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# Fungistatic effect of extracts and essential oils of *Lippia origanoides* H.B.K. and *Thymus vulgaris* L. as alternative management of *Botrytis cinerea* in strawberry

## Efecto fungistático de extractos y aceites esenciales de *Lippia origanoides* HBK y *Thymus vulgaris* L. como alternativas de manejo de *Botrytis cinerea* en fresa

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## Abstract

The strawberry gray mold caused by *Botrytis cinerea* is a disease that causes significant postharvest losses. In this study the fungistatic effect of extracts and essential oils of *Lippia origanoides* HBK and *Thymus vulgaris* L (at concentrations of 128, 256 and 500 mg/l) on the pathogenic fungus *Botrytis cinerea* under *in vitro* and *in vivo* conditions was evaluated. In the *in vitro* test, the percentage of inhibition of mycelial growth was determined. It was observed that the essential oil (EO) from *Lippia origanoides* showed the highest percentage of control (66.2%) of *Botrytis cinerea*. The evaluation *in vivo* showed that, the control percentage of the damage incidence from the pathogenic fungus (by an incidence value scale from 0 to 7 according to specific characteristics of deterioration of fruits) on bananas inoculated with *Botrytis cinerea* was measured. After 120 hours of monitoring, it was observed that the EO efficiently controlled the incidence of damage caused by the pathogen studied and no significant differences were observed when chemical control (fungicide Benomyl) was applied.

**Key words**: *Botrytis cinerea*, *Lippia origanoides* HBK, *Thymus vulgaris* L, *in vitro* and *in vivo* evaluations, essential oils, extracts, inhibition of mycelial growth.

## Resumen

El moho gris de la fresa causado por *Botrytis cinerea* es una enfermedad que produce importantes pérdidas poscosecha. En el estudio se evaluó el efecto fungistático de extractos y aceites esenciales de *Lippia origanoides* HBK y *Thymus vulgaris* L. en concentraciones de 128, 256 y 500 mg/lt sobre *B. cinerea* in vitro e in vivo. In vitro se determinó el porcentaje de inhibición del crecimiento micelial del hongo. En estas condiciones se observó que el aceite esencial (AE) de *L. origanoides* presentó el porcentaje de control más alto (66.2%) sobre *B. cinerea*. In vivo, se observó que en bananos inoculados con *B. cinérea* después de

120 los AE controlaron eficientemente la incidencia de daño causado por el patógeno estudiado y no se encontraron diferencias significativas con el control químico utilizando el fungicida Benomil.

**Palabras clave**: *Botrytis cinerea*, *Lippia origanoides* HBK, *Thymus vulgaris* L, evaluaciones *in vitro* e *in vivo*, aceites esenciales, extractos, inhibición de crecimiento micelial.

## Introduction

Strawberry (*Fragaria* sp.) is a crop with important potential for the regional and national economy of Colombia (Cano, 2012). However, it is a highly perishable product and it is susceptible to mechanical damages, water loss and physiological and microbiological deterioration caused by fungi as *Botrytis cinerea* (gray mold) that causes the fruit to rot and consequently to loss its economic value (Cruz *et al.*, 2008).

The gray mold in strawberry constitutes a disease that produces important losses at post-harvest (approximately 20%), affecting the economy of the producers, marketers and consumers (López *et al.*, 2006). Traditionally, the chemical fungicides have been the primary methods to control this pathogen but, its continuous application has generated public interest due to problems caused as toxicity, high costs, reduction in exports because product residues and, damage to the environment, farmers health and final consumer (Arturo, 2008; Montoro *et al.*, 2009). On the other hand, the plant pathogen microorganisms have generated resistance to the active ingredients of some synthetic fungicides, as a response to the high selection pressure by high doses and continuous applications without a previous study or control program, causing large economic loses (Leroch *et al.*, 2010; Wilson *et al.*, 1997).

Recently, the researches have been focused on the evaluation of several control alternatives to reduce the dependence on synthetic fungicides, among them, the antagonistic microorganisms, physical treatments, natural substances like essential oils (EO) and extract from different kinds. The EO and ethanolic extracts (EE) of the species of the genera *Lippia* and *Thymus* have biocontroller properties (Combrinck *et al.*, 2011; Lizcano, 2007; López *et al.*, 2006; Bolívar *et al.*, 2009; Bouchra *et al.*, 2003; Alzate *et al.*, 2009). The objective of this research was to evaluate the *in vitro* and *in vivo* fungistatic potential of the essential oils and ethanolic extracts of *L. origanoides* HBK and *T. vulgaris* as alternatives to control *B. cinerea*.

