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# *In vitro* effect of cocoa leachates on growth and development of *Moniliophthora roreri* ([Cif.] H.C. Evans *et al*.) isolated from *Theobroma cacao* (L.)

# Evaluación *in vitro* de lixiviados de cacao sobre el crecimiento y desarrollo de *Moniliophthora roreri* ([Cif.] H.C. Evans *et al*.) aislado de *Theobroma cacao* (L.)

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***Theobroma cacao* (L.) affected by *Moniliophthora roreri* in a commercial farm, Yacopi, Cundinamarca (Colombia).**

Photo: C.R. Beltrán-Acosta


## ABSTRACT

The cocoa crop is part of the peasant's economic activity and agroforestry systems in many regions of Colombia and the world. Its yields are diminished by phytosanitary problems, being the frosty pod rot caused by the fungus *Moniliophthora roreri,* the most limiting disease with decreases in production of over 90%. In this study, samples of cocoa leachates from commercial producing farms were characterized and the antifungal activity of sterilized, non-sterilized, and filtered cocoa leachates against *M. roreri* was evaluated under *in vitro* conditions, at concentrations of 1, 2, 5, 10, and 15%. Monosporic isolates of the pathogen were obtained from diseased fruits collected from cocoa-producing farms in the municipality of Yacopi in Cundinamarca, Colombia. The effect of leachates on *M. roreri* conidial germination, mycelial growth, and inoculum production was evaluated. It was found that non-sterilized cocoa leachates at all concentrations caused 100% reduction on the three variables evaluated, while sterilized leachates generated the same effect at concentrations of 10 and 15%. Filtered leachates showed no inhibitory effect on the radial growth of the pathogen, but they caused a reduction of conidial germination of 89.9, 90.5 and 95.9% at concentrations of 5, 10, and 15%. The presence of malic, citric, and ascorbic acids, compounds such as $NH_4$, $PO_4$, and $NO_3$, and elements such as Ca, K, Na, Mg, and Cl in the evaluated leachates was identified using high-efficiency liquid chromatography and physicochemical analysis. This research showed the potential of cocoa leachates, even at low concentrations,



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for the control of *M. roreri*, which can be attributed to the action of specific compounds such as those found in chromatographic tests.

**Additional keywords:** fungal mycelial growth; conidia germination; frosty pod rot disease; liquid products.


## RESUMEN

El cultivo de cacao es parte de la actividad económica campesina y de sistemas de agroforestería en muchas regiones de Colombia y del mundo. Su rendimiento se ve disminuido por problemas fitosanitarios, siendo la monilia causada por el hongo *Moniliophthora roreri,* la enfermedad más limitante causando disminuciones de producción de más del 90%. En este estudio, se caracterizaron muestras de lixiviados de cacao de fincas comerciales y la actividad antifúngica de estos lixiviados esterilizados, no esterilizados, y filtrados contra *M. roreri* fue evaluada bajo condiciones *in vitro* en concentraciones de 1, 2, 5, 10, y 15%. Aislamientos monospóricos del patógeno fueron obtenidos a partir de frutos enfermos colectados en fincas productoras de cacao del municipio de Yacopí en Cundinamarca, Colombia. El efecto de los lixiviados se evaluó sobre la germinación conidial de *M. roreri*, el crecimiento micelial, y la producción de inóculo. Se encontró que los lixiviados de cacao no esterilizados en todas las concentraciones generaron 100% de reducción de las tres variables evaluadas, mientras que los lixiviados esterilizados generaron el mismo efecto a concentraciones de 10 y 15%. Los lixiviados filtrados no mostraron efecto inhibitorio sobre el crecimiento radial del patógeno, pero presentaron una reducción de la germinación conidial de 89,9; 90,5 y 95,9% a las concentraciones de 5, 10, y 15%, respectivamente. La presencia de ácido málico, cítrico, y ascórbico, compuestos como $NH_4$, $PO_4$, y $NO_3$, y elementos como Ca, K, Na, Mg, y Cl fue identificada en los lixiviados evaluados usando cromatografía líquida de alta eficiencia y análisis fisicoquímicos. Esta investigación mostró el potencial de los lixiviados de cacao, aún a bajas concentraciones para el control de *M. roreri*, el cual puede ser atribuido a la acción de compuestos específicos como los encontrados en las evaluaciones cromatográficas.


**Palabras clave adicionales:** crecimiento micelial; germinación conidial; monilia; productos líquidos.

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## INTRODUCTION

The cocoa (*Theobroma cacao* L.) is a tropical species from which chocolate and its derivatives are obtained (Lozada *et al*., 2012). It is cultivated by 6 million farmers and is the source of income for more than 40 million people around the world (Fromm, 2016). The International Cocoa Organization (ICCO) estimated that more than 4.697 million metric tons of cocoa beans were produced globally between 2019 and 2020. According to this report, Africa was the first largest cocoa-producing continent with 75.7% (3.55 million tons), followed by the Americas with 18.4%, and Asia and Oceania with 5.9% (ICCO, 2020). According to Fedecacao (National Federation of Cocoa Producers), Colombia registered a production of 63,416 t for 2020, showing an increase of 6.1% compared to the production in 2019 (Fedecacao, 2021).

The cocoa crop is affected by limiting diseases, among which are the black pod caused by the oomycete *Phytophthora* spp., witches' broom disease (*Moniliophthora perniciosa* (Stahel) Aime and Phillips-Mora), and frosty pod rot (*Moniliophthora roreri* (Cif.) H.C. Evans *et al*., 1978) that can cause significant yield losses (Jaimes *et al*., 2016; Pérez-Vicente, 2018). Frosty pod rot is the most economically limiting disease in all the Latin American countries where cocoa is cultivated (Phillips-Mora *et al*., 2005; Sánchez-Mora *et al*., 2014). The center of origin of the disease is central/northeastern Colombia where the highest levels of genetic diversity of the pathogen have been found (Phillips-Mora *et al*., 2007b). *M. roreri* has progressively expanded from its center of origin in South America northwards through Central America and Mexico

and westwards across the Andes and Amazonian forests (Phillips-Mora *et al.*, 2007b). In Colombia, frosty pod rot has generated losses of over 90% (Phillips-Mora *et al*., 2007a; Jaimes and Aranzazu, 2010; Correa *et al*., 2014).

The pathogen only attacks the fruits at any stage of development causing external and internal damage and total pod loss (Jaimes *et al*., 2016). Pods of less than 2 months old are the more susceptible (Sánchez and Garcés, 2012). Initial infections are symptomless, except for tissue swelling causing deformations or bumps (Bailey *et al*., 2018). Subsequently, a brownish spots appears on the fruit's surface, on which millions of conidia develop, forming a cream-colored powder. These conidia are dispersed by the wind and when they fall on a healthy fruit, under appropriate conditions, they germinate penetrating the cuticle or stomata. The structures colonize tissues intercellularly invading the cortical parenchyma and starting the disease cycle again (Evans *et al*., 2003; Ramírez, 2013; Correa *et al*., 2014; Bailey *et al*., 2018).

Frosty pod rot management consists of sanitary pruning of fruits, planting of improved material, and biological control practices (Krauss *et al*., 2010; Suárez and Rangel, 2013). The application of systemic fungicides has shown low efficacy and increases production costs (Flood and Murphy, 2004; Bateman *et al*., 2005). Therefore, it is necessary to look for new alternatives to manage the disease. Liquid products of the decomposition of harvest residues, known as leachates (Weltzien, 1991; Bele *et al*., 2018) have been reported with the potential to control some plant diseases. The use of banana rachis leachates to control black leaf streak disease (Osorio *et al*., 2012) has shown the ability to inhibit mycelial growth and germination of *Botryodiplodia theobromae*, *Colletotrichum musae*, *Fusarium* sp., and *Musicillium theobromae* conidia, pathogens that cause postharvest diseases in banana (*Musa acuminata* Colla) in Ivory Coast (Bele *et al*., 2018). This last study showed that leachate concentrations of 5, 15, and 20% exhibited an antifungal activity, with the 20% concentration being the most effective and the one that obtained complete inhibition of mycelial growth and conidial germination of all the fungi evaluated (Bele *et al*., 2018). The *in vitro* and *in vivo* effects of banana rachis leachates have also been demonstrated for the control of southern blight in tomatoes caused by *Sclerotium rolfsii* in Porto-Novo, Benin (Africa). In that study, leachates inhibited mycelial growth and pathogen development in the field with effectiveness similar to that found for the fungicide Maneb 80 (Sikirou *et al*., 2010).

Kamel *et al*. (2014) report the use of fulvic acid extracted from banana rachis to control powdery mildew (*Pohosphaera fusca* (syn. *Sphaerotheca fuliginea*)) and downy mildew (*Pseudoperonospora cubensis*) in cucumber at the Agricultural Research station in Sakha, Egypt. Álvarez *et al*. (2015) mention that leachates contain a high concentration of potassium that induces resistance to some diseases. These authors also state that applications of 5% fulvic acids from banana leachates reduce the severity of powdery mildew in roses caused by *Sphaerotheca pannosa*. There are no reports of the use of cocoa leachates for the control of *M. roreri,* although vegetable extracts of grapefruit, pepper, marjoram, and linden have been evaluated for their *in vitro* control. All these extracts without sterilization and at a concentration of 25% generated a 100% inhibition of mycelial growth (Arcos-Méndez *et al*., 2019). In Colombia, leachates from banana rachis have been assessed *in vitro* and *in vivo* against *Mycosphaerella fijiensis* and *in vivo* for *S. pannosa* in rose crops (Álvarez *et al*., 2010; Álvarez *et al*., 2015). Nevertheless, its use by farmers is still empirical and some leachates have been mainly used as biofertilizers or biofungicides. Cocoa leachates are obtained from the bean fermentation process. The mucilage that covers the seeds is subjected to a fermentation process by the action of microorganisms, forming sugars and then acetic acid. During the first 24 h of this process, cocoa beans leach substances or leachates composed of amino acids, sugars, and other substances (Wacher, 2011). This study sought to (i) characterize cocoa leachate samples from commercial cocoa producing farms and (ii) evaluate, under *in vitro* conditions, the potential antifungal activity of cocoa leachates against *M. roreri* at variable concentrations as an alternative for disease management with low impact on the production system, to provide knowledge on the potential use of these leachates.

