Investigation note

In vitro organic control of *Phytophthora cinnamomi* with essential oils of oregano and clove

Yisa María Ochoa Fuentes[1]
Anselmo Hernández Pérez[1]
Juan Carlos Delgado Ortiz[2]
Omegar Hernández Bautista[2]
Ernesto Cerna Chavez[1]
Luis Alberto Aguirre Uribe[1]
Luis Mario Tapia Vargas[3§]

[1]Department of Parasitology-Autonomous Agrarian University Antonio Narro. Buenavista, Saltillo, Coahuila, Mexico. CP. 25315. (yisa8a@yahoo.com; jabaly1@yahoo.com; luisaguirreu@yahoo.com.mx). [2]CONACYT-UAAAN Autonomous Agrarian University Antonio Narro-Department of Parasitology, Buenavista, Saltillo, Coahuila, Mexico. CP. 25315. (hernandez.anselmo1@gmail.com, moe-788@hotmail.com). [3]Experimental Field Uruapan-INIFAP. Avenida Latinoamericana No. 1101, Colonia Revolucion, Uruapan, Michoacán, Mexico. CP. 60500.

[§]Corresponding author: mariotv60@hotmail.com.

Abstract

Michoacán is the leading avocado producing state in the world; however, root diseases decimate and damage the trees causing their death. The aim of the present investigation was to evaluate the organic control of the *in vitro* growth of *Phytophthora cinnamomi* with essential oils of oregano (*Lippia berlandieri*) and clove (*Syzygium aromaticum*). In the months of October and November 2016, root samples were collected from trees with symptoms of the disease in avocado (*Persea americana* Mill. Var. Hass), in the INIFAP experimental garden located in San Juan Nuevo Parangaricutiro, Michoacán. The isolates were identified morphologically and molecularly. The control of *P. cinnamomi* with essential oils of oregano and clove was evaluated determining the mean inhibitory concentration and its fiducial limits to 95% by means of a Probit regression by the method of maximum likelihood. The analyzes were carried out using the statistical program R 3.4. According to the results obtained, in relation to the inhibition of growth there is a reduction in the growth of *P. cinnamomi*. The essential oils of clove (*Syzygium aromaticum*) and oregano (*Lippia berlandieri*) are a natural alternative for the control of the oomycete *P. cinnamomi* for their fungicidal activity at low concentrations so they can be included in integrated disease management programs.

Keywords: *Persea americana* Mill. Var. Hass., essential oil, growth rate, inhibition.

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The avocado (*Persea americana* Mill.) Is the fruit of a tree native to Mexico and Central America (Teliz, 2000). The world production of the crop is around 4 700 000 tons and 70.3% of this production is provided by the American continent (FAOSTAT, 2015). The Mexican Republic is the most important producer and exporter of avocado in the world, with a production of almost 2 million tons, of which the state of Michoacán contributes 77% of the national production, making it the world capital of this crop (SIAP 2017).

However, there are different phytosanitary limits, which significantly reduce yield, causing malformations of the fruit, causing considerable losses in postharvest and even the death of the tree. Different pathogens are known to cause fungal diseases known as scab (*Sphaceloma perseae*), anthracnose (*Colletotrichum gloeosporioides*) and the cosmopolitan oomycete *Phytophthora cinnamomi*, which causes rotting of the root system and in the aerial part of the tree a wilt known as sadness of the avocado, *P. cinnamomi* is considered of greater economic importance (Zentmyer *et al*., 1994; Pérez, 2008).

This pathogen attacks all the varieties of avocado in the world, damaging the roots due to a plugging of the vascular bundles, which translates into the death of the tree (Coffey, 1992; Whiley *et al*., 2007). In Mexico, the presence of the disease known as avocado sadness has been detected in all the producing areas; emphasizing by the severity of the damages, as for example in the region of Atlixco, Puebla, where it caused the almost disappearance of this cultivation Reyna (1983). In the production region of Michoacán, it is considered that around 4 000 ha are affected by the disease, presenting an exponential trend (Téliz, 2000).

