Article

Evaluation of the *in vitro* antibacterial activity of essential oils of oregano and thyme against *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum (*Rs*), is a phytopathogenic bacterium of great importance worldwide known to cause the disease of bacterial wilt. This is a disease that devastates many crops, among them the potato. In order to find a natural alternative to control the *Rs* bacteria, the bactericidal power of the essential oils of oregano (*Thymus vulgaris*) and thyme (*Lippia graveolens*) was evaluated. The technique used for the analysis of the antibacterial activity was diffusion in agar, using sterile filter paper disks, 3 different dilutions were evaluated (1:1, 1:5 and 1:10) and 7.5, 10 and 15 µl were placed of each of the concentrations of the oils. The 70% alcohol was used in one of the filter paper discs as a negative control. In addition, a streptomycin (10 µg disc^{-1}) and ampicillin (10 µg disc^{-1}) were used as positive control. Dextrose agar medium and potato previously inoculated with the strain under study were used. After incubation, the halos of inhibition of bacterial growth were measured in millimeters. The analyzes were in triplicate. The essential oils of oregano and thyme showed inhibitory effects on the growth of *Rs* in the 1:1 dilution that turned out to be more effective than the rest of the dilutions evaluated, and the most effective amount applied was 15 µl of oregano essential oil and thyme compared the antibiotics used. Essential oils could be considered as an alternative for the control of *Rs* in plants.

Keywords: biobactericide, biocontrol, inhibition, bacterial wilt.

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Introduction

Ralstonia solanacearum (*Rs*) is a phytopathogenic bacterium of great importance worldwide known as bacterial wilt (González *et al*., 2009). The causative organism of this disease was previously referred to as *Pseudomonas solanacearum*. Bacterial stain is also known as brown rot. This bacterium lives in the soil (Zhou *et al*., 2008) and is dispersed mainly through it, surviving in this medium for long periods of time. In addition, it is possible to transmit it through water, equipment or infected materials.

It can also be spread by means of transplanting and propagating infected plants, when cutting is done without disinfecting the equipment that previously had contact with an infected plant and an important form of dissemination occurs through irrigation (Aphis and PPQ, 2004). *R. solanacearum*, is a Gram negative bacterium belonging to the Pseudomonadaceas family. With mobile rod morphology by means of one to four even more flagellum polar flagella (Sen Yan *et al*., 2005), it is a disease that devastates numerous crops of economic importance, depending on the breed (three races of *Rs* are known: race 1 that mainly affects Solanaceae and crops of ornamental interest, race 2 affects bananas, and race 3 affects potato) (Pradhanang *et al*., 2003). In this crop (*Solanum tuberosum* L.), the symptoms induced by *R. solanacearum* generally appear in the foliage of the plants.

These symptoms consist of the wilting of the younger leaves in the terminal parts of the branches, in addition, the initial symptoms of mild yellowing on only one side of the leaf or on one branch and not the next (Brader *et al*., 2007). In young stems, infected by *Rs*, they can become visible as long, narrow, dark brown rays. In advanced infections (severities> 60%), cross sections of the stem or stolon may reveal brown discoloration of the infected tissues (Swanson *et al*., 2005). Symptoms can be found in potato tubers. Infected potato tuber may reveal gray-brown discoloration.

As the infection progresses, discoloration can spread to the tuber cortex with a milky (mucilaginous) exudate, which indicates the presence of bacterial cells, it can also be observed in freshly cut sections of infected tubers bacterial exudate may also be visible in the eyes or at the point where the stolon connects to the tuber. These signs or symptoms may not be visible early in the development of the disease (Smith and Saddler, 2001).

When the tubercle is clearly infected, if it is cut transversely and light pressure is applied, white drops of bacterial exudate leave the vascular ring. This exudate is mixed with the soil and the mixture of bacterial exudate and soil dries and adheres to the surface of the tuber. The tubers left on the earth continue their rotting process; the bacteria continue to destroy the tissues surrounding the vascular ring and finally the skin breaks and cracks appear.

