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PLANT EXTRACTS FOR THE IN VITRO CONTROL OF COLLETOTRICHUM *GLOEOSPORIOIDES* P. ISOLATED FROM *CARICA PAPAYA* L.

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— Abstract—

Papaya is a crop of great importance worldwide; Mexico and Colombia are world leaders in its production. One of the main limitations for this crop is the diseases that include the anthracnose that affects pre and post-harvest, which is why the search for efficient, sustainable, and harmless alternatives in crop health is essential to reduce losses in production and thereby improve the income and quality of life of the producer and those involved in the production chain to the consumer with quality products and without contaminants that affect their health. The objective of this study was to evaluate plant extracts under two forms of extraction in the *in vitro* control of *Colletotrichum gloesporioides*. Therefore, the antifungal effect of plant extracts of oregano leaves (*Oreganum vulgare*), pepper fruits (*Pimienta dioica*), neem leaves (*Azadirachta indica*), and dried matarraton leaves (*Gliricidia sepium*) obtained from distillation and microwave methods was evaluated on *C. gloesporioides* isolated from *Carica papaya*. We used the technique of poisoned medium in Petri dishes with PDA medium; in the first phase, the concentration of 60% was tested for all extracts. Subsequently, the minimum inhibitory concentration was determined for those who in the first phase inhibited 100%. The antifungal effect was determined by mycelial growth, the number of total and germinated conidia. The results indicated that the extract of pepper fruit obtained by microwave in a concentration of 40% and M2 UNACH patent of 20 % inhibited *C. gloesporioides*. The extracts obtained by the microwave had a better antifungal effect than those obtained by distillation.

Keywords

Anthracnose; Pimienta dioica; microwave; antifungal.

Papaya is also called *lechosa* in Venezuela, *fruta bomba* in Cuba and *Mamao* in Brazil. It is one of the most popular tropical fruits because of its mild and pleasant flavor and the nutritional properties attributed to it. It is highly sought after by consumers who have a preference for fruits that are not only pleasant but also have a high nutrient content (Corporación Colombiana de Investigación Agropecuaria [Corpoica], 2000). The production of papaya (*Carica papaya L.*) is of great economic importance; Mexico ranks fifth as a world producer and first as an exporting country, while Colombia is in eighth place as a world producer (Evans, 2015). There is a relevant production volume for papaya cultivation in Colombia, according to the National Agricultural Survey (ENA) published by the National Administrative Department of Statistics (DANE, 2016), during 2015, Colombia produced 105,459 tons of papaya. The papaya crop is subject to various diseases during rainy periods; the most important of these are considered to be: papaya ringspot, followed by anthracnose, root and foot rot, and black spot (DANE, 2016). *Colletotrichum gloeosporioides P.*, is the pathogenic agent causing anthracnose on *Carica papaya L.*, a disease found in all areas where papaya is grown and becomes the main cause of postharvest fruit losses (Instituto de Investigaciones Agropecuarias [INIA], 2016), with estimated losses of 25-40% (Quiroga, 2016) and occurs in all producing regions of the world, causing huge losses in Brazil, Hawaii (USA), and Mexico (Ventura, et al., 2004).

Colletotrichum gloeosporioides (Penz.) Sacc. is a ubiquitous, prolific, and economically important pathogen, as it induces substantial yield losses by affecting vegetative parts and causing postharvest deterioration of fruits in temperate, subtropical, and tropical climates. In addition to affecting fruit, the pathogenic fungus also attacks other parts of the plant such as roots, flowers, and stems (DANE, 2016). Once in the fruit, the fungal spores germinate, and after 48 hours, they form a germ tube that directly penetrates the cuticle of the unripe fruit, where it remains dormant but the invasion is reactivated during fruit ripening (Zavala et al., 2011). One of the outstanding characteristics of *Colletotrichum sp*. is its ability to survive in dormancy or quiescence when environmental or physiological conditions of the host prevent it from developing (Parra, 2008), the fungus produces an appressoria that penetrates the fruit, degrading the cuticle and producing a dormant subcutaneous hypha that does not develop until the fruit ripens. The pathogen is activated in conditions with the prevalence of rainy seasons or high relative humidity (Rodriguez et al., 2009). It has been considered as one of the main pathogens attacking fruit tree plantations during pre-flowering, the disease remains asymptomatic until the degree of fruit ripening is such that the pathogen performs an intercellular invasion and the disease is expressed, such phenomenon is known as quiescent infection (Montaño and Lemus, 2015). For the control of anthracnose, integrated crop management

