

Antibacterial activity of mexican oregano essential oil (*Lippia berlandieri*) against the phytopathogenic bacterium *Xanthomonas euvesicatoria*

Actividad antibacteriana del aceite esencial de orégano (*Lippia berlandieri*) contra la bacteria fitopatógena *Xanthomonas euvesicatoria*

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Abstract

Xanthomonas euvesicatoria causes bacterial spot disease in leaves, roots, fruits and stems of pepper plants. Identification of this phytopathogen in jalapeno seeds from Delicias, Chihuahua, Mexico and diseased plants from New Mexico, USA, was carried out by isolation on semiselective media, pathogenicity assays and biochemical tests. Mexican oregano (*Lippia berlandieri*) essential oil was tested *in vitro* against *Xanthomonas euvesicatoria*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were performed and the oil showed an inhibition of bacterial growth in concentrations of 0.01 mg/ml and a bactericidal effect in concentrations of 0.05 mg/ml. Oregano essential oil is reported to have antimicrobial activities due to the effect of high content of carvacrol. Oregano oil had an MIC that was 10 times lower compared to pure carvacrol, since carvacrol content, measured by gas chromatography/mass spectrometry (GC/MS) was only 30%. The antimicrobial effect *in vivo* was tested using a randomized complete block design model in a greenhouse. Disease severity, xanthomonad incidence as well as chlorophyll indices were calculated showing a strong inhibition of the disease, when seeds or foliage were treated with oregano oil. These results demonstrate the current commonality of xanthomonad pathogens on both sides of the Mexican-American border, and that oregano oil has potent antibacterial activity.

Keywords: bacterial spot, minimum inhibitory concentration, Carvacrol.

Resumen

Xanthomonas euvesicatoria es la bacteria agente causal de la marchitez bacteriana en hojas, raíces y frutos de chile jalapeño. Se realizó la identificación de este patógeno en las semillas de chile jalapeño provenientes de Delicias, Chihuahua, México y plantas enfermas provenientes del estado de Nuevo Mexico, EUA; a través de cultivo en medios semi-selectivos, ensayos de patogenicidad y pruebas bioquímicas. El aceite esencial del orégano mexicano (*Lippia berlandieri*) fue probado *in vitro* contra *Xanthomonas euvesicatoria*. Pruebas de concentración mínima inhibitoria (CMI) y concentración mínima bactericida (CMB) fueron determinadas y el aceite mostró una inhibición de crecimiento a concentraciones de 0.01 mg/ml y un efecto bactericida a concentraciones de 0.05 mg/ml. El aceite esencial de orégano muestra actividades antibacterianas gracias al efecto de la alta concentración de carvacrol. El aceite de orégano mostró una CMI que fue 10 veces menor en comparación con el efecto de carvacrol puro, ya que la concentración determinada en el aceite por medio de cromatografía de gases/espectrometría de masas (GC/MS) fue de 30% de carvacrol. El efecto antibacteriano *in vivo* fue probado utilizando un diseño de bloques completos al azar en un invernadero. La severidad e incidencia de la enfermedad, así como los índices de clorofila, fueron calculados mostrando una inhibición de la enfermedad cuando las semillas u hojas de las plantas de chile se trataron con el aceite de orégano. Estos resultados demuestran la problemática de la bacteria *Xanthomonas* en las fronteras México-Americanas y que el aceite esencial de orégano ejerce una acción antibacteriana.

Palabras clave: marchitez bacteriana, concentración mínima inhibitoria, Carvacrol.

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Introduction

Xanthomonas euvesicatoria, also called Xanthomonas campestris pv. vesicatoria, has been identified as the causal agent of the disease known as bacterial leaf spot (Pohronesny et al., 1990; Bouzar et al., 1994; Kufлом and Diane, 1997; Ji-Liang and Orser, 2004). Bacterial spot is a destructive disease of pepper in climates with high temperatures and frequent rainfall (Pohronesny et al., 1990; Vorholter et al., 2008); seeds can be infested and serve as a major source of inoculum for bacterial spot, as well as the means for the long distance spread of the pathogen (Kufлом and Diane, 1997; Sanogo and Clary, 2008).

Bacterial leaf spot can cause serious yield loss in pepper crops through plant defoliation and fruit drop (Sanogo and Clary, 2008). This disease has a major economic impact in both the United States and Mexico. In northern Mexico, the disease has had an effect on quality and quantity of the fruit, resulting in a significant impact on market standards causing losses greater than nine million U.S. dollars (Velasquez-Valle and Amador-Ramirez, 2007).

Factors such as humidity, rainfall and wind play an important role in the occurrence of bacterial spot. It has also been reported that the pathogen can survive on dried seeds for many years and in infected crop debris in the soil (Andrade et al., 2008). At present, disease management practices using either antibiotics or pesticides, have been banned due to their undesirable attributes such as long degradation time, bioaccumulation and chronic and acute toxicity (Baricevic et al., 2001; Abbasi et al., 2002). Other treatments that have been used recently include copper sprays and plant activators (Abbasi et al., 2002), which are effective but sometimes induce systematic resistance. In addition to the toxic effects in the treatment of infectious plant diseases, pharmaceutical antibiotics are simple substances that show single modes of action and microbial resistance is easily developed (Elgayyar and Draughon, 2001). Neither the use of antibiotics nor pesticides provides satisfactory solutions to the persistent incidence of bacterial leaf spot; therefore, there is a need for alternative management of pathogenic *Xanthomonas euvesicatoria*.

