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Biosurfactants synthesized by endophytic *Bacillus* strains as control of *Moniliophthora perniciosa* and *Moniliophthora roreri*

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ABSTRACT: Ecuador is responsible for up to 70 % of the world's production of fine flavor cocoa. Although fungal diseases have reduced cocoa production, eco–friendly alternatives have been implemented to control the spread of phytopathogens. This study aimed to explore the potential of endophytic strains isolated from fine flavor Nacional cocoa trees to synthetize compounds with antimicrobial and surface active properties. It was determined that under *in vitro* conditions, seven strains (DS03, DS07, DS18, DS23, DS31, DS34 and DS50) exhibited antifungal activity that inhibited the mycelial growth of *Moniliophthora roreri* and *Moniliophthora perniciosa*. The strains were identified based on their morphological (pattern forming *Bacilli*) and genetic characteristics using the 16S SSU rRNA gene sequences. Furthermore, we determined that the endophytic strains contained genes that encoded the lipopeptides Fengycin (*fenA, fenB, fenC, fenE*), Iturin (*ituB, ituC, ituD*), and Bacyllomicin D (*bamC, bamD*). The production of biosurfactant compounds was determined by a reduction in surface tension in the mineral medium. Additionally, the biosurfactant crude extract (BCE) was tested for its inhibitory activity against *M. perniciosa* and *M. roreri*. The minimal inhibitory concentration (MIC) was determined to be 0.07 and 0.035 mg mL^{-1}, respectively, in both cases in a fungistatic action mode. The swelling granulation and fragmentation of both phytopathogens' hyphae were observed using the BCE treatment. These results suggested the biocontrol capacity of the endophytic strains, which under *in vitro* conditions, have the ability to synthesize antifungal and surface active compounds that might be used as an ecological alternative in cocoa plants to prevent infection by phytopathogens.

Keywords: antimicrobial biosurfactant, biocontrol, cocoa, endophytes microorganisms

Introduction

Cocoa is an agricultural, economic commodity considered essential to millions of people worldwide. More than 6 million smallholders cultivate cocoa trees, and more than 40 million people globally depend on cocoa production as their primary income source (Wickramasuriya and Dunwell, 2018). Ecuador is one of the world's leading cocoa bean growers, producing almost 300,000 MT in a calendar year (Vega and Beillard, 2015). Worldwide, cocoa bean production is severely limited by diseases that have caused a reduction in annual yield of approximately 20 %, although the percentage loss may vary across different production regions (Ploetz, 2016). Witches' broom (*Moniliophthora perniciosa*) and frosty pod rot (*Moniliophthora roreri*) are two of the world's most dangerous cocoa diseases. (Evans, 1981; McElroy et al., 2018). Although they have a narrow geographic distribution and are usually non–lethal diseases, they impact yields by rendering beans in affected pods commercially useless (Ploetz, 2016). Ecuador and Perú are among the top 10 cocoa–producing countries where devastating losses could occur if these phytopathogens were to spread (Evans, 2016; Ploetz, 2016).

The most common strategy for the control and prevention of both diseases is based on copper–based fungicides. (Bateman et al., 2005). Pesticides are known to produce declines in valuable natural resources, including biodiversity loss, soil depletion, and damage inflicted on people and the environment (Le Mire et al., 2016; López–Morales and Salaya–Domínguez, 2017), but nowadays more eco–friendly alternatives are available and have already been implemented. This change has encouraged the use of endophytic bacteria such as biocontrol agents receiving attention as an alternative strategy for the application of synthetic fungicides.

In cocoa crops, the potential of endophytic bacteria and fungi for controling certain cocoa diseases has been previously reported (Mejía et al., 2008; Melnick et al., 2011). For instance, many *Bacillus* strains have been extensively studied as they produce various antifungal lipopeptides (LPs), including surfactin, iturin, and fengycin that can elicit relevant antimicrobial properties to inhibit the growth of several phytopathogenic fungi (Torres et al., 2016). Thus, this study aimed to evaluate, under *in vitro* conditions, the potential antifungal activity of biosurfactant compounds synthesized by cocoa endophytic strains to inhibit the mycelial growth of *M. roreri* and *M. perniciosa*.

Materials and Methods

Isolation of endophytic strains and preliminary screening of their antifungal activity

Endophytic strains were isolated from disease–free cocoa cultivars, presenting high numbers of flowers and

healthy pods. Cocoa cultivars (75 fine flavour National variety and 75 CCN51) were located at two plantations in Naranjal (2°40′35.2″ S, 79°38′21.2″ W, altitude of 25 m. a.s.l.) and Balao (2°51′51.8″ S, 79°37′20.8″ W, altitude of 10 m. a.s.l.), Guayas–Ecuador. Bacterial strains were isolated using a protocol previously described by Silva et al. (2012). Before bacterial isolation, a brief disinfection procedure was carried out to discard epiphytic bacteria. Leaves were washed in running tap water to remove dust, then a 1 cm^2 piece was cut and sterilized in 70 % ethanol for one min, followed by immersion in 2 % sodium hypochlorite for 1 min and then washed in sterile water for 1 min. Subsequently, leaf tissue was macerated in a 0.85 % NaCl solution and plated on nutrient agar. After 24 h of incubation at 30 °C, morphologically distinct colonies were isolated and purified. The presence of *Bacillus*–like colonies was confirmed using standard methodologies (i.e. Gram′s staining, motility and phenotypic characteristics). Pure cultures were cryopreserved at –80 °C in Luria Bertani broth (LB, 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) supplemented with 20 % (v v^{-1}) of glycerol.

Bacterial isolates were screened for their antifungal activity against *M. roreri* and *M. perniciosa*. The pathogens were first cultured on fresh Potato Dextrose Agar (PDA) for 10 days at 28 °C, then 5 mm disks containing mycelia and agar of *M. roreri* and *M. perniciosa* were aseptically removed. The disks were placed in the center of PDA plates and with an inoculating loop, the isolated bacteria exhibiting typical characteristics of *Bacillus* were streaked on the opposite side of the Petri dish. The plates were then incubated for seven days at 28 °C and their ability to inhibit fungal growth was observed by the formation of inhibition zones; all tests were performed in triplicate.

Morphological and genetic identification of endophytic bacterial strains

Strains exhibiting antagonistic activity were selected for further characterization. Aliquots of 10 µL from each endophytic isolate were grown in 250 mL flasks containing 100 mL of LB broth. The flasks were incubated at 30 °C and 150 rpm for 24 h or till the mid–log phase had been reached. For colony formation, 2 µL of the liquid culture was inoculated in triplicate onto plates containing Lysogeny broth–glycerol–manganese (LBGM, LB plus 1 % [v v^{-1}] glycerol, 0.1 mM MnSO$_4$, and 1.5 % agar per liter) solid medium. Endophytic strains were grown on LBGM solid medium as it strongly promotes biofilm formation, sporulation, and pigment production (Shemesh and Chai, 2013). The plates, incubated at 30 °C for 72 h prior to analyzing the bacterial colony patterns, were photographed daily to observe differences in pattern formation between the isolates.