measures must be established, based on preventive and curative actions, oriented both in the field and postharvest stages (DANE, 2016). Chemical control is the most common due to the economic importance of the crop. Many chemicals are available, among the most used are compounds such as dithiocarbamate, benzimidazole, and triazoles; and other fungicides such as chlorothalonil, imazalil, and prochloraz (Parra, 2008).

According to Dirzo (2008), "The plant world is loaded with a surprising diversity of metabolites, most of which have no obvious involvement in the primary metabolic processes of the plant (photosynthesis, respiration, etc.). These compounds have therefore earned the name "secondary metabolites." It is estimated that more than 100,000 are produced by plants (Perez and Jimenez, 2011). Secondary compounds have no apparent function in primary metabolism but do have an ecological implication as a defense against herbivores, viruses, fungi, bacteria, as well as allelopathic substances (Echeverría, 2012).

Plants produce compounds with antimicrobial properties that can be used to control different diseases in fruit and vegetable products. The obtaining of plant extracts and the study of their active compounds allow their use against different phytopathogens. Under in vitro conditions, extracts inhibit pathogen growth, as well as sporulation and spore germination, thus helping to control fruit and vegetable diseases (Hernández et al., 2007).

The obtaining of vegetable extracts can be done by different methods, which allow the separation or purification of multiple substances, some commonly used are distillation, which is one of the oldest and most used methods to separate and purify liquids; the method consists of applying heat to a mixture of substances until one of them passes to the vapor phase, then the vapor is cooled by passing through a refrigerant where it condenses and the liquid is collected in a suitable container. Less volatile substances or impurities will remain in the initial vessel (Quercuslab, 2015). There is also the microwave technology, which has become during the last years a tool that improves the productivity of the processes; it works with electromagnetic radiations that are in the range of 0.3 to 300 GHz (λ = 1 to 0.001 m) (Martinez et al., 2010). Recently, the microwave-assisted method has been used as an alternative method of extraction at laboratory scale and has represented great advantages such as energy savings, short process times, higher yield, economical and environmentally friendly processes (Puertas, et al., 2013).

The objective of this research work was to determine the effectiveness of the use of plant extracts in the in vitro control of the pathogen causing anthracnose, to offer a sustainable alternative to this problem.

METHODOLOGY

The research was carried out at the Laboratorio de Agrotecnologías de la Agencia Universitaria para el Desarrollo (AUDES) Cacao-Chocolate of the Universidad Autónoma de Chiapas, located in Ciudad Universitaria, Tuxtla Gutierrez, Chiapas, Mexico.

The present research was developed in two phases: the first phase evaluated the antifungal effectiveness of extracts at a concentration of 60% on *C. gloeosporioides P.*; the second phase corresponding to the determination of the minimum inhibitory concentration (MIC) of extracts on *C. gloeosporioides*. In each phase, we worked with a strain of *Colletotrichum gloeosporioides P.* isolated from papaya fruits (*Carica papaya L.*) supplied by the Laboratorio de Agrotecnologías de la AUDES Cacao-Chocolate of the Universidad Autónoma de Chiapas, which was isolated from *C. papaya* fruits obtained directly from the field in the municipality of Villaflores, Chiapas in January 2018 and multiplication of the pathogen was carried out in this same laboratory through repotting in potato dextrose agar (PDA) culture medium, which was incubated at a temperature of 23°C ± 2°C for 12 days.

