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"El saber de mis hijos hará mi grandeza"

EFFECT OF ESSENTIAL OILS IN THE CONTROL OF THE *Clavibacter michiganensis* SUBESPECIE *michiganensis* IN TOMATO (*Lycopersicum esculentum* L.) PLANTS

EFECTO DE ACEITES ESENCIALES EN EL CONTROL DE *Clavibacter michiganensis* SUBESPECIE *michiganensis* EN PLANTAS DE TOMATE (*Lycopersicum esculentum* L.)

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ABSTRACT

Plants produce compounds with antimicrobial properties that can be used to control diseases of horticultural products, for this reason, the aim of this study was to enlarge the knowledge on the effect of essential oils as an alternative for control for bacterial canker *Clavibacter michiganensis* subsp. *michiganensis* (Smith) (*Cmm*). Essential oil of *Lippia palmeri* was obtained by hydridestillation from plants collected in Sonora, Mexico. Essential oils of *Origanum vulgare*, *Thymus vulgaris* and *Cinnamomum zeylanicum* were obtained from commercial stores in Hermosillo, Sonora, Mexico. To evaluate the effect of the essential oils on the bacterium *Cmm,* direct applications in two concentrations (1:1 and 1:5 (v/v)) on tomato plants foliage were carried out. Then, plants were inoculated with $1x10^{-7}$ CFU/mL of the bacterium and observed for 12 days. The evaluated parameters were incidence and severity. The essential oils and chemical compounds of *Origanum vulgare* and *Thymus vulgaris* had effect against *Clavibacter michiganensis* subsp. *michiganensis* up to 9 days after application in a 1:1 (v/v) concentration and up to 12 days after application with the 1:5 (v/v) concentration. The essential oil of *Lippia palmeri* only had effect until six days after the application with both concentrations; that is to say, only these three types of essential oils had bactericidal properties, since with the essential oil of *Cinnamomum zeylanicum* the inhibition of Cmm was very limited, both in incidence as in severity.

Keywords: Bacterial canker of tomato, *Lycopersicum esculentum*, antibiotic, resistance, organic control.

RESUMEN

Las plantas producen compuestos con propiedades antimicrobianas que pueden usarse para controlar enfermedades de productos hortícolas, por esta razón, el objetivo del presente consistió en ampliar el conocimiento sobre el efecto de los aceites esenciales en el control del cancro bacteriano *Clavibacter michiganensis* subsp. *michiganensis* (Smith) (*Cmm*). El aceite esencial de *Lippia palmeri* se obtuvo por hidrodestilación de plantas recolectadas en Sonora, México. Los aceites esenciales de *Origanum vulgare, Thymus vulgaris* y *Cinnamomum zeylanicum* se obtuvieron de tiendas comerciales en Hermosillo, Sonora, México. Para evaluar el efecto de los aceites esenciales en la bacteria Cmm, se llevaron a cabo aplicaciones directas en dos concentraciones (1: 1 y 1: 5 (v / v)) en el follaje de plantas de tomate. Luego las plantas se inocularon con $1x10^{-7}$ CFU/mL de la bacteria y se observaron durante 12 días. Los parámetros evaluados fueron incidencia y severidad. Los aceites esenciales y compuestos químicos de *Origanum vulgare* y *Thymus vulgaris* tuvieron efecto contra *Clavibacter michiganensis* subsp. *michiganensis* hasta 9 días después de la aplicación en una concentración 1: 1 (v / v) y hasta 12 días después de la aplicación con la concentración 1: 5 (v / v). Mientras que el aceite esencial de *Lippia palmeri* solo tuvo efecto hasta seis días después de la aplicación con ambas concentraciones; es decir, solo estos tres tipos de aceites esenciales tenían propiedades bactericidas, ya que con el aceite esencial de *Cinnamomum zeylanicum* la inhibición de Cmm era muy limitada, tanto en incidencia como en severidad.

Palabras clave: Cancro bacteriano*, Lycopersicum esculentum,* antibióticos, resistencia, control orgánico.