## Materials and methods

### Plant material

The samples of plant material of *L. origanoides* and *T. vulgaris* were obtained from plots of the working collection of medicinal and aromatic plants from the Experimental Center of the Universidad Nacional de Colombia - Palmira (CEUNP), where the predominant soil is Vertisol, the annual rainfall is 1000 mm, 25 °C and relative humidity is 83%.

The collected leaves from both species were placed on metallic trays to expose it to the environment for three days at the shade in the Plant Chemistry lab of the Universidad Nacional de Colombia - Palmira.

### Extraction of ethanolic extracts

To get the EE 50 g of leaves of each species were grinded after dehydration at the lab conditions (42% relative humidity), and were placed in two sample holders reservoirs of 10 cm diameter to which 180 ml of 98% ethanol were added. The reservoirs were sealed to avoid plant compound volatilization and stored for 3 days at an average temperature of 25 °C. After this time, the percolates or ethanolic concentrates were extracted, they were roto-evaporated with a Buchi model R-114 equipment. In this pro-

cess 176 ml of ethanol of each reservoir were recovered getting samples of *L. origanoides* and *T. vulgaris* and 10 g of dry matter of final extract in each sample were obtained.

**Extraction of essential oils**

The EO were obtained by hydro-distillation on a Clevenger equipment from 100 g of leaves (42% of humidity) of 1-3 cm size; they were places on a glass reservoir for hydro-distillation.

In all the runs or distillation stages 200 ml of distilled water were used, the water was contained on a round flask that was heated on an oven at constant temperature of 80 °C. Once the vapor of the process was condensated, for phases separation (hydrolate-essential oil) a Clevenger trap was used. Then, the oil was removed and for each mm of this 5 g of anhydrous sodium sulfate ($Na_2SO_4$) were added in order to dry out humidity residues, avoid oxidation and finally preserve it at 10 °C.

**Inoculation with *Botrytis cinerea***

The studied pathogen fungus was isolated from strawberry (*Fragaria* sp.) infected with *B. cinerea*. The samples were collected in a crop of a commercial farm of the town el Castillo, in Palmira, Valle and, preserved in humid chambers (RH 98%) to stimulate the pathogen growth.

The recognition of the fungal colonies was done by the imprint method (López *et al.*, 2006) before mounting the slides with cotton blue and observe them under the microscope at 40X magnification. To identify the fungal structures the Barnett (1972) and Pardo (1995) keys were used and the pure cultures were sown in PDA and refrigerated at 8 °C.

***In vitro* evaluation**

The minimal inhibitory concentration was determined by the dilution method in broth of the extracts with fungistatic activity, according to what is established in the Eucast document, ED 7.1 (Rodríguez–Tudela *et al.*, 2008), the evaluated concentrations were 128, 256 and 500 mg/ml. the inhibition of mycelial growth was determined by the Equation 1 proposed by Sztejnberg *et al.* (1987) (Alzate *et al.*, 2009):

$$Inhibition\ \% = \left[1 - \left\{\frac{Growth\ in\ the\ treatment\ (mm)}{Growth\ in\ the\ reference\ control\ (mm)}\right\}\right] x\ 100 \qquad Equation\ \mathbf{1}$$

***In vivo* evaluation**

For this evaluation fruits were selected at a scale 4 of maturity according to what is established in the NTC 4130 1997 standards for strawberry Chandler variety (Icontec, 197). The fruits were washed with distilled water and a 1% hypoclorite solution.

Following the methodology proposed by Alvarado *et al.* (2011) for each treatment five strawberry fruits were taken and perforated with a sterile needle to make 2 mm wide x 2 mm depth holes on the basal part of the fruit close to the peduncle. Later, these fruits were placed in beakers containing the evaluated treatments (256 and 500 mg/l) and covered completely for 5 seconds to then place them on a smooth surface on a flow chamber, there they were dried for 5 min at 25 °C till RH 69%. Finally, they were sprayed with a solution of 1.0 x $10^5$ spores/ml.

A similar process was performed for the control treatments –a treatment with 98% ethanol on a beaker and, a solution of the chemically synthesized fungicide Benomil at 100 µg/ml–. Finally, the fruits were distributed in stowed plastic containers, covered with sackcloth to perform a follow up for 120 h before qualifying the severity indexes and the control percentages.