The research was carried out using a Completely Randomized Design (CRD), starting in phase one with 11 treatments, each with four replicates. The treatments were distributed in eight plant extracts and three controls: one negative (PDA medium or absolute control) and two positives; the chemically synthesized fungicide (I.A. Ciprodinil 37.5% + Fludioxonil 25%) and a fungicide in the patent process (M2 UNACH). In phase two, the pepper fruit extract obtained by microwave and the M2 UNACHproduct were evaluated.

The experimental unit (EU) in phases I and II consisted of a Petri dish and there were four replicates for each treatment evaluated. For the first and second phases, a total of 44 EU were established.

Collection of plant material

Fresh oregano (*Oreganum vulgare*) leaves were collected from the municipality of Copainalá, Chiapas, dried pepper (*Pimienta dioica*) from Comalcalco, Tabasco, fresh neem (*Azadirachta indica*) leaves from Tuxtla Gutiérrez, Chiapas and dried leaves of matarratón (*Gliricidia sepium*) from the locality Emiliano Zapata in the municipality of Tecpatán, Mexico. The plant material was collected in August 2018 and taken to the Agrotechnology Laboratorio de Agrotecnologías de la Agencia Universitaria para el Desarrollo (AUDES) Cacao-Chocolate of the Universidad Autónoma de Chiapas, for selection and classification, avoiding those that were affected by insects and diseases. The leaves and fruits used were dried in the greenhouse and stored in a dry place to avoid wetting and contamination by insects or fungi.

Obtaining plant extracts

The plant extracts were obtained by two extraction methods, which are described below:

Distillation: we used the methodology described by Ramírez, et al., (2016), for which the plant material was used, either chopped or crushed and as solvent distilled water and ethyl alcohol (9:1). For this purpose, we used a distiller adapted to obtain the extract. The plant material was placed inside the distiller's kettle together with the solvent, it was hermetically covered to make the extraction process continuous by applying heat and constant pressure, the vapor was conducted to a condenser, and by cooling with running water, the distillate was obtained.

Microwave: the crushed plant material or in small pieces was placed in a cloth bag, which was immersed in a beaker with sterile distilled water and ethyl alcohol (9:1). Subsequently, the radiofrequency of a microwave was used for the extraction of metabolites, under the following conditions: 220 KW of power, a period of 150s, and 2,450 MHz (Ramírez, et al., 2016).

phase 1. Antifungal effectiveness of extracts at a 60% concentration on *C. gloeosporioides*: Petri boxes were prepared with Potato Dextrose Agar (PDA) culture medium, added with the extract at 60% (v/v) and the inoculation of the pathogen was performed. The cultures were maintained under controlled conditions (incubator) of 23°C ± 2°C. Eight plant extracts were evaluated using two extraction methods (distilled and microwave) and three controls; PDA was the negative control and the positive controls were the chemically synthesized fungicide (A.I. Cyprodinil 37.5% + Fludioxonil 25%) and the patented product M2 UNACH. The inhibitory effect was quantified every 24 hours for 12 days by mycelial diameter growth. The fungus was scraped superficially and washed with distilled water using the appropriate dilution. The production of total and germinated spores was quantified.

phase 2. Determination of the minimum inhibitory concentration on the *C. gloeosporioides* fungus strain: the extracts that presented total inhibition of the growth and development of the pathogen at a concentration of 60% (v/v) were used for the tests corresponding to this stage, in which the minimum inhibitory concentration (MIC) was determined; these products were evaluated at concentrations of 50%, 40%, 30%, 20% and 10% (v/v). A negative control (PDA) was included in all tests.

The culture medium was prepared with PDA to which each of the extracts was added at the concentrations to be evaluated. Subsequently, the pathogen was inoculated. As an indicator variable of the inhibitory effect, the mycelial growth diameter of the pathogen was measured every 24 hours for 12 days, and the production of total and germinated spores was also quantified using the Neubauer spore counting chamber, as described in Phase 1.