INTRODUCTION

The plant pathogen known as *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is a bacilli Gram-Positive bacterium of the Firmicutes family (Schaad, 2001), reported as Phytosanitary risk of type A2 (OEPP / EPPO, 2013). The main source of dissemination of the disease caused by Cmm is by seed, where the pathogen is found on the cover and inside the cover (Fatmi and Schaad, 1989). The number of bacteria

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that infests the seeds is variable, but with an average of bacterial inoculum of 10^2 and 10^4 Colony Forming Units (CFU) per seed is enough for the disease to proliferate (De Leon et al., 2011). Chang et al. (1991), report that a seed infection in the order of 0.01-0.05% (1-5 seeds in 10,000) was capable of initiating an epidemic in the Midwestern US. Some countries where affected from bacterial canker from 2000 to 2010 in Israel, Japan, Mexico, the Canary Islands and USA, as well as in Asia and South America (De Leon et al., 2011). The control of this disease is usually using antibiotics which are limited, and cupric compounds, which have been suspended in some countries of the European Union by the norm 473/2002 due to their impact on the environment (Lacobellis et al., 2005). Notwithstanding the above, these treatments have shown little efficiency in Cmm, which is the principal reason for the search of new products to combat this disease, as well to solve the problem of bacterial resistance, among others (Kaya et al., 2008, Zwenger and Bassu, 2008). The genera *Origanum, Thymus, Lippia* and *Cinnamomum* are aromatic plants that produce essential oils that show variations in antimicrobial activity, for the control of phytopathogenic bacteria to be used in the integrated management of tomato with less impact on the environment, food and health.

The antimicrobial properties of essential oils and components have been evaluated *vs* microorganisms including fungi, bacteria, viruses and insects (Basim et al., 2004). Carvacol, as an active compound which has been detected in the essential oil of *Origanum vulgare* and *Thymus vulgaris* in 60 to 70% and 45%, respectively; the inhibition of growth of numerous pathogens by carvacol has been previously reported; however, its mechanism of action has not been defined (Ultee, 2002). Based on the above described, the present investigation was conducted to enlarge the knowledge the antibacterial effects of essential oils of *Origanum vulgare* and *Lippia palmeri, Thyme vulgaris* and *Cinnamomum zeylanicum* against bacterial canker *Clavibacter michiganensis* subsp. *michiganensis* on tomato.

MATERIALS AND METHODS

Selection of essential oils

From a previous study evaluating the antibacterial activity against Cmm of six essential oils under *in vitro* conditions of *Cinnamomum zeylanicum*, three oils of oreganum (*Lippia palmeri* W.) collected from plants from three areas in Sonora, Mexico (Puerto Oregano = 29°54′15″N 112°40′59″O=, Alamos = 27°01′39″N 108°56′24″O= and Hermosillo = 29°05′44″N 110°57′03″O=), one sample from a comercial store (*Origanum vulgare* L. and *Thymus vulgaris* L.). Only four were selected considering their bactericidal activity and a review of the literature: Cinnamon (CA) (*Cinnamomum zeylanicum Brine*), (PO) Oreganum (*Lippia palmeri* W.), Oreganum (OC) (*Origanum vulgare* L), and (TO) Thyme (*Thymus vulgaris*). The quality of the oils were from 98% to 100% purity.

Obtaining essential oils

The *Lippia palmei* oreganum plants used in this study were identified according to Ortega (2005). The essential oils were obtained from leaves, with three days of drying at ambient temperature and in the shade until a humidity of 7.5% was obtained. The oil was extracted by steam distillation with the Clevenger apparatus for 2.5 H, following the official method of A.O.A.C. 6.006 (1975). After extraction oils were stored in amber bottles and stored at 4 °C for further use.

Inoculum preparation: Clavibacter michiganensis subsp. michiganensis (Cmm)

The Cmm strain used in the present study was previously identified according to Borboa et al. (2009), which was isolated in the agricultural areas of the state of Sonora, Mexico, and preserved in NBY broth plus 40% of glycerol, at 20 °C according to Schaad (2001). Suspensions of the inoculum were prepared from young 24 h colonies as follows: a NBY nutrient broth culture with shaking was centrifuged for 15 min at 1704 G-force (radius of rotor (100mm)) and the cell pellet was resuspended in 0.85% sterile saline solution to remove the culture medium fragments, centrifuged again and standardized the inoculum to a turbidity of the McFarland nephelometer tube of 0.5. A 1 mL aliquot was taken to make serial dilutions, to 10^{-7} and to determine the approximate number of viable cells. Dilutions were plated (0.1 mL) with NBY medium by diffusion, the bacterial solution was diffused with a pull rod and incubated at 30 °C/ 48 hours (Madigan et al., 2006). This suspension was used to evaluate the antibacterial effect of cinnamon (CA) (*Cinnamomum zeylanicum* Brine), Oregano (PO) (*Lippia palmeri* W.), Oregano (OC) (*Origanum vulgare* L) (*Thymus vulgaris* L), in tomato plants.

