

How to Cite:

Falquez, O. F. C., Torres, R. O. V., Chuez, R. V. G., & Mendoza, K. A. D. (2022). Antifungal activity of milkweed (*Euphorbia hirta* L.) in the control of moniliasis (*Moniliophthora roreri* Cif.) of cocoa (*Theobroma cacao* L.). *International Journal of Health Sciences*, 6(S1), 14245–14256.
<https://doi.org/10.53730/ijhs.v6nS1.8643>

Antifungal activity of milkweed (*Euphorbia hirta* L.) in the control of moniliasis (*Moniliophthora roreri* Cif.) of cocoa (*Theobroma cacao* L.)

Orly Fernando Cevallos Falquez

Facultad de Ciencias Pecuarias y Biológicas. Universidad Técnica Estatal de Quevedo. Los Ríos, Ecuador

Email: fcevallos@uteq.edu.ec

ORCID: <https://orcid.org/0000-0002-4137-7133>

Ronald Oswaldo Villamar Torres

Facultad de Ciencias Pecuarias y Biológicas. Universidad Técnica Estatal de Quevedo. Los Ríos, Ecuador

Email: rvillamart@uteq.edu.ec

ORCID: <https://orcid.org/0000-0003-2511-1789>

Raquel Verónica Guerrero Chuez

Facultad de Ciencias Pecuarias y Biológicas. Universidad Técnica Estatal de Quevedo. Los Ríos, Ecuador

Corresponding autor email: rguerrero@uteq.edu.ec

ORCID: <https://orcid.org/0000-0002-7609-3480>

Katia Anabel Díaz Mendoza

Facultad de Ciencias Pecuarias y Biológicas. Universidad Técnica Estatal de Quevedo. Los Ríos, Ecuador

Email: katia.diaz2016@uteq.edu.ec

ORCID: <https://orcid.org/0000-0002-8201-9367>

Abstract---The cocoa crop (*Theobroma cacao*) is constantly affected by diseases that can considerably compromise its production, among them moniliasis (*Moniliophthora roreri*). The search for alternative control measures to reduce its presence in the field is imperative; therefore, the objective of this study is to evaluate the effect of *Euphorbia hirta* extract on *M. roreri*, a cocoa pathogen. The study consisted of two phases: in vitro and in vivo in the field. The in vitro experiment was established under a completely randomized design with treatments using different concentrations of the extract (1, 2, 3, 4, 5, 6, 6, 7, 8, and 9%) to modify the culture medium and control

(T0). Spore germination and mycelial growth of *M. roreri* were evaluated. The results revealed that milky extract (*E. hirta*) at a concentration of 9% v/v inhibited 100% of spore germination and 79.78% of mycelial growth. In the field trial, a completely randomized block design was used where three treatments were applied to the fruit (less than three months old) and a control: *E. hirta* extract with concentrations of 10% (T1) and 5% (T2), a chemical control based on copper oxide (T3) and the control (T0). Trees treated with 10% extract of milkweed (*E. hirta*) showed lower percentages of fruit infected by *M. roreri* and other pathogens compared to the other treatments. Similarly, the percentage of healthy fruit in T1 was higher than in the other treatments. These results show the potential of species such as *E. hirta* to be used as a source of compounds that can be used as tools to reduce the impact of *M. roreri* in cocoa plantations.

Keywords---disease management, extracts, plant extracts, inhibition.

Introduction

Agricultural and horticultural crops are affected by various fungi that cause crop losses and health risks to consumers due to mycotoxins. As a control measure for this large group of microorganisms, the indiscriminate use of synthetic chemicals has become widespread. Authors such as Choudhury et al. (2018), argue that chemical control of phytopathogens has led to the development of pesticide resistance in some species of pathogens, and thus, has forced the use of higher concentrations with a consequent increase in toxicity in food products, affecting the health of consumers and the environment.

To reduce the use of chemical pesticides, it is necessary to study new alternatives, such as the use of natural products with antifungal activity (Singh et al. 2012). These products could be obtained from plants with biochemical components used naturally as defense mechanisms. Jiménez et al. (2019) indicate that these species can metabolize active compounds generating protection against insects and preventing the invasion of pathogens.

