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Effect of Emulsified Oregano Oil on Alternaria Alternata (In Vitro Tests) and On Lycopersicum Esculentum Mill Seedlings (In Vivo Tests)

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Abstract

Oil essence from aromatic plants has been considerable interest for antimicrobial properties in food and agriculture. This study evaluates the effect of oregano oil as a natural pesticide on a fungal pathogen (*Alternaria alternate*) isolated from red tomatoes (*in vitro* test) and effect on seedlings of it is under greenhouse condition (*in vivo* test). Microcosms tests were conducted used concentrations of 1%, 5% and 10% oregano oil emulsion over the growth of the fungus, showing a percentage of inhibition of 79.91%, 99.90% and 100%, respectively. Also, the fungicidal effect of the oregano oil was assessed in greenhouse tomato seedlings (n=20) with the same concentrations, the treatment of the 1% concentration showed the highest number of healthy leaves per plant performing even better than the chemical pesticides used.

Keywords: *Alternaria alternata,* biofungicide, *in vitro* trial, *in vivo* trial, *Lycopersicum esculentum* Mill, oregano oil.

1. Introduction

In recent years, efforts have been made in the implementation of additives and antioxidant bioactive elements derived from agricultural industry and essential plant oils which have proved to be useful to protect crops against pathogens during production **(Ruiz *et al.,* 2012).** These studies have been developed to improve the efficiency of resources exploitation and reduce the use of pesticides **(Pathak *et al.,* 2010; Badakhsh *et al.,* 2010).** Recently research shown the use of native plant in order to obtain natural products with antimicrobial and antifungal activity (**Mello *et al*., 2012; Saglam*et al*., 2013).**

Plant extracts are among the natural products that present antimicrobial, antifungal, and antiviral properties **(Cano *et al*., 2004)**. Some studies showed that essential oils from spice such as fennel, peppermint oregano had highest anti-microbial activity (**Abo-El Seoud*et al*.**, 2007;**Saglam*et al*., 2013**).Interest in such characteristics has increased in recent years because of the current tendency towards a "green" market free of harmful chemical compounds **(Arana-Sánchez *et al.,* 2010).**

In Mexico, oregano is primarily used as a food seasoning and in the pharmaceutical industry for its antioxidant properties in systems containing highly polyunsaturated fatty acids. The main antioxidant components of the oil are carvacrol and thymol (**Saglam*et al*., 2013),** besides phenolic compounds have been proven to have a natural ability to inhibit oxidation **(Terenina *et al*., 2011). Paredes *et al.* (2007)** related their antimicrobial properties to species type, each specie shown different effect due to the geographical conditions of the crop, harvest periods and extraction methods.

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Mexican oregano *(Lippia berlandieri Schauer*) was tested as bactericide, on five species of *Vibrio* highly pathogenic to humans: *V. cholerae, V. parahaemolyticus, V. vulnificus, V. mimicus and V. alginolyticus,*inhibiting to 50% of the population at 100 and 200 mg L^{-1} of oil oregano (**Paredes *et al.*,2007).**

In recent years the medicinal and specie cultivation in Puebla, Mexico is an activity that present high profitability for the inhabitants of different municipalities, in this way the use of oregano oil as biopesticide might be a favorable option towards a new market for the farmers, considering the new sustainable technologies and "green market"(**Herrera-Inforural, 2010**).

In addition, Mexico is a country with significant agricultural production; 60% of its territory is used for food production **(SAGARPA, 2010).** Among the most economically important crops is the red tomato (*Lycopersicum esculentum* Mill*)*; the tomato is the second most-planted crop after the green chile, and the first in terms of production volume, the state of Sinaloa being the main producer with 35% of the national total. Greenhouse-grown red tomatoes are important for their yield and price, which make them highly profitable among farming products **(SAGARPA, 2010).** Greenhouse production volume is 200 ton/ha and so it is considered the highest producing greenhouse crop **(De Santiago, 2008).** Nevertheless, due to physical and biological factors, primarily pests and diseases, production loss occurs and fluctuates between 5% and 20% of the yield **(INIFAP, 2010).**