Since ancient times, plant extracts including essential oils, have been used for a wide variety of purposes including their use as antimicrobials (Aureli et al., 1992; Biondi et al., 1993; Dorman and Deans, 2000; Chorianopoulos et al., 2004; Chorianopoulos et al., 2006). Essential oils have been screened for their potential applications as alternative remedies for many infectious diseases, and have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Dorman and Deans, 2000). Mexican oregano (*Lippia berlandieri*) has been proven to show *in vitro* potent antimicrobial activity (Portillo-Ruiz et al., 2005). The monoterpenic phenol carvacrol is the main constituent of commercial oreganos and should be given special emphasis since it demonstrates low toxicity and surprisingly broad antimicrobial activity (Chorianopoulos et al., 2006). This simple molecule is promising for the development of effective disease treatment not only in humans, but also for animals and plants (Veldhuizen et al., 2001). Mexican oregano is mainly composed of carvacrol; however, the antimicrobial activity is also attributed to other phenolic compounds found in the oil such as thymol and cymene (Vernin et al. 2001), and potent antibacterial activity might be attributed to a synergistic action of these compounds.

Few studies have focused on documenting the effectiveness of essential oil obtained from the Mexican oregano *Lippia* species. The main goal of this investigation was to evaluate the antimicrobial effect of *Lippia berlandieri* essential oil on *in vitro* MIC's and *in vivo* against

Xanthomonas euvesicatoria, in order to determine the potential of oregano oil as an alternative treatment for bacterial spot and its feasibility to replace the use of antibiotics and pesticides.

Materials and methods

Bacterial strains, isolation and identification of *Xanthomonas* in pepper seeds. Four strains of *Xanthomonas euvesicatoria* were used in this study, three from New Mexico, USA, and one from Chihuahua, Mexico. The new mexican strains were obtained from different farms located in New Mexico (kindly provided by Dr. S. Sanogo, College of Agricultural and Environmental Sciences; Entomology, Plant Pathology and Weed Sciences NMSU); these strains are named according to the farm from which they were isolated (Table 1). The mexican strain (Chihuahua, Mexico) was isolated from infected jalapeno pepper seed provided by the Cuerpo Academico CA-100 from Universidad Autonoma de Chihuahua located in Chihuahua Mexico.

Table 1. Bacterial strains used in this study.

Strain	Host	Location
Alvarez farm	Chile pepper	La Union, New Mexico
Provencio farm	Chile pepper	Chamberino, New Mexico
Kasparian farm	Bell pepper	Deming, New Mexico
Tula Seed strain	Jalapeno pepper	Chihuahua, Mexico

For isolation of the Mexican Tula seed pathogen, the standardized method described by the International Seed Federation was used (www.worldseed.org). This method focuses in the detection of viable *Xanthomonas campestris* pv. *vesicatoria* (*Xanthomonas euvesicatoria*) by dilution plating of seed extracts on semi-selective media, and the confirmation of suspected bacterial colonies by a pathogenicity assay. One gram of seeds were incubated in 3 ml of seed extraction buffer (0.05M PO₄ buffer pH 7.2; 8.5g/

L NaCl and 0.02% Tween 20) for 14 h at 4 °C. Then, 0.1 ml of the extract was spread onto two plates: Modified Tween medium B (mTMB, per liter composition: bacto peptone 10g, H₃BO₃ 0.1g, KBr 10g, CaCl₂ anhydrous 0.25g, bacto agar 15g, tween 80 10ml, cephalaxin 65mg, 5-fluorouracil 12mg, trobamycin sulphate 0.2mg, nystatin 35mg), and yeast dextrose calcium carbonate medium (YDC, per liter composition: yeast extract 10g, CaCO₃ 20g, d-glucose 20g, bacto agar 17g). It was necessary to dilute the extract 1:100 to obtain less than 300 colonies per plate. After plates were incubated at 28 °C for 5 days, pale yellow, mucoid colonies typical of *Xanthomonas* spp. developed, then they were transferred to a new plate of mTMB and isolated by the quadrant streaking technique. Isolated strains were used for biochemical characterization consisting of hydrolysis tests (casein, starch, lipids, gelatin, and cellulose), catalase production and oxidation/fermentation of glucose, lactose, and sucrose. Biochemical tests were incubated at 28 °C for 4-7 days, and if after 7 days no growth or color reaction was observed, the test was considered negative. In addition, PCR amplification of the intergenic 16S-23S ribosomal gene (Forward primer 5'-GTGCCAGCAGCCGCGGTAAT; Reverse primer 5'-TACTCCACCGCTTGTGCGGG), followed by sequencing (ABI3100) was carried out to confirm strain identity. In addition ClustalW algorithm was used for multiple sequence alignment.

Pathogenicity assays were performed using the seed isolates. Seedlings of a known susceptible jalapeno pepper cultivar (Early Jalapeno) were grown under greenhouse conditions until the 2-3 true leaf stage (4 weeks after germination); a small quantity of the selected colonies was transferred to a culture tube with 5 ml of nutrient broth; the inoculum was adjusted to an optical density of 0.1 at 625 nm (ca. 10⁸ CFU/ml).