Metabolites synthesized by plants comprise intermediate products of enzymatic reactions that occur as part of biological pathways in living organisms (Singh et al., 2012). In the case of primary metabolites, these are compounds that are associated with essential cellular functions which are directly involved in growth, development, and reproduction, they are usually indispensable for the viability of an organism; while secondary metabolites are compounds that are formed by metabolism but are no longer used for the formation of new cells (Avalos & Perez, 2009). In this regard, Gurjar et al. (2012) argue that plants can synthesize aromatic secondary metabolites, such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins, most of which have antifungal activity and can be used in agriculture because they are not harmful to health and contribute to environmental care.

In the cultivation of cocoa (*Theobroma cacao* L.) multiple efforts have been made to control diseases, these pathologies directly affect the economy of families who have the crop as a source of employment and income. Losses can reach 90% in the Ecuadorian Litoral region, the most important being: moniliasis (*Moniliophthora roreri*), witches' broom (*Moniliophthora perniciosa*), and Phytophthora (*Phytophthora spp.*); additionally, in a lower percentage, the so-called machete disease (*Ceratocystis cacaofunesta*) can occur. According to Ploetz (2016), moniliasis is the one that currently generates the greatest losses in the region.

Moniliasis control measures include the use of disease tolerant cultivars, cultural control, use of pesticides, and to a lesser extent (very little studied in the region) the use of biological controllers and plant extracts. Since there is no 100% effective strategy for disease control, it is important to seek new strategies for its control (Evans, 2016). For that reason, the study of plants containing components that are toxic to pathogens has potential. Ten Hoopen & Krauss (2016), state that there is evidence that when such compounds are extracted through plant extracts and applied to infested crops, they can stop or reduce the impact of pathogens on crops. With this background, the present research aimed to evaluate the effect of *Euphorbia hirta* extract on *Moniliophthora roreri* in vitro and in vivo, to improve crop health

Materials and Methods

Place of study

The research was carried out at laboratory and field levels. The in vitro phase was carried out at the Chemistry and Biochemistry Laboratory located at the "La María" campus, belonging to the State Technical University of Quevedo, located at km 7.5 of the Quevedo - El Empalme road, San Felipe campus, Mocache canton, Los Ríos province. The field trials were carried out in the four-year-old cocoa plantation CCN-51 of the farm "La Francia" located in the parish of Santa María del Toachi, canton Santo Domingo, province of Santo Domingo de Los Tsáchilas. The cocoa lot is located between the geographical coordinates of 0°37'14.9" South and 79°20'47.3" West longitude, at an altitude of 360 m.a.s.l. and has an average annual temperature of 22°C and humidity of 93%.

Experiment design and management

To achieve the objectives and determine the effect of *E. hirta* extract on *M. roreri* in vitro, nine doses of *E. hirta* hydroethanolic extract were evaluated to inhibit spore germination and mycelial development of *M. roreri*. The extract was prepared using dried samples of healthy plants of the species placed in a 1:1 hydroethanolic solution (hydroethanolic extract) at the rate of 140 g of crushed plant tissue per liter of solution. The maceration was carried out for eight days at a constant temperature of 25 °C. Subsequently, the macerate was filtered and subjected to alcohol elimination with the use of a rotary evaporator.

With the extract obtained, the potato dextrose agar (PDA) medium was amended at concentrations of 1, 2, 3, 4, 5, 6, 7, 8, and 9% v/v; additionally, the

unamended medium (0% control) was prepared. On the other hand, repopulations of the pathogen were obtained on a PDA medium and incubated for 21 days.

To evaluate spore germination, a spore suspension (100 μ L of suspension with a concentration of 1×10^6 spores/ mL + Tween® 20 0.01%) of the pathogen was inoculated in the Petri dishes containing the PDA culture medium and amended extract according to the treatments proposed. Ten Petri dishes were inoculated for each treatment. A 100 μ L of the suspension was inoculated and streaked on the surface of each Petri dish. The same procedure was repeated for the control. Incubation was carried out at a temperature of $25^\circ\text{C} \pm 2^\circ\text{C}$. Eight hours after inoculation, the progress of spore germination in the controls was observed under the microscope. Once 100% germination was reached in the controls, germination was stopped in the plates of the treatments by placing four drops of methyl blue in a Petri dish.

The evaluations were performed by microscopic observations (40 X objective lens). Ten optical fields were chosen to count one hundred spores (germinated and non-germinated), one field per Petri dish. With these data, germination percentages were determined. To evaluate the inhibition of mycelial development, the mycelial discs of the pathogen were inoculated in the Petri dishes containing the PDA culture medium amended with the extract according to the treatments, following the same procedure of preparation of the treatments already explained in the germination experiment.