Genomic DNA of the isolates was extracted using a slightly modified protocol as the one described by (Ausubel et al., 2003). The Universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT) were used to amplify the 16S SSU rRNA gene. Reactions were carried out in 20 µL containing 20 ng of genomic DNA, 0.4 µM of forwarding primer, 0.4 µM of reverse primer, 400 µM of dNTPs, and 0.4 U of Taq DNA polymerase. Polymerase chain reaction (PCR) conditions started with an initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1min and extension at 72 °C for 1 min. After a final extension at 72 °C for 3 min, the amplicons were analyzed by electrophoresis on 2 % (w v^{-1}) agarose gels. The PCR products were purified and sent for sequencing at Macrogen Inc. (Seoul, South Korea), and all sequences were deposited in the NCBI database under the following accession numbers: SUB8562136 (DS03), SUB8562600 (DS07), SUB8562609 (DS10), SUB8562620 (DS18), SUB8562685 (DS23) SUB8563474 (DS31), SUB8563654 (DS34), SUB8563688 (DS50). Sequence alignment and classification were performed using the SINA v1.2.11 aligner tool (Quast et al., 2013) and the ARB SILVA SSU NR 99 reference database (Release 132) (Pruesse et al., 2012). The sequence "Search and Classify" option was used to classify DS strains with the least common ancestor (LCA) method based on SILVA′s taxonomies. Default parameters were used with the only exception that the Bacteria variability profile was selected under advanced alignment parameters. For computing and reconstruction of the phylogenetic tree, the FastTree program was chosen with the GTR model and Gamma rate model options activated. The neighbors mentioned above and the default parameters were used. Additionally, the endophytic strains were screened for genes (Table 1) reported in the synthesis of lipopeptide biosurfactants using specific primers in order to determine the relationship between antifungal and surface activity compounds. PCRs were carried out in 20 µL reaction mixtures containing 10 µL of GoTaq® Green Master Mix, 0.4 µL of each primer, 3.6 µL of ultrapure water and 1 µL of template DNA (20 ng of bacterial genomic DNA). The reactions were generated under the following cycling conditions: initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 1 min, 55 °C or 52 °C for 1min, and 72 °C extensions for 1.5 min; and a final extension at 72 °C for 7 min. A total of 5 µL of each amplification reaction was analyzed by electrophoresis in a 1.5 % agarose gel stained with SYBR Safe.

Biosurfactant production and crude extract recovery

Biosurfactant production was evaluated using two different culture media: Potato dextrose broth (PDB) and Basal Mineral Media (BMM, 20 g, glucose; 8.5 g

Table 1 – Primer sequences used for the amplification of lipopeptide–related nonribosomal peptide–synthetase genes in DS strains.

Antibiotic	Gene(s)	Primers	Sequence	Polymerase chain reaction (PCR) product size
Bacyllomicin D	bamD	ITUD–F1	TTGAAYGTCAGYGCSCCTTT	482 bp
		ITUD–R1	TGCGMAAATAATGGSGTCGT	
	bamC	BAMC–F1	AGTAAATGAACGCGCCAATC	957 bp
		BAMC–R1	CCCTCTCCTGCCACATAGAG	
Bacilysin	bacD	BACD–F1	AAAAACAGTATTGGTYATCGCTGA	749 bp
		BACD–R1	CCATGATGCCTTCKATRCTGAT	
	bacAB	BACAB–F1	CTTCTCCAAGGGGTGAACAG	815 bp
		BACAB–R1	TGTAGGTTTCACCGGCTTTC	
Ericin	eriB	SPAB–ERIB–F1	GCACAGATGGAAAATCTGAAG	688 bp
		SPAB–ERIB–R1	GAAAATTGCTCCCCAAATGA	
	eriSa	ERISA–F1	TTCGATGARTTCGATTTGGA	357 bp
		ERISA–R1	GCAGCCCTTTTTCTTTTATTTC	
Fengycin	fenB	FENB–F1	CCTGGAGAAAGAATATACCGTACCY	670 bp
		FENB–R1	GCTGGTTCAGTTKGATCACAT	
	fenC, fenA, fenE	FENCEA–F1	CCCATCCGACYGTAGAAG	820 bp
		FENCEA–R1	GTGTAAGCRGCAAGYAGCAC	
Iturin	ituD	ITUD–F1	TTGAAYGTCAGYGCSCCTTT	482 bp
		ITUD–R1	TGCGMAAATAATGGSGTCGT	
	ituC	ITUC–F1	CCCCCTCGGTCAAGTGAATA	594 bp
		ITUC–R1	TTGGTTAAGCCCTGATGCTC	
	ituB	ITUB–F1	ATCCAGAATATCCGCAAGAC	
		ITUB–R1	GCTCGATATGCGGACTCAAT	
Mersacidin	mrsA	MRSA–F1	GGGTATATGCGGTATAAACTTATG	597 bp
		MRSA–R1	GTTTCCCCAATGATTTACCCTC	
	mrsM	MRSM–F1	AAATGACCCGGCATATGTTC	816 bp
		MRSM–R1	TGCTGACTAACTGGAATTGGAA	
Mycosubtilin	fenF	ITUD–F1	TTGAAYGTCAGYGCSCCTTT	482 bp
		ITUD–R1	TGCGMAAATAATGGSGTCGT	
	mycC	MYCC–F1	AATCAATTGGCACGAACCTT	1026 bp
		MYCC–R1	ATCGCCCGTTTTGTACATTC	
Sublancin	sunT	SUNT–F1	GCTTTGTTAGAAGGGGAGGAAT	974 bp
		SUNT–R1	CTTGTCCCAACCCATAGGATAA	
Subtilin	spaB	SPAB–ERIB–F1	GCACAGATGGAAAATCTGAAG	688 bp
		SPAB–ERIB–R1	GAAAATTGCTCCCCAAATGA	
	spaC, spaS	SPACS–F1	CCGGACAGGAGTATTTTAAGGA	460 bp
		SPACS–R1	GCAGTTACAAGTTAGTGTTTGAAGGA	
Subtilosin	albF	ALBF–F1	TCAACAGCTGGATGAACGAAC	888 bp
		ALBF–R1	AGGCGGTAYGTTTGCTGWATCT	
	albA	ALBA–F1	TTGTTTATAGAGCAGATGTTTCCA	625 bp
		ALBA–R1	GGCTCTCTTTTCGCATGAGT	
Surfactin	srfA	SRFA–F1	AGAGCACATTGAGCGTTACAAA	626 bp
		SRFA–R1	CAGCATCTCGTTCAACTTTCAC	
	sfP	SFP–F1	ATGAAGATTTACGGAATTTA	675 bp
		SFP–R1	TTATAAAAGCTCTTCGTACG	

NaNO$_3$; 4 g KH$_2$PO$_4$; 5.7 g Na$_2$HPO$_4$; 0.21 g MgSO$_4$.7H$_2$O; 7.10^{-6} g CaCl$_2$; 0,01 g FeSO$_4$.7H$_2$O; 1 g yeast extract per liter). 250 mL Erlenmeyer flasks containing 100 mL of each medium were inoculated with 2 % (v v^{-1}) of adjusted initial inoculums of 2.0 McFarland standard inoculums. The flasks were incubated for 24 h at 30 °C and 110 rpm in an orbital shaker. Next, the samples were centrifuged for 20 min at 4 °C and 3900 × g. The cell–free supernatant surface activity was evaluated by applying the oil drop assay. In this technique, 200 µL of the cell–free supernatant was added to a Petri dish containing a thin film of crude oil, the formation of clear zones on the oil surface was recorded. The cell–free supernatants were then used to measure the surface tension with a manual force tensiometer. The instrument was calibrated against water (γst 72 mM m^{-1}) to ensure accuracy over the entire range of surface tension. Surface tension distilled water (72 mN m^{-1}) was used as a negative control, and an uncultured medium was used to compare and determine the surface tension reduction

produced by each strain (Coronel–León et al., 2015). In addition, to obtain the biosurfactant crude extract (BCE) the bacterial cells were removed from the culture medium by centrifugation at 8,000 × g, for 15 min at 4 °C. Next, the cell–free supernatants were subjected to acid precipitation using concentrated hydrochloric acid (HCl) until pH 2 was reached, and BCE was left overnight at 4 °C. The BCE was subsequently collected by centrifugation for 20 min at 4 °C, and 11,000 × g) and washed twice with acid distilled water (pH 2) to eliminate any impurities. The BCE was dried in an oven at 40 °C to constant weight and then quantified by gravimetry (g L^{-1}) (Coronel–León et al., 2016a).