Determination of the antibacterial activity of essential oils

A total of 200 seeds of the Missouri variety previously disinfected with 3% sodium hypochlorite, were germinated in separate frigolit thermal vessels, containing sterile peat-moss substrate (Sunshine, Sun Gro Horticulture Canada, Ltd.). Seedlings were produced at 25 °C using sterile faucet running water for irrigation; 30 days after the emergency, 40 seedlings were first selected in terms of their vigor and health, then the essential oils of *Lippia palmeri, Origanum vulgare, Thymus vulgaris* and *Cinnamomum zeylanicum* were applied to each plant using the technique of direct spray on the foliage, in concentrations 1:1 and 1:5 (v/v); the application of the bacterial inoculum was by direct spray at a concentration of 10^7 UFC/mL. Another 40 plants were considered with the same process, without application of bacterial inoculum (negative control); another 40 as positive control with application of the bactericidal gentamicin sulfate and hydrochloride of oxytetracycline (4 gr/L) (Agronomic Chemistry of Mexico-Chihuahua, Mexico), and 40 more plants with the bacterial

inoculum of Cmm were considere as positive control plants. Then all seedlings were covered separately with polyethylene bags and placed in an incubation chamber with an 80-90% relative humidity and a temperature of 25 ± 2 °C for a period of four to seven days according to Lelliot et al. (1987), are apropiarte to induce signs of the disease.

The parameters evaluated from the day of the treatments applications were: incidence using the formula proposed by James (1983). Where: I (%) = (Number of plants with symptoms of Cmm/Total number of plants evaluated) x 100.

The severity of the primary symptoms produced by the pathogen was measured through the presence of wilting and curl of the leaves, established as a qualitative variable "degree of severity", for which four levels were set according to the progression of the disease in the plant according to Kempe and Sequeira (1983) (Table 1). Evaluations were performed for 15 days after the applications of the treatments.

Tabla 1. Escala de severidad de la enfermedad del cancro bacteriano en plantas de tomate (Kempe y Sequeira, 1983).
Table 1. Severity scale of bacterial canker disease in tomato plants (Kempe and Sequeira, 1983).

Grade	Percentage	Symptom
0	0	Healthy plant
1	0-10	Mild
2	10-50	Severe
3	50-75	Very stern
4	75-100	Death planta

Statistical analysis

A completely randomized design with factorial arrangements of 4x2x5 was developed, where factor A is the type of oil with 4 levels, factor B is the concentration of oils with 2 levels and factor C, the interval exposure of time with 5 levels. The data obtained were analyzed using the ANOVA GLM test. Significance was estimated at $P<0.05$. The comparison of means was performed for Tukey's multiple range test. The data were processed in the statistical package NCSS 2001.

RESULTS AND DISCUSSION

The technique of inoculation by direct spray on the foliage was effective finding that the incidence of the disease maintained in gradual increase. Essential oils of *Origanum vulgare* (OV), *Lippia palmeri* (LP), *Thymus vulgaris* (TV) y *Cinnamomum zeylanicum* (CZ), had a bactericidal effect on *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in the 1:1 (v/v) concentration, although the concentrations of 1:5 (v / v) was the less efficient. The incidence of disease increased with the time (Table 2); moreover was observed that plants inoculated with gentamicin sulfate and hydrochloride of oxytetracycline (GSHO), showed similar resoults like *Lipia palmeri* in the 1st observation (3 days) and and 6 dyas), however, after 12 days, the incidence was cloused to 100%; for its part the plants inoculated with Cmm (PIWOT), at 9 days, showed 100% of disease. The severity of the disease ended with the life of the plants at 9 and 12 days after inoculation (Table 3). This effect of severity was higher with the essential oil of OV in concentration 1:1 (v/v), which inhibited in 100% the development of the disease six days after the application; this effect was significative compared with the commercial bactericide ($P<0.05$) (Figure 1). The oils of *Origanum vulgare* and *Thymus vulgaris* in 1: 5 (v/v) concentration were effective only at three days with 0% damage in both oils (Table 3). In a study by Daferera et al. (2003), evaluating the efficacy of *Origanum, Thymus, Dictamnus,* and *Rosmarinus* essential oils in the growth of *Botritis cinerea*, *Fusarium* spp. and *Clavibacter michiganensis* subsp *michiganensis* in artificial growth media, they found total inhibition of pathogens with the essential oils of *Origanum, Thymus* and *Dictamnus* at relatively low concentrations (85-300 µg·ml). The same authors, indicated that the chemical composition of the oils was determined by gas chromatography-mass spectrometry (GC-MS) and thymol was the main component of *Origanum* oil, whereas for *Thymus* and *Dictamnus,* carvacol was principal element. The results generated by Daferera et al. (2003), are agree with results of our investigation, due *Origanum vulgare* (OV), *Lippia palmeri* (LP), *Thymus vulgaris* (TV) which are rich in thymol and carvacol. For it´s part, plants of negative control treatment and those sprayed with only oil´s essentials, no

