Article

In vitro evaluation of methods against *Botrytis cinerea*

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Abstract

Although experimental methods are available for the control of fungal diseases in strawberry cultivation, including chemical, organic and biological, other low-cost methods such as homeopathic substances are not ruled out, which have not been evaluated against *Botrytis cinerea*. The objective of the present study was to compare conventional treatments and homeopathic substances, under *in vitro* conditions, in the mycelial growth of *B. cinerea* as a previous reference to *in vivo* evaluation. Treatments: PDA medium, synthetic fungicide Switch® (Fludioxonil, Ciprodinil) 1 g L^{-1}, essential oil of *Tagetes lemmonii* (1%), commercial extract of *Larrea tridentata* (2 mL L^{-1}), homeopathic substances, arsenic 6 CH and nosode of *Botrytis* 7 CH and dual test with *Trichoderma harzianum*. Registered variables: mycelial diameter, growth rate and percentage of mycelial inhibition; in the dual test, days at first hyphal contact and type of antagonism were measured. The data were subjected to analysis of variance and multiple means comparison test (Tukey, $p \leq 0.05$). Switch® and *Tagetes* oil inhibited mycelial growth by 100%, with *Larrea* extract the inhibition was 65.8%, *T. harzianum* inhibited 36.2% and hyphal contact occurred on the sixth day (type II antagonism), controlling the pathogen, the homeopathic substances nosode 7 CH and arsenic 6 CH inhibited the fungus in 31.2 and 11.8%, respectively. The effect of nosode 7 CH was different ($p \leq 0.05$) from the control, but homeopathic arsenic was not.

Keywords: antifungal effect, fungal diseases, homeopathic substances, organic and biological methods, strawberry.

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Introduction

Strawberry (*Fragaria vesca* L.) is grown in Mexico in 12 states, but only Baja California, Guanajuato, and Michoacán generate 91.55% of total national production (Santoyo, 2009). Gray mold (*Botrytis cinerea* Pers. *ex* Fr.) is one of the main strawberry diseases; this plant pathogen has the capacity to act as a saprophyte and has been considered of high importance, since it affects a large number of crops around the world. The losses in the strawberry crop can be severe, up to 25% in the main harvest and around 37% in the second productive peak, so that without effective pre and post-harvest control it can cause large economic losses, affecting productivity in harvest quantity and quality (Ceredi *et al*., 2009).

For the control of *B. cinerea*, the use of modern fungicides that act on specific metabolic sites of the fungus has resulted in the formation of races resistant to certain chemicals, in this sense, the need has arisen to seek other resources to the integrated management of the disease (Cano, 2013).

Some methods explored in plants for fungal control consist of using microbial antagonists that regulate populations of phytopathogens (Chávez, 2004), the use of plant extracts has also been implemented to control fungal diseases, since they are a rich source of bioactive chemical products (Balandrin *et al*., 1985). These methods can currently be used as part of integrated disease management, and recently, the use of homeopathic substances is another promising resource for the biocontrol of phytopathogenic fungi and which, compared to previous methods, is attractive due to its low cost (Meneses, 2007).

The *in vitro* study model of substances or organisms to control fungal diseases is a useful methodological resource to select what is promising and to outline its evaluation *in vivo*. In this sense, *in vitro* tests of fungal sensitivity to fungicides help determine whether or not their biological activity is expected, either because the fungus develops resistance or due to aspects related to the quality and quantity of the synthetic product used (Mercier *et al*., 2010); therefore, the inclusion of control treatments and the verification of the fungicidal or fungistatic effect are necessary methodological aspects.

Regarding works on the use of plant extracts against fungi *in vitro* conditions, there are numerous investigations, but *Botrytis* highlights those related to extracts of *Larrea tridentata* (Lira, 2003) and *Lippia origanoide* (Taborda, 2015). In the case of extracts of governor plants (*L. tridentata*) its effect is positive for the control not only of *Botrytis* but also of *Fusarium*, *Pythium* and *Rhizoctonia*, this plant source of metabolites is distributed in arid regions of Durango and Coahuila (Lira, 2003).

In the case of essential oils against *Botrytis*, there are few studies on *Tagetes* that highlight the effect of total inhibition (Romagnoli *et al*., 2005), although in other works a positive effect against other fungus species is reported (Barajas *et al*., 2011 ; López *et al*., 2018). In general, there are few species of Tagetes explored for their effect against fungi and in the case of *Tagetes lemmonii*, a species distributed regionally in Sonora and Durango (Serrato, 2014), research is scarce.