## MATERIALS AND METHODS

### Characterization of cocoa leachates from two commercial producing farms in Cundinamarca

#### *Cocoa leachates production and collection*

The leachates obtained from the fermentation of cocoa beans were collected in sterile glass containers

24 h after the beginning of the fermentation process and kept refrigerated at 6°C. This period was selected due to the fact that the highest amount of leachates was produced at this time. To conduct the study, three samples (S) of leachates were obtained from two farms located at the municipality of Yacopi (Cundinamarca, Colombia). Leachates were obtained from the farms La Floresta (5° 31' 30.3'' N, 74° 20' 40.39'' W) (S1 and S3), and Filadelfia (5° 29' 18.3'' N, 74° 20' 3.7'' W) (S2). These farms were selected due to the fact that both were planted with cocoa clones CCN51, ICS95 TSH565, ICS60, EET8, YAC2, and farmers followed a similar fermentation procedure to obtain the cacao leachates.

### *Physicochemical and chromatographic analysis of cocoa leachates*

To determine the compounds that are present in the three leachate samples and their concentration, the physicochemical analysis of cocoa leachates was carried out at the Water and Soils Laboratory of the Faculty of Agricultural Sciences at Universidad Nacional de Colombia. The following variables were evaluated: pH, using the potentiometric method (Tello and Fernández, 2012); OH, $CaCO_3$, and $HCO_3$ by titration with $H_2SO_4$ (Roldán and Ramírez, 2008); chlorides by titration with $AgNO_3$ (Carrasquero and Castillo, 2002); sulfates using barium chloride by turbidimetric titration (Aguilera *et al*., 2010); phosphates and ammonium by colorimetric titration (D'Angelo *et al*., 2001); Ca, Mg, K and Na using atomic absorption spectrometry (Pohl *et al*., 2012), and B by potentiometric titration (Peña *et al*., 2012). The electrical conductivity (EC) was measured by reading with a conductivity meter (Schott, Lab960 and electrode of the same brand) at 25°C and the sodium adsorption ratio (SAR) that consists of the ratio between Na and Ca plus Mg (Peña *et al*., 2012).

The analysis by high-efficiency liquid chromatography was carried out at the Postharvest Laboratory of the Faculty of Agricultural Sciences at Universidad Nacional de Colombia. The main sugars and organic acids in the compound were determined following the methodology of Zheng *et al*. (2016). The samples were centrifuged (3,000 rpm, 5 min) to eliminate impurities and the supernatant was stored at 4°C. Then, 0.5 mL of the leachate sample was taken and completed to a volume of 5 mL with $H_2SO_4$ 1N solution. This solution was subjected to analysis on an Ultimate 3000 Dionex HPLC chromatograph (Zheng *et al.*, 2016).

## *In vitro* evaluation of the antifungal activity of cocoa leachates against *Moniliophthora roreri* at variable concentrations

### *Collection of affected plant material and obtaining of* M. roreri *isolates*

Fruits with symptoms associated with frosty pod rot were collected in the farms Filadelfia (5° 29' 18.3'' N, 74° 20' 3.7'' W) and El Porvenir (5° 31' 2.6'' N, 74° 20' 40.2'' W) located in the municipality of Yacopi, province of Rionegro, at the north of the department of Cundinamarca (Colombia). The conditions of these farms were as follows: an average temperature of 21.1°C (minimum of 18.8°C and maximum of 29°C), average annual rainfall of 2,592 mm, and relative humidity between 81 and 90% (Pinzón *et al*., 2012; Penagos, 2019). In each farm, 15 trees were randomly selected to collect fruits with the presence of the pathogen. These fruits were processed at the Plant Health Laboratory of the Faculty of Agricultural Sciences at Universidad Nacional de Colombia, Bogotá campus.

Five mm explants were taken from the diseased fruits collected, making deep cuts of the affected tissue at the lesion site. Explants were disinfected using 70% alcohol for 1 min, 2% sodium hypochlorite for 30 s, followed by three washes with sterile distilled water (Barrera and García, 2008). Then, they were seeded in Petri dishes with Potato Dextrose Agar (PDA) medium (Oxoid, Thermo Scientific, USA). The dishes were incubated for 14 d at 25°C until sporulation. Isolates of the pathogen were confirmed by macroscopic and microscopic characterization (Phillips-Mora *et al*., 2006a; Phillips-Mora *et al*., 2006b). Finally, monosporic cultures were carried out following the methodology described by Carrera-Sánchez *et al*. (2014).

Isolates were then cultured on PDA medium (Oxoid, Thermo Scientific, USA), and the diameter of the colony and sporulation were measured in eight isolates obtained (data not shown) for 14 d. The isolate with the highest rate of mycelial growth in time (mm $d^{-1}$) and the highest conidia production per $cm^2$ of the colony was selected.

### *Preparation of culture medium with cocoa leachates for* in vitro *evaluations*

Cocoa leachates were centrifuged at 3,000 rpm for 5 min to remove impurities (Sorvall Legend X1,

Thermo Scientific, USA). Subsequently, they were added to sterile PDA medium (120°C, 20 min) before solidifying, at concentrations of 1, 2, 5, 10, and 15% v/v in relation to the culture medium, following the agar diffusion method (Chang *et al*., 2008). Petri dishes with PDA and the growing pathogen were used as controls.

On a second evaluation, a PDA medium was prepared, and the cocoa leachates were added at the same concentrations mentioned above, before the sterilization process. The mixture of culture medium and cocoa leachates was then subjected to autoclave sterilization at 120°C for 45 min (Automatic electromechanical autoclave, Aravell, Colombia). Petri dishes with PDA and the growing pathogen were used as controls.

The third method evaluated consisted in filtering the leachates using sterile 0.20 $\mu$m filters (Millipore) after centrifugation (3,000 rpm, 5 min). The filtered leachates were added to the culture medium previously sterilized in an autoclave. The treatments were the same as those of the previous evaluations. Petri dishes with PDA and non-sterilized leachates and Petri dishes with PDA, both with the growing pathogen, were used as controls.

### *Evaluations of antifungal activity of cocoa leachates on* M. roreri

The antifungal activity of cocoa leachates to inhibit the radial growth of the fungus was determined by macroscopic observation, placing an agar/*M. roreri* mycelium disc (5 mm radius) from monosporic cultures of the 18-d-old pathogen in each of the Petri dishes with cocoa leachates at the evaluated concentrations according to the treatment (Chuah *et al*., 2010) and controls. Radial growth was determined by measuring the diameter (mm) of colonies for 14 d (time during which the pathogen in the control treatment completed growth in the Petri dishes).

#### Percentage of inhibition of *M. roreri* mycelial growth

The toxicity of cocoa leachates on *M. roreri* was expressed as the percentage of inhibition of mycelial growth (IMG) of *M. roreri*, according to the formula proposed by Yahyazadeh *et al*. (2007), after 14 d of growth on PDA culture medium (1).

$$IMG\ (\%) = [(C-T)/C] \times 100 \quad (1)$$

where, $C$ is the diameter of the pathogen colony in the control treatment without cocoa leachates, and $T$ is the diameter of the pathogen colony according to the cocoa leachate concentrations evaluated.

#### Evaluation of the effect of cocoa leachates on the germination of *M. roreri* conidia

Cocoa leachates were centrifuged at 3,000 rpm for 5 min to remove impurities (Sorvall Legend X1, Thermo Scientific, USA). Subsequently, they were added to a sterile water agar (WA) culture medium (Oxoid, Thermo Scientific, USA) before solidification at concentrations of 1, 2, 5, 10, and 15 v/v in relation to the culture medium.

For the second evaluation method, the leachates were added to the WA medium before sterilization at the same concentrations. Finally, for filtered leachates, the same concentrations mentioned above were added to the WA medium previously sterilized in an autoclave. To evaluate the effect of cocoa leachates on conidial germination, 200 $\mu$L of a $4.5 \cdot 10^4$ conidia/mL suspension of *M. roreri* was seeded on WA with the concentrations of leachates to be evaluated (prepared by the three methods previously described). Conidial germination was determined by microscopic counting (Olympus CX31, Olympus, Japan) every 24 h for 96 h. For this purpose, 100 conidia were evaluated and those conidia that showed a germ tube with twice the diameter of the conidia were considered germinated (Marín and Bustillo, 2002). Germination values were calculated as the ratio between germinated and the total number of evaluated conidia and then expressed as percentage.

### *Evaluation of the effect of cocoa leachates on* M. roreri *inoculum production*

Conidia formed on sterilized and non-sterilized leachate treatments and controls were collected 14 d after incubation by removal with 2 mL of sterile distilled water with Tween 80. The number of conidia produced was determined by quantification in a Hemocytometer (Boeco, Germany) (Petlamul and Prasertsan, 2012). The effect of filtered leachates on inoculum production was not evaluated. Filtration was considered as a complementary analysis to evaluate if the sterilization method may alter leachates effect on mycelial growth and conidia germination of *M. roreri*.

### *Determination of EC50 value for mycelial growth and conidia germination inhibition*

The effective concentration of cocoa leachates to reduce mycelial growth or conidia germination by 50% (EC50) was calculated by fitting the mycelial growth or conidia germination rate against the log transformed cocoa leachate concentrations using the best fitting model (lLL.4) in the R package "drc" v3.0.1 (Ritz *et al.,* 2015).

## Experimental design and statistical analysis

A completely randomized design was used to evaluate the antifungal activity of cocoa leachates. Five concentrations of leachates (1, 2, 5, 10, and 15% v/v) were evaluated for sterilized, non-sterilized and filtered leachates, for a total of 16 treatments including the control treatment (0%), in which no leachates were added to the culture media. Seven replicates were used for each combination of leachate concentration and sterilization method. Each replicate corresponded to one Petri dish with the PDA medium amended with the cocoa leachate concentration to be assessed. Filtered leachates were assessed in five replicates at each concentration due to contamination of two of the initially established replicates. Additional experiments were conducted to validate the obtained results. The free statistical software R 3.6.2 (R Development Core Team, 2019) (PBC, Boston, USA) and the packages dplyr (Wickham *et al.*, 2021), ggplot2 (Wickham, 2009) and car (Fox and Weisberg, 2019) were used for the analysis of radial growth, conidial germination, and inoculum production data. An ANOVA mean comparison analysis and Tukey's test were performed with a probability value of $P \leq 0.05$.

# RESULTS

## Physicochemical and chromatographic analysis of cocoa leachates evaluated on *M. roreri*

The analyses showed an acidic pH lower than 3.7 in the three leachate samples from the farms La Floresta (S1 and S3) and Filadelfia (S2) (Tab. 1); for this parameter, the three leachates showed similar values (3.66, 3.64 and 3.49, respectively).