Tabla 2. Efecto de aceites esenciales sobre la incidencia de la enfermedad del cancro bacteriano en diferentes tiempos.
Table 2. Effect of essential oils on the incidence of bacterial canker disease at different times.

Essential oil	Concentration									
	1:1					1:5				
Day´s	3	6	9	12	15	3	6	9	12	15
GSHO	9cd	20c	82b	89b	100a	8cd	19c	80b	88b	100a
PIWOT	62a	96a	100a	100	100a	59a	94a	99a	97a	100a
Lippia palmeri	11c	26c	83bc	100a	100a	46b	61b	87b	100a	100a
Origanum vulgare	0e	0d	80c	100a	100a	0c	20c	57c	89b	100a
Thymus vulgaris	51b	67b	89b	100a	100a	0c	12d	77c	82a	100a
Cinnamomum zeylanicum	16c	90a	100a	100a	100a	74a	100a	100a	100a	100a

Different literals indicate significant difference with $P \leq 0.05$.; GSHO= gentamicin sulfate and hydrochloride of oxytetracycline; PIWOT: plants inoculated with Cmm

Tabla 3. Efecto de aceites esenciales sobre la severidad de la enfermedad del cancro bacteriano en diferentes tiempos.
Table 3. Effect of essential oils on the severity of bacterial canker disease at different times.

Essential oil	Concentration									
			1:1					1:5		
Day´s	3	6	9	12	15	3	6	9	12	15
GSHO	6cd	17d	79b	89b	100a	5c	16d	73b	80b	100a
PIWOT	62a	87a	100a	100a	100a	59a	85a	100a	100a	100a
Lippia palmeri	5c	50c	100a	100a	100a	50b	75b	100a	100a	100a
Origanum vulgare	0e	0e	75c	100a	100a	0c	20cd	55b	100a	100a
Thymus vulgaris	40b	67b	89b	100a	100a	0c	30c	50b	100a	100a
Cinnamomum zeylanicum	10c	90a	100a	100a	100a	75a	100a	100a	100a	100a

Different literals indicate significant difference with $P \leq 0.05$. GSHO= gentamicin sulfate and hydrochloride of oxytetracycline; PIWOT: plants inoculated with Cmm

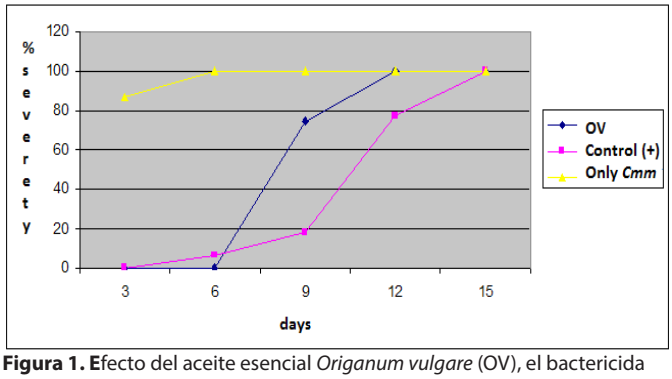

Figura 1. Efecto del aceite esencial *Origanum vulgare* (OV), el bactericida (sulfato de gentamicina y clorhidrato de oxitetraciclina = Control +) en el control del cancro bacteriano *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) en plantas de tomate en concentración 1:1.
Figure 1. Effect of essential oil *Origanum vulgare* (OV), bactericide (gentamycin sulfate and oxytetracycline hydrochloride = Control +) in canker disease *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) control in tomato plants at concentration 1: 1.