Three leaves were infiltrated (1-4 sq cm of the surface) with the suspension by gently forcing the liquid into the adaxial surface of a leaf using a sterile syringe without a needle;

sterile distilled water was used for the negative control as previously described by the International Seed Federation (ISF, <http://www.worldseed.org/isf/home.html>). Inoculated plants were incubated in a growth chamber at 30 °C with 10 h light per day covered with a plastic bag to retain and increase the humidity. Plants were observed daily for one week looking for the presence of chlorotic lesions. Non-pathogenic bacteria will produce a hypersensitive reaction in 24 h or will not develop lesions at all. This experiment was repeated to ensure reproducibility in pathogenicity assays.

GC-MS analysis of Lippia berlandieri essential oil. Oregano essential oil was obtained by steam distillation and provided by the Don Pablo Licon company located in Chihuahua, Mexico. Dry leaves and small stems of *L. berlandieri* were used for oil extraction. Components were identified by direct comparison with authentic standards (Sigma) on the basis of retention time, Kovats retention indices, and comparison with literature data (Adams, 2001).

Analysis of essential oil was performed by gas chromatography coupled to mass spectrometry using a CP-3800 Varian GS/MS. The GC was equipped with a capillary column Saturn 2200 (30 m x 0.25 mm fused silica capillary column, film thickness 0.25 mm), and Helium was used as a gas carrier at a flow rate of 55 ml/min. The GC oven temperature was initiated at 60 °C, then increased to 250 °C at a rate of 3 °C/minute, and kept constant for 5 min at 250 °C.

Determination of the antibacterial activity of Lippia berlandieri essential oil and pure carvacrol. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oil was estimated. Pure bacterial cultures were inoculated in 3 ml of Mueller Hinton Broth (MHB, which is the medium recommended for this assay according to the International Seed Federation) for 18 h at 37 °C, the culture was adjusted to OD = 0.1 at 625 nm, and 150 µl of each culture was added to a sterile 96-

microtiter dish (Corning 96 well plates, Sigma-Aldrich, CLS3628). Varying concentrations (but constant volumes) of oregano oil were added (using five wells per strain); the plates were covered and incubated at 37 °C in an orbital shaker for 18 h. After incubation, microplates were read at 625 nm in a plate reader (Bio-tek FL 800). Non-inoculated controls and DMSO and methanol blanks were also assayed. Before and after exposure to oregano oil, viable plate counts on Mueller Hinton Agar were carried out to determine the MBC. These experiments were repeated three times. The effect of pure carvacrol (Carvacrol 98% Sigma-Aldrich No. 282197) against *Xanthomonas euvesicatoria* strains was also carried out as described above with the same number of replications.

Testing of Lippia berlandieri essential oil's effectiveness in disease protection.

a) Sources of infested pepper seed: Three sets of seed were used (Table 2). The first set consisted of naturally infested jalapeno seed obtained from the diseased plant variety Tula (Cuerpo Academico CA-100 from the Universidad Autonoma de Chihuahua). The second set, obtained from the cultivar Early Jalapeno (Chile Pepper Institute, New Mexico State University), was subdivided in two lots, and seed in one lot was artificially infected with the Mexican strain of *X. euvesicatoria* isolated from the Mexican Tula seed, whereas seed in the other lot was artificially infected with a strain from New Mexico. Artificially infection of Early Jalapeno seeds was carried out based on the method proposed by Adam Bognadove, (2011) (<http://www.reu.iastate.edu/2002/papers/DerrickBarker.pdf>). Bacterial cultures were grown in Nutrient Broth at 30 °C for 24 h; after which the optical density was measured and adjusted to 0.1. One gram of pepper seeds was covered with a wet absorbent paper towel for three hours using deionized sterile water. Seeds were placed in a petri plate and mixed with 2 ml of the 0.1 O.D.₆₂₅ bacterial suspension for 25 min; this suspension was removed and the seeds were allowed to dry for 1 h. The process was repeated to ensure successful colonization.

b) *Seed treatment with Oregano oil:* In order to test the antibacterial activity of oregano oil, different treatments were tested including both seed and foliar treatments depending on the seed variety as indicated in Table 2.

Table 2. Treatments applied to seeds and plants of jalapeno varieties Tula and Early. Note that Tula seeds were already infected with a Mexican strain of *Xanthomonas*.

Jalapeno variety	Target	Treatment	Identity or Procedure
Tula	Seeds	Untreated (infected control)	Inected with ^a MX strain of <i>Xanthomonas</i>
	Seeds	Bleach (0.57% free chlorine)	Immersed for 20 min
	Seeds	Oregano oil (1 mg/ml)	Immersed for 20 min
	Foliar	Oregano oil (1 mg/ml)	Sprayed at 20 and 50 days after germination
	Foliar	Oregano oil (0.1 mg/ml)	Sprayed at 20 and 50 days after germination
Early	Seeds	Untreated (Non-infected control)	-
	Seeds	Inoculated with ^a MX strain	-
	Seeds	Inoculated with ^b NM strain	-
	Seeds/Foliar	Inoculated with ^a MX strain and sprayed w/oregano oil (1 mg/ml)	Oil sprayed at 20 and 50 days after germination
	Seeds/Foliar	Inoculated with ^b NM strain and sprayed w/oregano oil (1 mg/ml)	Oil sprayed at 20 and 50 days after germination

^aMX strain of *Xanthomonas* was isolated from Mexican-grown Tula seed.