The experimental design was completely randomized, to determine the effects of the treatments studied, an analysis of variance (ANOVA) was performed and in the cases where significant differences were found, Tukey's mean comparison test (P≤ 0.05) was applied, using the SPSS software version 17.0 for Windows.

RESULTS AND DISCUSSION

PHASE 1. Effectiveness of plant extracts at a concentration of 60%

The results obtained are shown in Table 1 and Image 1, where it can be seen that the treatments of pepper obtained by microwave and the M2 UNACH treatment were the only ones that did not allow the growth of the pathogen. The ANOVA performed indicated statistical differences between treatments for the variables growth and a total number of conidia. Tukey's test of means for the growth variable registered significant differences between pepper obtained by microwave and M2 patent, with all treatments including the positive control where the chemical synthesis product was used, which registered growth, as well as with the negative control, but this did not register differences with the extracts obtained from *matarraton* and oregano, obtained by the two methods and with neem obtained by distillation.

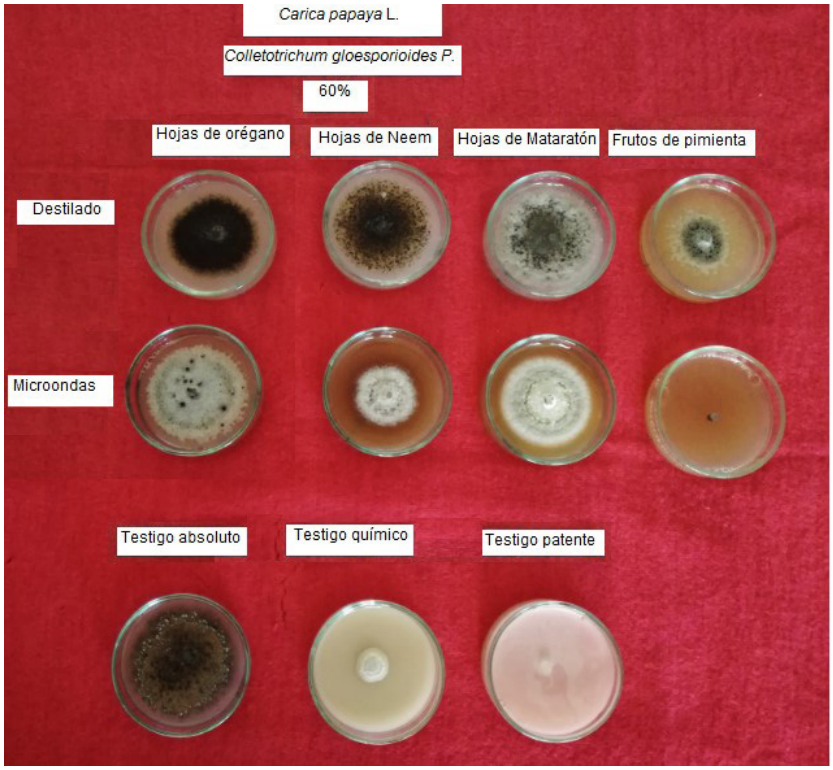

Image 1. In vitro effects of 60% plant extracts on *Colletotrichum gloesporioides* of *C. papaya*

It is observed that for the number of total conidia, the extracts of oregano (distilled and microwaved), neem obtained by microwaving, pepper obtained by distillation and the chemical control, although they registered growth, reduced the production of conidia between 96.08 and 99.9%, while the treatments of pepper obtained by microwaving and patent M2 did not register conidia formation, treatments that among them did not register statistical differences but did with the negative control and all with the *matarraton* obtained by distillation, which obtained the highest value surpassing the negative control in 21.55%, which indicates that the extract stimulated the development of the pathogen, which could make it potential as a culture medium in the in vitro production of the pathogen for future research, as did Verastegui (1995), who from his research on the analysis of the antifungal effect of 20 plant extracts concluded that some essential oils can inhibit growth, but others can stimulate it. Likewise, the chemical synthesis product control showed a slight mycelial growth of 13.75 mm and spore development, however, when observed under the microscope they were dehydrated or affected in their physical structure, as shown in Image 2, which showed the action of the product on the pathogen.

