

OPTIMIZACIÓN DE LA  
DISTILACIÓN *ORIGANUM  
VULGARE* L, CON EFECTO ANTIFUNGO  
EN *MONILIOPHTHORA  
RORERI* (CIF & PAR)

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## — Abstract—

*Moniliophthora roreri* (Cif & Par), since entering Mexico in 2005 (Lopez *et al.*, 2006) it significantly decreased grain production of dry cocoa, passing 43,974.52 to 22,405.01 ton of dry cocoa (Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food [SAGARPA], 2015). This pathogen became the main threat for cocoa producer; the measures were still scarce for sustainable management. The objective of this work is to optimize the extraction process by distilling of *Origanum vulgare* L, by evaluating *in vitro* its antifungal effect; eight were obtained hidrodestillation, these were obtained with fresh and sun-dried material and two solvents (Water: Alcohol) by using the technique of liquid culture medium in test tubes, by consisting of a solution of more cocoa extract water ratio (1:1 v/v) to which they were added fungus conidia. To this solution is added the hydrodistillation of *O. vulgare* L. in relation (1: 1 v / v), by determining its effect on the formation and germination of conidia of the fungus in five observation times (0, 24, 48, 72 and 96 hours). The results show that all hydrodistillation *O. vulgare* L metabolites present with inhibitory effects on the variables evaluated, being the best treatments. It is the best treatments of control over the formation of conidia the O7 and O3 (*O. vulgare* L. dry, 45 g L<sup>-1</sup> water ratio 10.0: alcohol and fresh *O. vulgare* L., 300 g L<sup>-1</sup> ratio of 10: 0 water: Alcohol) the bests, by reaching 68.3% at 48 hours and 65.6% after 72 hours respectively, compared with the control.

**Keywords**

*Plant extracts, hydrolate, Theobroma cacao.*

Cocoa's moniliasis caused by the fungus *Moniliophthora roreri* (Cif & Par) is the main problem in 11 countries of the American continent, including Mexico and Colombia (Phillips et al., 2007; Sánchez and Garcés, 2012). This pathogen under natural conditions exclusively attacks the fruit at any stage of development, with fruits up to three months of age being the most susceptible (López, 2015). Among the main symptoms are protuberances or "humps", oily spots, yellowing or premature maturation and chocolate spots (Merchán, 1980; Evans, 2002; Oliveira and Luz, 2005; López et al., 2006). It causes the total loss of its seeds or decrease in its organoleptic quality due to the degradation that it causes in the tissues (Ramírez, 2013).

In March 2005, it was detected for the first time in plantations of Pichucalco's municipality in the state of Chiapas - Mexico (López et al., 2006), leaving behind the demolition of large number of hectares planted, abandonment of plantations and great production casualties (Ramírez, 2008a; Ramírez et al., 2011a).

With Colombian origins; carried out by the departments of Santander and Antioquia (Phillips et al., 2005; Phillips, 2006; Grisales and Afanador, 2007; Jaimes and Aranzazu, 2010), up to this date there has been found five generic groups of this fungus (Phillips and Aime 2005; Alvarez et al., 2014), which has devastated the country and traditional control strategies have generated collateral results, such as changes in the organism leading to resistance to fungicides and mutations that have originated several strains in some regions (Meinhardt and Rincones, 2008, Meinhardt et al., 2014). The control of *M. roreri* through the use of chemical synthesis fungicides has been tested in several places; but, the results are not completely effective for the management of this disease; in addition, the high frequencies of application, the pollution they cause and their cost are also questioned, since it is often uneconomical for the cacao tree, despite having an effect on the decrease of the disease (Meza and León, 1972; Suárez, 1979; Achicanoy and Buritica, 1981, González, 1982, Cruz, 1986, Martí et al., 1987, Sánchez et al., 2003).

As part of their metabolism, plants have components that are known as secondary metabolites and their chemical properties have been extensively investigated since the mid-nineteenth century (Vergara, 1997; Croteau et al., 2000); which can be a useful tool for pest and disease control, with a very high potential to handle the main phytosanitary problems of agricultural production (Hernández et al., 2007; Barrera and Bautista, 2008).

In Mexico, research has been carried out with extracts of plants and it has been found a great diversity of plants (among them oregano (*Origanum vulgare L.*) with inhibitory effect on the growth and development of

pathogens including *Phytophthora spp.*, *Colletotrichum gloeosporioides* and *Moniliophthora roreri*, (Ramírez y López, 2006; Ramírez, 2008b; Ramírez *et al* 2011a). Oregano and ginger have shown an effect on the inhibition of various types of bacteria and fungi that cause diseases in animals and crop plants, both at the field as at the post-harvest period (Bertelli *et al.*, 2003; Nguiefack *et al.*, 2004; Kulisic *et al.*, 2004; Nostro *et al.*, 2004; Sahin *et al.*, 2004; Sacchetti *et al.*, 2005; Hersch *et al.*, 2005).