The concentrations of Cl, K, and Mg were close among the three leachate samples. The SAR also showed similar values for the three leachate samples evaluated (Tab. 1). On the other hand, the $NH_4$ concentration for samples S1 and S3 did not show differences between these two samples. However, a lower $NH_4$ concentration was obtained in sample S2 than in samples S1 and S3 (Tab. 1). This result shows close values of $NH_4$ concentration in the leachates from the farm La Floresta (S1 and S3) in contrast to those obtained in the second farm (Filadelfia, S2). In the case of S, leachate sample S1 showed a higher content (240 mg $L^{-1}$) than that found in samples S2 (74.1 mg $L^{-1}$) and S3 (134 mg $L^{-1}$). Sample Sl obtained a Ca concentration of 232 mg $L^{-1}$ compared to 67.5 mg $L^{-1}$ and 108 mg $L^{-1}$ in samples S2 and S3, respectively (Tab. 1). For this parameter, the higher values were observed in the leachates from the farm La Floresta (S1, S3) in contrast to those obtained from the second farm (Filadelfia, S2).

Simple and compound sugars such as fructose, glucose, and sucrose were found at different concentrations in each of the leachates evaluated. Values for fructose of 3,982, 1,793, and 6,332 mg $L^{-1}$ were determined in the leachates of samples S1, S2, and S3, respectively (Tab. 1). However, the results showed a wide variation in the concentration of this compound between leachates S2 and S3. In the case of glucose, close values were found among the evaluated samples (Tab. 1). Saccharose concentration showed variation between both farms, with close values in the samples S1 and S3 (Floresta) and the lowest values observed in S2 (Filadelfia).

The presence of citric, ascorbic, and malic acids was identified. Citric and malic acids were found at higher concentrations in sample S3. Additionally, the leachates of S2 showed a lower content of citric and ascorbic acids compared to the other two samples evaluated (Tab. 1).

### *Selection of the* M. roreri *isolate*

Monosporic cultures were obtained from each of the eight isolates, and the growth speed of the colonies *in vitro* and inoculum production capacity were evaluated for each of them. Based on this information, one isolate from the farm Filadelfia was selected for its higher growth speed (90 mm diameter of the colony

**Table 1. Presence and quantity of basic compounds, sugars and main acids in the cocoa leachate samples (S1 and S3 - La Floresta, S2 - Filadelfia), expressed as concentration in mg $L^{-1}$ of compound and/or elements obtained by physicochemical analysis of water and liquid compounds.**

| Parameter | Sample 1 (S1) | Sample 2 (S2) | Sample 3 (S3) |
|---|---|---|---|
| Chromatographic analysis | | | |
| Fructose | 3,982 | 1,793 | 6,332 |
| Glucose | 4,246 | 5,318 | 3,831 |
| Saccharose | 2,032 | 157 | 1,234 |
| Citric acid | 91,677 | 10,160 | 132,693 |
| Ascorbic acid | 697 | 143 | 340 |
| Malic acid | 79,783 | 125,208 | 138,014 |
| Physicochemical analysis | | | |
| pH | 3.66 | 3.64 | 3.49 |
| Cl* | 246 | 215 | 273 |
| $SO_4$* | 240 | 74.1 | 134 |
| $PO_4$* | 27.0 | 64.9 | 172 |
| $NO_3$* | 45.5 | 23.7 | 28.8 |
| Ca* | 232 | 67.5 | 108 |
| K* | 1,238 | 1,045 | 1,148 |
| Mg* | 186 | 151 | 132 |
| Na* | 5.43 | 2.41 | 4.29 |
| $NH_4$* | 25.3 | 8.35 | 30.9 |
| EC | 4.3 | 2.9 | 3.4 |
| SAR | 0.06 | 0.04 | 0.07 |

*: measurements in mg $L^{-1}$. EC: Electrical conductivity, SAR: Sodium absorption ratio.
S1 and S3: leachates obtained from the farm La Floresta (5º 31' 30.3'' N, 74º 20' 40.39'' W). S2: leachates obtained from the farm Filadelfia (5º 29' 18.3'' N, 74º 20' 3.7'' W). Both farms are located at Yacopi, Cundinamarca. Colombia.

12 d after incubation) and sporulation capacity ($9{\cdot}10^3$ to $1{\cdot}10^4$ conidia/$cm^2$).

## Effect of cocoa leachates on the *in vitro* growth of *M. roreri*

Non-sterilized leachates showed a 100% inhibitory effect on the radial growth of *M. roreri* ($P < 0.000$) from a concentration of 1 to 15% (Fig. 1A and 1B). These results were consistent with additional experiments conducted with other cocoa leachate samples in which 100% inhibitory effect was obtained (data not shown). The control treatment started mycelial growth from 18 to 24 h after seeding on the medium and continued during the 12 d of incubation, showing typical *M. roreri* colonies with a powdery appearance and beige to brown colors (Phillips-Mora *et al*., 2006a; Phillips-Mora *et al*., 2006b).

In the culture medium prepared with the different dilutions of non-sterilized leachates, the profuse growth of bacterial colonies of similar size was observed, both on the surface of the medium and deep inside. The colonies showed a creamy appearance, individual growth, whitish to cream color without prominent elevation on the culture medium.

Regarding the leachates sterilized by autoclaving, in the 1, 2, and 5% treatments the mycelial growth of *M. roreri* did not show significant differences compared to the control. In these treatments, the colonies completed the total area of the Petri dish at 12 d of incubation (Fig. 1C). However, an alteration in the morphology of the colony was observed with the treatment of 5%. A change in pigment (hue) in the central axis of the colony was registered, which was of clear color and not beige (due to lower sporulation), with a cottony and dense appearance compared

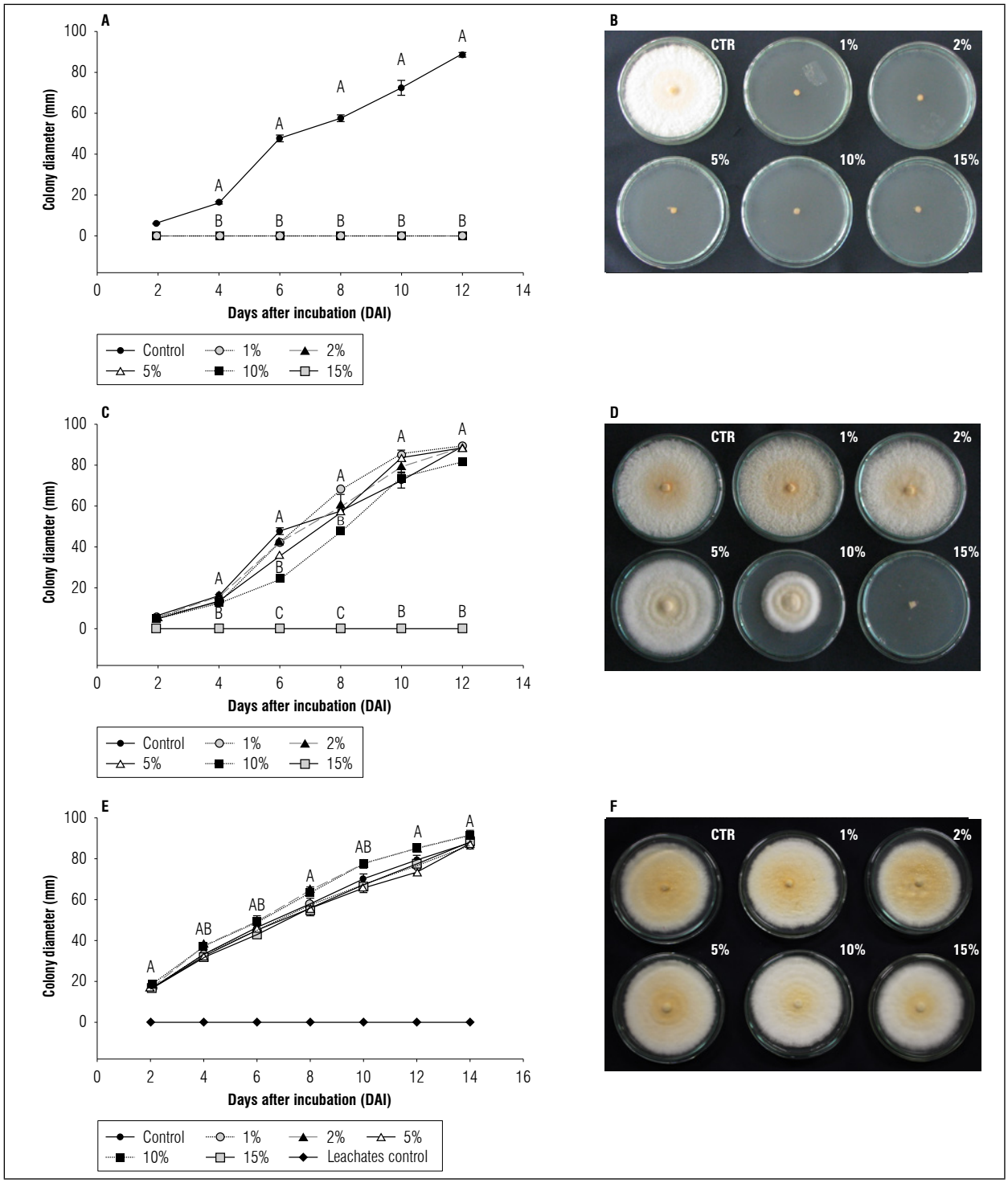


**Figure 1. Radial growth of *M. roreri* under five cocoa leachate dilutions on PDA culture medium incubated at 25 °C at 2, 4, 6, 8, 10, 12 and 14 days after incubation (DAI) and their effect on the appearance of colonies 12 DAI for non-sterilized and sterilized leachates treatments, and 14 DAI for filtered leachates treatment. A. and B. Non-sterilized leacheates. C and D. Sterilized leachates. E and F. Filtered leachates, control (*M. roreri* on PDA) and leachate control (*M. roreri* on PDA with 10% non-sterilized leachates). I). The data represent the mean value of seven Petri dishes ($n=7$) for non-sterilized and sterilized leachates and five Petri dishes ($n=5$) for filtered leachates ± standard error per treatment. Letters refer to significant differences between means according to Tukey's test ($P \leq 0.05$). Control: CTR.**

to the control (Fig. 1D). Significant differences were found for the evaluated concentrations of 10 and 15% compared to the control ($P<0.0001$ and $P<0.0001$, respectively). The sterilized cocoa leachates at a concentration of 10% inhibited about 80% of the radial growth of the *M. roreri* colony (Fig. 1C and 1D) and the concentration of 15% showed a 100% inhibitory effect on the mycelial growth of *M. roreri* (Fig. 1C and 1D). In additional experiments conducted with other cocoa leachate samples, an inhibitory effect of 88% of the radial growth for the concentration of 10%, and an inhibitory effect of 100% for the concentration of 15% were observed (data not shown).