Regarding the number of germinated conidia, all treatments showed lower values than those recorded by the negative control, reducing between 10.86% and 100% the amount of this type of structures; in the treatments of oregano and pepper obtained by microwave and patent M2 UNACH, they still exceeded the effectiveness of the positive control product of chemical synthesis, which allowed the germination of spores (1.04 x 103), while these three treatments inhibited it completely. In the case of microwaved oregano, no germinated conidia were found, although there was mycelial growth and the number of total conidia mostly showed damage to their structure when observed, which indicates some antifungal effect for *C. gloesporioides*, which reaffirms that it is a plant with fungal potential which in turn is corroborated with what was stated by Valverde (2017) who refers to Arcila, et al. (2004) and Tanackov (2013), which indicated that oregano essential oil has antifungal capacity against several microorganisms, among which the following stand out: *Candida albicans, C. tropicalis, Torulopsis, Glabrata, Aspergillus niger, Geotrichum*, and *Rhodotorula*. Verastegui (1995) stated that Davidson (1993) conducted a review of species and food flavorings with antimicrobial activity. Among those that demonstrated antifungal activity were cinnamon, cloves, oregano, garlic, and onion. In turn, the mechanism of action of oregano was studied and it was found that it produces alterations in microbial metabolism, particularly in respiration and sporulation, and it was established that this is due to the decrease in cellular energy.

Image 2. Effects exerted by Oregano Microonda extract and A.I. Ciprodinil 37.5% + Fludioxonil 25% on *C. gloesporioides* isolated from *C. papaya*. A) normal spore, B) spore dehydrated by exposure to plant extract C) hyaline spore caused by the effect of A.I. Ciprodinil 37.5% + Fludioxonil 25%.

The above results corroborate the research of Joya, et al. (2017) and Tamayo, et al. (2016), who found the antifungal activity of oregano both in the reduction of growth and in the formation and germination of conidia of *Moniliophthora roreri*, besides, the present research shows that the form of extraction used is of high importance because it can express in greater or lesser amounts the secondary metabolites and vary the percentage of phytopathogenic inhibition, likewise Ospina (2012) who cites Mahmoud, et al. (2011) documents a 50% decrease in antifungal activity when nimonol is extracted from the organic extract of neem leaves in evaluations with pathogenic fungi such as *Microsporum canis, Candida albicans*, and *Aspergillus sp*, concerning other types of extraction evaluated. Likewise, Ramirez, et al. (2016) reported that obtaining extracts assisted by microwaves allows obtaining a higher concentration of them, which managed to reduce the MIC in which they affect pathogens compared to the traditional distillation method.

Table 1
In vitro effects of 60% plant extracts on C. gloesporioides, isolated from C. papaya

Treatments	Mycelial growth (mm)	Total conidia (x 10^4 mL^{-1})	Germinated conidia (x 1 10^3 mL^{-1})
Distilled Oregano	47.75^{d}	41.14^{a}	1.04
Microwave Oregano	43.75^{d}	7.29^{a}	0
Distilled Neem	50.00^{d}	2080.72^{ab}	568.75
Microwave Neem	29.25^{c}	15.93^{a}	9.37
Distilled Matarraton	50.00^{d}	6261.87^{c}	1059.37
Microwave Matarraton	42.00^{d}	4.58^{a}	1.04
Distilled Pepper F	40.25c^{d}	201.56^{a}	26.041
Microwave Pepper F	0^{a}	0^{a}	0
Positive Control Patent M2 UNACH	0^{a}	0^{a}	0
Positive (Chemical) Control	13.75^{b}	20.41^{a}	1.04
Negative control (Absolute control)	50.00^{d}	5151.66^{bc}	1188.54

*Different letters in columns show significant differences (Tukey, P≤ 0.05)

phase 2. Determination of the minimum inhibitory concentration (mic)