The objective of the this research was to provide new alternatives that are environmentally friendly and effective in the management of *M. roreri*, considering the reports of Ramírez *et al.*, (2011a), it was intended to optimize the extraction process by distillation of *Origanum vulgare L.*, evaluating their antifungal capacity, on the formation and germination of *M. roreri* (Cif and Par) conidia isolated from cocoa pods from plantations in Mexico.

## EQUIPMENT AND METHODS

**Pathogen isolation:** The *M. roreri* fungus was multiplied from a fungus strain present in the Agrotechnologies Laboratory of the AUDES Cacao-Chocolate of the Universidad Autonoma de Chiapas, previously isolated from samples of diseased fruits in the state of spot, collected in cacao plantations of Comalcalco's municipality, in the state of Tabasco, Mexico; according to methodology described by Ramírez and collaborators (2011b).

**Hydrolates preparation:** The distillates were prepared in the Agrotechnologies Laboratory of the AUDES Cacao- Chocolate, from Orégano (*O. vulgare L.*), eight hydrolates were obtained, which are described in Table I:

**Table I.** Conventions for different treatments.

Treatments.		Plant	Quantity g L-1	Water: alcohol ratio
1	O <sub>1</sub>	Fresh Oregano	300	10:1
2	O <sub>2</sub>		600	10:1
3	O <sub>3</sub>		300	10:0
4	O <sub>4</sub>		600	10:0
5	O <sub>5</sub>	Dry Organo	45	10:1
6	O <sub>6</sub>		90	10:1
7	O <sub>7</sub>		45	10:0
8	O <sub>8</sub>		90	10:0
9	Absolute control (Abs. cont) Distillated water			

For the extraction process the plant material and the respective solvent were placed in the kettle and subjected to constant heating until the hydrodistillation was obtained in stainless steel distillation equipment made for this purpose.

**Liquid medium test in test tubes:** The methodology described by Ramírez, (2011a) was used to determine the total number of conidia and the number of conidia germinated in the presence of each treatment.

**Stock solution:** Four 50 mm Petri dishes containing 12 days of planting *M. roreri* crop were taken, a superficial scraping of the fungus was performed and washed with 50 ml of sterile distilled water, added in an Erlenmeyer and was added 50 ml of cocoa extract and one drop of tween 80.

**Hydrodistilled solution:** The stock solution was divided into six test tubes: three as a control (with 5 ml of distilled water + 5 ml of stock solution) and three containing the hydrolate to be analyzed (5 ml of hydrodistillate + 5 ml of stock solution); then vortexed to homogenize the mixture and readings were made in the Neubauer chamber, with three replicates, counting the number of total conidia and the number of germinated conidia. Tubes with treatments and their respective controls were incubated in the dark at 28°C +/- 2°C.

**Variables:** The variables that evaluated were: formation of total conidia and number of conidia germinated at 0, 24, 48, 72 and 96 hours, according to the methodology described by Ramírez (2013) and Ochoa (2015).

**Experimental design:** A completely randomized design (DCA) was used with nine treatments, three replicates per treatment at 0, 24, 48, 72 and 96 hours. A variance analysis was performed and, if significant differences were detected, Tukey's measures comparison test was applied at 5%, using the statistical software SPSS STATISTICS 2.0.

## RESULTS AND DISCUSSION

According to the statistical analysis, there were significant differences in the effect of treatments on conidia formation at 24, 48, 72, and 96 hours, while at 0 hours no significant differences were observed (Image 1). At 0 hours, the treatment with the highest conidia formation corresponded to treatment O6 (dry material ratio 10:1 water:alcohol), with 211,11 conidia x10<sup>4</sup> ml<sup>-1</sup>, and the treatment with the lowest formation of these structures was O8 with 143.61 conidia x10<sup>4</sup> ml<sup>-1</sup> (Image 1A). At 24 hours, the treatment with the highest conidia formation was the absolute control, presenting significant

differences with the other treatments, where the O1 treatment had less formation of these structures, with 82.92 conidia  $\times 10^4 \text{ ml}^{-1}$ , where all extracts inhibited the formation of conidia in comparison to the control (Image 1B). At 48 hours, the control treatment presented significant differences in relation to the other treatments, being O3, the one which presented less conidia formation with 86.11 conidia  $\times 10^4 \text{ ml}^{-1}$ , similar to O5 and O7, the last two obtained from dry material (Image 1C). At 72 hours the control presented greater conidia formation, where the O7 treatment had the best behavior with 72.22 conidia  $\times 10^4 \text{ ml}^{-1}$  similar to O5 and O8 (Image 1D). At 96 hours, the treatment with the best performance was O7, with 77.78 conidia  $\times 10^4 \text{ ml}^{-1}$ , presenting significant differences with the control (Image 1E).