Figura 2. Actividad antibacteriana de aceite *Origanum vulgare* sobre planta desarrollada de tomate inoculada con *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) al sexto día después de la inoculación.
Figure 2. Antibacterial activity of *Origanum vulgare* oil on tomato plant grown inoculated with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) at the sixth day after inoculation.

showed negative effects (Table 2). The severity of the disease was observed in all treatments, with no statistically significant differences in both oil concentrations and their interaction ($P<0.05$). The lowest severity was observed in the treatment of *Origanum vulgare* with 75% at 9 days in the 1:1 (v/v) combination, and 20% at 6 days in 1:5 (v/v) concentrations (Fig. 2). *Thymus vulgaris* showed a severity of 30% at 6 days in the 1:5 (v/v) concentration. The highest severity was observed in the treatment with *Cinnamomum zeylanicum* oil at all times and concentations (Table 3, Figure 3). These results indicate that Oregano and Thyme oils can exert a delay in time to appear the first symptoms of wilting in tomato plants, which it provides a margin in to adopt other preventive measures, like periodic applications at intervals of 6 days without increasing the concentration of the oil and stop the propagation of the inoculum to other healthy plants. *In vitro* studies by Dagmar et al. (2008), observed the effect of thirty-four essential oils to inhibit the growth of *Clavibacter michiganensis* subspecies *sepedonicus* (Cms) and *Clavibacter michiganesis* subspecies *insidiosus* (Cmi), detecting that the oils of *Origanum vulgare, O. compactum, Eugenia caryophyllata* and *Artemisia absintium* were the most efficient to control Cms and Cmi bacteria. The results of the present study agree with Dagmar et al. (2008), because the genus *Origanum* predominated in the inhibition of Cmm bacterium. Another important aspect to consider is the composition of the vegetable oils due to the environmental conditions influenced in the habitat where the plants developed (Sivropoulou et al., 1996). The essential oil of *Lippia palmeri* showed control in 50% at six days in concentration of 1: 1 (v/v), while in a concentration of 1: 5 (v/v) controlled up to 75%; the above indicates according the oil concentration increases, the severity decreases in relation to the exposure time (Figure 4). Previous studies have shown the ability of various species of oregano and thyme oils to retard and inhibit the growth of pathogenic bacteria of various plants such as *Agrobacteria tumefaciens, Clavibacter michiganensis* subspecies *michiganensis, Erwinia amylovora, E. carato-*

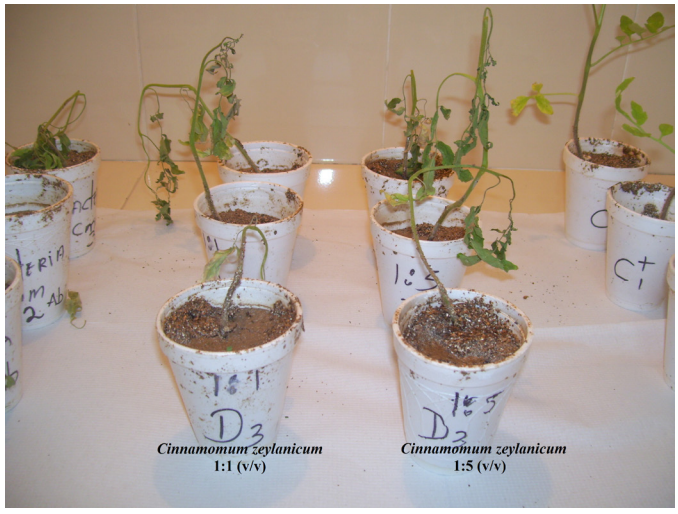

Figura 3. Actividad antibacteriana de aceite *Origanum vulgare* sobre planta desarrollada de tomate inoculada con *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) al sexto día después de la inoculación.
Figure 3. Antibacterial activity of *Origanum vulgare* oil on grown tomato plant inoculated with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) at the sixth day after inoculation.