For the determination of the minimum inhibitory concentration, those treatments that completely inhibited the development of the pathogen at a concentration of 60% were taken into account; subsequently, concentrations of 50, 40, 30, 20, and 10% (v:v) were evaluated on *C. gloeosporioides* isolated from papaya, these treatments were those corresponding to the pepper fruit extract and M2 UNACH patent in phase 1, the results obtained in this phase can be seen in Image 3 and Table 2, from the realization of the test it was found that there is an effect of fungal inhibition of the two products, it was observed that as the concentration of the plant extract or the patent decreased, the mycelial diameter increased at a faster rate, respectively, the coloration of the mycelium changed with the products used because as the concentration of the products decreased, a darker color was observed in the mycelium. This is directly related to the sporulation of the pathogen, that is to say, to the number of spores of the variable.

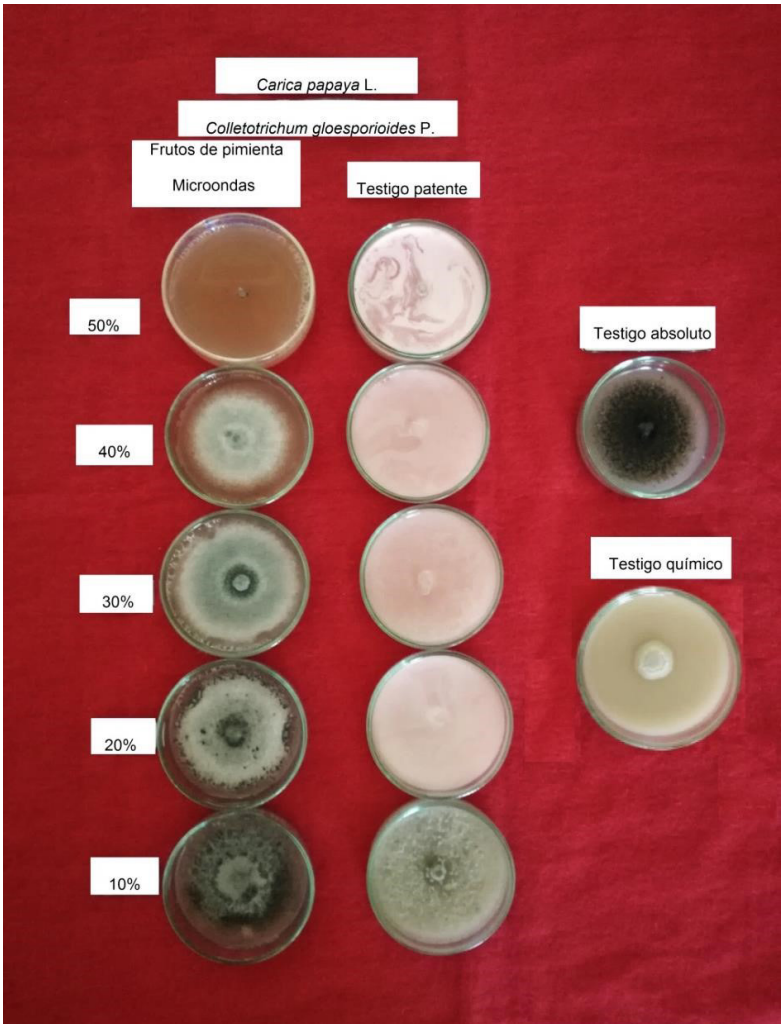

Image 3. Effects of pepper fruit extract and M2 UNACH patent at 50%, 40%, 30%, 20%, and 10% on *Colletotrichum gloesporioides* of *C. papaya*

Upon determining the MIC for the M2 UNACH patent, it was found that it inhibited the pathogen up to a concentration of 20%, in turn presenting significant differences concerning the 10% concentration and the negative control, according to Tukey's test of means (P≤0.05), as shown in Table 2. Regarding MIC, for the pepper fruit extract obtained by microwave on *C. gloesporioides*, it was found that it only inhibited completely at a concentration of 50%, from 40% mycelial growth was observed progressively approaching the negative control, likewise, in terms of spore formation, from 40% it was increasing, being statistically different from 20%, and this respectively different from the negative control, these results obtained differ to those presented by Jimenez (2018) who stated that the pepper fruit extract presented partial inhibition of 21.34% on the radial growth of the same fungus, while in total and germinated conidia recorded 66.80 to 91.49% inhibition respectively. In

turn, they are similar to the results presented by Ramírez, et al. (2016) who state that pepper fruit extracts obtained by the distillation and microwave-assisted method inhibited in vitro *C. gloeosporioides* isolated from papaya at concentrations of 30 and 20%.