Antifungal activity of biosurfactant compounds against *M. perniciosa* and *M. roreri*

Antifungal activity was determined using the poisoned food method (Imhof et al., 2003). Different concentrations of BCE (0.3 – 500 mg L^{-1}) were added to tubes containing 15 mL of molten PDA; the tubes were then mixed thoroughly and poured into Petri dishes. When the molten PDA was solidified, 5 mm discs of *M. perniciosa* and *M. roreri* were placed in the centre of the Petri dishes. Plates without biosurfactant compounds (only PDA) were used as positive controls, and each assay was performed in triplicate.

The fungal colonies' diameter was measured after seven days of incubation at 28 °C, and the mean diameters were calculated. Inhibition of fungal growth was estimated by measuring the fungal colony's diameter and expressed as a percentage of the inhibition of fungal growth divided by the growth obtained in the control plates. On the other hand, in the case of the BCE a morphological comparison of the pathogen's hyphae in the contaminated medium was used to evaluate the biosurfactant effect. The pathogen's hyphae were fixed with scotch tape on a drop of cotton blue on a slide. The structures were observed under a microscope and compared with the forms in the water control.

Results

Isolation of endophytic bacterial strains and preliminary screening of their antifungal activity

Endophytic strains were isolated from Naranjal and Balao cocoa plantations located on the Ecuadorian coast, where fine flavour National and CCN51 cocoa varieties are commonly cultivated. One hundred and fifty trees exhibiting subtle morphological differences were monitored, 78.4 % of the trees showed no signs of infection and 21.6 % showed disease symptoms. In total, 251 endophytic bacteria were isolated exclusively from disease–free cultivars presenting high numbers of flowers and healthy pods. All isolates were tested against *M. roreri* and *M. perniciosa* to determine their potential antifungal activity (Figure 1).

Only 51 bacterial isolates exhibited promising antifungal activity by being able to inhibit the mycelial growth of *M. roreri* and *M. perniciosa*. In this context, the *in vitro* test showed two pools of strains with sufficient antibiosis capacity; in the first group, the isolates DS03, DS07, DS18, DS23, DS31, DS34, and DS50 produced a zone of inhibition against *M. roreri* by not allowing the pathogen hyphae to develop normally. Out of this first pool strains DS03 and DS34 were the most efficient with an inhibition zone of 3.67 ± 0.20 cm. In the second group, strains DS50, DS03, DS23 and DS31 also inhibited *M. perniciosa* but to a lesser extent. Isolates DS50 and DS03 showed an inhibition zone of 3.30 ± 0.20 cm. Finally, it was found that strains DS07, DS18 and DS19 did not exert any inhibitory effect against *M. roreri*, although medium antifungal activity against *M. perniciosa* was detected (Figure 2).

Morphological and genetic identification of endophytic bacterial strains

The preliminary screening performed to determine the potential antifungal activity of the isolates to inhibit the mycelial growth of *M. roreri* and *M. perniciosa*, allowed for the identification of seven promising bioactive
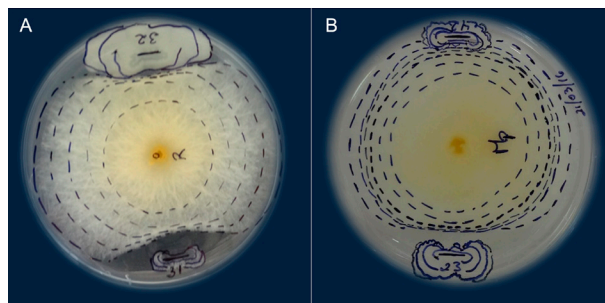

Figure 1 – Dual plate culture assay showing the antagonistic effect of cocoa endophytic strains against *Moniliophthora roreri* (A) and *Moniliophthora perniciosa* (B).
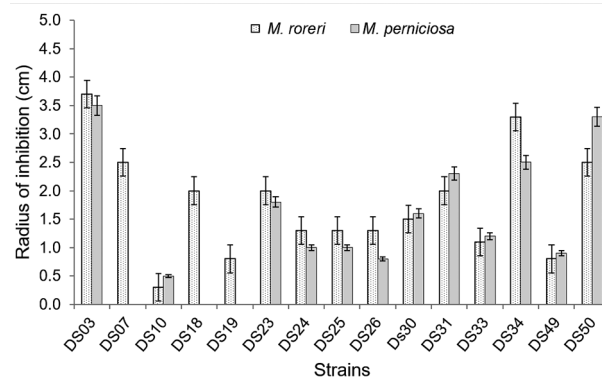

Figure 2 – Antifungal activity of the cocoa endophytic strains against the phytophatogens *Moniliophthora roreri* and *Moniliophthora perniciosa*.

bacterial strains (DS03, DS23, DS31, DS34, DS50, DS07 and DS18). These strains were subjected to further studies to confirm their morphotypes and phylogenetic position. In order to elicit the patterned growth of the endophytic isolates, two high–nutrient agars were chosen (LB broth and LBGM solid medium). In this study, three out of the five morphotypes were reported on. Strains DS03 and DS07 developed the B morphotype, which is characterized by presenting typical compact round colonial growth with rough edges; strain DS18 seemed to exhibit a tip–splitting, tree branch growth, characteristic of the T morphotype; and strain DS23 looked like a spiral vortex (SV) morphotype (Figure 3). The morphotypes of the strains DS31 and DS50 could
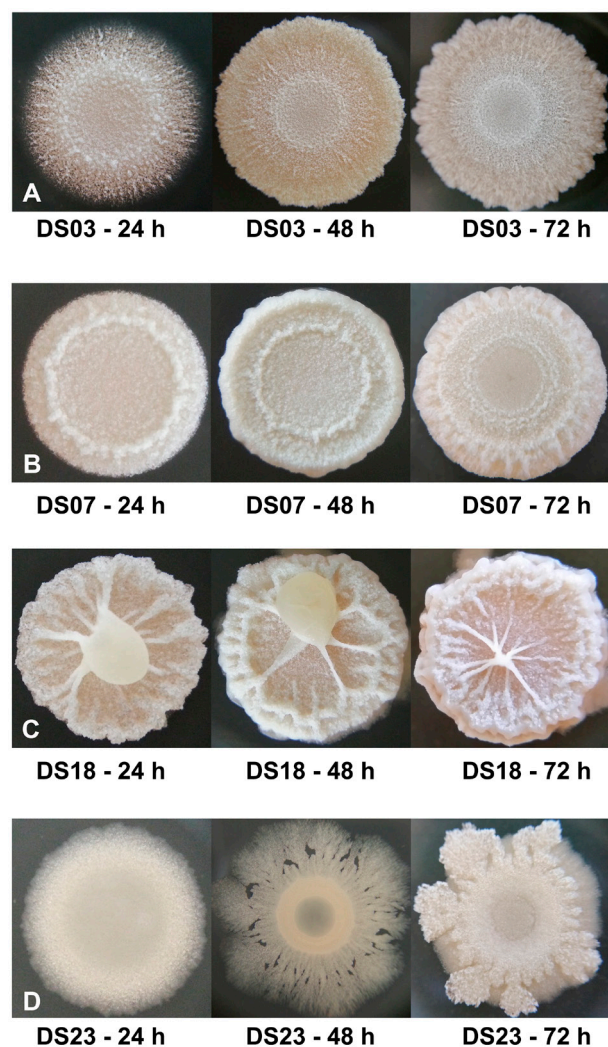

