Antifungal properties of essential oil of mexican oregano (*Lippia berlandieri*) against *Fusarium oxysporum* f. sp. *lycopersici*

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Propiedades antifúngicas del aceite esencial de orégano mexicano (*Lippia berlandieri*) contra *Fusarium oxysporum* f. sp. *lycopersici*

Resumen. Se determinó la actividad antifúngica del aceite esencial del orégano mexicano (*Lippia berlandieri*) contra *Fusarium oxysporum* evaluando sus efectos sobre el crecimiento micelial del hongo por medio de las fases de contacto y volátil y en términos de producción de biomasa. También fue evaluada la capacidad del aceite esencial para desinfectar semillas de tomate infestadas con *F. oxysporum*. Las concentraciones mínimas inhibitorias para la fase de contacto y volátil fueron de 0.2 µl ml^{-1} de medio y 0.15 µl ml^{-1} aire respectivamente y la producción de biomasa fue totalmente inhibida a la concentración de 0.2 µl ml^{-1} de caldo. El nivel mínimo de inóculo requerido para producir el 100% de infestación en las semillas fue de 10^5 esporas ml^{-1}, mientras que la concentración de 0.5 % de aceite esencial inhibió completamente la colonización de las semillas sin afectar su capacidad de germinación. Estos resultados muestran el potencial del aceite esencial del orégano mexicano como un importante inhibidor así como un agente fungicida de *F. oxysporum* en semillas de tomate.
Palabras clave: Antimicrobianos naturales, compuestos fenólicos, Verbenaceae, patógenos de plantas, semilla de tomate.

Abstract. Antifungal activity of essential oil of mexican oregano (*Lippia berlandieri*) against *Fusarium oxysporum* was determined by measuring mycelia growth and biomass production using direct contact as well as volatile phase of the essential oil. The capacity of the essential oil as a fungicide against *F. oxysporum* infected tomato seeds was evaluated too. The minimum inhibitory concentrations of essential oil under contact and volatile phase were 0.2 µl ml^{-1} of medium and 0.15 µl ml^{-1} air respectively and biomass production was totally inhibited at 0.2 µl ml^{-1} of broth. A minimum inoculum of 10^5 spores ml^{-1} is required to produce 100% of infestation and 0.5% of oregano essential oil completely inhibited the colonization of the fungus without affecting seed germination rates. These results showed the potential of essential oil of mexican oregano as an important inhibitor as well as a fungicide agent against *F. oxysporum* on tomato seeds.
Key words: Natural antimicrobials, phenolic compounds, Verbenaceae, plant pathogens, tomato seed.

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Introduction

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans is one of the most prevalent and damaging diseases of tomato that causes considerable losses, especially in susceptible varieties and under favorable weather conditions (Agrios, 2007). Although *F. oxysporum* is widely reported as a soil-borne pathogen, Reis and Boiteux (2007) concluded that this fungus can infest tomato crops via contaminated seeds. Several studies have indicated that infested seeds may contribute significantly to dissemination of another *Fusarium* species that are plant pathogenic fungi (Menzies and Jarvis, 1994; García-Garza, *et al.*, 1999; Ochoa and Ellis, 2002; Garibaldi *et al.,* 2004; Bennet *et al.*, 2008). Fungicides are used to control this problem. Unfortunately, these chemicals are not readily biodegradable; tend to persist for years in the environment and some fungi have developed resistance to them (Bajwa *et al.,* 2003). One alternative to this problem could be the use of essential oils and plant extracts to control plant pathogens. These products have been studied for many years and different spices of oregano have shown antifungal (Daferera *et al*., 2003; Viuda-Martos *et al*., 2007), antibacterial (Burt and Reinders, 2003; Chorianopoulos *et al.,* 2004; Zivanovic *et al.,* 2005), insecticidal (Pavela, 2005) nematicidal (Oka *et al.,* 2000) and antioxidant (Sánchez *et al.,* 2003; Skerget *et al.,* 2004) activities. The most common genus of the oregano species are *Origanum*, native of Europe and *Lippia*, native of Mexico. Their composition depends of species, climate, altitude and stage of growth (Arcila-Lozano *et al.,* 2004). There are some differences between these species; mexican oregano has larger leaves and stronger flavor than european species. The stronger flavor is attributed to its higher essential oil content than other varieties (Dunford and Silva, 2005). Mexican oregano (*Lippia graveolens* HBK, *Lippia berlandieri* Shauer) is classified as belonging to the family *Verbenaceae* and more than twenty four volatile compounds have been identified including β-myrcene, α-terpinene, γ-terpinene, *p*-cymene, cineole, carvacrol and thymol (Yousif *et al*., 2000; Vazquez and Dunford, 2005). The essential oil of this plant has been recognized as an important antioxidant agent (Rocha-Guzmán *et al.,* 2007). Portillo-Ruiz *et al*. (2005) demonstrated the antifungal activity of mexican oregano versus food-contaminant fungi and Hernández *et al*. (2003) confirmed antibacterial effects of hexane extracts of mexican oregano against Gram-positive and Gram-negative bacteria. In addition, essential oil of mexican oregano has been shown to have bactericidal activity against five species of *Vibrio* (Paredes-Aguilar *et al.,* 2007) and is reported to inhibit *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (Avila-Sosa *et al.,* 2010). In this context, the antifungal properties of essential oil of mexican oregano against *F. oxysporum*, was evaluated in this study.