Therefore, a viscous mass of unpleasant odor remains (Pastrik and Maiss, 2000). The producers with the purpose of controlling the disease and being able to compete commercially, as the main alternative resort to the use of agrochemicals. However, one of its main disadvantages is the resistance that pests acquire to chemical products, reporting that resistance is not acquired only to some “active substances” but also to chemosterilizing substances, antibiotics, bacterial toxins,

fungicides, herbicides, anticoagulants, methyl bromide, phosphamine and other agents. Resistance to pesticides is currently the main problem in agricultural production worldwide, since 1990 there have been reports of plants resistant to herbicides and fungi with resistance to fungicides in addition to resistance in insects and mites (Champoiseau *et al*., 2009).

On the other hand, the society to the agricultural sector demands that the use of chemical products as agro-inputs be increasingly smaller, so that alternative measures are sought to help reduce the incidence of pests and diseases, without affecting the health of the consumer and the environment (Palacio *et al*., 2009). In recent years, there has been a growing interest in the use of biologically active organic compounds, extracted from plant species that have the ability to eliminate pathogenic microorganisms by themselves, this is mainly due to the resistance that microorganisms have developed to the antibiotics (Daferera *et al*., 2003). The agriculture of the new millennium should establish new control alternatives that produce a lower environmental impact, since day by day the percentage of consumers who demand healthy food and free of chemical products is increasing (Ponce *et al*., 2004).

Essential oils obtained from plants are an alternative for the control of bacteria, because they are mixtures of substances obtained from plants whose main characteristics are their complex chemical composition and strongly aromatic character (Sánchez, 2006); They are biodegradable, friendly to the environment and have low or non-existent levels of toxicity, which allows them to be used in any environment (Cheng *et al*., 2009), they have insecticidal, antioxidant, antibacterial, antifungal and antiviral characteristics (Burt, 2004; Kordali *et al*., 2005) and be extracted by different methods.

The most commonly used technique is steam distillation, where the plant sample is placed in a container (closed clavenger type) and subjected to a stream of water vapor, so the essence is entrained and then condensed, collected and separated from the aqueous fraction (Ortega, 2011). Aromatic plants are important for obtaining essential oils such as thyme (*Thymus vulgaris* L.) and oregano (*Lippia graveolens* L.)

Regarding thyme, this species belongs to the Labiatae Family (Muñoz, 1996), with medicinal importance in the pharmaceutical sector; it has antiseptic, disinfectant, antispasmodic, deodorant and sedative properties and its main components are thymol and carvacol (Muñoz, 1993, El-Hela, 2007). For its part, Mexican oregano (*Lippia graveolens*) is characterized by being a perennial plant, of the *verbenaceae* family, which grows in semi-arid climates and is traditionally used as an intestinal antiseptic, antispasmodic, analgesic and anti-inflammatory and has antibacterial properties (Castillo *et al*., 2007; Osorno *et al*., 2009). It has been shown that the genera of *Lippia* and *Thymus*, among other aromatic plants, have antioxidant properties, related to phenolic compounds, carvacrol and thymol that can be used under certain conditions such as fungicides and bactericides (Aballa and Rosen, 2001).

Nowadays, essential oils represent a new alternative for the control of phytopathogenic bacteria and for the management of the potato crop, since it does not cause damage to the environment or to health. Based on the above, the present investigation arose, where the bactericidal effect of the essential oils of oregano (*Lippia graveolens*) and thyme (*Thymus vulgaris*) was evaluated against the bacterium *Ralstonia solanacearum* that causes the disease of bacterial wilt in the crop of potato.

Materials and methods

The evaluation of the antibacterial activity of two essential oils was carried out in the phytopathology laboratory of the Center for Biological Research of the Northwest (CIBNOR), by in vitro tests under controlled conditions of temperature at 30 ºC and humidity of 90%.