The methanolic extract of *T. lemmonii* is antifungal in *Botrytis* (Bojorquez, 2017) and the essential oil causes inhibition of *Fusarium oxysporun* f. sp. *ricini* (Jarquin, 2016), the latter has not been explored against *B. cinerea*. With respect to *Trichoderma harzianum*, it is referred to as *Botrytis* antagonist (Merchan, 2014) and in works carried out under *in vitro* conditions, the total inhibition of the growth of the fungus is evident (Calvo, 2012).

In relation to the effect of homeopathic substances against fungal diseases in plants, there are several studies carried out under *in vivo* conditions (Oliveira *et al*., 2013; Hanif and Dewar, 2015; Lorenzetti *et al*., 2016; Rissato *et al*., 2016; Oliveira *et al*., 2017) and only six *in vitro* (Carneiro and Bueno, 2010; Gama *et al*., 2015; Lorenzetti *et al*., 2016; Rissato *et al*., 2016; Toledo *et al*., 2016; Serrato *et al*., 2018) among the latter, two of them refer to nosodes and four to various substances, showing direct antifungal effect and none of them evaluated against *B. cinerea*.

From the results of the application of homeopathic substances *in vitro* against fungi, the homeopathic principle stands out *similia similibus curentur* related to substances such as arsenic, phosphorus, sulfur, *Arnica montana*, etc. (Tichavsky, 2007), which inhibit fungi such as: *Aspergillus niger*, *Alternaria solani* and *Sclerotinia scleroturum* (Gama *et al*., 2015; Toledo *et al*., 2016; Rissato *et al*., 2016) also highlights another homeopathic principle, the most similar one , which corresponds to the elaboration of nosode, a homeopathic substance derived from the organism that causes disease or damage, as in the cases of *Alternaria solani*, *Fusarium solani* and *Sclerotinia scleroturum* evaluated *in vitro* (Carneiro and Bueno, 2010; Rissato *et al*., 2016).

Under *in vivo* conditions, the application of homeopathic substances against fungi presupposes signals that the plant receives preparing it to face the establishment of the pathogen; however, the incorporation of the homeopathic substance (dynamized substance or nosode) into the *in vitro* culture medium can directly influence the pathogen; that is, influencing it by contact, a biological response that has been little argued in the agrohomeopathic phenomenon (Carneiro and Bueno, 2010; Lorenzetti *et al*., 2016; Rissato *et al*., 2016).

Although comparative studies of chemical, organic and biological treatments under *in vitro* conditions against phytopathogenic fungi are reported (Jarquin, 2016), there are relatively few that include homeopathic substances (Oliveira *et al*., 2013; Gama *et al*., 2015; Lorenzetti *et al*., 2016; Serrato *et al*., 2018). Considering the limited exploration of *T. lemmonni* oil as an antifungal, the limited information on homeopathic substances with a direct effect on fungi and its limited inclusion in comparative studies of antifungal efficiency in the *in vitro* model, as well as the importance of reducing Costs in the control of strawberry *B. cinerea*. In this study, conventional treatments and homeopathic substances were compared *in vitro* against *B. cinerea* in order to generate useful information for verification *in vivo*.

Materials and methods

The present investigation consisted of carrying out an *in vitro* evaluation of a chemical fungicide, two vegetable substances, an antagonistic biological agent and two homeopathic solutions (Table 1). The experiment was carried out in the Laboratory of the Master in Plant Protection at the Autonomous University Chapingo (UACH) in 2018.

Table 1. Treatments for evaluation in the bioassay with B. cinerea.

Treatment	Dose	Response variables
PDA (absolute control)	39 g L^{-1}	Growth rate (mm·day^{-1}), inhibition percentage (%) y growth rate (%)
Tagetes lemmonii	1%	
Homeopathic arsenic	1 drop L^{-1}	
Botrytis homeopathic	1 drop L^{-1}	
Chemical (Switch®)	1 g L^{-1}	
Governor's Extract	2 mL L^{-1}	
Trichoderma harzianum	3.5 mm	

Isolation of phytopathogenic fungi

The phytopathogenic fungus *B. cinerea* was isolated from strawberry plants in a greenhouse in the UACH Experimental Field. Plants with damaged fruits, showing a brown cover with a spore carpet texture, a peculiar symptom of *Botrytis* presence in that berry, were selected as a source of inoculum. Fragments of infected tissue were established under aseptic conditions in Petri dishes with Papa-Dextrose agar culture medium (PDA) and incubated in a growth chamber at 17 °C and 80% relative humidity for eight days and 1 hr of light daily.