Materials and methods

Plant material and extraction of essential oil of oregano

Oregano plants were collected from El Barreal de Guadalupe, Durango, México, and were identified by the Department of Botany of Universidad Autónoma Agraria Antonio Narro Unidad Laguna (ANUL), where the sample specimens have been deposited. Leaves and flowers of oregano were used for extraction of essential oil. It was obtained by steam distillation for 3 h. The oil yield was 4% and the density was 0.96 g ml^{-1} at room temperature. The oil was separated and dried over anhydrous sodium sulfate and stored in an amber bottle at 4°C for further use.

Pathogen fungal and inoculum production

F. oxysporum was isolated from diseased tomato plants collected from an infested field in Ejido Monterreycito, Durango, México. Parts of plants with symptoms of *F. oxysporum* infection were surface sterilized by immersion in 0.3% sodium hypochlorite (NaOCl) for 10 min, rinsed in sterile distilled water, transferred to potato dextrose agar (PDA) medium in 90 x 15 mm Petri dishes and incubated in dark at 28°C for 7 days. Fungal isolate was tested for pathogenicity on tomato seedlings (Reis and Boiteux, 2007) and found to be pathogenic. Spores were collected in sterile distilled water after 7 days growth in PDA medium, adjusted to 10^5 spores ml^{-1} using a hematocytometer and used as inoculum for further studies. This fungal pathogen was identified based on its colony morphology and conidial characteristics (Summerell *et al*., 2003) and has been deposited in Laboratorio de Fitopatología. Instituto Nacional de Investigación Forestal, Agrícola y Pecuaria. Matamoros, Coahuila, México**.**

Assessment of antifungal effects of contact and volatile phases of essential oil on mycelial growth

The antifungal effect of contact and volatile phase was evaluated according to the method proposed by Soylu *et al*. (2007)**.** Different concentrations of essential oil were prepared by dissolving the appropriate volumes in sterile Tween 20 (0.5%, v/v) and added aseptically to flasks with sterile molten PDA medium to obtain concentrations of 0.025, 0.05, 0.1, 0.15, 0.20 and 0.25 µl ml^{-1} of medium and 20 ml of this mix was poured in Petri dishes (90x15 mm). Agar disks of 5 mm diameter from actively growing mycelium of *F. oxysporum* were placed at the center of each Petri dish and incubated for 7 days at 28°C. Petri dishes with only PDA and Tween 20 were used as positive growth control. In order to determine the effectiveness of the volatile compounds of the essential oil, Petri dishes (20 ml of PDA and 50 ml of air) were filled with PDA medium and placed with 5 mm diameter agar discs from 7-days-old *F. oxysporum* culture. Different amounts of essential oil (1.0, 2.5, 5.0, 7.5, 10.0 y 12.5 µl) equivalent of 0.02, 0.05, 0.1, 0.15, 0.20 and 0.25 µl essential oil ml^{-1} air were placed directly on the cover inside the Petri dishes. After the oil was applied, Petri dishes were sealed and incubated 7 days at 28°C. Petri dishes containing only PDA medium were used as positive growth control. In both methods, the diameter of the colony was measured and expressed as percentage of mycelial growth inhibition (MGI) by using the formula, MGI (%) = $[(d_c - d_t)/d_c]$ x 100, where d_c = mean the mycelial growth diameter in control Petri dishes and d_t = mean the mycelial growth diameter from Petri dishes with essential oil. Experiments were conducted in triplicate. The minimum inhibitory concentration (MIC) of the oil was considered to be the lowest concentration of the oil that gave 100% inhibition on mycelial growth (Daouk *et al*., 1995).