Significant differences were not found in the diameter of the colony between the different concentrations of filtered leachates and the control treatment of the fungus growing on PDA. Significant differences were found in the diameter of the colony between the concentrations of filtered leachates and the treatment of non-sterilized leachates at a concentration of 10% (leachate control) ($P<0.0001$). The obtained result confirmed the inhibitory effect of non-sterilized leachates on the mycelial growth of the fungus (Fig. 1E and 1F).

### *Percentage of inhibition of* M. roreri *mycelial growth*

Non-sterilized leachates were the most effective, with 100% inhibition of the mycelial growth of the pathogen ($P<0.0000$) (Tab. 2). Their inhibitory effect was maintained at all evaluated concentrations and moments of assessment.

Autoclave-sterilized leachates were effective in inhibiting mycelial growth of *M. roreri* only at the 15% dose with a 100% inhibitory effect ($P<0.000$), while the 10% dose showed an inhibition percentage of 8.03% ($P<0.000$). The doses of 1, 2, and 5% did not significantly inhibit the growth of the pathogen ($P>0.975$).

Filter-sterilized leachates were not effective in inhibiting the mycelial growth of *M. roreri* ($P>0.428$) and the doses of 2, 5, 10 and 15% even generated an increase in *M. roreri* growth.

### *Effect of cocoa leachates on the germination of* M. roreri *conidia*

Non-sterilized cocoa leachates inhibited the conidial germination of the pathogen at all the concentrations evaluated ($P<0.000$), while the control showed germination percentages between 6 and 27% (Fig. 2A). These results were consistent with additional experiments conducted with other cocoa leachate samples in which 100% inhibitory effect was obtained on the germination of *M. roreri* conidia (data not shown).

Sterilized cocoa leachates showed a significant effect on conidial germination at concentrations of 10 and 15% ($P<0.001$ and $P<0.000$). At the 10% concentration, conidial germination was between 2 and 12%, while the control showed values between 6% and 37%. At the 15% concentration, conidial germination was less than 2% at 24 h after incubation without germination at 48 h. The lowest concentrations (1, 2, and 5%) did not show differences compared to the control (Fig. 2B). In additional experiments conducted with other cocoa leachate samples, the conidial germination was 15.2% for the concentration of 10%. At the 15% concentration, conidial germination was 0% at 24 h after incubation (data not shown).

On the other hand, filtered cocoa leachates showed an inhibitory effect on conidial germination with a germination percentage between 3 and 10% at concentrations of 5, 10 and 15% from 24 to 96 h after seeding. These results indicate an inhibitory activity, although at a lower proportion than that evidenced with non-sterilized leachates (Fig. 2C).

**Table 2. Percentage of inhibition of *M. roreri* mycelial growth *in vitro* on PDA medium amended with cocoa leachates at five concentrations.**

| Treatment | Concentration | | | | |
|---|---|---|---|---|---|
| | 1% | 2% | 5% | 10% | 15% |
| Sterilized leachates by autoclaving | -0.830±0.1 c | 0.538±1.0 c | 0.202±0.4 c | 8.029±1.4 b | 100±0 a |
| Non-sterilized leachates | 100±0 a | 100±0 a | 100±0 a | 100±0 a | 100±0 a |
| Filtered leachates | 2.667±0.3 a | -0.734±0.9 a | -4.036±0.5 a | -4.671±1.4 a | -0.621±0 a |

The data represent the mean value of seven Petri dishes ($n=7$) for non-sterilized and sterilized leachates and five Petri dishes ($n=5$) for filtered leachates. Letters refer to significant differences between rows according to Tukey's test ($P\leq0.05$). Mean values ± standard errors are presented.

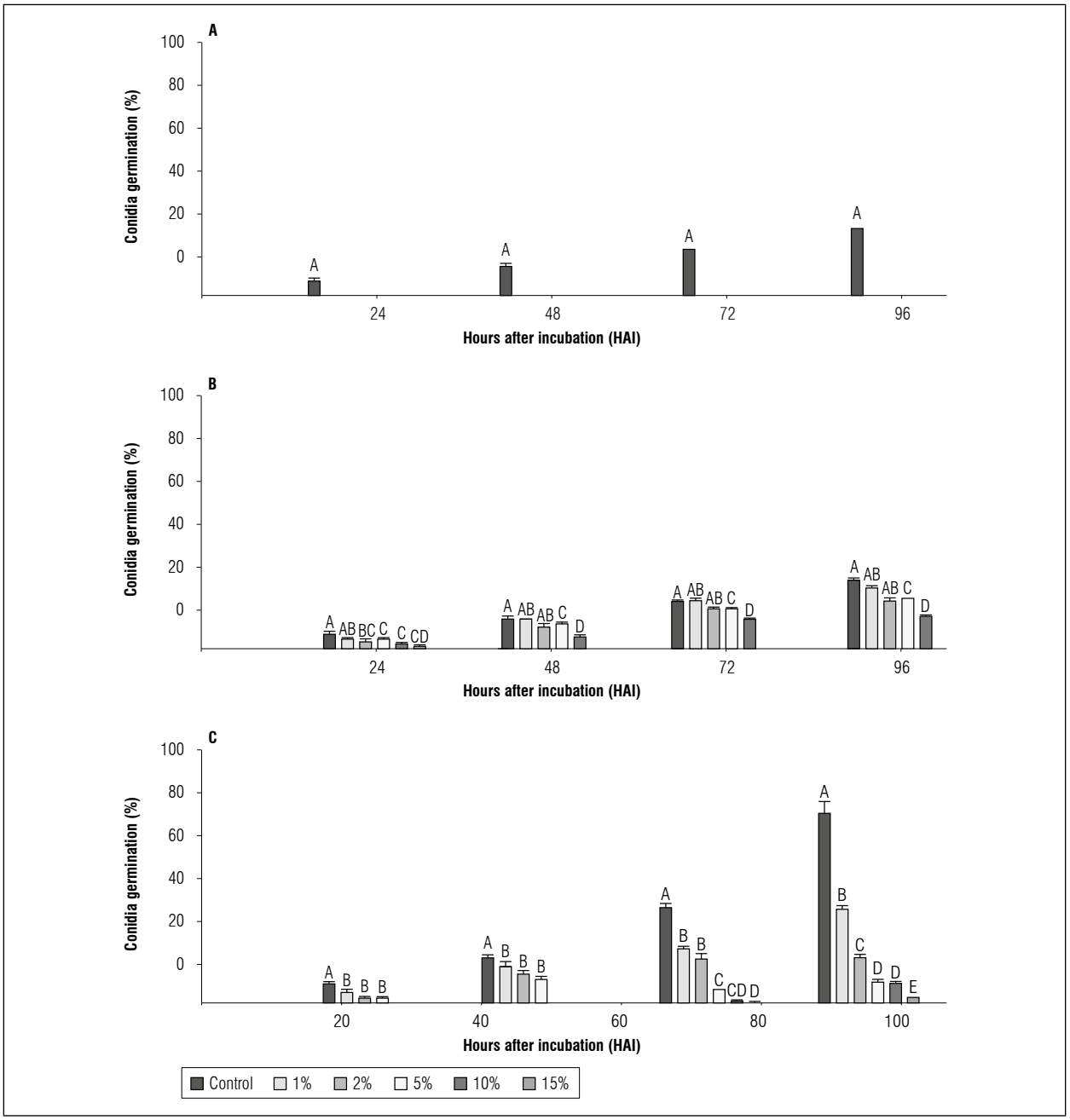


**Figure 2. Effect of cocoa leachates on the germination of *M. roreri* conidia 24, 48, 72 and 96 hours after incubation (HAI) under five concentrations of cocoa leachate and three methods of incorporation into the Water Agar culture medium incubated at 25 ºC. A. Non-sterilized cocoa leachates. B. Autoclave-sterilized cocoa leachates, and C. Filtered cocoa leachates. Each bar represents the mean of seven values ± standard error ($n=7$) or five values ± standard error ($n=5$) for autoclave-sterilized and filtered cocoa leachate respectively. Bars followed by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).**

### *Effect of leachates on* M. roreri *inoculum production*

Non-sterilized leachates at all the concentrations evaluated inhibited *M. roreri* conidia production ($P<0.000$); in this case, the mycelial inhibition of the pathogen was 100%, then no conidia production was registered. In contrast, the control produced an average of 10,000 conidia/cm$^2$ of the colony. The same

results were observed in additional experiments conducted with other cocoa leachate samples (data not shown).

Regarding sterilized leachates, a total inhibition in the production of inoculum was observed from the concentration of 10% ($P<0.000$). The concentrations of 1, 2 and 5% showed a lower inoculum production compared to the control, with values between $8.2 \cdot 10^3$ and $8.5 \cdot 10^3$ conidia/cm$^2$, but without significant differences with the control treatment ($1.1 \cdot 10^4$ conidia/cm$^2$) ($P>0.575$) (Fig. 3). The absence of sporulation at the highest concentration of sterilized cocoa leachates (15%) was also observed in additional experiments conducted with other cocoa leachate samples. Therefore, the results confirm the effect of these concentrations on the reduction in the production of *M. roreri* inoculum.

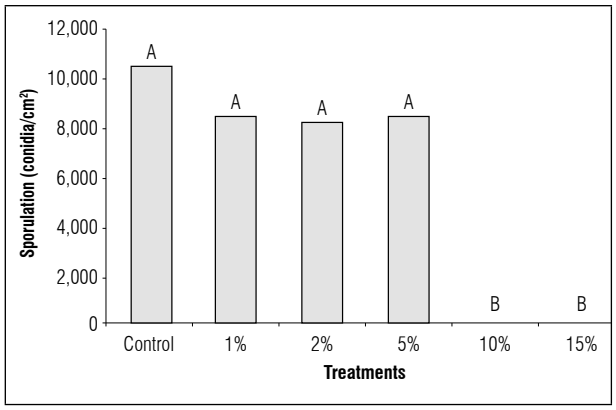


**Figure 3. Production of *M. roreri* inoculum under five concentrations of sterilized leachates on PDA culture medium incubated at 25 °C on day 14 after incubation. The data represent the mean of seven Petri dishes ($n=7$). Values with different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).**

## EC50 value for mycelial growth and conidia germination inhibition

The estimation of EC50 showed that the effective cocoa leachates concentration to reduce mycelial growth rate by 50% was 11.25% for the sterilized leachates, 0.974% for the non-sterilized leachates and 2.039% for the filtered leachates. The EC50 obtained for conidia germination inhibition assay was 10.25% for the sterilized leachates, 0.980% for the non-sterilized leachates, and 1.011% for the filtered leachates (Tab. 3).