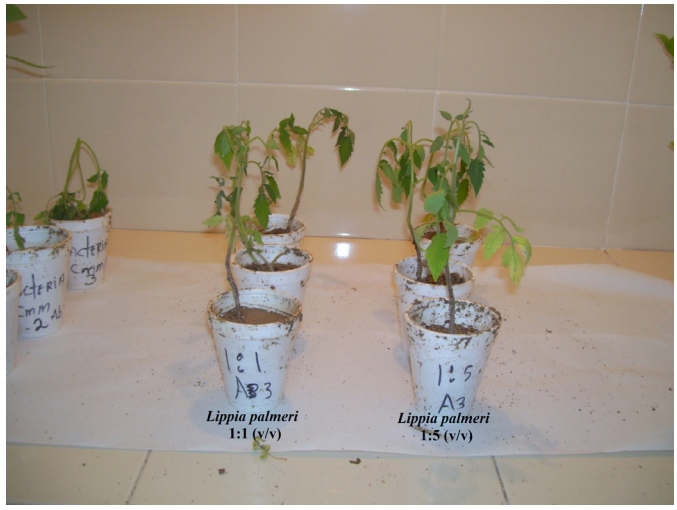

Figura 4. Actividad antibacteriana de aceite *Lippia palmeri* sobre planta desarrollada de tomate inoculada con *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) al sexto día después de la inoculación.
Figure 4. *Lippia palmeri* oil antibacterial activity on tomato plants developed, and inoculated with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) at the the sixth day after inoculation.

vora, Pseudomonas syringae, P. viridiflava, Axonopodis picovoltio, Xanthomonas vesicatoria (Sivropoulou et al., 1996; Vokou et al., 1993; Smith et al., 1997; Yıldız et al., 2001; Soylu et al., 2003). Likewise, similar results were obtained in the present study where a delay of appearance of the Cmm symptom was detected in plants treated with oregano and thyme oils. The mechanisms of action of the various organic compounds of plants are variable. The toxicity of phenols is attributed to the oxidation of compounds in the cell (Cowan, 1999). From carvacol, it has been reported to be present in essential oil of oregano in 60 to 70% and in thyme 45%, inhibiting the growth of many pathogens; however, the exact mechanism of action of this oil has not been defined (Ultee, 2002). With respect to thyme oil, it is mentioned that it may be a membrane break through lipophilic compounds (Friedman et al., 2002). The alcoholic triterpenes can alter the colloidal medium of the cellular protoplasm causing its death (Pelczar, 1992). Also, the application of essential oils from oregano and thyme, among others, has been studied to control the diseases caused by post-harvest fungi of the tomato, *Ralstonia stolonifer, Botrytis cinerea, Alternaria arboressens and Geotrichum candidum*. On the other hand, the essential oils from oregano and thyme, when studied with phytopathogenic fungi, the results have been variable depending on the fungus and oil evaluated (Dagmar et al., 2008); i.e. essential oils of oregano, thyme, lavender, rosemary and *Dictamnus* were tested by Daferera et al. (2003), to know its efficiency of control against *Botritis cinerea* and *Fusarium* sp. in artificial means of growth. The chemical composition of the oils was determined by gas-mass chromatography (GC-MS). The growth of *Botritis cinerea* and *Fusarium* spp. was totally inhibited by essential oils of oregano, thyme and *Dictamnus* spp. at low concentrations (85-300 µg / ml). Thymol was the main component of oregano oil, whereas thyme oils and Dictamnus spp., were rich in carvacol. The concentration of metabolites in the essential oil may be variable as in the case of the cinnamaldehyde concentration of the essential oil from cinnamon that can vary between 60 and 75% (Duke, 1986), whereas thymol and carbachol from the thyme can vary between 3 and 60% of the total (Lawrence, 1984). In the current study, using low doses of the oil, could lead to a decrease for inhibition of the bacteria, observing an initial tendency of major to minor in the control of the bacterium Cmm. The previously and described results indicate that the antibacterial activity of the essential oils may also depend on the relative concentration of the active components. However, the possible chemical interaction between the components is not excluded with synergistic and / or antagonistic effects

CONCLUSIONS

According results obtained, the essential oils and chemical compounds of *Origanum vulgare* and *Thymus vulgaris* had effect against *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) up to 9 days after application in a 1:1 (v/v) concentration and up to 12 days after application with the 1:5 (v/v) concentration. While the essential oil of *Lippia palmeri* only had effect until six days after the application with both concentrations; that is to say, only these three types of essential oils had bactericidal properties, since with the essential oil of *Cinnamomum zeylanicum* the inhibition of Cmm was very limited, both in incidence as in severity.

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