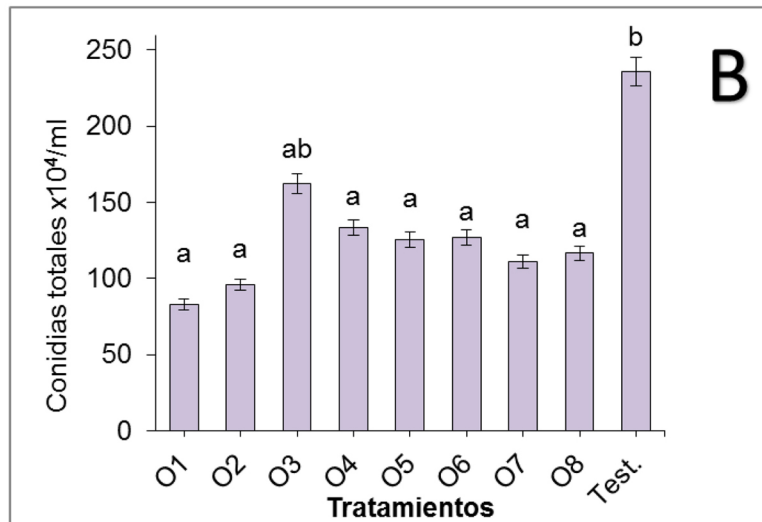
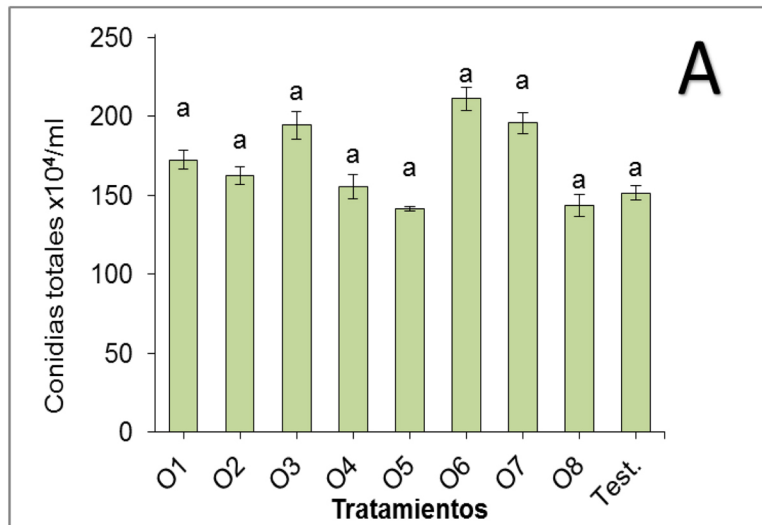
After 48 hours, the O7 treatment presented low values in the formation of conidia, reaching 68.3% at 72 hours.