**Table 3. EC50 values (%) estimated using mycelial growth inhibition and conidia germination.**

| Leachates | Mycelial growth | | Conidia germination | |
|---|---|---|---|---|
| | EC50 | SE | EC50 | SE |
| Sterilized | 11.252 | 1.09 | 10.250 | 41.18 |
| Non-sterilized | 0.974 | 0.54 | 0.980 | 1.01 |
| Filtered | 2.039 | 0.46 | 1.011 | 0.11 |

The data represent the effective concentration values of cocoa leachates to reduce mycelial growth or conidia germination by 50% (EC50). EC50 and SE (standard error) values were obtained by fitting the mycelial growth or conidia germination rate against the log transformed cocoa leachate concentrations using the best fitting model (ILL.4).

# DISCUSSION

The physicochemical and chromatographic analysis of cocoa leachates showed variation for the different evaluated parameters. In general, glucose and fructose showed higher concentrations in contrast to saccharose. The concentrations of citric and malic acids tended to be higher in comparison to those of ascorbic acid. Cl, $NO_3$, Mg and K tended to show similar values between leachate samples, and $PO_4$ and Ca showed a higher variation. Concerning the origin of the leachates, although variations were found among samples, a tendency to have similar concentrations was observed in the leachates obtained from the same farm at different times. S1 and S3 (Floresta) showed contrast with S2 (Filadelfia) regarding the contents of fructose, saccharose, ascorbic and citric acids, glucose, Na, EC, Cl, $SO_4$, Ca and K; glucose concentration was higher in S2 leachates.

Pacheco-Montealegre *et al.* (2020) stated that the fermentation process in cacao varies depending on the environmental conditions, fermentation protocol of the farm and genetics of the plant material, among others. Therefore, the content variations observed in the leachates assessed in this study, regardless of the similarities between both farms, may be due to factors found by Pacheco-Montealegre *et al.* (2020) in their study. Despite the variation observed, according to our results, the effect of the leachates on the *in vitro* growth of *M. roreri* seems not to depend on the content variations but on the compounds (fructose, saccharose, glucose, ascorbic and citric acids, glucose, Na, EC, Cl, $SO_4$, Ca and K) present in the liquid products. These compounds were the same in the three leachate samples evaluated in this study. The observed inhibition of *M. roreri* may be not only related

to the presence of the compounds mentioned above but also to the microbiological composition of the leachates. The high percentage (100%) of inhibition of the pathogen obtained with non-sterilized leachates at the five concentrations and the low effect observed with sterilized leachates by autoclaving and filtered leachates indicate that both, chemical and microbiological compositions of the cacao leachates may play a role in the inhibition of *M. roreri*.

The antifungal activity of some types of leachates has been previously reported by authors such as Özer *et al*. (2002), who demonstrated that leachates from composted alfalfa and sunflower stalks inhibit the production of pectolytic enzymes and isoenzymes of *Aspergillus niger* in treated onion seeds. Huang *et al*. (2012) and Yang *et al*. (2015), using *in vitro* analyses, demonstrated that aqueous leachates and volatile compounds produced by Chinese leek (*Allium tuberosum*) inhibit the growth of *Fusarium oxysporum* f. sp. *cubense* race 4 (*Foc* TR4), conidia proliferation and germination, and the activity of cell wall-degrading enzymes produced by the fungus.

The inhibitory activity of cocoa leachates on the germination of *M. roreri* propagules observed in this study showed a similar effect to that reported by Mogollón and Castaño-Zapata (2010), who obtained a total inhibition of conidial germination of the pathogen when using banana rachis leachates at concentrations of 90% for the *in vitro* control of *Paracercospora fijiensis* (Morelet) Deighton. In this study, non-sterilized cocoa leachates showed an inhibitory effect on conidial germination from a concentration of 1%. This could suggest variable effectiveness of leachate concentrations depending on the plant species these leachates are obtained from and the target pathogen.

Banana rachis leachates have been used in foliar sprays for the control of fungal diseases in plants. Álvarez *et al*. (2001) mention that applications of 5% fulvic acid from banana leachates have generated a decrease in the severity of powdery mildew in roses caused by *Sphaerotheca pannosa*. Arenas *et al*. (2004) report the use of fulvic acid for the control of *Mycosphaerella fijensis* and *Ralstonia solanacearum* Race 2 (causal agent of banana moko disease), achieving reductions of 31.6% in the incidence of the disease. In this study, an inhibitory effect on *M. roreri* of 100% (Tab. 1) was generated from the lowest concentration of non-sterilized cocoa leachates, while an inhibitory effect of 8.0% on the radial growth of *M. roreri* was observed from the concentration of 10% in the sterilized leachates. This suggests that compounds in cocoa leachates like ascorbic and citric acids may contribute to the inhibition of the pathogen through mechanisms that need to be evaluated in subsequent studies. Recent studies have reported the fungicidal and bactericidal effect of citric acid, which was shown to inhibit growth of bacteria *Shigella* sp. (In *et al.*, 2013), and phytopathogenic fungi *Colletotrichum* sp., the causal agent of anthracnose of cucumber and gourds (Kang *et al*., 2003; Morgunov *et al*., 2017). Additionally, exogenous applications of ascorbic acid have been found to induce disease resistance in some pathosystems (Boubakri, 2017). The application of ascorbic acid along with jasmonic acid enhanced the accumulation of phytoalexins in rice, and ascorbic acid alone induced resistance against Huanglongbing in citrus plants (Li *et al*., 2016). The application of derivative compounds of ascorbic acid induced resistance against *Turnip mosaic virus* in turnip (Fujiwara *et al*., 2013). Ascorbic acid has also been found to be active against fungal pathogens such as the rice blast fungus *Magnaporthe oryzae,* which decreased the percentage of normal appressorium formation after treatment with this acid (Egan *et al*., 2007). Both ascorbic and citric acids were found to be present in the cocoa leachates evaluated, which can suggest a possible effect of these compounds on the inhibition of *M. roreri.*

The high effectiveness of non-sterilized cocoa leachates that completely inhibited radial growth and conidial germination of *M. roreri* can be explained based on results of previous studies conducted with leachates from different plant species. In cocoa, microbial communities in the fermentation process are mainly *Enterobacteriaceae* related bacteria, Lactic Acid Bacteria, Acid Acetic Bacteria and yeasts (Camu *et al*., 2007; Okiyama *et al*., 2017; Pacheco-Montealegre *et al*., 2020). Ceballos *et al*. (2012) found that during the fermentation processes to obtain leachates from banana rachis, many microorganisms are present in these substances and are responsible for the segregation of numerous secondary metabolites that can act as biocides on other microorganisms. Bacteria involved in fermentation processes such as *Bacillus subtilis* and *B. amyloliquefaciens* can produce metabolites (chitinolytic and glucanolytic enzymes) that alter the structure of the mycelium cell wall and fungal ascospores, inhibiting the development of pathogenic microorganisms under *in vitro* conditions (Ceballos *et al*., 2012). Ouattara *et al*. (2011) reported that *Bacillus* strains are involved in the production of pectinolytic enzymes during cocoa fermentation. They identified

six species of *Bacillus,* including *Bacillus subtilis*. Cocoa fermentation microbiota also vary according to the geographic area, but most of the *Bacillus* strains have been reported as constituents of cocoa fermentation microbiota in Brazil (Schwan *et al*., 1986), Trinidad (Ostovar and Keeney, 1973) and Ghana (Carr *et al*., 1979; Nielsen *et al*., 2007). This could suggest that the combined action of microorganisms and substances (citric and ascorbic acids found in our samples) present in non-sterilized cocoa leachates increases the effectiveness of the compound to inhibit *M. roreri* compared to sterilized cocoa leachates. Nevertheless, bacterial species with antifungal effects that are present in the cocoa fermentation process have to be determined in further studies.

In addition to the above, authors such as Garcia-Armisen *et al*. (2010) detected bacteria of the genera *Tatumella, Pantoea,* and *Erwinia* during the fermentation process. Although some of these genera may be plant pathogens, these authors think they may contribute to citrate consumption since they are microorganisms that compete against other plant pathogens for space and nutrients. The analysis conducted by Pacheco-Montealegre *et al*. (2020) also showed that bacteria of these genera may be present in the cacao fermentation process. Additionally, Viesser *et al.* (2021) reported three major microbial groups in the cocoa fermentation process: lactic acid bacteria, acetic acid bacteria, and yeasts. These authors found that *B. subtilis* strains present in the cocoa fermentation process were associated with the production of pectinase and secondary metabolites such as lactic and acetic acids. Although we did not measure the acetic acid in our leachate samples, this compound has been reported as a key component for an efficient fermentation of cocoa beans (Soumahoro *et al*., 2020). Also, acetic acid is recognized for its antifungal and antibacterial properties, and has been applied for control of plant pathogens, such as seed-borne fungi (Dorna *et al*., 2021). This could explain the greater inhibitory effect of non-sterilized cocoa leachates on *M. roreri*, which can result from the combined action of microorganisms and compounds typically found in the leachates such as acetic acid.

The inhibitory effect of the filtered leachates on *M. roreri* was not observed during the test. From the 2% concentration, the leachates even contributed to the growth of the fungus at percentages between 0.6 and 4.7%. This could be due to the elimination of microorganisms that compete with the pathogen for space and nutrients, thus limiting its growth. Filter sterilization can eliminate microorganisms producing compounds with the potential to control *M. roreri*, reducing the control effect of leachates. On the other hand, an inhibitory effect of the filtered leachates was not observed at concentrations of 10 and 15%, as observed in the sterilized leachates. Although the heating at high temperatures of certain substances in the leachates eliminates important microorganisms, it may also generate some chemical reaction in their compounds that contributes to maintaining their capacity to control *M. roreri*. The degradation or oxidation of products such as ascorbic acid at heating temperatures of 100°C was reported to produce compounds such as furfural, 2-furoic acid, and 3-hydroxy-2-pyrone (Yin *et al.,* 2022). Among these products, furfural is the main degradation product of ascorbic acid. This compound is reported as a very effective fungicide and it was found that as little as 0.5% furfural is sufficient to entirely prevent the growth of the mold *Penicillium*. Furfural was observed to be particularly effective in inhibiting the growth of wheat smut (*Tilletis foetens*) (Zeitsch, 2000). Similarly, furfural from pine needle extract was shown to inhibit the growth of *Alternaria mali* the causal agent of Alternaria blotch of apple. The authors of that study mention that although furfural itself can not be completely substituted for an antifungal agrochemical, a partial mixture of furfural and antifungal agrochemical is effective as a fungicide (Jung *et al.,* 2007).