The red tomato is the most profitable crop in Mexico (**Financiera Rural, 2008**), however farmers incur significant loses in production. For the most important food and cash crops, these losses are estimated to be around 20% (**Jalander and Gachander, 2012).** Pathogenic fungi constitute one of the main causes of this lose in production. Consequently, producers have to invest from up to 20 %of their capital in pest management (**Ramírez *et al*., 2004).**

There are about 200 illness identified in the red tomato, of which 30 are economically important, e.g. canker, leaf spot, wilts, blights and root rots caused by fungi and bacteria. Studies carried out by **Mehmood *et al.* (2014)** show the pathogenic potential of the fungus *Alternaria alternata,* onearly blight of tomato, stem canker, black mold rot, leaf spot, and black shoulder.

In Mexico, many producers of greenhouse tomatoes choose natural or organic pesticides which allow them to sell their produce on national and international markets ***(*Cano *et al*., 2004).*** This also carries less risk of intoxication from pesticide exposure and enables the production of less perishable produce without the use of synthetic additives which cause adverse health effects ***(*Cano *et al*., 2004; Mehmood *et al*., 2014).** The objective of this study was to evaluate the effects of emulsified oregano oil, in two stages; in the first stage, was evaluated as a fungicide agent using *Alternaria alternata* under laboratory conditions; in a second step the effect on tomato seedlings was evaluate under greenhouse conditions as a sustainable alternative for growers.

Material and Methods

Fungus isolation

Sample taking was done at Centro de Innovación Tecnologica en Agricultura Protegida in Mexico (CITAP-UPAEP). Samples were taken from red tomato seedlings in a previously sterilized plastic bag. Subsequently, a bacteriological loop was used to take samples from the surface of the leaves visibly infected by fungi, which were grown on potato-dextrose-agar (PDA) medium [Bioxon, Mexico] under dark conditions at room temperature, for five days, the most dominant fungus strain was selected and reseeded until a pure culture was obtained.

For the molecular identification tests, genomic DNA of the isolated fungal strain was extracted using the ZR Fungal/bacterial DNA Kit (Zymo Research, USA) The intergenetic transcriptional regions (ITS1, ITS2) of fungal 18S ribosomal DNA, which includes the 5.8s rRNA region, was amplified using the primers ITS5 (5´-GAAGTAAAAGTCGTAACAAGG-3´) and ITS4B (5´-TCCTCCGCTTATTGATATGC-3´). The amplification program consisted of 30 cycles of 95°C for 45 s, 50°C for 45 s and 72°C for 45 s, with a final extension stage of 72°C for 7 min, using an iCycler (BIO-RAD) thermal cycler. The PCR products were analyzed by agarose gel electrophoresis for ITS bands and then purified using the QIAquick PCR purification kit (QIAgen). The samples were sequenced and deposited in the laboratories of the National Autonomous University of Mexico (Universidad Nacional Autónoma de México http://www.ibt.unam.mx/sintesis/secuenciacion.html).

The sequencing results were compared by alignment with available sequences from the GenBank database using the BLAST platform (http://blast.ncbi.nlm.nih.gov/) for identification of the fungal species.

Effect of oregano oil on fungal structure (*In vitro* tests)

Oregano oil was obtained from Agroindustrial Don Pablos, in Chihuahua, Mexico (http://www.grupoalianzaempresarial.com/). Oregano oil was emulsified by homogenization in water with Tween 80 (5%) as detergent.

To observe the effect of the oregano oil emulsions on the structure of fungi, micro cultures were made following Redill's technique. We applied 500 μL of oregano oil onto previously sterilized 1 x 1 cm filter paper at concentrations of percentages, 5%, and 10%. A 1.5 x 1.5 cm^2 of PDA agar (Bioxon) was placed over the filter paper and inoculated by stabbing. Fungi strain was compared with a blank micro culture which consisted of placing the PDA medium without filter paper, without oregano oil. After a 5 days incubation period under sterile conditions, the micro cultures were stained with cotton blue and observations were made using a microscope.