Figure 3 – Observed patterns of colonies grown on Lysogeny broth–glycerol–manganese (LBGM) medium. Endophytic strains morphotypes on nutrient rich, soft agar plates, from top to bottom pictures A and B depict a typical growing pattern of morphotype B, picture C seems to exhibit a morphotype T, and picture D, apparently, looks like a spiral vortex (SV) morphotype.

not be identified using LBGM solid medium as they did not exhibit any apparent pattern growth (Di Franco et al., 2002; Rudner et al., 1998; Shemesh and Chai, 2013).

In addition, the seven strains were genetically characterized using the 16S SSU rRNA gene sequence, generating sequences of 1,381bp, except for DS18 which presented problems during the sequencing service and only developed a 789bp sequence length. Due to issues during the 16S rRNA gene sequencing sample DS18 was excluded from further genetic analysis to avoid undesirable results. The 26 unique strains sequence matches obtained from the SINA "Search and classify," and 6 DS sequences (DS18 was excluded) were aligned using the SINA aligner to compare and identify differences within the sequences. The phylogenetic tree generated by the SILVA platform is shown in Figure 4. The phylogenetic analysis showed the six isolated strains DS03, DS07, DS23, DS31, DS34, and DS50 clustered together. All reports showed the same alignment scores for the 16S rRNA sequences of the six DS endophytic strains and *Bacillus velezensis*, *B. amyloliquefaciens* and *B. subtilis* groups, showing the high genetic similarity of the *Bacillus subtilis* species complex. Thus, it can be challenging to define the species or specific strain we were working with solely based on the 16S rRNA gene sequence. In addition to the 16S rRNA gene sequences, there are other gene sequences (*gyrA*, *gyrB*, *rpoB*, *polC*, *groEL*) that can be used for the comparison and discrimination of the *Bacillus* strains, although they were not considered for this study and a phenetical approach was selected.

Biosurfactant production and crude extract recovery

To determine the relationship between antifungal and surface activity, the amplified 16S rRNA gene sequence (~1400 bp) was used to do a Blast. The analysis of the sequences revealed a high level of similarity between the different *Bacillus* species. The screening of genes involved in the biosynthesis of *Bacillus* spp. antimicrobial peptides showed that all the evaluated strains had ten genes in common (*bamD*, *bamC*, *bacD*, *bacAB*, *fenB*, *ituB*, *ituC*, *ituD*, *fenF, and albF*). Although most genes were present in all strains, the genes *fenC*, *fenA*, and *fenE* were not detected in strain DS34. Gene *mycC* was not amplified in strains DS03 and DS08. Finally, genes *eriB*, *eriSa*, *mrsA*, *mrsM*, *sunT*, *spaB*, *spaC*, *spaS*, *albA*, *srfA* and *sfP* were not detected in any of the isolates. Table 2 depicts the presence and distribution of genes per strain.

A significant group of peptides with antimicrobial activity from *Bacillus* genus are microbial surfactants or biosurfactants; for this reason, the capacity of the strains to synthetize biosurfactants was first tested using the oil drop assay. All tested drops of the selected
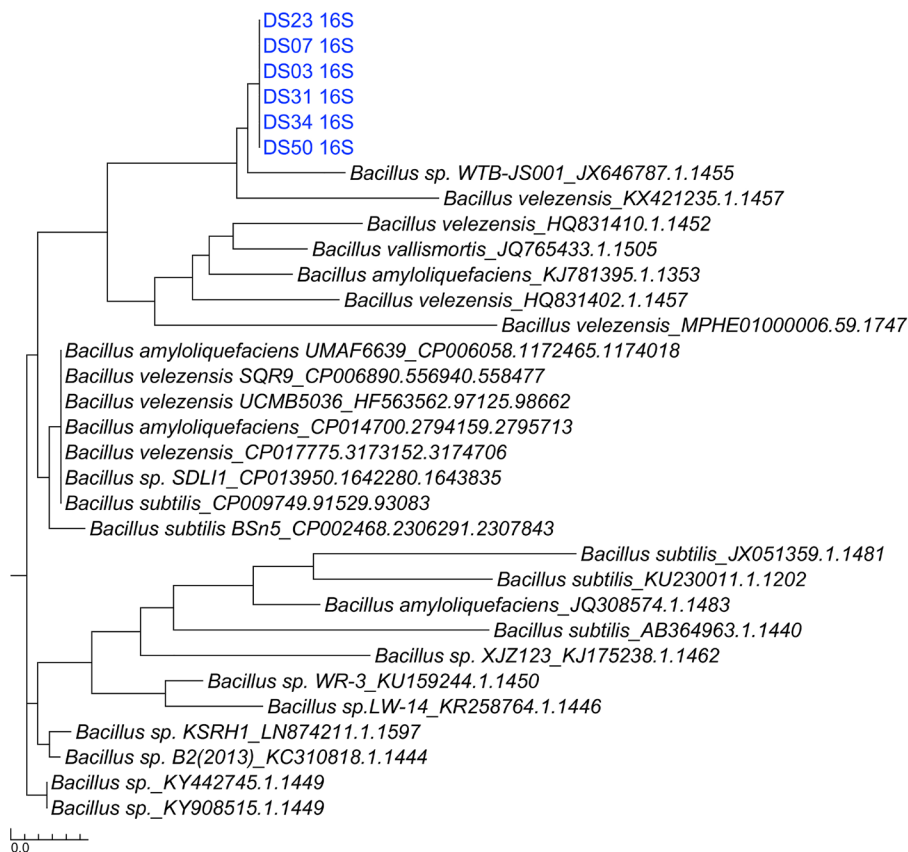
Figure 4 – FastTree inferred maximum likelihood phylogeny tree from the alignment of the 16S rRNA gene sequences of the *Bacillus* sp; Six DS strains and the nearest neighbours from the SINA "search and classify" tool, using the generalized time–reversible (GTR) models of nucleotide evolution from the ACT tool from SILVA; Blue = DS strains.

strains collapsed in this assay whereas non–surfactant–containing drops (water) remained stable. The surface tension measurements of the cell–free supernatant of each strain in different medium are shown in Table 3. All strains decreased the surface tension on the cell–free cultures when a mineral medium (BMM) was used. The surface tension reduction by each strain was more than 20 mN m^{-1} after 24h culture, whereas the control medium's surface tension was 60.0 ± 2.0 mN m^{-1}. The value of the reduction in surface tension was around 31.0 ± 2.0 mN m^{-1} for all strains (cell–free supernatant). On the other hand, the supernatants from PDB cultures showed no further surface tension reduction. The next step was to determine the antifungal activity of all strains.