Studies conducted to evaluate the microbial composition associated to the cocoa fermentation in different cocoa production areas in Asia, Africa, South America (Ecuador and Colombia) and Central America (Mexico) have reported bacteria in the families *Enterobacteriaceae*, *Lactobacillaceae* and *Acetobacteraceae* and yeast as such as *Hanseniaspora opuntiae*, *Picha* sp., *Pichia kudriavzevi*, and *Wickerhamomyces pijperi* (Ardhana and Fleet, 2003; Garcia-Armisen *et al*., 2010; Papalexandratou *et al*., 2011; Papalexandratou *et al*., 2013; Arana-Sánchez *et al*., 2015; Pereira *et al*., 2016; Pacheco-Montealegre *et al*., 2020). Therefore, it is highly probable that the bacteria and yeast present in the cacao leachates used in this study belong to these taxonomic groups.

Pacheco-Montealegre *et al*. (2020) stated that the dynamics of microbial fermentation in cacao vary between farms, protocols used and time of the process that changes the bacterial composition during fermentation. Nevertheless, our study evaluated cacao leachates at 24 h of the addition of fresh harvested

seeds of similar genetic plant material. Additionally, the fermentation process was conducted following similar procedures at the farm level and under comparable environmental conditions. In this sense, the obtained results in terms of the effects of the leachates on the *in vitro* growth of *M. roreri* seem to be more influenced by the leachates condition (filtered, sterile or non-sterile) than by their source of origin. This, despite the variation observed in the chemical composition of the leachates between farms. Our results may be explained by the findings reported by Pacheco-Montealegre *et al*. (2020) who showed that, although there is heterogeneity in the microbial transitions in the cacao fermentation process from a general perspective, the functional groups present over time may be predictable. Those microorganisms potentially involved in the *in vitro* control of *M. roreri* may belong to these groups.

Banana leachates have been reported to be a mixture of humic and non-humic substances. Within the non-humic substances, we can find compounds such as sugars, amino acids, polysaccharides, and proteins, whereas the mixture of different macromolecular complexes is reported within the humic substances (Arenas *et al*., 2004; Álvarez *et al*., 2015). Additionally, the use of plant leachates involves the action of microorganisms in charge of microbial decomposition and the production of natural compounds that act as factors inhibiting the pathogen, since they prevent the germination of conidia and the penetration of potential fungal pathogens (Arenas *et al*., 2004; Özer and Köycü, 2006). The inhibitory effect of leachates can be attributed to the action of biochemical compounds with antimicrobial effects such as phenolic acids, saponins, essential oils, naphthoquinones, and terpenoids. It can also be attributed to direct antagonism due to antibiosis, parasitism, or competition (for space or nutrients) (Mainer, 2009; Bubici *et al*., 2019). This suggests that certain acids present in leachates, such as citric acid and ascorbic acid, that were found in cocoa leachates during this study, may exert a control activity on fungal pathogens as previously demonstrated in research analysis conducted on some pathosystems. Therefore, future studies should analyze the control potential of this type of compound.

The use of cocoa leachates as a method to control *M. roreri* could be a great advantage for the farmer since it is a product generated naturally by the transformation processes of cocoa beans on the farm. Additionally, leachates, especially without sterilization, are natural products that exert effective control of the pathogen from low concentrations. However, it is necessary to carry out more studies to confirm that the microorganisms present in leachates do not represent a risk for human consumption and can be safely applied to cocoa fruits. It is important to note that this is the first study to evaluate the activity of cocoa leachates for the *in vitro* control of *M. roreri*. For this reason, the results obtained were contrasted and discussed with studies carried out with leachates from other vegetable species. Therefore, further studies must deepen in the compounds and microbiological composition of cacao leachates that may be involved in the inhibition of *M. roreri*. Additionally, it is necessary to conduct research that confirms the effect on the pathogen found, but using cacao leachates obtained under variable fermentation procedures at the farm level.

## CONCLUSIONS

The cocoa leachates showed variation for the different evaluated parameters. The effect of the leachates on the *in vitro* growth of *M. roreri* seems not to depend on the content variations but on the compounds present in the liquid products. The highest inhibition of *M. roreri* radial growth and conidial germination was obtained with non-sterilized leachates at all concentrations evaluated, and with autoclave-sterilized leachates at concentrations of 10 and 15%. The antifungal ability of non-sterilized cocoa leachates from low concentrations can be attributed to the combined action of the compounds and microorganisms present in the substance. The inhibitory effect on *M. roreri* of sterilized cocoa leachates is possibly attributed to specific compounds such as citric and ascorbic acids. The antifungal activity of these acids has been reported in the literature and these compounds were found in the cocoa leachates using chromatographic tests. The filtered leachates proved not to be an efficient method for the *in vitro* control of *M. roreri* in comparison with the other two leachates evaluated. The results obtained in this study show the control potential of cocoa leachates on *M. roreri in vitro*, which is a limiting pathogen of the crop. This suggests that further studies are required to clarify the microbiological and chemical effects and composition of the cocoa leachates. Additionally, *in vivo* evaluation needs to be conducted to confirm cocoa leachates as an alternative for commercial cacao crops.

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## Author's contributions

All authors contributed to the study conceptualization. Material preparation and methodology design were performed by SVGG, SGC and CBA. Data collection and analysis was conducted by SVGG. The first draft of the manuscript was written by SVGG and all authors commented on previous versions of the manuscript approving its final version. Funding of this research was acquired by SGC and CBA.

**Conflict of interest:** On behalf of all authors, the corresponding author states that there is no conflict of interest.

## BIBLIOGRAPHY REFERENCES

Aguilera Rodríguez, I., R.M. Pérez Silva, and A. Marañón Reyes. 2010. Determinación de sulfato por el método turbidimétrico en aguas y aguas residuales. Validación del método. Rev. Cuba. Quim. 22(3), 39-44.

Álvarez, E., J. Cortés, and G. Ceballos. 2010. Alternativas para el manejo de la Sigatoka negra en plátano Dominico Hartón (AAB) mediante el uso de lixiviados y productos biológicos. MusaLAC 1(2), 3-5.

Álvarez, E., C. Grajales, J. Villegas, and J. Loke. 2001. CIAT informe anual. Control del mildeo polvoso (*Sphaerotheca panosa* var. *rosae*) en rosa, usando un lixiviado de compost del raquis de plátano (*Musa* AAB). CIAT, Palmira, Colombia.

Álvarez, E., A. Pantoja, L. Gañán, and G. Ceballos. 2015. Current status of Moko disease and black sigatoka in Latin America and the Caribbean, and options for managing them. Publication 404. CIAT, Cali, Colombia.

Arana-Sánchez, A., L.E. Segura-García, M. Kirchmayr, I. Orozco-Ávila, E. Lugo-Cervantes, and A. Gschaedler-Mathis. 2015. Identification of predominant yeasts associated with artisan Mexican cocoa fermentations using culture-dependent and culture-independent approaches. World J. Microbiol. Biotechnol. 31, 359-369. Doi: https://doi.org/10.1007/s11274-014-1788-8

Arenas, A., D. López, E. Álvarez, G. Llano, and J. Loke. 2004. Efecto de prácticas ecológicas sobre la población de *Ralstonia solanacearum* Smith, causante de Moko de plátano. Fitopatol. Colomb. 28(2), 76-80.

Arcos-Méndez, M.C., L. Martínez-Bolaños, G. Ortíz-Gil, M. Martínez-Bolaños, and C.H. Avendaño-Arrazate. 2019. Efecto *in vitro* de extractos vegetales contra la moniliasis (*Moniliophthora roreri*) del cacao (*Theobroma cacao* L.). Agric. Trop. 5(1), 19-24.

Ardhana, M.M. and G.H. Fleet. 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. 86(1-2), 87-99. Doi: https://doi.org/10.1016/S0168-1605(03)00081-3

Barrera Necha, L.L. and L.J. García Barrera. 2008. Actividad antifúngica de aceites esenciales y sus compuestos sobre el crecimiento de *Fusarium* sp. aislado de papaya (*Carica papaya*). Rev. UDO Agrícola 8(1), 33-41.

Bailey, B.A., H.C. Evans, W. Phillips-Mora, S.S. Ali, and L.W. Meinhardt. 2018. *Moniliophthora roreri*, causal agent of cacao frosty pod rot. Mol. Plant Pathol. 19(7), 1580-1594. Doi: https://doi.org/10.1111/mpp.12648

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

Bele, L., D. Kra Kouamé, K. Patrice Assiri, and H. Atta Diallo. 2018. Antifungal activity of banana rachis leachate on some fungi responsible for banana (*Musa acuminate* Colla) post-harvest diseases. Int. J. Environ. Agric. Res. 4(3), 1-6. Doi: https://doi.org/10.5281/zenodo.1213546

Boubakri, H. 2017. The role of ascorbic acid in plant–pathogen interactions. pp. 255-271. In: Anwar Hossain, M., S. Munné-Bosch, D.J. Burritt, P. Diaz-Vivancos, M. Fujita, and A. Lorence (eds.). Ascorbic acid in plant growth, development and stress tolerance. Springer, Cham, Switzerland. Doi: https://doi.org/10.1007/978-3-319-74057-7_10

Bubici, G., M. Kaushal, M.I. Prigigallo, C. Gómez-Lama, and J. Mercado-Blanco. 2019. Biological control agents against *Fusarium* wilt of banana. Front. Microbiol. 10, 616. Doi: https://doi.org/10.3389/fmicb.2019.00616

Camu, N., T. De Winter, K. Verbrugghe, I. Cleenwerck, P. Vandamme, J.S. Takrama, M. Vancanneyt, and L. De Vuyst. 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Appl. Environ. Microbiol. 73(6), 1809-1824. Doi: https://doi.org/10.1128/AEM.02189-06

Carr, J.G., P.A. Davies, and J. Dougan. 1979. Cocoa fermentation in Ghana and Malaysia: Further microbial methods and results. University of Bristol Research Station, London.

Carrasquero D., A. and P. Castillo. 2002. Determinación conductimétrica de cloruros en extractos de saturación de suelos. Agron. Trop. 52(4), 555-563.

Carrera-Sánchez, K., L. Mosquera Paredes, and M. Leiva-Mora. 2014. Protocolo para el aislamiento de *Moniliophthora roreri* (Cif y Par) Evans et al. en frutos de cacao cv. 'Nacional' de la Amazonía ecuatoriana. Biotecnol. Vegetal 14(3), 147-150.