Inhibition test by conidia count

To evaluate the effect of oregano oil on *Alternaria Alternata* method spore count was performed. The fungus was previously grown on potato dextrose agar medium (PDA), after it is was cut ring of 0.5 cm diameter and put onto previously sterilized filter paper (of 2.5 cm diameter) at the center of the Petri box with PDA medium. The filter papers contained 1%, 5%, and 10% concentrations of emulsified oregano oil; control fungi were also grown without oregano oil.5 ml of a solution of Tween 80 at 0.1% was used to wash the conidia, ensuring the full extension of the petridish was covered by circular movements. We then took an aliquot of 200 μL of the conidia wash liquid, added it to the Neubauer chamber and examined it under microscope using the technique described by Zhang *et al.* (2008). The results were presented according to the following formula:

$$\% \text{ Inhibition} = 100 \times [(E\text{control} - Ei) / E\text{control}]$$

Where E control = the number of conidia without oregano (conidia/μL) and Ei = number of conidia with the different concentrations of oregano (conidia/μL)

Effect of oregano oil on tomato seedling (*In vivo* tests)

Twenty seedlings were usedfor each treatment (n=20). The evaluated treatments were: T1 (control) without oregano oil or chemical pesticide; T2 (control) with chemical pesticide (Oximet 2 g/L and Cumaricin 2 g/L)[Bayer, Mexico]; T3 with oregano oil at a concentration of 1%; T4 with oregano oil at 5%; T5 with oregano oil at 10%. The seedlings were given the same maintenance using a nutritive solution to feed them and the greenhouse temperature varied between 20° and 24°C. The response variable was the number of healthy leaves that presented no fungal growth and kept their green color. The results were analyzed with a test medium using variance analysis to evaluate whether the different emulsions had had a significant effect on the number of healthy leaves. The statistical analysis of the data was done using the MINITAB 16 statistics package. A mean test was performed using variance analysis in order to evaluate whether the different emulsions had a significant effect on the number of healthy leaves.

Results and Discussion

Fungal pathogen strain identification

Following fungus isolation from tomato seedlings, an evaluation freproductive structures revealed many similarities with *Alternaria alternata* (Figure 1).In these micro culture was observe conidia structure and septate hyphae which are typical of this species**(Green *et al*., 2005**), they develop in chains of 10 or more highly branched conidia from short conidiophores **(Carrillo, 2003)**. Subsequently was made molecular identification, the result of Gen bank confirmed 100% similarity in base sequence.

Effect of oregano oil emulsified on *Alternaria alternata* (*In vitro* test)

The effect of oregano oil on the fungus *A. alternate* was evaluated using spore count and micro culture; it was conducted in order to know the physiological response of the fungus during exposure of oregano oil in concentrations 1, 5 and 10%. The results of spore count display in Figure 2, fungal inhibition is observed on the treatment with the different concentrations of emulsified oregano oil.

The results shows that at a concentration of 1% of oregano oil the fungus still developed (80% of inhibition), however, at concentrations of 5% and 10% there was no fungal growth at all (Table 1). **Cueto *et al.* (2010)** evaluated and characterized the essential oil from Mexican oregano as a fungicide against *F. oxysporum,* they conducted *in vitro* trial on infected tomato seeds, observing that a concentration of 0.20 and 0.25 µl of essential oregano oil achieved total inhibition of fungal development on the first day, with no adverse effect on seed germination, the authors attributed its fungicidal property to the high thymol content (40-60%) present in its main volatile components, followed by carvacrol (5-25%).

Table 1: Inhibition of conidia production at different concentrations of oregano oil

Treatments	Conidia count (mL^{-1})	Inhibition (%)
Control	142500 ± 3593.76	0
1%	28625 ± 4098.72	79.91
5%	120 ± 25.88	99.90
10%	5 ± 2.70	100

In the micro culture trial was applied 500 µL oregano oil onto previously sterilized filter paper at concentrations of l %, 5% and 10%, the experiments were conducted to observe the effect of oregano oil on the reproductive structures. In the Figure 2 was observed that the control (without oregano oil) the reproductive structures of the conidiophores and the intact conidia can be seen. At a concentration of 1% there is a reduction of conidiophores, however, at concentrations of 5% and 10% only a few conidia can be seen. These experiments showed that the presence of oregano oil (put onto the filter paper) damaged the reproductive structures of the fungus. Similar results were obtained by **Feng and Zheng (2007),** who conducted *in vitro* tests using *A. alternata* showing inhibition in spore germination and elongation of the germinative tube at 500 ppm on exposure to cassia essential oil.