^bNM strain of *Xanthomonas* was isolated from the Provenencia farm, Las Cruces, NM.

Potted Early and Tula Jalapeno were placed on a greenhouse bench in a randomized complete block design with three replications. Plants were covered with a plastic bag after the first foliar delivery treatment in order to increase humidity to encourage disease development. Plants were watered every three days and observed everyday to record any disease symptom.

After symptoms were detected, *X. euvesicatoria* was isolated from diseased leaves as follows: Five spots were cut from symptomatic leaves with a razor blade and macerated with a sterile mortar and pestle in 100 µl of sterile deionized water. The mixture was centrifuged at 800 rpm for 1 min in order to precipitate leaf debris and suspend bacterial cells in the supernatant. A 10 µl aliquot of supernatant was streaked on a nutrient agar plate (please, describe the composition of nutrient medium). Plates were incubated for 5 days at 28 °C. Biochemical tests (described previously) were carried out for identification of the pathogen.

c) *Assessment of treatment efficacy.* The variables disease severity/incidence index, chlorophyll counting units, and matter yield were measured to determine the efficacy of oil treatment on disease development from infested seeds: Disease severity (s) was calculated using the Stover and Dickson scale. Different grades were assigned to each leaf according to the area spotted: grade 1 (< 5% area spotted), grade 2 (5-15% area spotted), grade 3 (16-33% area spotted), and grade 4 (> 33% area spotted). The proportion of diseased leaves, or disease incidence (I), was calculated as the number of diseased leaves divided by the total number of leaves in the plant.

Chlorophyll was measured using CCM-200 chlorophyll content meter; each plant was divided in three sections, three leaves from each section were measured for chlorophyll and three measurements from each leaf, corresponding to a total of 27 measurements per plant.

Results and discussions

Isolation and identification of Xanthomonas euvesicatoria. Biochemical tests were performed on yellow mucoid colonies from the YDC and mTMB media, and these resulted in positive reactions for all bacterial strains (Table 3). However, the time required to develop a positive reaction was different between strains: Alvarez and Kasparian strains developed positive biochemical reactions after 6 days of incubation; while Provencio and Tula Seed strains developed positive reactions after 3 days of incubation.

Amplification of the intergenic 16S-23S ribosomal gene was carried out for all presumptive xanthomonad strains, and the presence of a 430 bp band was the first step towards sequencing. BLAST results revealed that *X. euvesicatoria* (no. GeneBank AM039952.1) was a common match at the highest similarity (98%) for all the isolates. Partial sequence of the intergenic region was used to compare isolate distribution (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>), and all the strains were unique from each other (data not shown).

Table 3. Biochemical tests for the different *Xanthomonas euvesicatoria* strains (W indicates a weak positive test).

Characteristic	<i>X. euvesicatoria</i> (19)	Xanthomonas euvesicatoria strains			
		Alvarez	Provencio	Kasparian	Tula
Morphology in mTMB media		Large yellow mucoid circular colonies, a small crystalline halo around the yellow colony was present.		Medium yellow mucoid circular colonies, the halo was absent.	
Morphology in YDC media		Medium yellow mucoid circular colonies.			
Gram stain	Gram-negative	Gram-negative rods of 2 µm in size, commonly in pairs.			
Catalase production	+	+	+	+	+
Hydrolysis tests:					
Casein	+	+	+	+	+
Starch	+	+	+	+	+
Lipids	+	W	W	W	+
Gelatin	+	+	+	W	+
Cellulose	+	+	+	W	+
O/F basal medium (Hugh Lefson)					
Glucose oxidation	+	+	+	+	+
Lactose Oxidation	+	+	+	+	+
Sucrose Oxidation	+	+	+	+	+
Glucose	+	W	W	W	+
Fermentation	+	W	W	W	+
Lactose Fermentation	+	W	W	W	+
Sucrose					
Fermentation					

Four weeks after germination, Early variety of Jalapeno was inoculated with the four xanthomonad isolates, but only two strains, Provencio and Tula, caused symptoms one week after inoculation (Figure 1). The other two strains and negative controls showed no symptoms. Both virulent strains were re-isolated and verified to be xanthomonads by biochemical tests.

GC-MS analysis of *Lippia berlandieri* essential oil.

Essential oil was analyzed with the purpose of detecting the different components that were present in the oregano oil; the chromatogram (Figure 2) showed the seven major peaks that represent the most prevalent components in the essential oil. Total running time in the GS-MS was of 63.3 min. The analysis was performed in triplicate to ensure analytical repeatability.

In vitro determination of antibacterial activity of *Lippia berlandieri* essential oil. Minimum inhibitory (MIC) and bactericidal (MBC) activities were determined for each strain of *X. euvesicatoria*. Bactericidal effects were observed at higher concentrations (0.05 mg/ml) whereas inhibition of growth (MIC) was observed at concentrations of 0.01 mg/ml (Table 4) and repeated experiments showed similar results.