Ceballos, I., S. Mosquera, M. Ángulo, J.J. Mira, L.E. Argel, D. Uribe-Velez, M. Romero-Tabarez, S. Orduz-Peralta, and V. Villegas. 2012. Cultivable bacteria population associated with leaves of banana and plantain plants and their antagonistic activity against *Mycosphaerella fijiensis*. Microb. Ecol. 64, 641-653. Doi: https://doi.org/10.1007/s00248-012-0052-8

Chang, H.-T., Y.-H. Cheng, C.-L. Wu, S.-T. Chang, T.-T. Chang, and Y.-C. Su. 2008. Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin leaf against plant pathogenic fungi. Bioresour. Technol. 99, 6266-6270. Doi: https://doi.org/10.1016/j.biortech.2007.12.005

Chuah, T.S., Y.Y. Tan, and B.S. Ismail. 2010. *In vitro* evaluation of the antifungal activity of some essential oils on post-harvest fungal pathogens of tropical fruits. Plant Prot. Q. 25(4), 162-164.

Correa Álvarez, J., S. Castro Martínez, and J. Coy. 2014. Estado de la moniliasis del cacao causada por *Moniliophthora roreri* en Colombia. Acta Agron. 63(4), 388-399. Doi: https://doi.org/10.15446/acag.v63n4.42747

D'Angelo, E., J. Crutchfield, and M. Vandiviere. 2001. Rapid, sensitive, microscale determination of phosphate in water and soil. J. Environ. Qual. 30(6), 2206-2209. Doi: https://doi.org/10.2134/jeq2001.2206

Dorna, H., A. Rosińska, and D. Szopińska. 2021. The effect of acetic acid treatments on the quality of stored carrot (*Daucus carota* L.) seeds. Agronomy 11(6), 1176. Doi: https://doi.org/10.3390/agronomy11061176

Egan, M.J., Z.-Y. Wang, M.A. Jones, N. Smirnoff, and N.J. Talbot. 2007. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proc. Natl. Acad. Sci. USA 104(28), 11772-11777. Doi: https://doi.org/10.1073/pnas.0700574104

Evans, H.C., K.A. Holmes, and A.P. Reid. 2003. Phylogeny of the frosty pod rot pathogen of cocoa. Plant Pathol. 52(4), 476-485. Doi: https://doi.org/10.1046/j.1365-3059.2003.00867.x

Fedecacao. 2021. Así se comportó la producción de cacao por departamentos en el 2020. In: https://www.fedecacao.com.co/post/copy-of-design-a-stunning-blog; consulted: March, 2021.

Flood, J. and R. Murphy (eds.). 2004. Cocoa futures—A source book of some important issues confronting the cocoa industry. Commodities Press, Chinchina, Colombia.

Fox, J. and S. Weisberg. 2019. An R companion to applied regression. 3rd ed. Sage, Oaks, CA. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Fromm, I. 2016. From small chocolatiers to multinationals to sustainable sourcing: a historical review of the Swiss chocolate industry. pp. 71-87. In: Squicciarini, M.P. and J. Swinnen (eds.). The economics of chocolate. Oxford University Press, Oxford, UK.

Fujiwara, A., H. Shimura, C. Masuta, S. Sano, and T. Inukai. 2013. Exogenous ascorbic acid derivatives and dehydroascorbic acid are effective antiviral agents against *Turnip mosaic virus* in *Brassica rapa*. J. Gen. Plant Pathol. 79, 198-204. Doi: https://doi.org/10.1007/s10327-013-0439-5

Garcia-Armisen, T., Z. Papalexandratou, H. Hendryckx, N. Camu, G. Vrancken, L. De Vuyst, and P. Cornelis. 2010. Diversity of the total bacterial community associated with Ghanaian and Brazilian cocoa bean fermentation samples as revealed by a 16S rRNA gene clone library. Appl. Microbiol. Biotechnol. 87, 2281-2292. Doi: https://doi.org/10.1007/s00253-010-2698-9

Huang, Y.H., R.C. Wang, C.H. Li, C.W. Zuo, Y.R. Wei, L. Zhang, and G.J. Yi. 2012. Control of Fusarium wilt in banana with Chinese leek. Eur. J. Plant Pathol. 134, 87-95. Doi: https://doi.org/10.1007/s10658-012-0024-3

ICCO, International Cocoa Organization. 2020. Production of cocoa beans. Quarterly Bulletin of Cocoa Statistics 46(4), In: https://www.icco.org/wp-content/uploads/Production-QBCS-XLVI-No-4.pdf; consulted: February, 2019.

In, Y.-W., J.-J. Kim, H.-J. Kim, and S.-W. Oh. 2013. Antimicrobial activities of acetic acid, citric acid and lactic acid against *Shigella* species. J. Food Saf. 33(1), 79-85. Doi: https://doi.org/10.1111/jfs.12025

Jaimes, Y.Y., C. González, J. Rojas, O.E. Cornejo, M.F. Mideros, S. Restrepo, C. Cilas, and E.L. Furtado. 2016. Geographic differentiation and population genetic structure of *Moniliophthora roreri* in the principal cocoa production areas in Colombia. Plant Dis. 100(8), 1548-1558. Doi: https://doi.org/10.1094/PDIS-12-15-1498-RE

Jaimes Suárez, Y. and F. Aranzazu Hernández. 2010. Manejo de las enfermedades del cacao (*Theobroma cacao* L.) en Colombia, con énfasis en monilia (*Moniliophthora roreri*). Corpoica-La Suiza, Rionegro, Colombia.

Jung, K.-H., S.K. Yoo, S.-K. Moon, and U.-S. Lee. 2007. Furfural from pine needle extract inhibits the growth of a plant pathogenic fungus, *Alternaria mali*. Mycobiology 35(1), 39-43.

Kamel, S.M., M.M.I. Afifi, F.S. El-shoraky, and M.M. El-Sawy. 2014. Fulvic acid: A tool for controlling powdery and downy mildews in cucumber plants. Int. J. Phytopathol. 3(2), 101-108. Doi: https://doi.org/10.33687/phytopath.003.02.0866

Kang, H.-C., Y.-H. Park, and S.-J. Go. 2003. Growth inhibition of a phytopathogenic fungus, *Colletotrichum* species by acetic acid. Microbiol. Res. 158(4), 321-326. Doi: https://doi.org/10.1078/0944-5013-00211

Krauss, U., E. Hidalgo, R. Bateman, V. Adonijah, C. Arroyo, J. García, J. Crozier, N.A. Brown, G.M. ten Hoopen, and K.A. Holmes. 2010. Improving the formulation and timing of application of endophytic biocontrol and chemical agents against frosty pod rot (*Moniliophthora roreri*) in cocoa (*Theobroma cacao*). Biol. Control 54(3), 230-240. Doi: https://doi.org/10.1016/j.biocontrol.2010.05.011

Li, J., P. Trivedi, and N. Wang. 2016. Field evaluation of plant defense inducers for the control of citrus Huanglongbing. Phytopathology 106(1), 37.46. Doi: https://doi.org/10.1094/PHYTO-08-15-0196-R

Lozada, B.S., L.V. Herrera, J.A. Perea, E. Stashenko, and P. Escobar. 2012. Efecto *in vitro* de aceites esenciales de tres especies de *Lippia* sobre *Moniliophthora roreri* (Cif. y Par.) Evans *et al*., agente causante de la moniliasis del cacao (*Theobroma cacao* L.). Acta Agron. 61(2), 102-110.

Mainer, Y. 2009. Control de la Sigatoka negra (*Mycosphaerella fijiensis*) del plátano con productos naturales (lixiviado y antagonistas). MSc. thesis. Institut National d'Horticulture et du Paysage, Université d'Angers, Angers, France.

Marín Marín, P. and A.E. Bustillo Parley. 2002. Pruebas microbiológicas y fisico - químicas para el control de calidad de los hongos entomopatógenos. pp. 72-89. In: Mem. Curso Internacional Teórico- Práctico sobre Entomopatógenos, Parasitoides y otros Enemigos Naturales de la Broca del Café. Cenicafé, Chinchiná, Colombia.

Mogollón Ortiz, A.M. and J. Castaño-Zapata. 2010. Evaluación *in vitro* de lixiviados del raquis del plátano sobre *Paracercospora fijiensis* (Morelet) Deighton. Agronomía 18(2), 17-23.

Morgunov, I.G., S.V. Kamzolova, E.G. Dedyukhina, T.I. Chistyakova, J.N. Lunina, A.A. Mironov, N.N. Stepanova, O.N. Shemshura, and M.B. Vanshtein. 2017. Application of organic acids for plant protection against phytopathogens. Appl. Microbiol. Biotechnol. 101, 921-932. Doi: https://doi.org/10.1007/s00253-016-8067-6

Nielsen, D.S., O.D. Teniola, L. Ban-Koffi, M. Owusu, T.S. Anderson, and W.H. Holzapfel. 2007. The microbiology of Ghanaian cocoa fermentations analyzed using culture-dependent and culture-independent methods. Int. J. Food Microbiol. 114(2), 168-186. Doi: https://doi.org/10.1016/j.ijfoodmicro.2006.09.010

Okiyama, D.C.G., S.L.B. Navarro, and C.E.C. Rodrigues. 2017. Cocoa shell and its compounds: applications in the food industry. Trends Food Sci. Technol. 63, 103-112. Doi: https://doi.org/10.1016/j.tifs.2017.03.007

Osorio Gutiérrez, L.A., J. Castaño-Zapata, and L.B. Gutiérrez Ríos. 2012. Eficacia *in-vitro* de lixiviados de plátano sobre *Fusarium oxysporum* Schlecht, causante de la pudrición de raíces de arveja (*Pisum sativum* Linneo). Agronomía 20(1), 17-25.

Ostovar, K. and P.G. Keeney. 1973. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cocoa beans. J. Food Sci. 38(4), 611-617. Doi: https://doi.org/10.1111/j.1365-2621.1973.tb02826.x

Ouattara, H.G., S. Reverchon, S.L. Niamke, and W. Nasser. 2011. Molecular identification and pectate lyase production by *Bacillus* strains involved in cocoa fermentation. Food Microbiol. 28(1), 1-8. Doi: https://doi.org/10.1016/j.fm.2010.07.020

Özer, N., M. Mirik, and A. Citir. 2002. Effects of extracts from some medicinal plants and composts on pectolytic enzymes produced in liquid culture and on onion seeds by *Aspergillus niger* and *Fusarium oxysporum* f. sp. *cepae*. J. Turk. Phytopathol. 31(3), 137-154.