Antifungal activity of biosurfactant compounds against *M. perniciosa* and *M. roreri*

The minimum inhibitory concentration (MIC) of the biosufactant compounds ranged between 35 and 70 mg L^{-1} for *M. roreri* and *M. perniciosa*. The quantitative test for antagonistic activity showed that after seven days of incubation, the vegetative mycelium of *M. roreri* did not

grow on concentrations above 35 mg L^{-1}. Similarly, the fungal disc of *M. perniciosa* showed no signs of growth at 70 mg L^{-1} concentration for all seven DS strains evaluated. However, below this concentration, a slight development of mycelial structures was observed. In general, BCE showed antagonistic activity against *M. roreri* and *M. perniciosa* across a broad concentration in a dose–response manner (Figure 5). Based on these results and those shown in Figure 2, isolate DS03 had the most efficient antagonism against both cocoa pathogens and was, therefore, selected for further studies.

Cellular damage inflicted by BCE (35 mg L^{-1}) against both phytopathogenic fungi was evaluated through *in vitro* tests. The hyphae in *M. roreri* and *M. perniciosa* showed degradation, granulation and fragmentation originating from the action of the biosurfactant compounds secreted by isolate DS03 as seen in Figure 6. The most compelling evidence for the production of vesicles by the fungus was observed in *M. perniciosa* as a consequenc of exposure to the bioproduct, which was evident from the deformation of the fungal hyphae. This result strongly suggests the biological control effect that this strain exerted against the phytopathogen, *M. perniciosa*.

Table 2 – Amplicon detection of genes involved in the biosynthesis of lipopeptides.

Antimicrobial peptide	Gene name	DS 03	DS 07	DS 18	DS 23	DS 31	DS 34	DS 50
Bacyllomicin D	*bamD*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	bamC	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Bacilysin	*bacD*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	bacAB	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ericin	*eriB*	–	–	–	–	–	–	–
	eriSa	–	–	–	–	–	–	–
Fengycin	*fenB*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	fenC, fenA, fenE	Yes	Yes	Yes	Yes	Yes	–	Yes
Iturin	*ituD*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	ituC	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	ituB	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Mersacidin	*mrsA*	–	–	–	–	–	–	–
	mrsM	–	–	–	–	–	–	–
Mycosubtilin	*fenF*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	mycC	–	Yes	–	Yes	Yes	Yes	Yes
Sublancin	*sunT*	–	–	–	–	–	–	–
Subtilin	*spaB*	–	–	–	–	–	–	–
	spaC, spaS	–	–	–	–	–	–	–
Subtilosin	*albF*	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	albA	–	–	–	–	–	–	–
Surfactin	*srfA*	–	–	–	–	–	–	–
	srfP	–	–	–	–	–	–	–

Table 3 – Biosurfactant activity measurement from the strains cultured in basal mineral media (BMM) and Potato Dextrose Broth.

Strain	BMM	PDB	Drop test
	———————— mN m^{-1} ————————		
C	60.0	62.0	–
DS03	30.5	62	+
DS07	31.0	62	+++
DS18	30.0	60	+++
DS23	32.0	62	+++
DS31	30.0	60	+++
DS34	31.0	62.5	+++
DS50	30.5	62	+++

Figure 5 – Antifungal activity of the biosurfactant crude extract obtained from the strain DS03 to inhibit the mycelial growth of *Moniliophthora roreri* and *Moniliophthora perniciosa*. The control without biosurfactant effect was included.

Discussion

In the present study, 150 cocoa trees (75 National and 75 CCN51 cultivars) located at two plantations in Naranjal and Balao, Guayas–Ecuador were monitored. It was found that 78.4 % of the trees showed no signs of Witches' broom nor frosty pod rot diseases and 21.6 % showed disease symptoms. In total, 251 endophytic bacteria were isolated from disease–free cultivars presenting high numbers of flowers and healthy pods. Thus, we reasoned that trees colonized by endophytic microorganism might have been protected against phytopathogen infection. There is growing evidence indicating that beneficial microorganisms can suppress the growth of phytopathogens through a number of mechanisms including direct inhibition of pathogen growth by producing antibiotics, toxins, hydrogen cyanide and hydrolytic enzymes 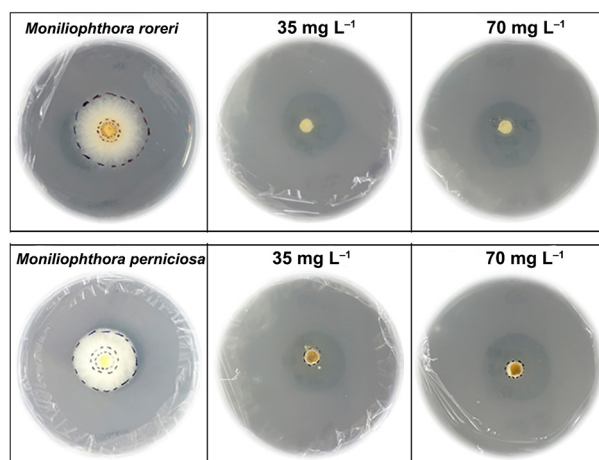(chitinases, proteases and lipases) which can degrade virulence factors or pathogen cell–wall components (Whipps, 2001). It has been suggested that endophytic microorganisms living in cocoa trees control the growth of pathogens by secreting extracellular compounds such as antimicrobial peptides and biosurfactants. To the best of our knowledge, this represents the first report of cocoa endophytic *Bacillus* synthesizing bioactive compounds with antifungal and surfactant properties that have been applied to control under *in vitro* conditions, the mycelial growth of *M. roreri* and *M. perniciosa,* the causal
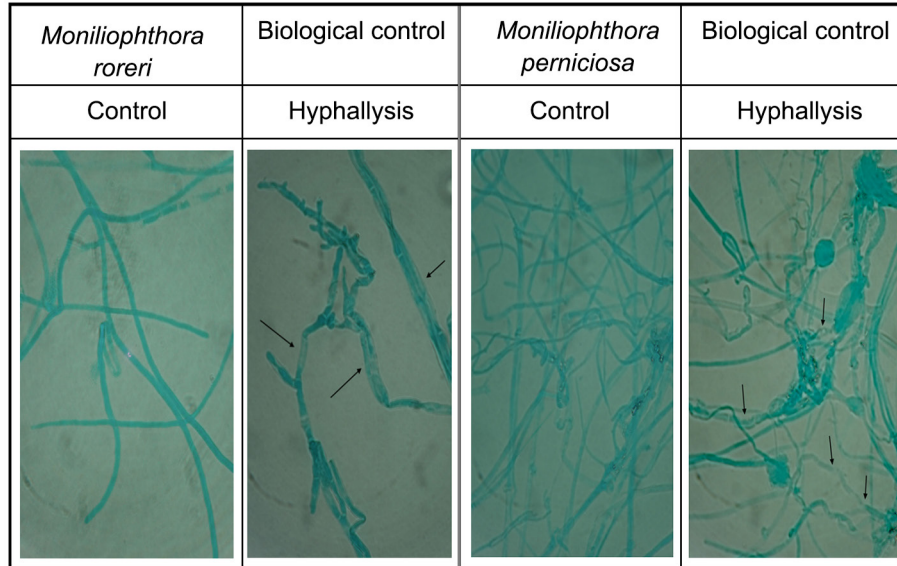
Moniliophthora roreri	Biological control	*Moniliophthora perniciosa*	Biological control
Control	Hyphallysis	Control	Hyphallysis

Figure 6 – Effect of biosurfactant crude extract obtained from strain DS03 on *Moniliophthora. roreri* and *Moniliophthora perniciosa* hyphae. Changes in hyphae morphology (black arrows) were evaluated by microscopy after 5 days of incubation of the fungal disks treated with DS03 crude extract supernatant, 40X.

agents of frosty pod rot and Witches' broom diseases, respectively. Antagonistic assays showed that seven out of the 51 endophytic strains evaluated inhibited mycelial growth in both pathogens; these results suggested that these endophytes may be able to synthetize bioactive lipopeptides with antifungal properties.