The mycelial discs were obtained with a sterile metal punch and 5 mm diameter discs were obtained. In each repetition, a mycelial disk was placed in the center of the Petri dish with an amended culture medium. Ten boxes were inoculated for each treatment and ten for the control (unamended). Incubation was carried out at a temperature of $25^\circ\text{C} \pm 2^\circ\text{C}$ and once in the control the pathogen colonies covered the entire surface of the plate (9 cm in diameter), the diameters of the colonies (cm) were measured in each treatment/repetition. With the values obtained, the percentages of inhibition of the pathogen were calculated using the following formula:

$$\text{Percentage of inhibition} = \frac{\text{Core diameter} - \text{Treatment diameter}}{\text{Core diameter}} \times 100$$

From cultures of *M. roreri* in a PDA medium, the percentage inhibition of spore germination and mycelial growth of the phytopathogen was measured; and in the field phase, where the extract was applied on plants with pods less than three months old and the following were evaluated: percentage of *M. roreri* infection in pods, percentage of healthy pods in plants treated with *E. hirta* extracts and additionally, percentages of pods affected with other problems not associated with moniliasis.

For the field trial, the cocoa plantation was prepared in advance through sanitary pruning and fertilization for the stimulation of flower-bearing production. The extract of *E. hirta* was applied at doses of 5 and 10% v/v. In addition, a control (water) and a commercial product recommended for disease control formulated with copper oxide were used. All treatments and controls were applied with a

soybean lecithin-based protective adjuvant at a dose of 0.05%. The preparation of suspensions or dilutions for application was carried out considering an application volume of 120 liters per hectare.

The treatments were applied by atomization with manual pressure between 16H00 and 17H00, to avoid the degradation of the compounds by ultraviolet rays. Applications were made every three weeks until the cobs were four months old. Infection data (number of cobs/tree with oily spots, protuberances, necrosis, and sporulation characteristic of *M. royeri*), healthy cobs/tree, cobs with other infections or disorders not related to *M. royeri* (witches' broom, black cob, or cherelles wilt) were recorded monthly for each tree.

Samples were collected from cobs with symptoms associated with *M. royeri* and from those whose symptoms did not correspond to the pathogen and were taken to the laboratory where isolations were made from which their reproductive structures were observed under the microscope to identify the pathogens morphologically. With the values of cobs obtained, the percentages of cobs infected by *M. royeri*, other infections (cobs showing symptoms not attributed to moniliasis), and the percentages of healthy cobs harvested for each tree were calculated.

Experimental design and statistical analysis

In the laboratory phase, a Completely Randomized Design (CRD) was applied with ten observation units for each treatment. In the field phase, a Completely Randomized Block Design (CSBD) was used. In the plantation, each plot consisted of four trees, and five blocks were established, where each tree corresponded to one repetition. To establish differences between the means of the treatments, a Tukey's multiple range test was performed ($P \leq 0.05$).

The data obtained were analyzed using the STATISTICA program (StatSoft Inc.).

Results and Discussion

Effect of *Euphorbia hirta* extract on *M. royeri* spore germination

The results obtained in the laboratory test indicate that significant differences were found between the different doses of the extract evaluated. Table 1 shows the results obtained when evaluating the effect of the different doses of *E. hirta* extract on the germination of *M. royeri* spores, showing that as the concentration of the extract increases, the percentage of spore germination decreases. This behavior of the pathogen before the concentrations of the extract indicates that indeed the extract of *E. hirta* harms the germination process of *M. royeri* spores. The results of this test had a coefficient of variation of 2.17.

Table 1. Effect of *E. hirta* plant extract doses on spore germination of *M. royeri* pathogen

Treatments	Concentration (%)	Germinated spores (%)	
T0	0	100	F
T1	1	100	F

T2	2	100		F
T3	3	100		F
T4	4	97		E
T5	5	96		E
T6	6	90		D
T7	7	81		C
T8	8	55		B
T9	9	0	A	
Value of P		<0.05		

Means with the same letter in each group of data did not differ statistically according to Tukey's test at 5%.

The treatment with the greatest effect on spore germination was T9 (9%v/v), which completely inhibited germination (0% germinated spores). When counting, germinated spores were observed in the remaining eight treatments and the control (T0). This increase in the case of T8 (8%v/v) was considerable, where it was observed that with a 1% reduction in the concentration of the extract, the percentage of germinated spores increased from 0% to 55%. Consequently, as the extract concentration decreased in the following treatments, it was observed that the amount of germinated spores increased, exceeding 80% germination from T1 (1%v/v) to T7 (7%v/v). No significant differences were found between treatments T1 (1%v/v), T2 (2%v/v), and T3 (3%v/v) with the control without extract (T0), which would indicate that media amended at low concentrations have no spore inhibition effect.