Özer, N. and N.D. Köycü. 2006. The ability of plant compost leachates to control black mold (*Aspergillus niger*) and to induce the accumulation of antifungal compounds in onion following seed treatment. BioControl 51, 229-243. Doi: https://doi.org/10.1007/s10526-005-1035-1

Pacheco-Montealegre, M.E., L.L. Dávila-Mora, L.M. Botero-Rute, A. Reyes, and A. Caro-Quintero. 2020. Fine resolution analysis of microbial communities provides insights into the variability of cocoa bean fermentation. Front. Microbiol. 11, 650. Doi: https://doi.org/10.3389/fmicb.2020.00650

Papalexandratou, Z., G. Falony, E. Romanens, J.C. Jimenez, F. Amores, H.-M. Daniel, and L. De Vuyst. 2011. Species diversity, community dynamics, and metabolite kinetics of the microbiota associated with traditional Ecuadorian spontaneous cocoa bean fermentations. Appl. Environ. Microbiol. 77(21), 7698-7714. Doi: https://doi.org/10.1128/AEM.05523-11

Papalexandratou, Z., T. Lefeber, B. Bahrim, O.S. Lee, H.M. Daniel, and L. De Vuyst. 2013. *Hanseniaspora opuntiae, Saccharomyces cerevisiae, Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process. Food Microbiol. 35(2), 73-85. Doi: https://doi.org/10.1016/j.fm.2013.02.015

Penagos Muñetón, A., A. Parra Coronado, A.L. Leguizamon García, A.O. Herrera Arévalo, C.R. Beltran Acosta, C.S. Pinilla Dativa, C.M. Sánchez Sáenz, E. Baquero

López, E. Torres Rojas, H.A. Cordoba Novoa, J. Torres Bazurto, J.A. Cáceres Zambrano, J.E. Ospina Noreña, J.A. Ladino Orjuela, J.F. Soler Arias, J.C. Barrientos Fuentes, L.A. Valderrama Flórez, L.E. Ramírez Chamorro, M.C. Henao Toro, M.F. Bejarano Hernández, P.A. Bermeo Fúquene, R. Villalobos Rebolledo, S. Gómez Caro, S.V. Gómez Gutiérrez, and S. Magnitskiy. 2019. Guía técnica para el cultivo de cacao en los municipios Nilo y Yacopí (Cundinamarca). Universidad Nacional de Colombia; Agrosavia. Bogota.

Peña Hernández, Y., G. Santacruz de León, and H. Charcas Salazar. 2012. Calidad del agua en pozos de la red de monitoreo del acuífero del valle de San Luis Potosí, México. Aqua-LAC 4(1), 49-59. Doi: https://doi.org/10.29104/phi-aqualac/2012-v4-1-06

Pereira, G.V.M., V.T. Soccol, and C.R. Soccol. 2016. Current state of research on cocoa and coffee fermentations. Curr. Opin. Food Sci. 7, 50-57. Doi: https://doi.org/10.1016/j.cofs.2015.11.001

Pérez-Vicente, L.F. 2018. *Moniliophthora roreri* H.C. Evans *et al*. and *Moniliophthora perniciosa* (Stahel) Aime: impact, symptoms, diagnosis, epidemiology and management. Rev. Protec. Veg. 33(1), 1-13.

Petlamul, W. and P. Prasertsan. 2012. Evaluation of strains of *Metarhizium anisophilae* and *Beauveria bassiana* against *Spodoptera litura* in the basis of their virulence, germination rate, conidia production, radial growth and enzyme activity. Mycobiology 40(2), 111-116. Doi: https://doi.org/10.5941/MYCO.2012.40.2.111

Phillips-Mora, W., M.C. Aime, and M.J. Wilkinson. 2007. Biodiversity and biogeography of the cacao (*Theobroma cacao*) pathogen *Moniliophthora roreri* in tropical America. Plant Pathol. 56(6), 911-922. Doi: https://doi.org/10.1111/j.1365-3059.2007.01646.x

Phillips-Mora, W., J. Castillo, U. Krauss, E. Rodríguez, and M. Wilkinson. 2005. Evaluation of cacao (*Theobroma cacao*) clones against seven Colombian isolates of *Moniliophthora roreri* from four pathogen genetic groups. Plant Pathol. 54(4), 483-490. Doi: https://doi.org/10.1111/j.1365-3059.2005.01210.x

Phillips-Mora, W., J. Castillo, W., A. Coutiño, C.F. Ortiz, A.P. López, J. Hernández, and M.C. Aime. 2006. First report of *Moniliophthora roreri* causing frosty pod rot (moniliasis disease) of cocoa in Mexico. Plant Pathol. 55(4), 584-584. Doi: https://doi.org/10.1111/j.1365-3059.2006.01418.x

Phillips-Mora, W., J. Cawich, W. Garnett, and M.C. Aime. 2006b. First report of frosty pod rot (moniliasis disease) caused by *Moniliophthora roreri* on cacao in Belize. Plant Pathol. 55(4), 584-584. Doi: https://doi.org/10.1111/j.1365-3059.2006.01378.x

Phillips-Mora, W. and M.J. Wilkinson. 2007. Frosty pod of cacao: A disease with a limited geographic range but unlimited potential for damage. Phytopath. 97(12), 1644-1647. Doi: https://doi.org/10.1094/PHYTO-97-12-1644

Pinzón Usuche, J.O., J. Rojas Ardila, F. Rojas, O.D. Ramírez, F. Moreno, and G. Antolinez Castro. 2012. Guía técnica para el cultivo del cacao. 5th ed. Fedecacao, Bogota.

Pohl, P., H. Stecka, and P. Jamroz. 2012. Fast and interference free determination of calcium and magnesium in honeys by solid phase extraction followed by flame atomic absorption spectrometry. J. Braz. Chem. Soc. 23(4), 710-717. Doi: https://doi.org/10.1590/S0103-50532012000400017

R Development Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. In: http://www.R-project.org/

Ramírez, S. 2013. Efectividad de extractos vegetales en el manejo de la Moniliasis (*Moniliophthora roreri)* del cacao (*Theobroma cacao* L.) en México. PhD thesis. Universidad Nacional de Costa Rica, Heredia, Costa Rica.

Ritz, C., F. Baty, J.C. Streibig, and D. Gerhard. 2015. Dose-response analysis using R. PLos ONE 10(12), e0146021. Doi: https://doi.org/10.1371/journal.pone.0146021

Roldán Pérez, G. and J.J. Ramírez Restrepo. 2008. Fundamentos de limnología neotropical. 2nd ed. Editorial Universidad de Antioquia; Universidad Católica de Oriente; ACCEFYN, Medellin, Colombia.

Sánchez Mora, F.D. and F.R. Garcés Fiallos. 2012. *Moniliophthora roreri* (Cif. y Par.) Evans *et al.* en el cultivo de cacao. Sci. Agropecu. 3(3), 249-258. Doi: https://doi.org/10.17268/sci.agropecu.2012.03.06

Sánchez-Mora, F., J. Zambrano Montufar, J. Vera Chang, R. Ramos Remache, F. Garcés Fiallos, and G. Vásconez Montúfar. 2014. Productividad de clones de cacao tipo nacional en una zona del bosque húmedo tropical de la provincia de Los Ríos, Ecuador. Cienc. Tecnol. 7(1), 33-41. Doi: https://doi.org/10.18779/cyt.v7i1.134

Schwan, R.F., M.C.D. Vanetti, D.O. Silva, A. Lopez, and C.A. Moraes. 1986. Characterization and distribution of aerobic, spore-forming bacteria from cacao fermentation in Bahia. J. Food Sci. 51(6), 1583-1584. Doi: https://doi.org/10.1111/j.1365-2621.1986.tb13872.x

Sikirou, R., A. Zannou, G. Gbèhounou, F. Tosso, and F.A. Komlan. 2010. Fungicide effect of banana column juice on tomato southern blight caused by *Sclerotium rolfsii*: Technical and economic efficiency. Afr. J. Agric. Res. 5(23), 3230-3238.

Soumahoro, S., H.G. Ouattara, M. Droux, W. Nasser, S.L. Niamke, and S. Reverchon. 2020. Acetic acid bacteria (AAB) involved in cocoa fermentation from Ivory Coast: species diversity and performance in acetic acid production. J. Food Sci. Technol. 57(5), 1904-1916. Doi: https://doi.org/10.1007/s13197-019-04226-2

Suárez Contreras, L.Y. and A.L. Rangel Riaño. 2013. Aislamiento de microorganismos para control biológico de *Moniliophthora roreri*. Acta Agron. 62(4), 370-378.

Tello Espinoza, P. and G. Fernández Villagómez. 2012. Evaluación de la generación de lixiviados en pacas impermeabilizadas de residuos sólidos urbanos. Experimento a gran escala. Rev. Int. Contam. Ambie. 28(Suppl. 1), 83-87.

Viesser, J.A., G.V. Melo Pereira, D.P. Carvalho Neto, G.R. Favero, J.C. Carvalho, A. Goés-Neto, H. Rogez, and C.R. Soccol. 2021. Global cocoa fermentation microbiome: revealing new taxa and microbial functions by next generation sequencing technologies. World. J. Microbiol. Biotechnol. 37, 118. Doi: https://doi.org/10.1007/s11274-021-03079-2

Wacher Rodarte, M.C. 2011. Microorganismos y chocolate. Revista Digital Universitaria 12(4), 3-9.

Weltzien, H. 1991. Biocontrol of foliar fungal diseases with compost extracts. pp. 430-450. In: Andrews, J.H. and S.S. Hirano (eds.). Microbial ecology of leaves. Springer Verlag, New York. Doi: https://doi.org/10.1007/978-1-4612-3168-4_22

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. Springer-Verlag, New York.

Wickham, H., R. François, L. Henry, and K. Müller. 2021. dplyr: A grammar of data manipulation. R package version 1.0.6. https://CRAN.R-project.org/package=dplyr

Yang, J., J. Huang, C. Wang, and Y. Feng. 2015. Effects of *Allium tuberosum* juice on the activities of two cell wall degrading enzymes produced by *Fusarium oxysporum* f. sp. *cubense*. J. Zhongkai Univ. Agric. Eng. 28, 64-66.

Yin, X., K. Chen, H. Cheng, X. Chen, S. Feng, Y. Song, and L. Liang. 2022. Chemical stability of ascorbic acid integrated into commercial products: A review on bioactivity and delivery technology. Antioxidants 11(1), 153. Doi: https://doi.org/10.3390/antiox11010153

Zeitsch, K. 2000. Applications of furfural. pp. 98-103. In: Zeitsch, K.J. (ed.) Sugar series. Vol. 13. Elsevier, Amsterdam. Doi: https://doi.org/10.1016/S0167-7675(00)80014-7

Zheng, H., Q. Zhang, J. Quan, Q. Zheng, and W. Xi. 2016. Determination of sugars, organic acids, aroma components, and carotenoids in grapefruit pulps. Food Chem. 205, 112-121. Doi: https://doi.org/10.1016/j.foodchem.2016.03.007