**Severity index and control percentages**

According to the model proposed by Alvarado *et al.* (2011) a pictographic diagram was elaborated for the severity index caused

by the pathogen in the fruit. The model is based on the follow up and observation of the diameter of the fruit injuries each 12 h during 5 days, recording the partial advance of the infection in comparison to the total infection of the fruit over the time.

This scale was used to follow the progress of the infection at 48, 96 and 120 hours in the inoculated fruits. Additionally, the control percentage of each treatment on the pathogenic fungus evaluated was determined, in function to the infection percentage presented by the negative control, as it is calculated in Equation 2.

$$In\ vivo\ control\ \% = \left[1 - \left\{\frac{Infection\ in\ the\ treated\ fruits\ at\ 120\ h\ (\%)}{Infection\ in\ the\ negative\ control\ at\ 120\ h\ (\%)}\right\}\right] x\ 100 \qquad Equation\ \mathbf{2}$$

**Experimental design and data analysis**

The treatments in the *in vitro* experiment were arranged as an augmented factorial design $2^2$ x 3 + controls, where the evaluated factors were: plant species (2) (*L. origanoides* and *T. vulgaris*), extract types (2) (EO and EE) and concentrations (3) (128, 256 y 500 mg/l) an as control, the application of 100 µg/ml of the commercial fungicide Benomil. In the *in vivo* experiment two concentration were evaluated (256 and 500 mg/l). The data was analyzed by the statistical software SAS (Statistical Analysis System Version 9.3.1, 2012).

## Results and discussion

***In vitro* evaluation**

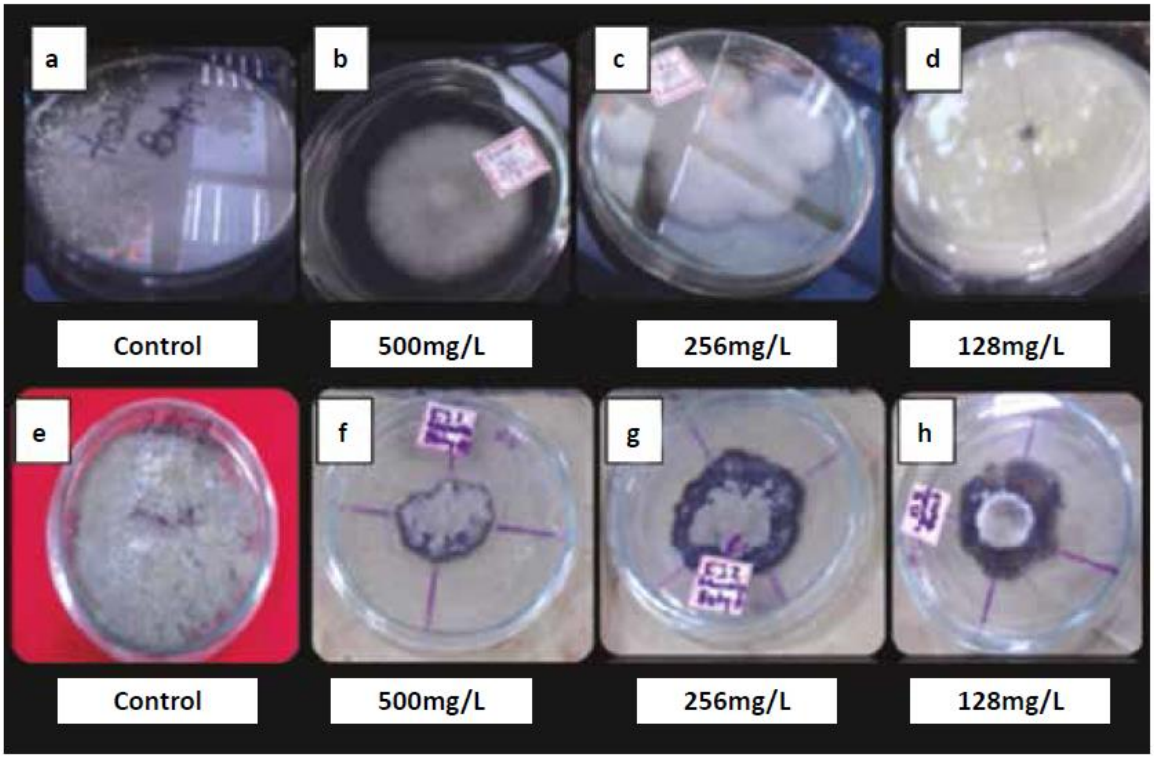
The *L. origanoides* extracts showed a higher average percentage of inhibition of mycelial growth (44%) than the ones of *T. vulgaris* (37%). This difference is due to the concentration of the complementary metabolites with inhibitory biological activity. According to Ruiz *et al.* (2007) the thymol of *L. origanoides* HBK is found on a concentration of 68% and according to Maqtari *et al.* (2011) in *T. vulgaris* the concentration is 54%.