The spore count tests (Table 1) showed that at a concentration of 1% these were inhibited up to 80%, while at concentrations of 5% and 10% there was 100% inhibition. **Guerrero *et al.* (2007)** evaluated the effect of the extract of fresh senna leaves (*Flourensia cernua)* on the mycelial inhibition and sporulation of *Alternaria alternata*, *in vitro*. Other studies using the essential oil *Origanum majorana L* found mycelium growth inhibition of 91.5%, in species *P. digitatum* and *A. Niger* at a concentration of 10 µl/ml. These species predominate in the microflora associated with the contamination of fruit juices and foodstuffs, with phytopathogenic and postharvest importance **(Helal *et al.,* 2006**).

Rao *et al.* (2010) attributed the antifungal activity of oregano oil to phenolic terpenoids in the mixture of chemical compounds of which it is constituted, adding to its hydrophobic nature which facilitates its diffusion through the lipid membrane. Moreover, the authors explain that chemical structure of carvacrol and thymol is characterized by containing a free hydroxyl group and an aromatic ring, responsible for its toxicity. Thus, the system of delocalized electrons in the carvacrol facilitates the dissociation of H + from the free -OH group. Protons and monovalent cations are transferred by differential gradient across the membrane, being similar to the K+ ion, gradually changing the pH, depolarizing the cell membrane and increasing its permeability. The accelerated ion flux of Ca^{2+} transients from the extracellular medium and other intracellular compartments (*e.g.* vacuole), and the change in pH are associated with the exposure of the lipid membrane to carvacrol, incurring a catastrophic lesion to the cell since it expands and increases the fluidity of the cellular membrane.