Biological evaluation substances and organisms

The commercial chemical Switch® (Fludioxonil, Ciprodinil) was obtained from an agrochemical store in the municipality of Texcoco, State of Mexico. The commercial organic product Prograníc® Mega (*Larrea tridentata* extract) was also purchased. The antagonistic organism (*Trichoderma harzianum*), in mycelium, was provided by Dr. Roney Vidal Solano, Department of Agricultural Parasitology, UACH.

From *T. lemmonii* A. Grey plants. (voucher 2012-199; Herbarium of the University of Sonora) Var. Limon (SAGARPA-SNICS, file 1423, record 1629) from a plantation established in the UACH Experimental Field in 2015 (19° 29.547' north latitude, 98° 52.470' west longitude and 2 267 masl, Cw1 climate type) were harvested stems in bloom in September 2018. This fresh biomass in quantity of 100 kg was subjected to a process of hydro-distillation by means of a stainless-steel distiller with a distillation capacity of 200 kg, the oil obtained was stored at 18 °C in the dark.

The homeopathic substances were prepared by Dr. Felipe de Jesús Ruiz Espinoza from the Regional University Center of Anahuac of the Autonomous University Chapingo (UACH), for the preparation of the mushroom nosode, the latter was isolated from a strawberry fruit and prepared by grinding with sugar. For the preparation, 0.05 g of the fungus sample was taken, which was placed in a 4-inch porcelain mortar. In three plastic bags of 6 x 10 cm, 5 g of sugar was added to each one, labeled with the name of *Botritis cinerea* T1C (bag 1), writing down the date. To bag 2, *B. cinerea* T2C and to bag 3, *B. cinerea* T3C.

Bag 1 was divided into three thirds, adding the first third and starting the crushing as follows: it was ground or crushed for 6 min, then, with a stainless steel coffee spoon, the pestle and mortar were scraped for 4 min. This was repeated by grinding 6 min and scraping that third for 4 min. When finished, the second third of the first bag was added. Grinding and scraping was repeated for 6 and 4 min, respectively. At the end, the last third was added, which was ground for 6 min and scraped for 4 min, repeating this sequence until completing 1 h.

From there the crushed was stored in the T1C bag. From the T1C crushing, 0.05 g was taken to start bag 2, which was prepared according to the times of the first bag and when the second bag was finished, the third bag was saved and started, when finished, it was saved and from there it was taken 0.05 g of the 3C trituration, which was added to a bottle containing 50 drops of distilled water and 50 drops of alcohol, stirred for 2 min and allowed to stand for 2 min.

At the end, this preparation constituted the 4C dilution or potency, hence the 5C, 6C and 7C were elaborated, according to the Hanamanianna centesimal scale, as mentioned by Sandoval (1961). This sequence of operations was done for arsenic, according to rule 7 of homeopathy (Zepeda, 2002), arsenic was obtained from the National Homeopathic Pharmacy located in the plinth of Mexico City and the characteristics of this substance are described in the work de García (1984).

Preparation and evaluation in Petri dishes

For the bioassay, the poisoned agar method was used, which consists of integrating the treatment substances into the culture medium. PDA culture medium was prepared in seven 250 mL Erlenmeyer flasks. For sterilization, they were autoclaved for 20 min at 120 °C and 15 lb pressure. When the autoclave temperature and pressure dropped to around 40 °C, the flasks were removed. The PDA medium plus the substances corresponding to the treatments were stirred before they were poured into the Petri dishes in order to homogenize the mixture.

The preparation was emptied into sterile 90 mm Petri dishes under aseptic conditions and allowed to cool and solidify at room temperature for 24 h. The next day, a PDA disc with the *B. cinerea* inoculum obtained with a sterile 3.5 mm diameter punch was placed and placed inverted in the center of the box. Petri dishes were incubated at 18 ± 2 °C in an oven. From the seeding of the fungus in the Petri dish and for 8 days, the diameter of the mycelium was measured with a digital vernier.