Determination of fungal biomass production

Different concentrations of essential oil (0.05, 0.1, 0.15, 0.175, 0.20 and 0.25 µl ml^{-1} of broth) were added into the flasks with potato dextrose broth (PDB) inoculated with a suspension of 10^5 spores ml^{-1}. All flasks were incubated at 28°C in a shaking incubator at 150 rpm for 7 days, and were periodically determined at every 24 h. Then cultures were centrifuged at 3000 rpm for 10 min at 4°C in a refrigerated centrifuge and washed twice with sterile distilled water and dried at 60°C until constant weight. Flasks which did not receive essential oil were used as controls. The percentage of inhibition of fungal biomass production was calculated using the following formula: % Inhibition = [(C – T)/C] x 100, where C and T represent the weight of mycelia from control and flasks with essential oil, respectively. Triplicates were maintained.

Optimum level of inoculum and determination of antifungal effect of the essential oil of oregano on the development of *F. oxysporum* in tomato seeds

To determine the minimal concentration of inoculum needed to infest 100% of seeds, different concentrations, viz., 10^2 – 10^7 spores ml^{-1} were prepared. Lots of seeds were immersed for 5 min in each spore suspensions. The wetted seeds were

blotted on sterile paper towels, placed on a sterile filter paper onto Petri dishes and kept in desiccator for 5 days (Punja and Parker, 2000). The inoculated seeds were plated in PDA medium and incubated at 28°C for 7 days to observe the growth of *F. oxysporum* around the seeds. Seeds treated with sterile Tween 20 (0.5% v/v) and a commercial fungicide were used as controls. To determine antifungal effect of essential oil of oregano on the development of *F. oxysporum* in tomato seeds, disinfested seeds were immersed for 5 min in a suspension of 10^5 spores ml^{-1}. After this period seeds were placed on sterile filter paper in Petri dishes and kept in desiccator for 5 days. On the other hand, different concentrations of essential oil (0.1, 0.25, 0.5, 0.75 and 1.0%) were prepared by dissolving the requisite amounts in sterile Tween 20 (0.5% v/v) solution and agitated at high speed until completely dispersed. After this, inoculated seeds were submerged for 10 min at all test concentrations. Finally seeds were placed in PDA medium and incubated at 28°C for 7 days to observe the development of the fungi around the seed. Seeds treated with Tween-20 and with commercial fungicide were used as controls. In addition, non-treated and treated seeds with the different concentrations of essential oil were tested for germination. Ten seeds were used for each replication and triplicates were maintained for all treatments.

Data analysis

All experiments were performed using completely randomized design. Data were analyzed using analysis of variance (ANOVA). Mean separation was performed by the Protected LSD method. Statistical analysis was performed with the SAS version 8.1 (SAS Institute, Inc., Cary, NC).

Results

Mycelial growth inhibition (MGI) of *F. oxysporum*

The mycelial growth inhibition percentage by oregano essential oil by direct contact as well as in volatile phase is presented in Figure 1. It was observed that essential oil showed antifungal effects at all concentrations tested. There were significant differences (P>0.05) between the different concentrations in the direct contact method, *viz*., 0.025, 0.5, 0.1 and 0.15 µl ml^{-1} of medium corresponding to 4.2, 16.8, 53.6 and 93.8 % of mycelial growth inhibition. Total inhibition of mycelial growth was recorded at 0.2 and 0.25 µl ml^{-1} of essential oil. In the case of volatile phase too significant differences (P>0.05) were observed between concentrations of 0.02, 0.05 and 0.1 µl essential oil ml^{-1} air corresponding to 35, 64 and 88.3 % inhibition of mycelial growth. It can be observed that the volatile phase produced stronger antifungal effects than contact phase at the same concentrations. The minimal concentration of essential oil required for total inhibition of mycelial growth was 0.15 µl essential oil ml^{-1} air, while it was 0.2 µl ml^{-1} of medium in the case of contact method. These results were considered to represent the MIC values.