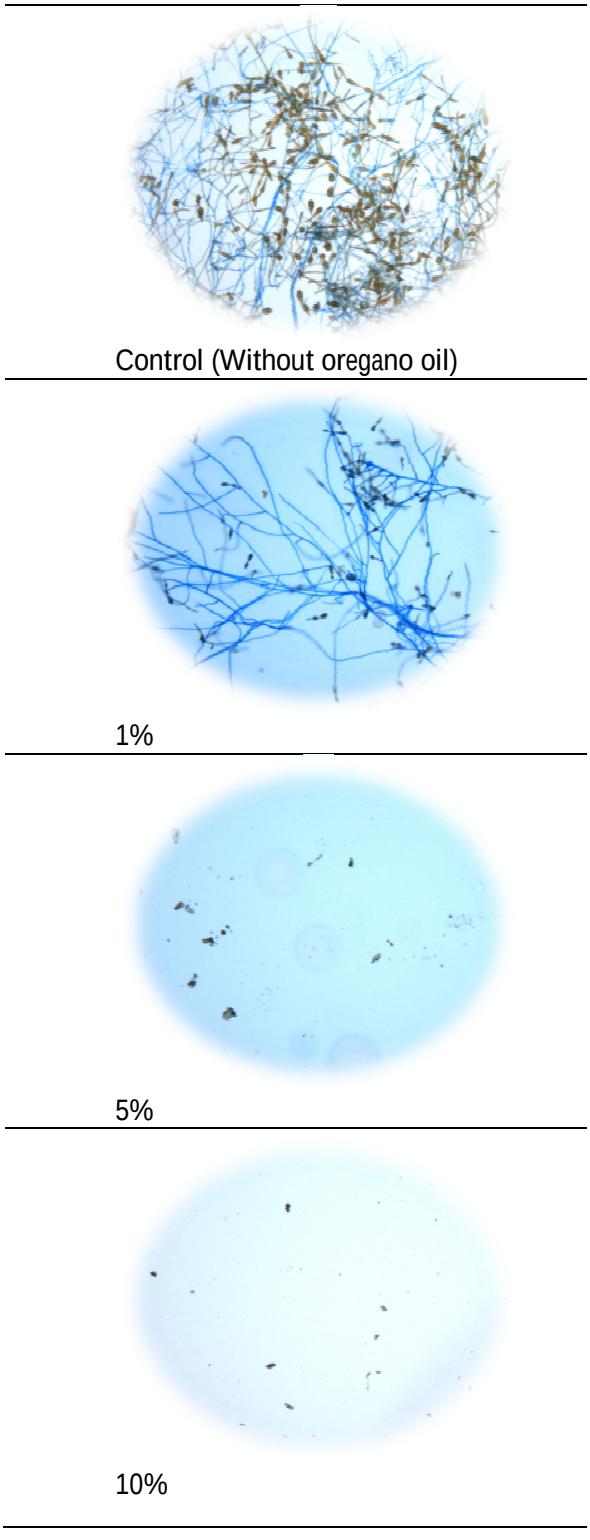

Figure 1: Micrographs of different oil oregano concentrations in the fungus reproduction structure.

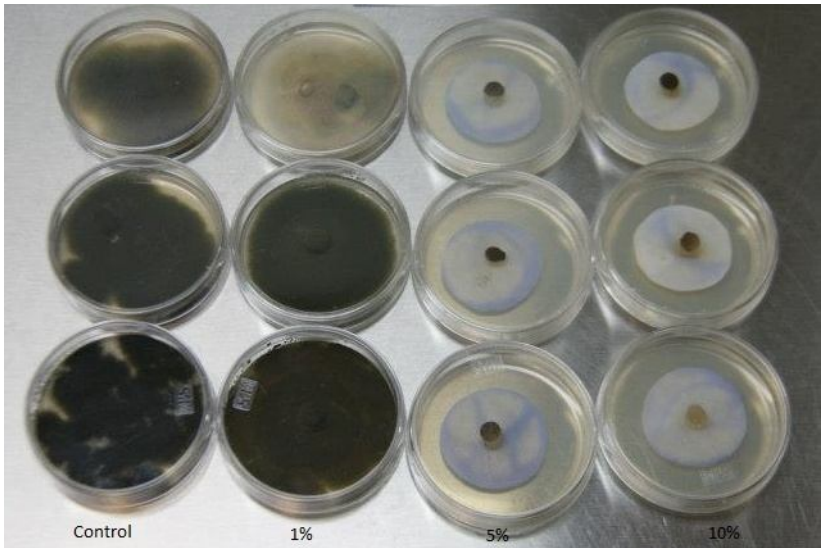

Figure 2: Growth inhibition of *Alternaria alternata* at different concentration of oregano oil exposure

Effect of oregano oil emulsified on tomato seedlings*In vivo* tests.

The effectiveness of oregano oil micro emulsions was evaluated by the number of healthy leaves in the tomato seedlings which had no fungal growth and maintained their green color. This indicator of physiological development adapted to the seedling was chosen based on the evidence described by**Smith *et al.* (1992)** who established that *Alternaria alternata* is able to penetrate through healthy, green leaves. *Table 2* shows the results of the healthy leaf count. In contrast to the *in vitro* experiments, the best treatment is seen to be at 1%, significantly better than the seedlings sprayed with chemical pesticides. It is important to highlight the scaling of the *in vitro* procedures since there are many physiological variables of the seedlings to be considered in order to choose the right concentration. In our work we observed that applying the micro emulsions at 10% oregano oil, the tomato seedlings turned yellow causing a mild necrosis in the leaves. However, at a concentration of 1% they were not affected by insects or bacteria from the environment. The application of oregano oil to greenhouse tomato seedlings significantly improved tomato production given that *A. alternata* did represent a factor of postharvest loss. In the study conducted by **Jia *et al.* (2013)** on tomatoes affected by the formation of dark brown blight on the stems and necrosis of the leaf tissue, it was recognized that the group of microtoxins produced by *A. alternata* are the principal factor of pathogenicity, observing that they are structurally similar to sphinganines. Sphinganines are an intermediate of the biosynthesis of sphingosine and sphingolipid complexes such as ceramides, which is evidence that the presence of these mycotoxins leads to the interruption of sphingolipid metabolism, inhibiting the reaction catalyzed by the sphingosine N-acetyltransferase (ceramid synthase) in the fruit of green tomato, this process induce programmed cell death in susceptible plant cells **(Zhang *et al*., 2011).**