Obtaining oils

The two essential oils used were: oregano (*Lippia graveolens*) and thyme (*Thymus vulgaris* L.).

Oregano (*Lippia graveolens*) was collected from plants from Naica, Chihuahua, located at the geographic coordinates at 27º 51' 17" north latitude and 105º 29' 33" west longitude. Oregano essential oil was obtained by steam drag, following the Official Method of the Association of Chemical Analysis (AOAC) 6.006 (1975), dividing the volatile compounds of the essential oil and using a Clavender distiller of 2 liters' capacity (Castillo *et al*., 2007).

The quality of the essential oils selected for this study ranged between 98 and 99% of purity according to (Ortega *et al*., 2005; Yesil *et al*., 2007). Regarding thyme, a commercial sample was used, which was purchased from the local store located in Hermosillo, Sonora, the thyme essential oil is from the Soria Natural brand and distributed by the Herbofarm house in Madrid, Spain, which are obtained by large scale water vapor drag with a purity level of 99%.

Bacterial cultures

The bacterial strain used in the present study was *Ralstonia solanacearum*, which was isolated and characterized from isolations of potato tubers from commercial food stores in the state of Sonora, Mexico (Alvarado, 2011).

Determination of the antibacterial activity of essential oils

Inoculum preparation

The bacterial strain was grown in a culture of 24 h at 30 °C, in nutritive broth (Difco, Sparks, MD) (beef extract 3 g and peptone 5 g) and adjusted to a concentration of $1x10^8$ UFC ml^{-1} with phosphate buffer saline (PBS). The bacterial inoculum was seeded massively on dextrose and potato agar plates, using a sterile cotton swab, to achieve uniform microbial growth (Borboa *et al*., 2010).

Disk diffusion method

Once the plates were inoculated with the bacteria, filter paper discs of approximately 10 mm in diameter were placed in the center thereof, in which different quantities of the essential oils under study were applied.

Antimicrobial activity

The essential oils were prepared at different concentrations using 70% ethyl alcohol as a diluent. The dilutions used were 1:1, 1:5 and 1:10. Aseptically, 7.5, 10, 15 µl of each of the concentrations of the essential oils were placed on the filter paper discs.

The 70% alcohol was used in one of the filter paper discs as a negative control and to rule out the antimicrobial activity thereof. In addition, a streptomycin (10 µg $disc^{-1}$) and ampicillin (10 µg $disc^{-1}$) disc were used as positive reference controls. After impregnating the discs with the respective treatment, the plates were incubated at 30 °C for 24 h. After the incubation period, the halos of bacterial growth inhibition were measured in millimeters using a ruler. The analyzes were carried out in triplicate.

Statistical analysis

The experimental design was trifactorial A∗B∗C, where the factor A is the 2 oils (oregano and thyme); factor B: the three dilutions (1:1, 1:5, 1:10) and the factor C: the 3 amounts applied 7.5, 10 and 15 µl. An Anova GLM was performed on the obtained data, significantly estimated at one ($p \leq 0.05$). The comparison of means was also performed using Tukey's multiple range test. All data were processed in the statistical package NCSS (2001) and (Jerry and Kaysuville, 2007). With the results obtained, the percentage of inhibition was calculated by previously transforming the values with arcosene (Sokal and Rohfl, 1988).

Results and discussion

Both the essential oils of oregano and thyme presented halos of inhibition, as proof of antimicrobial activity against *Ralstonia solanacearum*, in all concentrations and amounts applied.