The results show that *E. hirta* extract at concentrations of 1% to 10% can affect the development of the pathogen *M. roreri*; however, it should be noted that the higher the concentration of the extract, the better the potential for inhibition of germination and mycelial growth of the fungus under study. These data are similar to the results reported by Guerrero et al. (2020) who studied six different plant extracts and their effect on germination and in vitro development of *M. roreri*. These authors found that *A. bleado* and *E. hirta* species inhibited 100% of germination at the concentration of 10%.

Effect of *E. hirta* extract on mycelial development of *M. roreri*.

Regarding the effect of the different doses of *E. hirta* extract on the inhibition of the mycelial growth of *M. roreri*, the results of the in vitro study show that all the doses evaluated affected the mycelial development of the phytopathogen. The results indicate that the treatments with higher concentrations of extract increase the percentage of inhibition of mycelial development of *M. roreri* (Table 2). In this parameter, the results obtained showed a coefficient of variation of 3.40.

When analyzing the results, it was observed that the doses of the extracts that had the greatest inhibition on mycelial growth were T9 (9%v/v), inhibiting up to 79.78%, followed by T8 (8%v/v) with 68.44% inhibition, while T7 (7%v/v) and T6 (6%v/v) did not present statistical differences between them, inhibiting 56.33% and 54.44%, respectively. On the other hand, T5 (5%v/v) presented a mycelium development inhibition percentage of 45.44%, and T4 (4%v/v) had a value of 37.00%. Finally, the extract doses that inhibited mycelial growth in the lowest

percentage were T2 (2%v/v) and T1 (1%v/v) with 31.82% and 20.11%, respectively.

Table 2. Effect of *E. hirta* extracts at different doses on mycelial growth inhibition of *M. roreri*.

Treatments	Inhibition of the mycelial growth of <i>M. roreri</i> (%)	
T1 (1%v/v)	20.11	H
T2(2%v/v)	31.82	G
T3 (3%v/v)	34.22	F
T4 (4%v/v)	37.00	F
T5 (5%v/v)	45.44	E
T6 (6%v/v)	54.44	D
T7 (7%v/v)	56.33	C
T8 (8%v/v)	68.44	B
T9 (9%v/v)	79.78	A
Value of P	<0.05	

Means with the same letter in each group of data do not differ statistically according to Tukey's test at 5%.

When the mycelial development of the pathogen was evaluated at concentrations of less than 10%, it was observed that the milkweed extract was able to inhibit mycelial growth by up to 20%. The effect of the extract on the pathogen increases gradually with increasing concentration, so they would be directly related. These results are similar to those reported by Quezada (2021) who applied a 2% ethanolic extract of garlic in culture media where he inoculated *M. roreri* and managed to inhibit the mycelial growth of the phytopathogenic fungus.

In similar research conducted by Ramírez et al. (2011), the authors evaluated extracts (in the form of hydrolase) of *Origanum vulgare* (oregano) on the development of *M. roreri*. The results showed that the *O. Vulgare* extract was able to completely inhibit the growth and development of the microorganism.

Similar results on the effect of plant extracts on other phytopathogens are regularly published. An example of this is the study conducted by Chávez and Aquino (2012) who, when evaluating garlic extract (*Allium sativum*) at concentrations of 20% with potato dextrose agar (PDA) medium under in vitro conditions, obtained a high inhibitory effect on the mycelial growth of *Rhizoctonia sp.*, *Fusarium sp.* and *Sclerotium sp.* fungi.

Studying the effect of an extract on several species of the same pathogen genus is quite common, and results presented by Moo-Koh et al. (2014) reported that the aqueous extract of *Bonellia flammea* at a concentration of 3% presented antifungal effectiveness on seven isolated strains of *Curvularia spp.* finding 100% mycelial growth inhibition in *C. verruculosos*, 87% in *C. lunata*, 82% in *E. rostratum*, 50% in *C. cassicola*, and 20 to 28% in other strains. The results obtained open the possibility of studying the effect of *E. hirta* extract on another

Moniliophthora species of special interest to those who depend on cocoa cultivation, such as *M. perniciosa*, the causal agent of witches' broom.