In this sense, it is necessary to have extensive knowledge of the *in vitro* behavior of *P. cinnamomi* as well as of organic alternatives for its control. Therefore, it is necessary to start from alternative culture media to accelerate their development and therefore be able to make rapid assessments regarding their growth, since this phytopathogen is affected in its in vitro development by different factors such as temperature. The control of fungal diseases has depended to a great extent on the treatments with agrochemicals; however, its use represents a severe risk to human health and contributes to the increase in pollution to the environment (Abdel-Monahim *et al*., 2011).

To reduce this problem, there is a need to research, generate, validate, transfer and adopt strategies that are accessible, simple to apply and non-toxic for humans and animals (Naeini *et al*., 2010). Today, natural products are widely accepted and increasingly replace chemical synthesis products.

In response to this trend, there has been growing interest in research into the possible use of essential oils and plant extracts as natural fungicides, which are not harmful to the environment (Benites *et al*., 2009; Bajpai and Kang, 2010). It has been shown that essential oils and their compounds have a fungicidal effect (Wilson *et al*., 1997; Gogoi *et al*., 1997), which has increased the interest in the application of this type of products as natural antimicrobial agents in food and agricultural crops (Celis *et al*., 2012). Therefore, the agriculture of the new millennium must establish new control alternatives that produce a lower environmental impact, since day by day the percentage of consumers who demand safe food and free of chemical waste, safe for human health is increasing (Ponce *et al*., 2004).

Therefore, the objective of the present investigation was to evaluate the dynamics of *in vitro* growth of *P. cinnamomi* in alternative culture media and its control with essential oils of oregano (*Lippia berlandieri*) and clove (*Syzygium aromaticum*).

Sampling: in the months of October and November 2016, root samples were collected in avocado trees (*Persea americana* Mill. var. Hass) under inoculum pressure that presented characteristic symptomatology of descending death known as avocado sadness. The collection site was the experimental garden of INIFAP located in San Juan Nuevo Parangaricutiro, Michoacán, whose semi-warm, sub-humid climatic conditions with summer rains oscillate between 1 200 to 1 600 mm of precipitation and temperatures of 10 to 28 °C (García, 1981).

A directed sampling was carried out close to the drip area at a depth of 30 cm at four equidistant points. With the help of a straight shovel, root samples were taken (2 to 6 mm in diameter), with the presence of damage (necrotic dark brown tissue) and placed in polyethylene bags previously labeled with the data from the orchard, municipality and georeferencing, later they were transported to the phytopathology laboratory of the Department of Agricultural Parasitology at the Autonomous Agrarian University Antonio Narro (UAAAN).

Isolates of phytopathogens: the roots were washed with sterile distilled water to fragment them in pieces no greater than 0.5 cm, with a sterile scalpel a longitudinal cut was made selecting the limits of healthy and diseased tissue, the root cuts were washed in solution of 1% sodium hypochlorite for 3 min, followed by three washes with sterile distilled water and placed on absorbent paper previously sterilized. Subsequently, V8®-PARPH was planted in selective medium placing four pieces of roots horizontally in Petri dishes of 8.5 cm in diameter; three boxes per sample, giving a total of 12 roots per tree sampled and finally the isolates were incubated at 28 °C for three days in total darkness (Fierro, 2011).

Purification and multiplication: V8® agar (Erwin and Ribeiro, 1996), strains with characteristic growth of *P. cinnamomi*, were transferred to culture medium. The purification technique used was by tip of hypha in triplicate, placed in the center of the Petri dish, sealed with plastic sealing film (cling wrap) and incubated at 28 °C in a bioclimatic chamber for 72 h in the Phytopathology laboratory. Department of Agricultural Parasitology.

Morphological and molecular identification: the sporulation of the pathogen was induced with incubation time of 7 days and controlled temperature of 28 °C ±2, were identified morphologically considering cenocytic mycelia, hyaline, with the presence of oogonium and coraloid growth, coinciding with that reported by Erwin and Ribeiro (1996) and molecularly using the PCR-ITS technique, extracting DNA according to the methodology of Doyle and Doyle (1990), from 0.2 g of mycelium from the pure culture with lysis buffer (EDTA 50 mM, pH 8.5; Tris HCl 100 mM, pH 8; NaCl 50 mM; SDS 2%).

DNA visualization was performed on a 2% agarose gel stained with GelRed (GenScript®). The amplification of the ITS region was carried out with the primers ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'). In the same way, the product of the reaction was visualized by means of electrophoresis in 2% agarose gel stained with GelRed (GenScript®) and the PCR product was sent to the phytosanitary diagnostic laboratory UA-LAB.