The effect of pure carvacrol on two different *X. euvesicatoria* strains was tested (Provencio and Tula). Interestingly, its antibacterial effect was not as potent as oregano oil, despite the fact that the oregano oil was measured to have 30% of carvacrol content according to the GC/MS data.

In vivo (in the plant host) determination of antibacterial activity of *Lippia berlandieri* essential oil. Early jalapeno and Tula jalapeno varieties were planted on December 2nd 2007 following the seed treatments and foliar applications described before. Plants were examined three times per week for disease symptoms, including chlorotic or necrotic zones in leaves. The data collected is presented based in observations of the leaves in each plant; total number of diseased leaves were counted and classified according to the severity of the disease based on the parameters used by Chuang, 1987.

To calculate the amount of leaf spot disease severity (s) the modified Stover and Dickson scale (Chuang and Jeger, 1987) was used:

$$s = (0.05x + 0.15y + 0.35z + 0.25w) / n$$

in which x, y, z, and w represent the number of leaves with disease grades 1, 2, 3 and 4 respectively, and n is the total number of leaves.

The disease incidence (I), was calculated as the number of diseased leaves divided by the total number of leaves on the plant. Results are summarized in Figures 3 and 4. When severity indices are calculated, the range can vary from 0 (absence of spots in the leaves) to 0.15 (medium severity/incidence index); incidence varied from 0 (no diseased leaves)

to 0.5 (the most severe index). When Early jalapeno variety was inoculated with either the xanthomond isolates from Provencio, NM or from Tula, plants exhibited disease symptoms (Figure 4). When inoculated plants were sprayed twice with 1 mg/mL oregano oil (20 and 50 days post germination), disease incidence and severity both significantly dropped (Figure 4). The Tula jalapeno variety developed bacterial spot symptoms without inoculation due to the seeds already being infected with *Xanthomonas* (Figure 5). When oregano oil was applied to either the Tula seed or to the foliage, disease symptoms decreased significantly (Figure 4).

Table 4. MIC and MBC of different *Xanthomonas euvesicatoria* strains when exposed to different concentrations of oregano (*Lippia berlandieri*) essential oil and carvacrol.

<i>X. campestris</i> strains	Agent	^a MIC* (mg/ml)	^a MBC** (mg/ml)
Alvarez	Oregano oil	0.01+0.005	0.05+0.005
Provencio	Oregano oil	0.01+0.004	0.05+0.006
Kasparian	Oregano oil	0.01+0.004	0.05+0.006
	Carvacrol	0.10+0.020	1.00+0.082
Tula	Oregano oil	0.01+0.00	0.05+0.005
	Carvacrol	0.10+0.0304	1.5+0.064

^a Values represent the means of three separate experiments with \pm one standard deviation shown.

* Significant inhibitory effect ($p < 0.01$).

** Significant bactericidal effect ($p < 0.001$).

Figure 1. Pathogenicity assay. A=Alvarez strain, B=Kasparian strain, C=Provencio strain, D=Tula. Arrows indicate the leaf that was inoculated.