Effect of *E. hirta* extract on *M. roreri* infection and the number of healthy cobs in the field.

E. hirta extract had a positive effect on the number of healthy cobs on applied plants. The number of healthy cobs transformed into percentages in each of the treatments is shown in Figure 1. In these results, a coefficient of variation of 2.67 was obtained.

The results obtained indicate that there were statistical differences between the treatments with *E. hirta* and the controls (chemical and without application or absolute). The treatment with the highest percentage of healthy cobs was T1 (with the application of 10% v/v of milkweed extract), where the average value of cobs without infection was 95.05%. The treatment with the application of 5% (v/v) milky extract showed intermediate values between T1 and the controls (T3 and T0) with a percentage of healthy cobs of 91.50%.

The preventive effect of the cupric fungicide (copper oxide) on the percentage of healthy cobs did not differ statistically from the control, presenting average values of 87.64% and 87.32% of healthy cobs, respectively. The results indicate, therefore, that the plots with the potential to achieve better yields would be those treated with milkweed extract at 10% v/v.

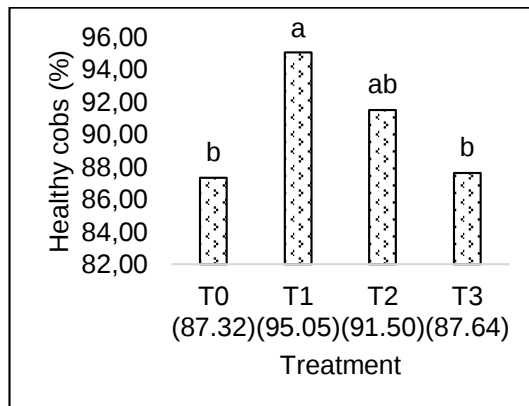


Figure 1. Percentage of healthy cobs in the field with the effect of the different treatments, means with a common letter are not significantly different (Tukey $p > 0.05$).

Infection of cobs with *M. roreri* was present in all treatments studied. However, the percentages of cobs infected by this phytopathogen were statistically different among all the treatments with extract, the copper-based commercial product (T3), and the control without application (T0), as can be seen in Figure 2.

The lowest percentage of infection by *M. roreri* was recorded in the treatment with the application of 10% milkweed extract (v/v) or T1, where the percentage of cobs with symptoms was 2.05%, followed by T2 with the application of milkweed

extract at 5% (v/v), where an average infection value of 3.43% was recorded. To a lesser extent, the impact of the pathogen on the cobs was reduced by the treatment with cupric fungicide (T3), where an average infection rate of 4.94% was recorded. The control without application (T0) showed an average infection rate of 6.52%, approximately three times higher than that achieved by T1.

The values of ear infection caused by *M. royeri*, even in the control, were low, considering that this is a pathology present in the plantations and that it usually presents itself aggressively. Therefore, in the presence of diseased cobs with other symptoms not associated with infections caused by *M. royeri*, the values were recorded in each treatment and the percentages were analyzed (Figure 2).

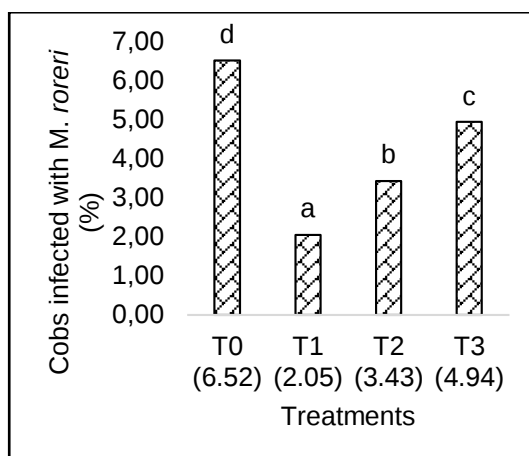


Figure 2. Percentage of cobs infected with *M. royeri* in the field, means with a common letter are not significantly different (Tukey $p > 0,05$).

From the samples of the cobs with other infections with symptoms not associated with *M. royeri*, after isolation and morphological identification in the laboratory, it was determined that the pathogen present in 90% of the samples was *Phytophthora spp.* In these results, a coefficient of variation of 37.74% was obtained.