Morphological analysis suggested that the endophytic strains belong to the genus *Bacillus*. Further genetic analysis using the small ribosome subunit gene sequence confirmed these results; nonetheless, the 16S rRNA gene sequencing did not provide enough evidence to discriminate between *Bacillus* species as most of them exhibited almost 99 % similarity to other *Bacillus* species as shown in Figure 4. The phylogenetic tree suggests that the endophytic bacterial strains belong to the *B. subtilis* species complex, and even more specifically to the "operational group *B. amyloliquefaciens*", as described by Fan et al. (2017). According to these results, the *B. velezensis* and *B. amyloliquefaciens* analysed species appear to be the most genetically related species to the endophytic DS strains studied and are consistent with the idea that our cocoa isolated endophytic strains belonged to the "operational group B. amyloliquefaciens". According to Fan et al. (2017), the members of the "operational group *B. amyloliquefaciens* are distinguished from *B. subtilis* by their ability to synthesize nonribosomal lipopeptides such as bacillomycin D or iturin A. Based on the results shown in Table 2 from the amplicon detection of genes involved in lipopeptides' biosynthesis, all of the seven DS strains were positive in the detection of two nonribosomal peptide–synthetase genes corresponding to *bamC* and *bamD*. These results corroborate our earlier observations, which suggested the DS strains belong to the "operational group *B. amyloliquefaciens*".

Furthermore, a second morphological analysis clearly showed differences between the DS strains, discarding the duplication of results (Figure 3). The colony formation assays allowed us to compare patterns of bacteria growing on agar plates. Differences shown in bacteria multicellularity morphology could have different causes: wider spreading in the plate due to effective use of nutrients or coordination of biochemical activities (Shapiro, 1998). It has long been known that in natural environments, bacteria most commonly grow as organized multicellular populations that form complex pattern. (Shapiro, 1998). These patterns are characterized by organized and readily identifiable geometric and predictable morphologies (morphotypes), which can be observed on solid and semi-solid surfaces (Rudner et al., 1998). It has been noted that *B. subtilis* growing either on nutrient–poor hard agar surfaces or on nutrient-rich soft agar, exhibit characteristic growth patterns. Certain authors have proposed that this pattern formation might occur because the colony tries to alter its shape to survive in adverse environments (Ben–Jacob et al., 1994; Shapiro, 1998), i.e. where high humidity percentages are prevalent. Currently, five morphotypes have been described for *B. subtilis* strains which were grown under demanding agar conditions (up to 4 % agar concentration) and low levels of nutrients (Ben–Jacob et al., 1994). Although it was previously thought that these features were specific to *B. subtilis* morphotypes, they have been observed in several other Bacilli strains, which suggest the presence of universal growing patterns within this genus (Salhi and Mendelson, 1993). These findings made evident that the *Bacillus* species complex cannot be simply understood by comparing the 16S rRNA sequence and it is somewhat necessary to apply other identification methods (Fritze,

2004). Thus, the nonribosomal peptide synthetase (NRPS) genes were analyzed so as to characterize the endophytic strains due to their high frequency as antibiotic compounds and surfactant activities produced by *Bacillus* (Romero et al., 2007).

Moreover, NRPS molecular analysis helps us to understand the antagonistic mechanism of endophytic strains (Ongena and Jacques, 2008). The seven *Bacillus* sp., strains selected were found to have sequences related to the genes responsible for producing fengycin, iturin, bacillomycin D, bacilysin, mycosubtilin and subtilisin antimicrobial peptides (Table 2). It has been reported that *Bacillus* sp., may produce a variety of antimicrobial peptides under complex regulation influenced by environmental conditions and the presence of competing organisms (Perez et al., 2017; Benitez et al., 2011).

The analyses carried out on the endophytic strains suggest their ability to produce extracellular compounds with antifungal activity. The potential of *Bacillus* sp., to produce a wide variety of metabolites with antibacterial and antifungal activities has been described elsewhere (Shoda, 2000). Some of these bacteria (*B. subtilis, B. pumilus, B. amyloliquefaciens* and *B. licheniformis*) have been commercialized and applied to the control of crop diseases (Haas and Défago, 2005). *Bacillus* genus is an impressive producer of lipopeptides, which are found in microbial surfactants. In view of this, our study demonstrates that the seven DS strains had the capacity to produce biosurfactants due to a decrease in the surface activity of water (72 mN m^{-1} to 30 mN m^{-1}). The antifungal activity shown by the BCE tested, suggested that different antimicrobial molecules could be produced, including lipopeptides. The genus *Bacillus* is well known for producing cyclic lipopeptide families with biosurfactants properties such as surfactins, iturins, fengycins and kurstakins (Jacques, 2011). Biosurfactants have been reported to exhibit antibiotic, fungicide, insecticide, antiviral and antitumor activities. Biosurfactant lipopeptides produced by *B. subtilis* such as bacillomycin (Besson et al., 1977), iturin A (Yu et al., 2002), mycosubtilin (Peypoux et al., 1986), lichenysin (Grangemard et al., 1999), and fengycin (Kim et al., 2004) have been used as ecological alternatives to synthetic agrochemicals. Plaza et al., (2013) reported that a strain of *B. subtilis* growing on molasses produced surfactin, a biosurfactant with antifungal activity against *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Colletotrichum gloeosporioides*, and *Phoma complanata*, achieved more than 40 % of growth inhibition. Other authors have also studied the antimicrobial potential of biosurfactants produced by *B. subtilis* (Ghribi et al., 2012; Melnick et al., 2011); they reported that the evaluated strain was able to suppress the growth of *Penicillium, Aspergillus, Rhizopus, Alternaria, Puccinia, Peronospora, Candida, Staphylococcus, Enterococcus* and *Klebsiella*. Additionally, Mnif et al. (2015) reported that the lipopeptide mixture exhibited strong antifungal activity against *Rhizoctonia solani* and *Rhizoctonia bataticola* with an MIC of 4 mg mL^{-1} and 0.04 mg mL^{-1}, respectively. Toral et al. (2018) explored the high

antifungal activity against *B. cinerea* of a patented strain of *Bacillus* that produces lipopeptides; these compounds are thought to trigger antioxidant activity in fruit. It is evident that the information published about lipopeptides and their effects against phytopathogens considers them as a mixture of molecules. Therefore, the production of various lipopeptides by a single strain could be advantageous as a synergistic effect may result. Simultaneous production of certain lipopeptides in many *B. subtilis* strains (Tsuge et al., 2005) such as iturin A and surfactin (Ahimou et al., 2000) has also been reported. However, the co-production of three or more lipopeptide antibiotics is unusual (Perez et al., 2017). Iturins and fengycins are mainly catalogued as antifungal lipopeptides. Thus, the presence of these genes in the *Bacillus* sp., DS strains can explain the potent antifungal activity shown in the dual-culture assays.

Furthermore, lipopeptides from the families of fengycins and surfactins can act as inducers of a systemic resistance response through stimulation of the plant cell's immune response (Ongena and Jacques, 2008). This phenomenon has been demonstrated in other edible cultivars such as beans and tomatos. As mentioned above, the cocoa trees from where the *Bacillus* strains were collected did not show disease symptoms, even though they were collected from a farm with high incidences of *M. roreri* and *M. perniciosa* infection. The lack of Witches's broom and frosty pod rot symptoms on these trees could be explained by the induction of systemic resistance caused by the presence of the *Bacillus* sp., DS endophytic strains.