Oregano

The average percentage antibacterial activity obtained from all concentrations and different amounts of oregano extract applied was 47.65%. The average percentages of inhibition for the concentrations studied (1:1, 1:5 and 1:10) were 67.6, 39.33 and 36.33% respectively (Figure 1 and Table 1). In a similar study (Borboa *et al*., 2010), in which the antibacterial activity of the oils of four different varieties of oregano was evaluated on the bacterium *Clavibacter michiganensis* subsp. *michiganensis*, average values of 47.5, 35.6 and 30.8 mm were found diameter for the same concentrations and in which the applied amount of 15 µl was used. The inhibition results obtained in the present study are greater than those found by Borboa *et al*. (2010), this may be due not only to the different sensitivity of the bacteria under study, but also to the different composition of the oregano oils used (Pradhanang *et al*., 2003).

The concentration of oregano essential oil that showed a higher antibacterial activity against the *Rs* bacteria was 1:1 in the different amounts applied of 15 µl with 65%, 10 µl with 68% mm and 7.5 µl with 65% inhibition (Figure 1). For its part in the applied concentrations of 7.5, 10 and 15 µl in

the dilutions of 1:5 and 1:10, the numerical percentage values show no significant difference, except for the amount of 15 µl in the dilution 1:5 that showed a higher value of 52% inhibition, 10 to 18% higher than the other amounts applied in the present study (Table 1).

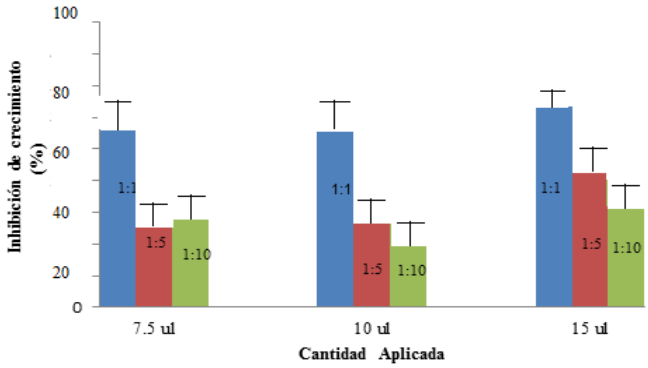

Figure 1. Percentage of growth inhibition of *Ralstonia solanacearum*, by effect of oregano essential oil in dilutions 1: 1, 1: 5, 1:10 at different amounts applied 7.5, 10 and 15 µl.

Table 1. Percentage values (%) of growth inhibition of *Ralstonia solanacearum* due to the effect of essential oils (trifactorial AXBXC).

Aceite	Dilutions	Amount applied	Inhibition percentage (%)
Oregano	1:1	15 µl	70.4 a
Thyme	1:1	15 µl	70.2 a
Oregano	1:1	10 µl	68.1 ab
Thyme	1:1	10 µl	65.5 abc
Oregano	1:1	7.5 µl	65.2 abc
Thyme	1:5	15 µl	63.4 abcd
Thyme	1:1	7.5 µl	62.5 abcde
Thyme	1:5	10 µl	61.9 abcdef
Thyme	1:10	15 µl	55.7 bcdef
Oregano	1:5	15 µl	52.1 cdef
Thyme	1:5	7.5 µl	45.4 cdef
Oregano	1:10	15 µl	40.2 def
Oregano	1:10	7.5 µl	39.1 ef
Thyme	1:10	10 µl	35.5 efg
Oregano	1:5	10 µl	34.4 efg
Oregano	1:5	7.5 µl	32.2 fg
Oregano	1:10	10 µl	30.4 fg
Thyme	1:10	7.5 µl	15.4 g
*Alcohol 70%			0 h
+Estreptomicina (10 µg disc^{-1})	(10 µg disc^{-1})	(10 µg disc^{-1})	67.8 ab
+Ampicilina (10 µg disc^{-1})	(10 µg disc^{-1})	(10 µg disc^{-1})	66.2 abc

*= negative control: inoculation by the fungus to be evaluated without presence of extract; += positive reference controls. Different literals indicate significant difference with $p< 0.05$.

