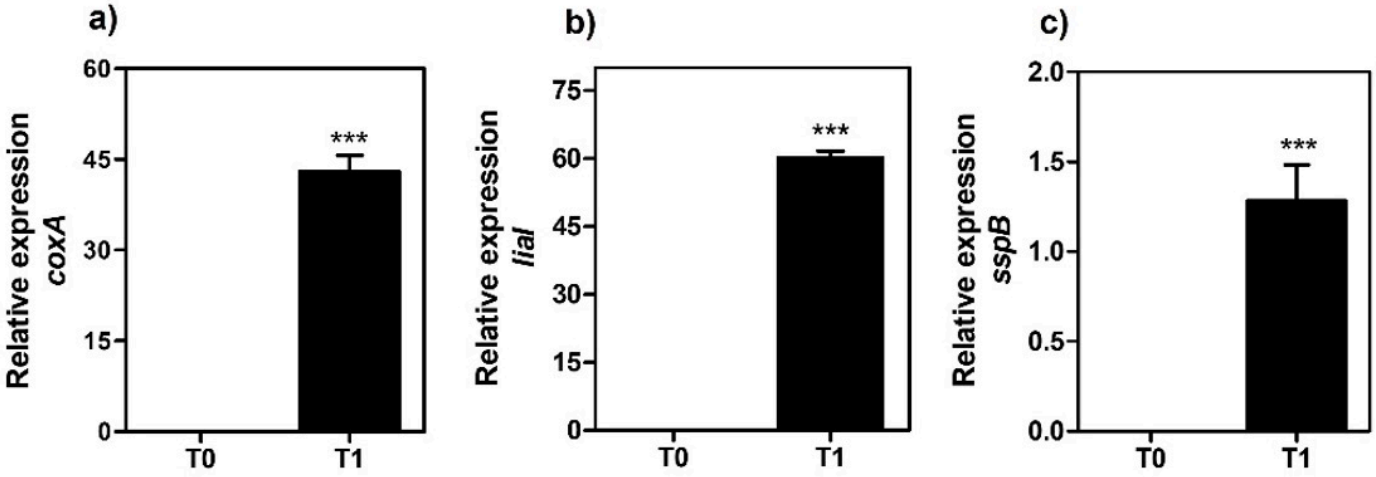


Figure 3

Relative expression of a) *coxA*, b) *lial*, and c) *sspB* in the interaction system of *B. licheniformis* M2-7 and *C. lunata* SY17. ***Indicate significant statistical differences (*P*<0.05) Student's t-test. The error bars indicate the standard deviation (SD) of three independent experiments (n=3).