In order to analyse the antifungal mechanism of the BCE, *M. roreri* and *M. perniciosa,* two microorganisms particularly problematic for the cocoa industry in Ecuador, were selected. The degree of metabolic activity is one of the parameters used to evaluate an antimicrobial compound's effect: the lower the metabolic activity, the greater the chance of surviving the exposure to an antimicrobial agent (Coronel-León et al., 2016 b). With this in mind, the cellular effect of exposure to BCE was assessed. The cocoa endophytic strain DS03 showed the highest antifungal activity; this strain caused both phytopathogens' swelling hyphae due to biosurfactant reaction over the fungus cell wall. In connection with this, Coronel-León et al. (2016c) mentioned that the mechanism of action lipopeptides from *Bacillus* was based on osmotic perturbation, including ion-pore formation; according to this information, results suggest that liquid accumulated in the periphery of the hyphae due to increased permeability of the cell membrane (Figure 6). These results are consistent with those published by Mnif et al. (2015) who described the loss of the granulation and fragmentation of hyphal mycelia of the pathogenic fungi *Ralstonia bataticola* when treated with antifungal metabolites produced by *B. subtilis* SPB1.

This study demonstrated that the endophytic strain DS03 exhibited strong antifungal activity against the cocoa phytopathogens *M. roreri* and *M. perniciosa.* These results are important as they provide the basis for future studies where the antifungal activity of the biosurfactants will be tested under greenhouse conditions to corroborate

the *in vitro* results. In addition, it is essential to note that in this study we used the crude extract biosurfactants which can be considered an economical alternative to be applied in agricultural fields and result in the reduction in phytopathogen incidences.

Conclusion

The endophytic strain isolated from healthy cocoa leaves, named isolate DS03, exhibited strong antifungal activity under in vitro conditions against the cocoa phytopathogens *M. roreri* and *M. perniciosa*. Scientific evidence has been provided to demonstrate that biosurfactant crude extract inhibits the growth of these pathogens. Thus, this strain may become a potential biological control agent that might reduce the incidence of frosty pod and Witches′ broom disease in cocoa farms.

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Authors' Contributions

Conceptualization: Coronel–León, J.R. **Data acquisition:** Serrano, L.; Sosa Moreno, A; Sosa Del Castillo, D. **Data analysis:** Serrano, L.; Sosa Moreno, A.; Bonilla, J.; Coronel–León, J.R. **Design of methodology:** Serrano, L.; Sosa Del Castillo, D.; Coronel–León, J.R. **Writing and editing:** Serrano, L.; Sosa Moreno, A.; Sosa Del Castillo, D.; Bonilla, J.; Romero, C.A.; Galarza, L.L.; Coronel–León, J.R.

References

Bateman, R.P.; Hidalgo, E.; García, J.; Arroyo, C.; Ten Hoopen, G.M.; Adonijah, V.; Krauss, U. 2005. Application of chemical and biological agents for the management of frosty pod rot (*Moniliophthora roreri*) in Costa Rican cocoa (*Theobroma cacao*). Annals of Applied Biology 147: 129-138. https://doi.org/10.1111/j.1744-7348.2005.00012.x

Ben–Jacob, E.; Schochet, O.; Tenenbaum, A. Cohen, I.; Czirók, A.; Vicsek, T. 1994. Generic modelling of cooperative growth patterns in bacterial colonies. Nature 368: 46-49. https://doi.org/10.1038/368046a0

Benitez, L.B.; Caumo, K.; Brandelli, A.; Rott, M.B. 2011. Bacteriocin–like substance from *Bacillus amyloliquefaciens* shows remarkable inhibition of Acanthamoeba polyphaga. Parasitology Research 108: 687-691.

Besson, F.; Peypoux, F.; Michel, G.; Delcambe, L. 1977. Structure of bacillomycin L, an antibiotic from *Bacillus subtilis*. European Journal of Biochemistry 77: 61-67.

Coronel–León, J.; de Grau, G.; Grau–Campistany, A.; Farfan, M.; Rabanal, F.; Manresa, A.; Marqués, A. 2015. Biosurfactant production by AL 1.1, a Bacillus licheniformis strain isolated from Antarctica: production, chemical characterization and properties. Annals of Microbiology 65: 2065-2078.

Coronel–León, J.; Marqués, A.; Bastida, J.; Manresa, A. 2016a. Optimizing the production of the biosurfactant lichenysin and its application in biofilm control. Journal of Applied Microbiology 120: 99–111

Coronel–León, J.; López, A.; Espuny, M.J.; Beltran, M.T.; Molinos–Gómez, A. Rocabayera, X. Manresa, A. 2016b. Assessment of antimicrobial activity of Nα -lauroyl arginate ethylester (LAE®) against Yersinia enterocolitica and Lactobacillus plantarum by flow cytometry and transmission electron microscopy. Food Control 63: 1-10.

Coronel–León, J.; Aranda, F.J.; Teruel, J.A. Marqués, A.; Manresa, Á.; Ortiz, A. 2016c. Kinetic and Structural Aspects of the permeabilization of biological and model membranes by Lichenysin. Langmuir 32: 78-87.

Evans, H. 1981. Pod rot of cacao caused by *Moniliophthora (Monilia) roreri*. Phytopathological Papers 24: 1-44.

Evans, H.C. 2016. Witches′ broom disease (*Moniliophthora perniciosa*): history and biology. p. 137-177. In: Bailey, B.; Meinhardt, L., eds. Cacao diseases: a history of old enemies and new encounters. Springer, Cham, Switzerland.

Fan, B.; Blom, J.; Klenk, H.P.; Borriss, R. 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "Operational Group *B. amyloliquefaciens*" within the *B. subtilis* species complex. Frontiers in Microbiology 8: 22. https://doi.org/10.3389/fmicb.2017.00022

Di Franco, C.; Beccari, E.; Santini, T.; Pisaneschi, G.; Tecce, G. 2002. Colony shape as a genetic trait in the pattern–forming *Bacillus mycoides*. BMC Microbiology. 2: 33. https://doi.org/10.1186/1471-2180-2-33

Fritze, D. 2004. Taxonomy of the genus *Bacillus* and related genera: the aerobic endospore–forming bacteria. Phytopathology 94: 1245-1248. https://doi.org/10.1094/PHYTO.2004.94.11.1245

Ghribi, D.; Abdelkefi–Mesrati, L.; Mnif, I, Kammoun, R.; Ayadi, I.; Saadaoui, I.; Maktouf, S.; Chaabouni–Ellouze, S. 2012. Investigation of antimicrobial activity and statistical optimization of *B. subtilis* SPB1 biosurfactant production in solid–state fermentation. Journal of Biomedicine and Biotechnology 2012: 373-682. https://doi.org/10.1155/2012/373682

Grangemard, I.; Bonmatin, J.; Bernillon, J.; Das, B.; Peypoux, F. 1999. Lichenysin G. A novel family of lipopeptide biosurfactants from *Bacillus licheniformis* IM 1307: production, isolation and structural evaluation by NMR and mass spectrometry. The Journal of Antibiotics 52: 363-373.

Haas, D.; Défago, G. 2005. Biological control of soil–borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3: 307-319. https://doi.org/10.1038/nrmicro1129

Imhof, A.; Balajee, S.; Mar, K. 2003. New methods to assess susceptibilities of *Aspergillus* isolates to Caspofungin. Journal of Clinical Microbiology 41: 5683-5688.