Regarding the positive and negative controls (alcohol as negative control and streptomycin and ampicillin as positive reference controls), the values indicate that streptomycin was higher than ampicillin but lower than oregano treatments in the amounts of 15 and 10 µl in the dilutions of 1:1 and thyme in the amount of 15 µl of the 1:1 dilution (Table 1), which leads to consider these dilutions as possible concentrations suitable for the control of *Rs*; however, it is important to indicate that in addition to these biological effectiveness studies, phyto toxicity studies should be conducted to evaluate whether these concentrations do not have an adverse effect on the proper development of the plant throughout the phenological cycle (Castro, 2004; Osorno *et al*., 2009).

Thyme

The average percentage antibacterial activity obtained from all the concentrations and different amounts of thyme extract evaluated was 46.64%. The average values of inhibition found were 65.83, 38.56 and 35.53% inhibition, for the concentrations 1:1, 1:5 and 1:10, respectively (Figure 2). These results coincide in relation to the study developed by Borboa *et al*. (2010) for the bacteria *Clavibacter michiganensis* subsp. *michiganensis*, who obtained percentages of 50.3, 33 and 21 for the same concentrations. However, it should be noted that for *Rs* the 1:1 dilution with dilutions of 7.5, 10 and 15 µl behaved numerically superior to those presented by the same author Borboa *et al*. (2010).

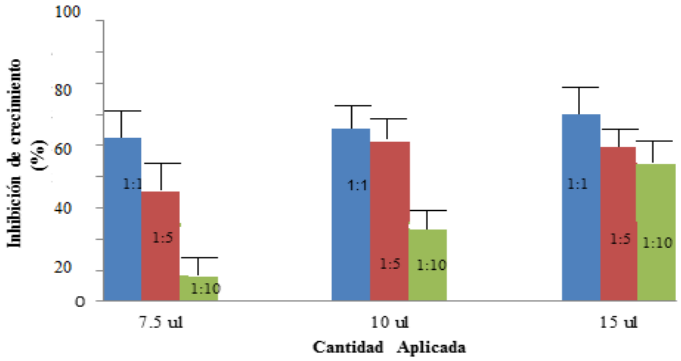

Figure 2. Percentage of growth inhibition of *Ralstonia solanacearum*, by effect of thyme essential oil in dilutions 1:1, 1:5, 1:10 at different amounts applied 7.5, 10 and 15 µl.

However, even though in the present study the oils under study have not been characterized, the results obtained suggest that it is due to the participation of secondary metabolites that have the ability to inhibit the growth of pathogens (Corella-Bernal and Ortega-Nieblas, 2013; Silva-Vázquez *et al*., 2015). Studies by Arango-Bedoya *et al*. (2015), report among the major components Carvacrol, Timol, p-cimeno and cineol 1.8 as responsible for the inhibitory activity. Under in vitro conditions they tested the essential oil of oregano (*Lippia origanoides*) vs the phytopathogen *Phytophthora infestans*, obtaining significant results (20.53 µg ml^{-1}) in an inhibition in the growth of the pathogen of 50% (CE50).

On the other hand, Ortega-Nieblas *et al*. (2011), report the antimicrobial activity of essential oils of *Lippia palmeri* against four Gram-positive bacteria and six Gram-negative bacteria, the results of these authors show an increased activity against *Escherichia coli* O157: H7 and

Staphylococcus aureus, important bacteria that are affecting human health and have also been reported present in horticultural products. The results obtained in the present study further support the use of plant extracts such as *Lippia palmeri* for support in the control of fungi and pathogenic bacteria.

When making the comparison between the essential oils of oregano (*Lippia graveolens*) and thyme (*Thymus vulgaris* L.), of the average percentage values of inhibition in the growth of *Ralstonia solanacearum*, it can be seen that there is no significant difference in oregano and thyme. The two oils are considered to have an inhibitory effect on the growth of *Ralstonia solanacearum*, an effect that in other researches has proven their growth inhibitory abilities in pathogenic bacteria of plants such as: *Agrobacteria tumefaciens*, *C. michiganensis* subsp. *michiganensis*, *Erwinia amylovora*, *E. caratovora* and *Xanthomonas vesicatoria* (Sivropoulou *et al*., 1996; Smith *et al*., 1997; Soylu *et al*., 2006).