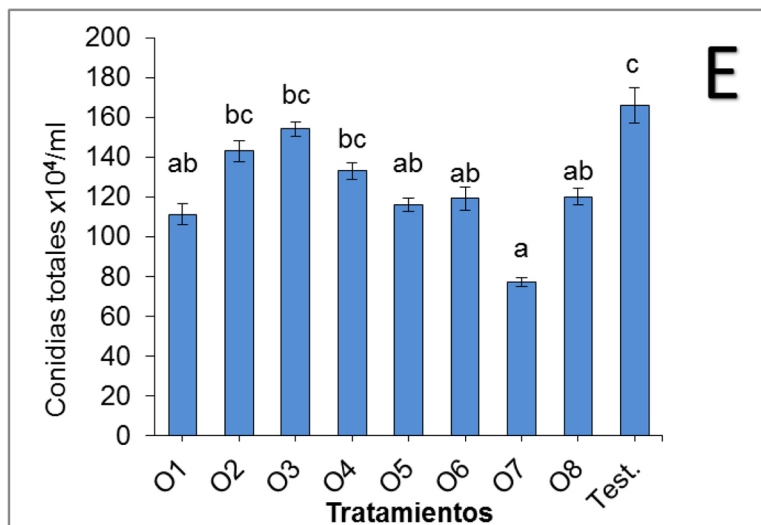
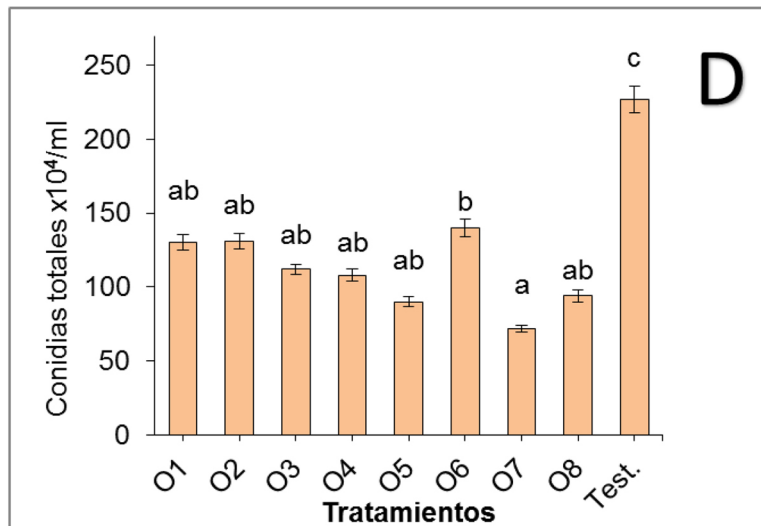
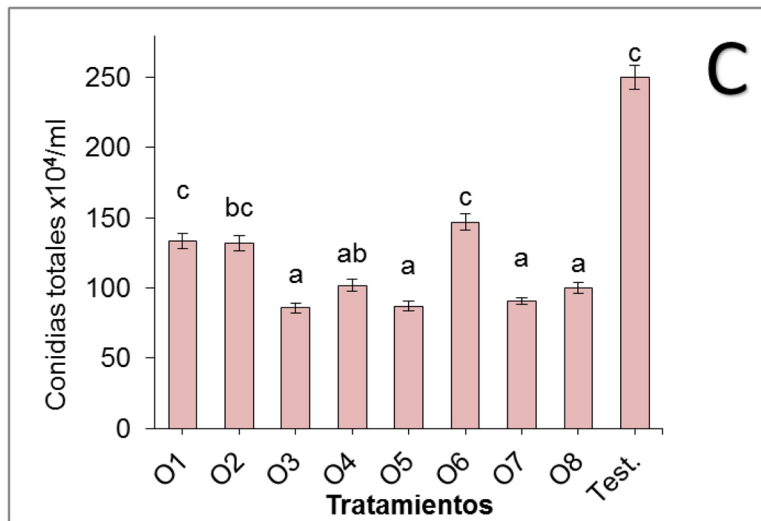
The results obtained indicate that the oregano hydrolate has a regulatory effect of *M. roreri*, as reported by Ramírez (2013), adding to the reports of antibacterial and antioxidant activities, as well as having an effect as a natural food preservative (Hersch *et al.*, 2005; Kulisic *et al.*, 2004; Nostro *et al.*, 2004).

As for its antimicrobial activity, the research results confirm that the oregano's essential oil has multiple effects, including antimicrobial against gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* and on gram negative bacteria (Albado *et al.*, 2001), besides observing an antifungal activity for the inhibition of the formation and germination of conidia.

According to García *et al.*, (2006) cinnamon's essential oils (*Cinnamomum zeylanicum*) and oregano (*Origanum vulgare*) were evaluated for their antifungal activity against *Aspergillus flavus* and the production of aflatoxins in pecans. Both oils had fungicidal activity in vitro against *A. flavus*, the oregano's essential oil from 1000 ml.  $\text{L}^{-1}$ .

**Image 1.** Oregano extracts' effect, on the number of total conidia  $\times 10^4 \text{ ml}^{-1}$ . A: 0 horas. B: 24 hours. C: 48 hours. D: 72 hours. E: 96 hours. Treatments with different letters indicate significant differences according to Tukey's test ( $P \leq 0.05$ )







According to Ramírez *et al.*, (2011a), the extraction form of *O. vulgare* influences the extraction of active metabolites, since the hydrolate presented total inhibition of the growth and conidia formation of the pathogen. Therefore, according to Sahin *et al.*, (2004) the most important component of oregano is essential oil, containing 60-75% volatile phenols, particularly thymol and carvacrol, which have a similar chemical structure (carvacrol naturally is a thymol isomer) and an antimicrobial effect.

For the conidia germination variable, none of the oregano treatments presented statistical differences at any moment of observation, although at 48 hours none of the oregano treatments present germinated conidia, the absolute control (distilled water) presented 1.39 germinated conidia  $\times 10^4$  ml<sup>-1</sup>.

## CONCLUSIONS

All oregano treatments presented significant differences with respect to the control (distilled water) after 24 hours, inhibiting in different percentages the formation and germination of *M. roreri*, conidia, being able to be associated to the production of secondary metabolites that have effect antifungal activity affecting the formation of structures that produce cocoa moniliasis.

The O7 treatment (dry oregano, 45 g/L 10:0 water: alcohol ratio) was the best hydrolyzate of the eight evaluated, showing an inhibitory effect after 48 hours, which over time presented the best levels of control in the formation and germination of *M. roreri* conidia with a percentage of inhibition of 68.3% at 72 hours; therefore, it constitutes a potential alternative for the management of cocoa moniliasis, both for conventional and organic production systems.

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