Jacques, P. 2011. Surfactin and other lipopeptides from *Bacillus spp.* p. 57-91. In: Soberon–Chavez, G., ed. Biosurfactants: from genes to applications. Springer, Berlin Germany.

Kim, P.; Bai, H.; Bai, D.; Chae, H.; Chung, S.; Kim, Y.; Park, R.; Chi, Y. 2004. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. Journal of Applied Microbiology 97: 942-949.

Le Mire, G.; Nguyen, M.L.; Fassotte, B.; du Jardin, P.; Verheggen, F.; Delaplace, P.; Jijakli, M.H. 2016. Review: Implementing plant biostimulants and biocontrol strategies in the agroecological management of cultivated ecosystems. Biotechnology, Agronomy, Society and Environment 20: 299-313.

López–Morales, D.; Salaya–Domínguez, J.M. 2017. Papel de *Trichoderma* en los sistemas agroforestales–cacaotal como un agente antagónico (Paper on Trichoderma in agroforestra-cacaotal systems as an antagonistic agent). Tropical and Subtropical Agroecosystems 20: 91-100.

McElroy, M.S.; Navarro, A.J.; Mustiga, G.; Stack, C.; Gezan, S.; Peña, G.; Sarabia, W.; Saquicela, D.; Sotomayor, I.; Douglas, G. M.; Migicovsky, Z.; Amores, F.; Tarqui, O. Myles, S.; Motamayor, J. C. 2018. Prediction of cacao (*Theobroma cacao*) resistance to *Moniliophthora* spp. diseases via genome–wide association analysis and genomic selection. Frontiers in Plant Science 9: 1-12. https://doi.org/10.3389/fpls.2018.00343

Mejía, L.C.; Rojas, E.I.; Maynard, Z.; Bael, S.V.; Arnold, A.E.; Hebbar, P.; Samuels, G. J.; Robbins, N.; Herre, E.A. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biological Control 46: 4-14. https://doi.org/10.1016/J.BIOCONTROL.2008.01.012

Melnick, R.L.; Suárez, C.; Bailey, B.A.; Backman, P.A. 2011. Isolation of endophytic endospore–forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. Biological Control 57: 236-245. https://doi.org/10.1016/j.biocontrol.2011.03.005

Mnif, I.; Grau–Campistany, A.; Coronel–León, J.; Hammami, I.; Ali, M.; Manresa, A. Ghribi, D. 2015. Purification and identification of Bacillus subtilis SPB1 lipopeptide biosurfactant exhibiting antifungal activity against Rhizoctonia bataticola and Rhizoctonia solani. Environmental Science and Pollution Research 23: 6690-6699. https://doi.org/10.1007/s11356-015-5826-3

Ongena, M.; Jacques, P. 2007. *Bacillus lipopeptides*: versatile weapons for plant disease biocontrol. Trends in Microbiology 16: 115-125.

Perez, K.J.; Santos, J.; Lopes, F.C.; Pereira, J.Q.; Santos, D.M.; Oliveira, J.S.; Velho, R. V.; Crispim, S.M.; Nicoli, J.R.; Brandelli, A.; Nardi, R.M.D. 2017. *Bacillus* spp. isolated from puba as a source of biosurfactants and antimicrobial lipopeptides. Frontiers in Microbiology 8: 61. https://doi.org/10.3389/fmicb.2017.00061

Peypoux, F.; Pommier, M.; Marion, D.; Ptak, M.; Das, B.; Griffin, W. 1986. Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. The Journal of Antibiotics 39: 636-641.

Plaza, G.; Turek, A.; Krol, E.; Szczygloska, R. 2013. Antifungal and antibacterial properties of surfactant isolated from *Bacillus subtilis* growing on molasses. African Journal of Microbiology Research 7: 3165-3170.

Ploetz, R. 2016. The impact of diseases on cacao production: a global overview. p. 33-59. In: Bailey, B.; Meinhardt, L., (eds.). Cacao diseases: a history of old enemies and new encounters. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-319-24789-2

Pruesse, E.; Peplies, J.; Glockner, F. O. 2012. SINA: accurate high–throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics 28: 1823-1829. https://doi.org/10.1093/bioinformatics/bts252.

Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web–based tools. Nucleic Acids Research 41: D590-D596.

Romero, D.; Vicente, A.; Rakotoaly, R.; Dufour, S.; Veening, J.; Arrebola, E.; Cazorla, F.; Kuipers, O.; Paquot, M.; Pérez–García, A. 2007. The Iturin and Fengycin families of Lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Molecular Plant–Microbe Interactions 20: 430-40.

Rudner, R.; Martsinkevich, O.; Leung, W.; Jarvis E. D. 1998. Classification and genetic characterization of pattern-forming Bacilli. Molecular Microbiology 27: 687-703. https://doi.org/10.1046/j.1365-2958.1998.00717.x

Salhi, B.; Mendelson, N.H. 1993. Patterns of gene expression in Bacillus subtilis colonies. Journal of bacteriology 175: 5000-5008. https://doi.org/10.1128/jb.175.16.5000-5008.1993

Shapiro, J. 1998. Thinking about bacterial populations as multicellular organisms. Annual Review of Microbiology 52: 81-104. https://doi.org/10.1146/annurev.micro.52.1.81

Shemesh, M.; Chai, Y. 2013. A combination of glycerol and manganese promotes biofilm formation in *Bacillus subtilis* via histidine kinase KinD signaling. Journal of Bacteriology 19: 2747-2754. https://doi.org/10.1128/JB.00028-13

Shoda, M. 2000. Bacterial control of plant diseases. Journal of Bioscience and Bioengineering 89: 515-521. https://doi.org/10.1016/S1389-1723(00)80049-3

Silva, H.S.A.; Tozzi, J.P.L.; Terrasan, C.R.F.; Bettiol, W. 2012. Endophytic microorganisms from coffee tissues as plant growth promoters and biocontrol agents of coffee leaf rust. Biological Control 63: 62–67. https://doi.org/10.1016/j.biocontrol.2012.06.005

Toral, L.; Rodríguez, M.; Béjar, V.; Sampedro, I. 2018. Antifungal activity of Lipopeptides from *Bacillus* XT1 CECT 8661 against Botrytis cinerea. Frontiers in Microbiology 9: 1315. https://doi.org/10.3389/fmicb.2018.01315

Tsuge, K.; Inoue, S.; Ano, T.; Itaya, M.; Shoda, M. 2005. Horizontal transfer of iturin A operon, itu, to *Bacillus subtilis* 168 and conversion into an iturin A producer. Antimicrobial Agents and Chemotherapy 49: 4641-4648. https://doi.org/10.1128/AAC.49.11.4641-4648.2005

Vega, H.; Beillard, M.J. 2015. Ecuador cocoa update and outlook. USDA, Washington, DC, USA. Available at: https://www.fas.usda.gov/data/ecuador-ecuador-cocoa-update-and-outlook. (Report, EC15002) [Accessed Oct 10, 2019]

Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52: 487-511. https://doi.org/10.1093/jexbot/52.suppl_1.487

Wickramasuriya, A.; Dunwell, J. 2018. Cacao biotechnology: current status and future prospects. Plant Biotechnology Journal 16: 4-17. https://doi.org/10.1111/pbi.12848

Yu, G.; Sinclair, J.; Hartman, G.; Bertagnolli, B. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry 34: 955-963. https://doi.org/10.1016/S0038-0717(02)00027-5