Differences (P < 0.05) were observed among the evaluated extracts. The EO showed an average percentage of growth inhibition of *B. cinerea* of 55.9%, being higher than the one obtained with ethanolic extracts (37.1%) (Table 1). Hammami *et al.*, (2011) found similar results to the ones of this study when evaluating the biocontroller potential of EO and EE of *Viola odorata* on *B. cinerea* grown *in vitro*, however the first ones showed a higher inhibitory activity than the EE.

Similarly, differences among the evaluated concentrations were found (P < 0.05). The highest concentration (500 mg/l) showed the highest inhibition percentage (58.3%). The concentrations 256 and 128 mg/l inhibited 41 and 40.1% of the mycelial growth of *B. cinerea*, respectively (P > 0.05). The EO at the highest concentrations (500 mg/l) showed the highest inhibition percentages (66.2%), (Picture 1 and Table 1). These results are similar to that one of Lizcano (2007) with ethanolic extracts of *T. vulgaris* in three dilutions, where the 500 mg/l treatment also showed the highest inhibition of mycelial growth of *B. cinerea* grown *in vitro*.

***In vivo* evaluation**

In this growth media, the *L. origanoides* extracts showed high averages for mycelial growth inhibition (90.5%) and are statistically similar to the ones obtained with *T. vulgaris* (89%). The average of inhibition of mycelial growth of the fungus was higher in the EO (92.2%) in comparison to the results obtained with EE (87.4%) (P < 0.05) (Table 2), which agrees with the results of the *in vitro* evaluations of this study and with the results obtained by Fang *et al.* (2011) who evaluated the potential biocontroller effect of EO and EE of *V. odorata* in the control of *B. cinerea in vitro* and *in vivo*, finding in




**Picture 1.** a, b, c, d = Inhibition of the *in vitro* mycelial growth of *Botrytis cinerea* under three concentrations of ethanolic extract of *Lippia origanoides*. e, f, g, h = under three concentration of essential oils of *Thymus vulgaris*.

both cases, that the EO have higher antifungal activity than the ethanolic extracts.

These results are explainable, due to the fact that the volatile secondary metabolites (complementary) with bio-controller promising activity are found as concentrates in the EO and are obtained by the volatilization-condensation method, while the ethanol extraction necessarily means the isolation of other plant compounds at high concentrations that can affect the potential of the biocontroller (Hammami *et al.*, 2011).

Although, in average, the *L. origanoides* EO at high concentrations (500 mg/l) showed slightly higher control percentages in comparison to lower concentrations, there were no differences (P > 0.05) among the evaluated concentrations. This result is opposite to the one obtained in the *in vitro*

**Table 1.** General inhibition averages (%) of the *in vitro* mycelial growth of *Botrytis cinerea* in ethanolic extracts and essential oils of *L. origanoides* and *T. Vulgaris* in several concentrations.

| Concentration (mg/l) | Type of extract | | Average |
|---|---|---|---|
| | EE | EO | |
| | Inhibition (%) | | |
| 128 | 24.2 | 56.0 | 40.1 b* |
| 256 | 36.5 | 45.5 | 41.0 b |
| 500 | 50.5 | 66.2 | 58.3 a |
| Average | 37.1 b* | 55.9 a | – |

[a]. EE: Ethanolic extracts; EO: essential oils.
*Averages with similar letters on the same column are not statistically different (P > 0.05).