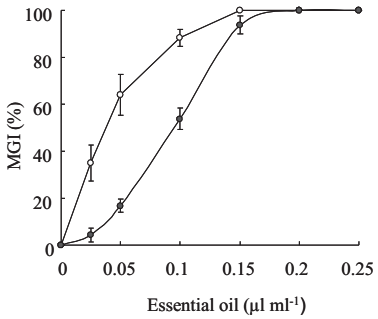

Figure 1. Effect of different concentrations of oregano essential oil on mycelial growth inhibition percentage (MGI) of *F. oxysporum* in direct contact (—●—) and in volatile phase (—○—) in µl ml^{-1} of medium and µl ml^{-1} of air respectively. Data are the average of three replicates.

Determination of fungal biomass production

The results clearly showed that biomass production of *F. oxysporum* was influenced by the presence of oregano essential oil in the culture medium. It can be observed that all concentrations markedly inhibited the fungal biomass production. There was significant difference in the biomass production between treatments containing 0.05 µl ml^{-1} of essential oil and control and no significant difference was observed on seventh day between 0.1 and 0.15 µl ml^{-1}. It can be observed that the growth of the fungus was similar at all concentrations for all 7 days of growth. However it can be noted from Figure 2 that 0.05, 0.10 and 0.15 µl ml^{-1} of oregano essential oil recorded significant inhibition of fungus biomass production during the first day in relation to the rest of the days. No significant difference was observed between all concentrations between fourth and seventh day and concentrations of 0.20 and 0.25 µl ml^{-1} showed 100% inhibition of fungal biomass production all days.

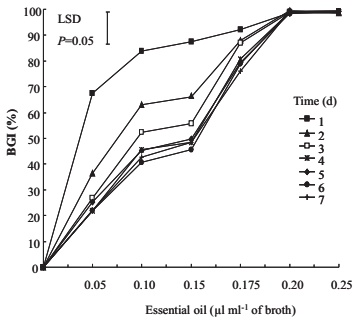

Figure 2. Effect of different concentrations of oregano essential oil on percentage inhibition of *F. oxysporum* biomass production. Data are the average (± SD) of three replicates.

Determination of the level of inoculum and antifungal effect of the essential oil of oregano in tomato seed infested with *F. oxysporum*

It was observed in this study that the lowest level of inoculum required to produce 100% of infestation was 10^5 spores ml^{-1} (Figure 3). There were significant differences (P>0.05) between inoculum levels of 10^2, 10^3 y10^4 spores ml^{-1}, which presented 12.5, 20 and 75 % infestation respectively. With respect to the antifungal effect of the essential oil on the development of *F. oxysporum* inoculated on tomato seeds, it was found that different concentrations of essential oil significantly inhibited the development of the fungus when compared with untreated controls. It was established that at 0.5% concentration, seeds were well protected against this fungus infestation since it completely inhibited the colonization of the seeds (Figure 4).

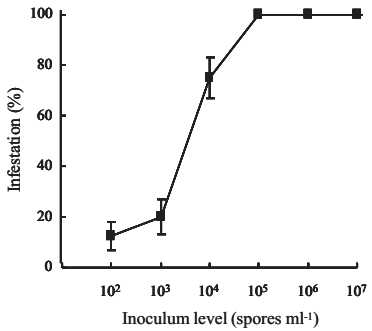

Figure 3. Influence of different inoculum levels on infestation percentage of tomato seeds by *F. oxysporum*. Data are the average (± SD) of three replicates.