The infection values of infected cobs not associated with *M. royeri* show that the T1 treatment (10% v/v extract) was the one that presented the lowest infection percentages, with an average of 2.90%, a value that differs statistically from the other treatments. The other treatment with milky extract (T2 5% v/v) and the control without application showed no statistical differences between them, and their infection values were intermediate between T1 and T3 (copper oxide), which presented the highest infection percentage with 7.42% (Figure 3), indicating that the fungicide did not affect the percentage of diseased cobs not attributed to *M. royeri*.

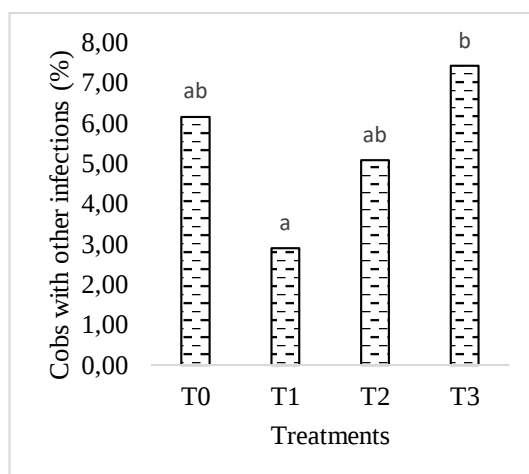


Figure 3. Percentage of cobs with symptomatology not related to *M. roreri*, means with a common letter are not significantly different (Tukey $p > 0,05$).

The results found indicate that the application of *E. hirta* extract on cobs not only protected them from infection caused by *M. roreri* but also the percentage of diseased cobs by other pathogens was lower than in the other treatments and controls. This effect is probably because plant extracts can have antifungal activity on several genera of phytopathogens, and additionally, have an effect on oomycetes such as *Phytophthora* spp. Results of a study conducted by Valero et al. (2014), revealed that when evaluating the inhibitory effect of aqueous extracts of *Larrea tridentata*, *Flourensia cernua* and *Quercus pungens*, at concentrations of 10% and 20%, the extracts managed to inhibit the mycelial growth of five phytopathogenic fungi such as *Phytophthora capsici*, *Botrytis* spp, *Alternaria solani*, *Aspergillus flavus* and *Rhizopus* sp., demonstrating the effectiveness of the extracts on different genera of phytopathogens.

The protection of the cobs with the application of *E. hirta* extract, and the consequent percentage of healthy cobs is due to the concentration of secondary metabolites that have an inhibitory effect on the development of fungi and bacteria. The secondary metabolites reported in *E. hirta* species are diterpene esters, triterpenoids, and palmitates, compounds that have antifungal activity (Barla et al., 2006). The literature reports that most plants with inhibitory effects on pathogens have in their biochemical composition compounds such as phenolic compounds, terpenoids, tocopherols, flavonoids, indole, and glucosinolates (Al-Askar, 2012). Multiple studies reported, indicate that plant species have diverse capacities to inhibit the content of their secondary metabolites (Valero et al., 2014).

When *E. hirta* extract was applied to cocoa trees in the field at concentrations of 5% and 10%, it was found that the percentages of infection by the pathogen *M. roreri* and other pathogens that affect the crop showed lower values, demonstrating the potential of the extract to protect the cocoa pods in early stages. Results of a study conducted by Cazar et al. (2014) showed that when evaluating the inhibitory effect of ethanolic extracts from eucalyptus (*Eucalyptus globulus*) against *Alternaria* sp. in field and greenhouse crops at concentrations of

25% and 50%, they showed antifungal activity compared to the chemical control, due to secondary metabolites such as saponins, tannins, quinones, and flavonoids.

Similar investigations where the effect of ethanolic extracts on other phytopathogens was evaluated are reported by Alvarez (2014) who when evaluating the ethanolic extract of strawberry (*Fragaria spp.*) in field conditions on *Botrytis cinerea* achieved an inhibitory effect by significantly reducing mycelial growth by up to 73.8%. However, when the concentration level of the extract was reduced, there was a significant increase in mycelial growth, which shows that the higher the concentration, the greater the antifungal effect.

Conclusion

The *E. hirta* extract evaluated at the concentrations used in this study harmed spore germination, inhibiting 100% of spore germination and mycelium development with 79.78% of *M. roleri* when used at a concentration of 9% v/v extract. Trees treated with 10% v/v *E. hirta* extract had lower percentages of infection caused by the pathogen *M. roleri* and other pathogens while showing higher percentages of healthy cobs. These results indicate that the species extract has the potential both to improve crop health and to be used as an integrated management tool to reduce the use of chemical pesticides.

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