Bioassays: the essential oils (AE) were obtained from leaves of oregano (*Lippia berlandieri*) (T1) and flower buds of cloves (*Syzygium aromaticum*) (T2), by means of the steam trawl technique (Ortuño, 2006). The biological effectiveness of the AE on *P. cinnamomi* was determined with the methodology of the poisoned V8®-Agar culture medium with different concentrations 5, 45, 80, 200, 400 and 800 ppm with four repetitions each and an absolute control, added alcohol, tween 80 and xanthan gum as emulsifying agents.

The sowing was carried out after 24 h placing 5 mm diameter explants in the center of the Petri dishes and they were incubated in total darkness at 28 ±2 ºC. To record the mycelial growth, the radial growth was measured every 24 h at the four cardinal points of the boxes and ended when the absolute controls (TA) of the phytopathogen filled the Petri dish. The percentage of inhibition in *P. cinnamomi* was calculated using the formula used by Ochoa *et al*. (2012) which consists of determining the percentage by the reason of the difference of the treatments and the control, with respect to the growth of the control.

Statistical analysis: in the bioassays, the mean inhibitory concentration and its fiducial limits were determined at 95% by means of a Probit regression by the maximum likelihood method. The analyzes were carried out using the statistical program R 3.4.

Derived from the isolation and morphological and molecular identification of the phytopathogens obtained from the samples taken in the experimental garden under inoculum pressure, different strains of the same species were obtained, coinciding with the identification keys of Erwin and Ribeiro (1996) for *P. cinnamomi*, confirming the identification by comparing the products of the sequencing with the records of the GenBank database (Table 1).

Table 1. Molecular characterization of the isolates of the sequences reported in the gene bank with the intergenic sequences (ITS) of the rDNA genes.

Strain[1]	Access No.[2]	Species	Similarity[3]	Origin[4]
Pc-33	LN846114.1	*Phytophthora cinnamomi*	99%	Canary Islands
Pc-41	LN846114.1	*Phytophthora cinnamomi*	99%	Canary Islands
Pc-42	KP183223.1	*Phytophthora cinnamomi*	99%	Australia B

[1]= nomenclature for the different isolations; [2]= number of access in the NCBI database (National Center of Biotechnology Information); [3]= index of similarity between the sequences of the isolated species and the species compared; [4]= geographic origin of the isolates.

Regarding the inhibition obtained with the essential oils, the regressions are highly significant in the treatments evaluated (α= 0.05) because at a higher concentration a reduction in the growth of *P. cinnamomi* was observed (Figure 1). The average inhibitory concentration estimated for the essential oil of oregano was 59.36 ppm, obtaining a 100% inhibition against the mycelial growth of the Oomycete at a concentration of 800 ppm.

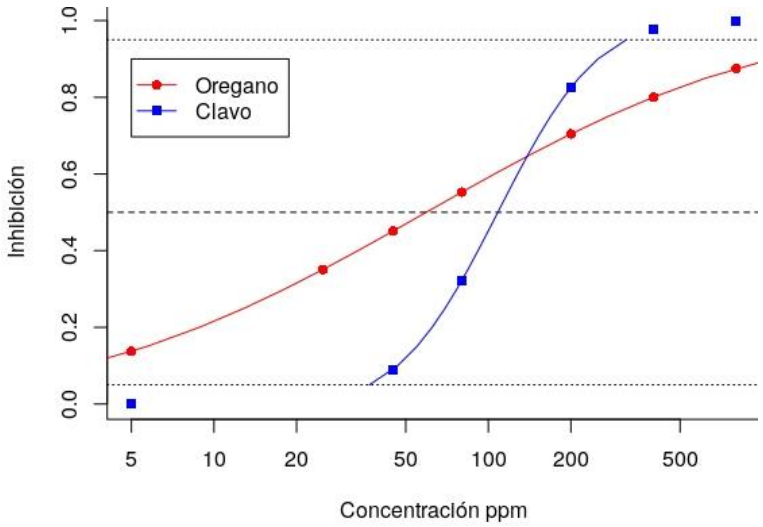

Figure 1. Inhibition of *P. cinnamomi* with oregano and clove essential oil at different concentrations.