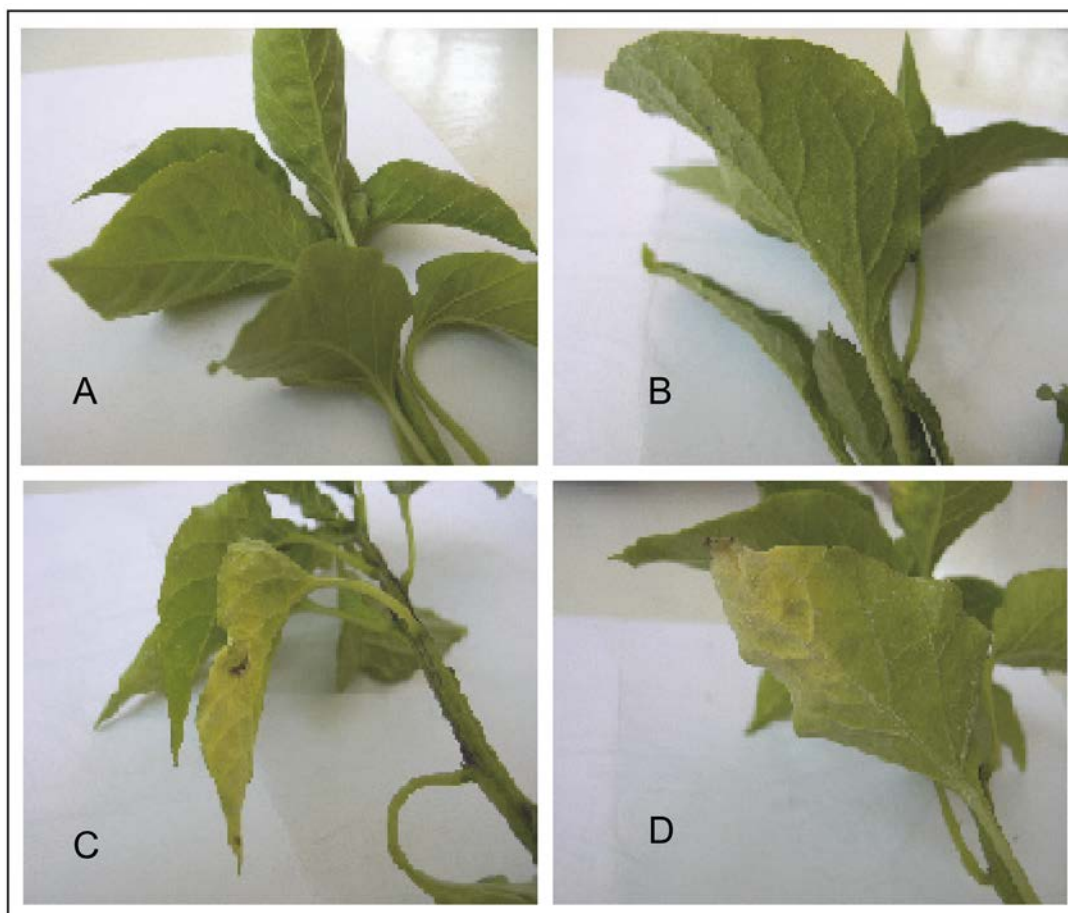


Figure 2. Chromatogram of the oregano essential oil: «x» axes indicates the retention time and «y» axes indicates the peak height in K counts. Numbers indicate the analytes detected according to the retention time: Cyclohexene-1-methyl-4-cymene (1), Cymeole (2), Carvacrol (3), Caryophyllene (4), Caryophyllene oxide (5), Alpha-terpinene (6), 3-Carene (7). Retention times matched with separately run standards, and the mass spectra matched those in the Adams 2001 library.

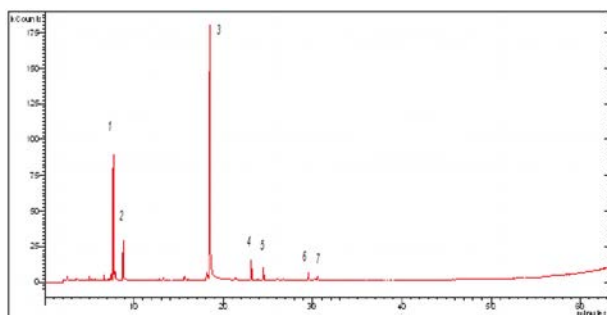
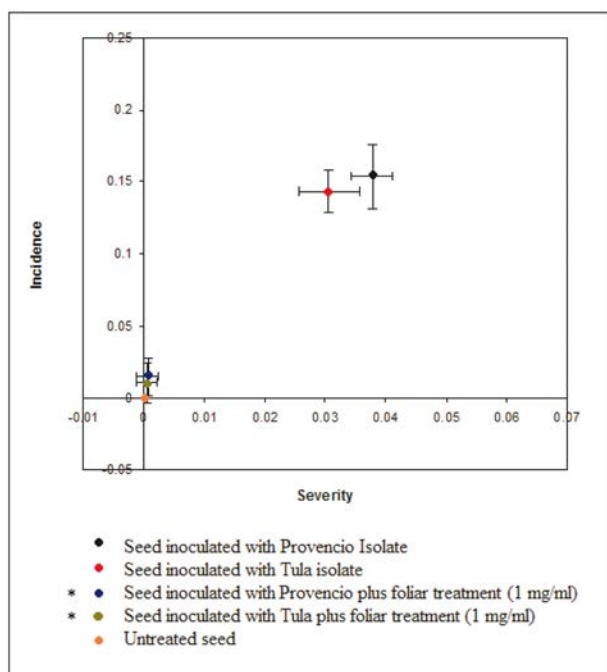


Figure 3. Severity and Incidence indices when Early jalapeno variety was inoculated with *Xanthomonas* and then treated with oregano oil. Values represent the means of three replicates, and error bars represent one standard deviation.

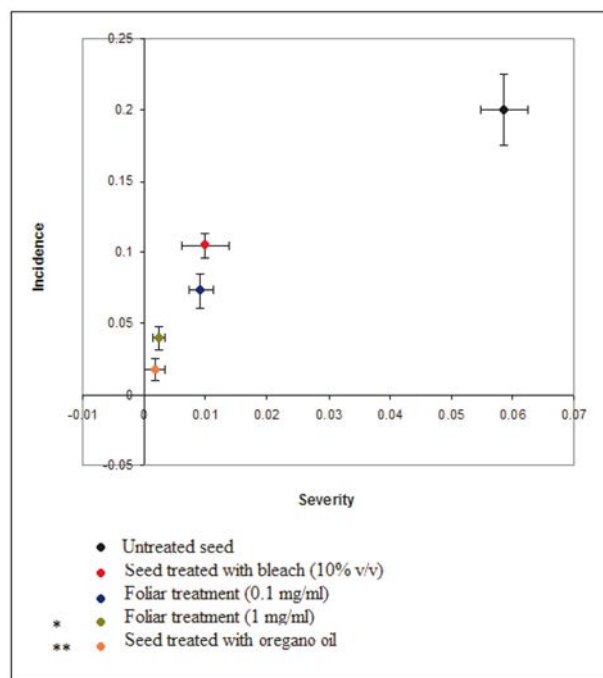


*Significant difference between infected plants and infected plants that were foliar-treated ($p < 0.01$).