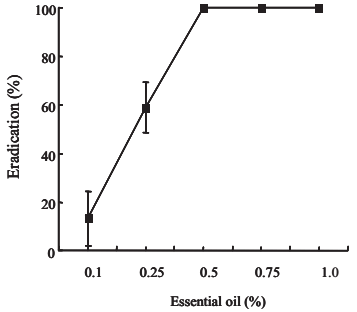

Figure 4. Percentage of antifungal effect of different concentrations of essential oil against tomato seeds infested with 10^5 spores ml^{-1} of *F. oxysporum*. Data are the average (± SD) of three replicates.

Discussion

Different species of oregano have been studied extensively and are reported to contain different as well as important inhibitors against many agents that infect several foods and crops. However, the efficiency of essential oil of oregano as a biocide has been done on oregano belonging to european species. It is observed that chemical composition of mexican and european species is similar, but, principal active components are present at different concentrations. mexican oregano is characterized by its high content of thymol, present as the main constituent at 40% to 60% of total volatiles, and carvacrol at 5 to 25% (Lawrence, 1984). On the contrary, the principal active components of *O. vulgare* are carvacrol (59%) and thymol (6.5%) (Lee *et al*., 2007). With regard to their biocide effect, results presented in this study clearly showed that essential oil of mexican oregano remarkably inhibited the development of *F. oxysporum.* Earlier Daferera *et al*. (2003) confirmed the effectiveness of essential oil of *O. vulgare* against *Botrytis cinerea, Fusarium solani* var. *coeruleum* and *Clavibacter michiganensis.* Later Velluti *et al.* (2004) evaluated the ability of 37 essential oils at different water activity (a_w) and temperature conditions and reported that the essential oil of the same oregano showed antifungal activity against *Fusarium verticillioides, F. proliferatum* and *F. graminearum*. On the other hand, differences were recorded between the two methods of treatment, *viz*., direct contact and volatile phase of essential oil with regard to the inhibition of mycelial growth of *F. oxysporum.* It was observed that the inhibition by the volatile phase of essential oil was greater than direct contact method. Similar to the results obtained in this study, Soylu *et al.* (2007) too reported that the volatile phase of essential oil of *O. syriacum* var. *bavanii* showed stronger antifungal activity than the contact phase against the mycelial growth of *Sclerotinia sclerotium* under *in vitro* conditions. Inouye *et al*. (2000) established that fungicidal and fungistatic action of oregano essential oil is due to the absorbance of vapors of the essential oil by fungal mycelium. It can be observed from our study that different concentrations of essential oil of mexican oregano caused a significant decrease in biomass production and concentrations of 0.20 and 0.25 µl ml^{-1} showed 100% inhibition of fungal biomass production since the first day. These results demonstrated that this oil create an inappropriate environment for the growth of *F. oxysporum* in liquid medium. These results are in accordance with a previous study (Daouk *et al.,* 1995) that reported antifungal action of essential oil of *Origanum syriacum* L. against *Aspergillus niger, F.oxysporum* and *Penicillium* species in yeast extract sucrose broth (YES). It was also observed that the minimum concentration of inoculum required to infest 100% of seeds was 10^5 spores ml^{-1}. At 0.5% concentration, the essential oil of oregano exerted the antifungal effect on the infested tomato seeds. No adverse effects were observed on the germination of the seeds due to the application of essential oil. The results of this study gains importance since there is evidence that some plant pathogens can infest new growing areas via contaminated seeds (Menzies and Jarvis, 1994; García-Garza *et al*., 1999; Ochoa and Ellis, 2002; Garibaldi *et al.,* 2004; Reis and Boiteux, 2007; Bennet *et al.*, 2008).The capacity of inhibition of different species of oregano has been attributed to the compounds with phenolic structures that are present in the oil such carvacrol and thymol. It was found that these compounds are highly active against different genera of bacteria (Dorman and Deans, 2000). Lambert *et al.* (2001) showed that the mechanism of inhibition of these components against *Staphylococcus aureus* and *Pseudomonas aureginosa* is due to the damage to the membrane integrity, which further affects pH homeostasis and equilibrium of inorganic ions. The results of this study clearly showed the capacity of essential oil of mexican oregano as an important inhibitor as well as fungicide agent against *F. oxysporum* on infested tomato seeds.

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