In *P. infestans*, phytopathogen belonging to the same genus, Soylu *et al*. (2006) Reported total inhibition with oregano essential oil at a dose of 0.3 µg ml^{-1}, similar investigations refer to the use of this oil for the control of Deuteromycetes, Cueto *et al*. (2010) presents results of the fungicidal action of oil and ethanolic extract of oregano on *Fusarium oxysporum*, however the antifungal action may vary according to the biology of the pathogen: for example, García *et al*. (2006) reported a 100% inhibition at a concentration of 100 ppm in *Aspergillus flavus*, whose IC_{50} value is lower than in our research. On the other hand, Carrillo *et al*. (2010) attributes its fungicidal effect associating it with the content of thymol and carvacrol.

These compounds of terpene units present in the essential oils of some species of the family Lamiaceae, act causing alterations in the morphology and hyphal aggregates, causing a reduction of growth by lysis between the wall and the cell membrane of the pathogen agent (Kordal *et al*., 2008).

Carvacrol increases the fluidity of the membrane causing leakage of protons and potassium ions, resulting in a collapse of the membrane potential and the inhibition of ATP synthesis (Fisher and Phillips, 2008). Regarding the essential oil AE nail (T2), presented an IC_{50} higher than AE oregano being 1.824x higher (Table 2); however, when observing its IC_{95}, this value is lower with respect to T1 (AE oregano), considering Figure 1, this is due to the homogeneity of the susceptibility of the pathogen to the essential oil (T2), which is found with lesser amount of oil, different from T1, which requires a higher concentration to increase inhibition. Currently, this essential oil is used in agriculture to counteract other phytopathogens such as *Phytophthora nicotianae* (Browers and Locke, 2004). *S. aromaticum* obtained by traditional distillation and assisted by microwaves, is efficient to inhibit the development of *Alternaria solani* and *Colletotrichum gloesporioides* in values of 30 and 10% isolated from tomato and papaya, respectively (Ramírez *et al*., 2016). On the other hand, Damián *et al*. (2010) reported that the extract of *Artemisa* sp., inhibits 100% of the mycelial growth of *Phytophthora cactorum*, *P. capsici*, *P. cinnamomi* and *P. mirabilis*, as well as 60% of *P. infestans*, at a dose 100 ppm, this information differs from the results of the present study, higher concentrations are needed for the control of *P. cinnamomi*.

Table 2. Mean inhibitory concentration of essential oils on *P. cinnamomi*.

Treatment	n	gl	ppm				Pred. Eq.	P value
			IC_{50}	LFI - LFS	IC_{05}	IC_{95}		
Lippia berlandieri	24	5	59.36	26.84-114.09	1.437	2451.75	y= -1.8+1.01	$6.50e^{-06}$
Syzygium aromaticum	24	5	108.3	87.29-134.06	36.862	318.208	y= -7.15+3.51	$1.71e^{-11}$

N= number of repetitions; gl= degrees of freedom; IC= inhibitory concentration, LFI and LFS upper fiducial limit and lower 95%; Ec. Pred.= Prediction equation, P value, probability value (α= 0.05).

Raina (2001) by gas chromatography evaluated the composition of the clove oil, noting that the alilbenzene eugenol is the main compound with 94%, whose mode of action promotes the disruption of the cytoplasmic activity of the membrane increasing its non-specific permeability, in addition suggests that eugenol possesses ATPase inhibitory activity (Gutiérrez *et al*., 2017). Both treatments are potential control agents that can be included in avocado sadness management programs caused by *P. cinnamomi*, the compounds and secondary metabolites of the different botanical species play an important role in their resistance against pests, therefore, research on the antimicrobial properties of essential oils allows the discovery of new agents for the control of phytopathogens in organic form (Kordali *et al*., 2007; Lee, 2007).

Conclusions

According to the results obtained, the Centeno-agar culture medium was the most efficient for the isolation and proliferation of *Phytophthora cinnamomi* obtaining a greater growth of morphological structures. The essential oils of clove (*Syzygium aromaticum*) and oregano (*Lippia berlandieri*) are a botanical alternative for the control of the oomycete *P. cinnamomi* for their fungicidal activity so they can be included in integrated disease management programs, at a lower cost than conventional agrochemicals.

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