In this greenhouse study, chlorophyll content decreased by xanthomonad infection, and chlorophyll content was maintained in

oregano-treated plants (Figure 5). For example, when the Early variety was infected with either the Tula or Provencio strain, chlorophyll content decreased by a factor of 3, and when plants were foliar treated with oil, chlorophyll content increased to control levels in a two-fold order (Figure 5A). Similar results were observed with the Early variety (Figure 5B).

Figure 4. Severity and Incidence indices when Tula jalapeno variety was treated with oregano oil. Values represent the means of three replicates, and error bars represent one standard deviation.



* Significant effect on Foliar treatment with 1 mg/ml when comparing with controls (untreated seed and seed treated with bleach) and foliar treatment at 0.1 mg/ml ($p < 0.01$).

** Significant effect on Seeds treated with oregano oil when compared with controls and foliar treatments ($p < 0.01$).

Bacterial spot disease along the U.S.-Mexican border in peppers and tomato has represented a major problem in the last decade causing crop losses with a serious economic impact on producers. One of the goals of this study was to isolate the causal agent of a spot disease in pepper seeds, using the method proposed by the International Seed Federation, which involves the use of semi-selective media

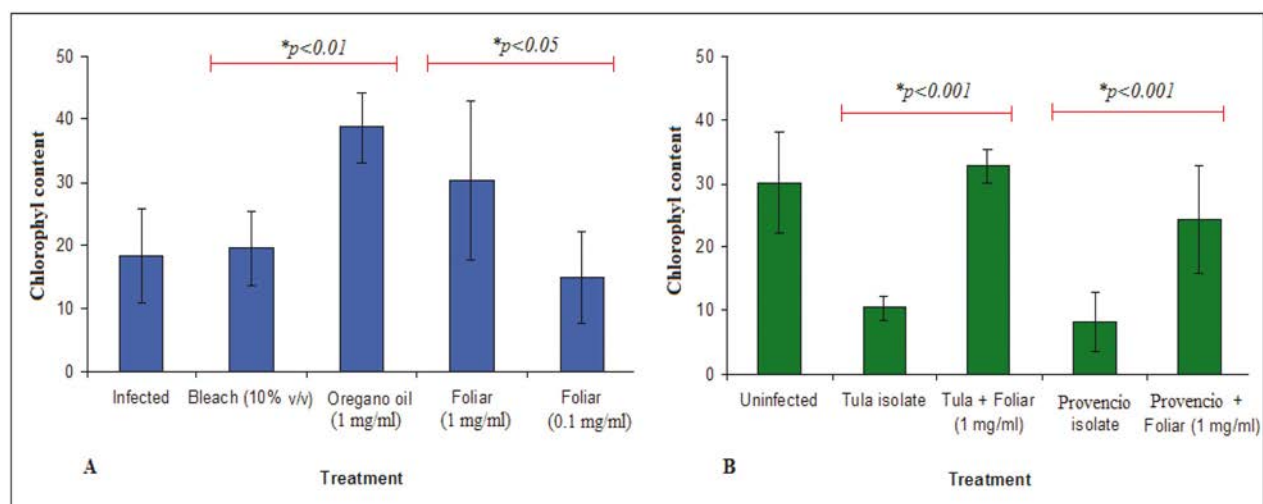
and pathogenicity assays. Several methods have been proposed as means for identification *X. euvesicatoria*; one of which (Alvarez *et al.*, 1985) consists in producing monoclonal antibodies (MCAs) that rapidly identifies *X. campestris pv vesicaria* from other pathovars. In a different approach (17), *X. campestris pv campestris* was detected using immunofluorescence microscopy when polyclonal and monoclonal antibodies were produced from flagellar extracts, however, both methods produced cross-reactivity with different non-xanthomonad strains and with different pathovars. Specific primers that detect specific genes in the pathogen represent a promising method. In this study, the amplification of the 16S intergenic space sequences was used in combination with sequence data, biochemical tests, and the isolation protocol proposed by the International Seed Federation. The combined approach resulted in isolation and identification of xanthomonads and allowed their screening for virulence on pepper plant hosts. Tula variety seeds were obtained during a Mexican outbreak of bacterial spot on infected pepper plants, and

using these techniques *X. euvesicatoria* was isolated from the seeds and verified to be the causal agent of the disease.

Two other bacteria are likely to cause similar symptoms to the ones caused by *Xanthomonas* and biochemical tests are helpful to differentiate them. *Pseudomonas* is an oxidative non-fermentative bacterium, so the positive sugar fermentations were helpful in eliminating this possibility. *Xylella* has protease activity (capable to hydrolyze casein and gelatin) (Fedatto, 2006), the difference with *Xanthomonas* is that it is non motile, does not ferment glucose and does not produce lipase.

According to the results obtained in the present investigation, among monoterpenes and aromatic hydrocarbons detected in oregano oil, cyclohexene-1-methyl-4-cymene was predominant which was expected since it is a precursor of carvacrol and thymol. Although carvacrol and cymene were the major essential oil components, 1-8-cineole and alpha-terpinene were also present, consistent with other results reported (Duschastki *et al.*, 1999).