**Table 2.** Inhibition (%) of the *in vivo* mycelial growth of *Botrytis cinerea* in strawberry fruits.

| Extract (specie) | Concentration | EE | EO | Average |
|---|---|---|---|---|
| | | Growth inhibition (%) | | |
| *L.origanoides* | 256 (mg/l) | 87.7 | 92.3 | 90.0 |
| | 500 (mg/l) | 88.0 | 94.3 | 91.1 |
| *T.vulgaris* | 256 (mg/l) | 87.0 | 88.6 | 87.8 |
| | 500 (mg/l) | 87.0 | 93.6 | 90.3 |
| Average | | 87.4 | 92.2 | 89.8 |
| Controls | | | | |
| Benomil | 100 (µg/ml) | — | — | 89.6 |
| Ethanol | 98 (%) | — | — | 95.3 |
| Abs. control | | — | — | 34.3 |

[a] EE: Ethanolic extracts; EO: essential oils.
*Averages with similar letters on the same column are not statistically different (P > 0.05).

experiments with the same species and the results obtained by Soylu *et al.* (2010) who evaluated three concentrations (37, 75 and 100 mg/l) of EO of *Origanum syriacum* to control *in vivo B. cinerea* and, found a high correlation between the EO concentration and the control percentage of the pathogen.

On the other hand, the evaluations at 48, 96 and 120 h after inoculation demonstrated that the control percentage is over 80%, comparable to the inhibition indexes for mycelial growth obtained with the chemical control (Benomil) (Figure 1), confirming the potential of these plant species as efficient alternatives to control this pathogen, this reduces the impact on the environment and, health damages in the field workers at harvest and post-harvest activities and, therefore, in the final consumer (Jacometti *et al.*, 2010) (Wilson *et*

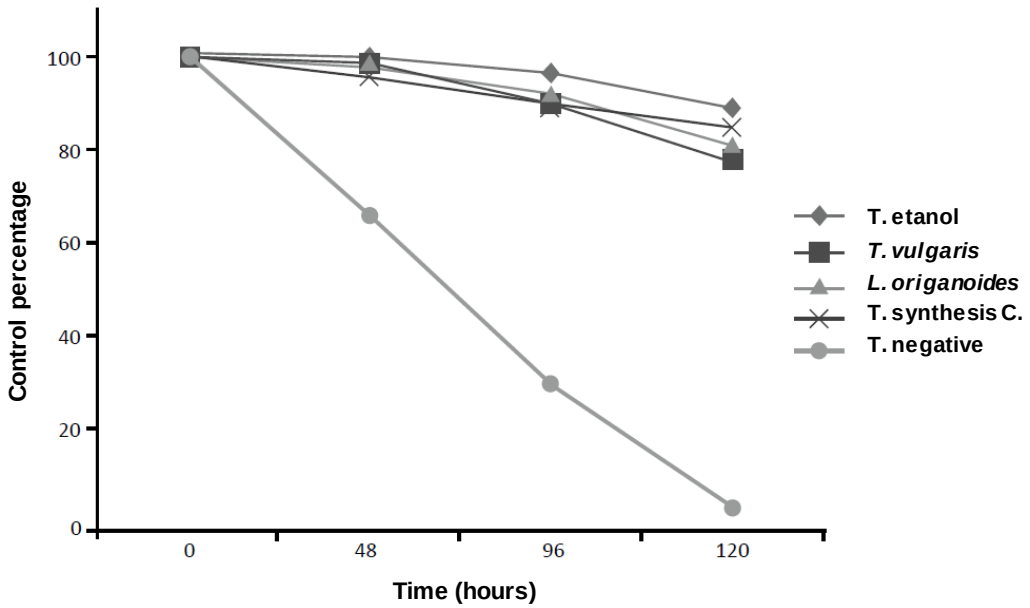



**Figure 1.** Progress in time (48, 96 and 120 h) of the percentage control *in vivo* of *Botrytis cinerea* by extracts of the plant species *L. origanoides* and *T. vulgaris* and three controls. T. synthesis C. = Benomil.

*al.*, 1997).

## Conclusions

- The essential oils and the ethanolic extracts *Lippia origanoides* showed higher inhibition on the mycelial growth of the pathogenic fungus *Botrytis cinerea* in strawberry, when compared to the results obtained with the same products from *Thymus vulgaris*.
- The essential oils of *L. origanoides* and *T. vulgaris* presented the highest average inhibitions of mycelial growth, both, *in vitro* and *in vivo* of the pathogenic fungus *B. cinerea*, common to the strawberry fruits.
- The essential oils and ethanolic extracts of *L. origanoides* and *T. vulgaris* are promising and efficient alternatives in the control of the pathogenic fungus *B. cinerea*.
- The *in vitro* results demonstrated a direct proportionality between the evaluated concentrations and the control percentage over *B. cinerea* in strawberry, being 500 mg/l the concentration that showed higher inhibition percentages.

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