Although spore production was observed, as the concentration increased, the pathogen was progressively inhibited, which makes the patented product M2 UNACH and the vegetable extract of pepper fruit obtained by microwaving, potentially effective products for the control of the pathogen at the appropriate concentration. Likewise, in the treatments that presented mycelial growth, conidia formation and germination were recorded, which showed statistical differences for the control, inhibiting the formation of conidia between 47.09 and 100% and germination between 72.54 and 100%, besides, in observations made under the microscope, it was observed that in the treatments with high concentrations of product, conidia were observed with damages in their structure or form.

Barros, et al., (20 20), identified by gas chromatography coupled to mass spectrometry (GC-MS) the compounds of *P. dioica* oil obtained by hydrodistillation, obtaining that it contained 76.88% of eugenol, which inhibited the mycelial development of fungi up to 97.78%.

Padrón (2010) reports that in general, essential oils possess strong antibacterial properties because they contain a high percentage of phenolic compounds such as eugenol. This suggests that their mechanism of action is similar to that of other phenolic compounds by altering the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport, and coagulation of cellular contents.

Sublethal concentrations of eugenol have been found to inhibit the production of *B. cereus* amylase and proteases, impairing the cell wall, leading to cell lysis. It is believed that the hydroxyl group of eugenol, to which certain proteins bind, prevents enzymatic action in *E. aerogenes* (Denyer & Hugo, 1991b; Sikkema, et al., 1995; Davidson, 1997; Wendakoon & Sakaguchi, 1995 cited by Padrón), so the action shown by the extract obtained by microwaving *P. dioica* on *C. gloesporioides* isolated from papaya may be due to this compound.

Table 2

In vitro determination of the MIC of pepper fruit and Patent M2 UNACH on the growth and development of Colletotrichum gloesporioides isolated from C. papaya

Treatments	Mycelial growth (mm)	Total conidia (x 10^4 mL^1)	Germinated conidia (x 1 10^3 mL^{-1})
Patent t. 50%	0^a	0^a	0^a
Patent t. 40%	0^a	0^a	0^a
Patent t. 30%	0^a	0^a	0^a
Patent t. 20%	0^a	0^a	0^a
Patent t. 10%	50.00^c	70.52^a	6.25^a
Micr Pepper F 50%	0^a	0^a	0^a
Micr Pepper F 40%	42.50^b	524.58^{ab}	34.37^a
Micr Pepper F 30%	50.00^c	910.52^{abc}	16.35^a
Micr Pepper F 20%	50.00^c	3636.61^c	76.4^a
Micr Pepper F 10%	50.00^c	3088.22^{bc}	92.18^a
Negative control	50.00^c	6873.43^d	335.72^b

*Different letters in columns show significant differences (Tukey, P≤ 0.05)

CONCLUSIONS

The extract obtained from fruits of *Pimienta dioica* by the microwave method at a concentration of 50% v/v proved to be effective in the *in vitro* control of *C. gloesporioides* isolated from *Carica papaya*.

The microwave extraction form of *A. indica, O. vulgare, G. sepium*, and *P. dioica* proved to be more efficient than by distillation since at the same concentration they inhibited the growth and the formation and germination of conidia of *C. gloesporioides* isolated from *C. papaya*.

The extract obtained by microwave of *P. dioica* and the M2 UNACH patent product has the potential to be an alternative for the control of papaya anthracnose, by reducing the growth and development of the pathogen.

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