Figure 5. Chlorophyll content in infected Tula jalapeno variety (A) and in Early variety inoculated with the Tula and Provencio xanthomonads (B), and effect of foliar and seed-applied oregano oil. Values represent the mean of three replicates, and standard deviations were calculated using the unbiased estimator for the mean.



It has been reported that various agronomic conditions such as duration of daylight, temperature, water stress, and the plant growth phase affect the development of the oregano plant and its essential oil composition (Velasquez-Valle and Amador-Ramirez, 2007). For instance, water stress decreased fresh weight and oil content while increasing thymol and carvacrol content in the oil obtained from *Origanum vulgare* species; flowering also increased the oil yield (Stover and Dickson, 1970; Vernin *et al.*, 2001); young plants have a higher amount of thymol whereas older plants have a higher content of carvacrol (Stover and Dickson, 1970). According to GC/MS analysis of this study, carvacrol content in the oregano oil sample corresponded to 30% (v/v) which is a significantly higher amount when compared with essential oil obtained from *Origanum* spp.

Previous work has demonstrated the efficacy of oregano oil as antimicrobial, and most of these studies have focused on the food industry, testing the effects against some food-borne pathogens as well as other human pathogens (Aureli *et al.*, 1992; Biondi *et al.*, 1993; Force *et al.*, 2000; Baricevic *et al.*, 2001; Arcila-Lozano *et al.*, 2004; Choriantopoulos *et al.*, 2004; O'Mahony *et al.*, 2005; Choriantopoulos *et al.*, 2006). Nevertheless, few studies have considered the potential effect expected of oregano essential oil against animal or plant pathogens, specifically using *Lippia* essential oil or other oregano-like plants.

The concentration of oregano oil that is needed to inhibit bacterial growth (MIC) and to kill 99% of bacterial cells (MBC) is surprisingly low, while the effect of pure phenol carvacrol is not as effective as the oil (Table 4); this suggests that the components found in oregano oil act synergistically to potentially increase the antimicrobial effect. Future studies are required to test this hypothesis.

A plant disease, in its broad sense, is any growth or developmental condition that is not «normal» to that plant and can usually reduce its economic or aesthetic value (Sanogo and Clary, 2005). For a presumptive diagnosis of

diseases in plants, we depend on symptoms and signs of the disease that can be measured with different parameters described before (severity/incidence indices, chlorophyll amount, and dry weight).

Seeds of the Early variety of jalapeno were pathogen-free and were infected with strains that exhibited virulence in both jalapeno and bell peppers. The varieties were chosen for two reasons: the first one was in order to test if the method used to infect Early jalapeno seeds was successful, and the second reason was to compare if the treatments for avoiding disease worked in both varieties.

Among the two types of infected seeds, the incidence/severity indices were similar for both native-infected seeds (Tula variety) and inoculated (Early variety) with two different strains, Early jalapeno inoculated with both pathogenic strains, showed lower incidence/severity indices than the already infected Tula jalapeno when foliar treatment at the same concentrations was applied.

It can also be stated that the effect of the extract is more effective at the seed level because once the disease is present in adult plants; the pathogen can be more resistant to treatment and more difficult to control. It is also more convenient in the sense that it does not consume as much time as foliar treatment, and it is performed only once with less quantity of essential oil.

When emphasizing strain virulence, as observed in Figure 3, it seems that infection with the Provencio isolate produces higher severity/incidence than infection with the Tula seed isolate for that particular variety of jalapeno; however, to control both pathogens the single oil concentrations appears to be equally effective in eliminating pathogenesis, which indicates the broad effectiveness of the oregano oil. Since *Xanthomonas euvesicatoria* produces chlorosis in leaf tissue, chlorophyll content was an effective parameter to track infection. Chlorophyll content changes represent both plant xanthomonad infection and plant recovery due to oregano oil treatment.

In summary, Koch's postulates were proven in that a xanthomonad was isolated from infected seed (Tula), was inoculated on to healthy plants, causing disease, and was re-isolated. Oregano oil showed great *in vitro* activity against the strains of *Xanthomonas* used in this study, with MICs averaging 0.01 mg/ml. Surprisingly, the oregano oil (with 30% carvacrol content) was significantly more potent than a commercial source of carvacrol (98%), indicating that in oregano essential oil there are other components that work synergistically with carvacrol. This study is the first to show the effectiveness of oregano oil in controlling bacterial spot disease in peppers, and emphasizes the importance of natural treatments in crop production that could replace toxic and ineffective alternatives.

Conclusions


The phytopathogen *X. euvesicatoria* was identified by a simple method in infected seeds and leaves of jalapeno. We examined the antibacterial activity of oregano oil against strains of *X. euvesicatoria* from different origins. The susceptibility of the plant pathogen was more remarkable in the seed treatment, but it was also efficient in higher concentrations when a foliar application was performed. Among the antimicrobial compounds identified, carvacrol predominated in the oil composition; however, when antibacterial test were performed with pure carvacrol, oregano oil demonstrated a higher antibacterial activity. Mexican oregano oil has proven to be effective and it is a potential candidate for future studies of synergism, compatibility and activity in other pathogens (human, plant or animal).

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