Table 2: Number of healthy leaves of tomato seedlings with different concentrati ons of emulsified oregano oil

Treatmts	healthy leaves Mean (n=20)
Control (Without pesticide, oil oregano without)	0.70±0.2 **a**
With pesticide	11.30 ± 3.01**b**
1%	16.45 ± 3.47 **c**
5%	10.60 ± 4.71 **b**
10%	3.00 ± 1.50 **a**

* Same letter are not significantly different ($p<0.05$)

Alternaria alternata is considered a necrotrophic fungus which kills the host cells by parasitizing the plant from the early stages, accessing the tissue through the microfissures of the fruit causing localized rotting **(Blancard *et al*., 2011)**. The toxins produced by the necrotrophic fungus is quickly translocated outward through the vascular system, causing electrolyte leakage induces and necrotic lesions along the veins **(Chung, 2012).** It also changes the physiology of its host, generating an increase in RNA levels and hydrolyitic enzymes. The presence of the fungus in the crop causes tissue necrosis and the acceleration of senescence and foliar lesions.

The concentration of oregano oil at 10% had a growth range from 2-9 leaves per seedling, with a mean of 1.50 healthy leaves (Tab 2); after a few days the growth of healthy leaves was null. This effect was similar to the control treatment (without pesticide and without oregano oil) with a mean of 0.70 healthy leaves (Tab 2); after 2 weeks, in both cases the growth of healthy leaves stopped and the seedlings died. The damage to the plant (herbicide effect) is due to the accumulation of lipids in the cytoplasm and the reduction of some organelles such as mitochondria by the inhibition of DNA synthesis or the degradation of the nuclear membrane **(Rolim de Almedia *et al.,* 2010).**

For the 5% concentration, the healthy leaf count was from 5-18 leaves remaining constant. After one week an improvement was seen in the growth indices for number of healthy leaves, however, a prolonged and constant exposure to oregano oil at 5% caused a considerable decline in the optimal development of the seedlings. In the 1% oregano oil concentrate there was a control and an ideal growth, having seedlings of up to 28 healthy leaves due to the proper assimilation of nutrients, which was reflected in the recovery of firmness. However, in some leaves the curling and slight yellow pigmentation persisted.

The use of oregano oil allowed an improvement in the development of healthy leaves without the presence of fungus in the blank. The concentration at 1% after 2 weeks with a mean of 16.45 healthy leaves (Tab 2) shows the best results, since it permits the highest growth of healthy leaves even compared to the chemical pesticides used in the UPAEP greenhouse.

In the oregano oil study on greenhouse tomato seedlings it was decided to use microemulsions with Tween 80 (emulsifying agent), since the physical structure of oregano, oil could have made it difficult to apply to the seedlings. Microemulsions are thermostable and of low viscosity favoring their dispersion in the oil phase, and also preserve their bactericidal and antifungal properties **(Zhang *et al.*, 2008, 2009).**

Conclusion

Oregano oil has certain properties that are attributed to its principal compound, carvacrol, which is a volatile, phytotoxic monotherpene. This can act as a natural fungicide and at high concentrations can be used as an herbicide since it inhibits the germination and root growth of seedlings. This study allowed us to do a scaling and analyze the effects on native flora, finding that the product at 1% continued to be slightly toxic to the seedlings, but with no pathogenic growth on them. The 10% concentration gave the best *in vitro* results, but when applied to seedlings physiological damage was seen causing inhibition and even death. The properties of carvacrol were evidenced in both experiments, due to the fact that it inhibited the growth of fungi and the germination of the tomato seedlings. Due to the scaling, we were able to see the natural response of the seedlings in different concentrations of oregano oil.

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