Regarding the comparison of the mean values of the dilutions (1:1.1:5, 1:10 µl.) of the essential oils of oregano and thyme in the percentage of growth inhibition of *Ralstonia solanacearum*, there were significant differences in the 1:1 dilution unlike the following dilutions 1:5 and 1:10. In addition, a similar result with the 1:1 dilution was obtained in the growth inhibition study of *Clavibacter michiganensis* subsp. *michiganensis* with oregano and thyme oils (Borboa *et al*., 2010). In the comparison of percentage values of inhibition of the amounts applied (7.5, 10, 15 µl.) of oregano and thyme vs *Ralstonia solanacearum* growth, there were significant differences in the amount applied of 15 µl, 7.5 µl and 10 µl (Table 1). Figures 3 and 4, are a representation of the inhibitory effect of the oils evaluated.

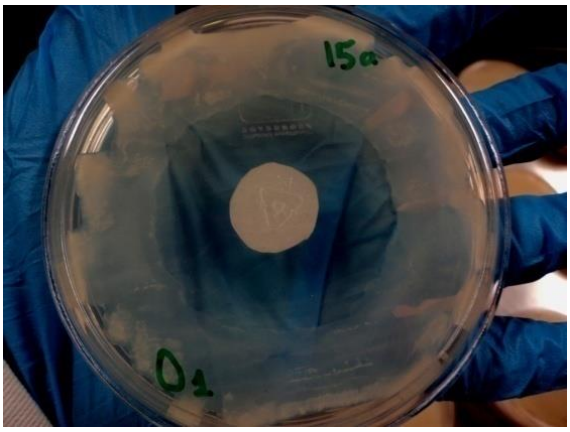

Figure 3. Growth inhibition halo of *Ralstonia solanacearum* with oil essential oregano (*Lippia graveolens*) dilution 1:1, 15 µl.

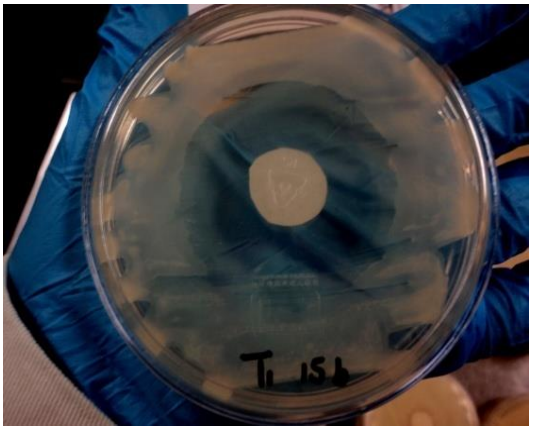

Figure 4. Growth inhibition halo of *Ralstonia solanacearum* with oil essential Thyme (*Thymus vulgaris* L.) dilution 1:1, 15 µl.

Regarding the positive and negative controls applied in the present study (alcohol as negative control and streptomycin and ampicillin as positive reference controls), the values show a similar behavior to that indicated with oregano, but with the particularity that thyme was superior in the amounts of 15 µl at the 1:1 dilution, vs. positive reference controls (Table 1).

Conclusions

The essential oils of oregano and thyme showed inhibitory effects on the growth of the bacterium *Ralstonia solanacearum* in the dilution 1:1 and in the amount of 15 µl. The essential oils of oregano and thyme showed better inhibitory effect than the antibiotics used streptomycin (10 µg) and ampicillin (10 µg), which leads to consider them as an alternative of adequate biological control vs *Rs*; however, it is important to indicate that phyto-toxicity studies are suggested to evaluate whether these concentrations do not have an adverse effect on the proper development of the plant throughout the phenological cycle.

Gratefulness

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