Preparation of treatments

The dual preparation with *T. harzianum* was carried out on sterile culture medium and placing a 3.5 mm diameter disk at one end of the Petri dish previously divided in half and then placing the mycelium of *B. cinerea* at the other end. The pure *T. lemmonii* essential oil was prepared at 1%, for which 0.1 mL of Tween 20 surfactant was added, shaking circularly to obtain an emulsion, and then it was homogeneously dissolved in 100 mL of PDA, then it was poured into a 250-ml flask mL. The same procedure was used to prepare the governor extract, where the 100 mL solution of culture medium was prepared with 2 mL of the organic product.

For the synthetic product Switch®, 1 g of the product was added in one liter of water and waited until it dissolved, stirring, transferring 100 mL of mixture to the flask to be sterilized. In the case of homeopathic substances, one drop of each homeopathic solution, *Botrytis* 7 CH and arsenic 6 CH, was added to one liter of distilled water, and then vigorously succussed the bottle for 2 min. Subsequently, the required quantity (100 mL) was deposited in the 250 mL Erlenmeyer flasks.

Experimental design

The experiment was analyzed by a completely randomized experimental design with six treatments each with five replications and an absolute control, the experimental unit consisted of a 90 mm diameter Petri dish with PDA culture medium incorporating the treatments to be evaluated.

Registered variables

The radial growth of the fungus was measured with a frequency of 24 h, ending until the Petri dish without treatment (control) was completely covered with mycelium (8 days), based on this time, evaluations of the dual growth *Trichoderma-Botrytis* were also performed. Measurements were made with a digital vernier. The growth rate of the mycelium (VC) was calculated with the formula of Sinclair and Cantero (1989).

$$VC=\frac{Df-Di}{Tf-Ti}$$

Where: VC = growth rate (mm day^{-1}); Df = final growth diameter (mm); Di= initial growth diameter (mm); Ti = initial growth time (days); Tf = final growth time (days).

The percentage of inhibition (% I) was determined by applying the formula of Fokkema (1973), cited by Kagezi *et al.* (2015).

$$\%\ I=\frac{D1-D2}{D1}\ (100)$$

Where: % I= percentage of inhibition of mycelial growth; D1= diameter of the mycelial growth of the control (mm); D2= diameter of mycelial growth of the influenced (mm).

To calculate the percentage of growth the formula was used.

$$\%C=\frac{CI\ (100)}{CT}$$

Where: %C= percentage of mycelial growth; CI= influenced growth (mm); CT= control growth (mm).

To determine the class of antagonism under dual growth conditions, the scale proposed by Bell *et al*. (1982): 1. The antagonist overgrows the plant pathogen and covers 100% of the Petri dish; 2. The antagonist covers 75% of the Petri dish, stops the plant pathogen and can overgrow and sporulate on it; 3. No organism is dominant, each one covers 50% of the surface (antagonist and pathogen); 4. The phytopathogen covers 75% of the Petri dish and stops the growth of the antagonist and can overgrow and sporulate it; and 5. The plant pathogen overgrows the antagonist and covers 100% of the Petri dish.

Statistical analysis

For the statistical evaluation of the experiment, the data were subjected to a combined analysis of variance and Tukey's mean comparison test ($p \leq 0.05$) corresponding to a completely randomized experimental design with five replications. This analysis was carried out using the Statistical Analysis System software (SAS 9.0) with the Proc Anova procedure.

Results

The antagonistic agent *T. harzianum* controlled the growth of the mycelium of *B. cinerea* by inhibiting it in 36%, although its growth rate was lower (7.44 mm day^{-1}) than that of the pathogen in the control treatment (9.8 mm day^{-1}) (Table 2), in the *Botrytis-Trichoderma* treatment, *Trichoderma* managed to stop the development of *Botrytis* at the moment of contact (Figure 1). The coefficients of variation were 6 to 17%.

Table 2. Comparison of growth responses of *T. harzianum vs. B. cinerea*.

Treatment	Dose	VCD	t(VCD)	INH	CREC
Botrytis	0	9.8	3.28 a^{z}	0 b	100 a
Trichoderma + Botrytis	-	7.44	2.9 b	36.25 a	63.75 b
CV	-	14.5	6.4	17	5.9
DMS	-	1.83	0.29	7.14	7.14

VCD= diametral growth rate (mm day^{-1}); t(VCD)= VCD data transformation; INH= growth inhibition percentage; CREC= diametral growth percentage; DMS= minimal significant difference (Tukey, $p \leq 0.05$); CV= coefficient of variation; ^{z}= averages with the same letter within the column are statistically the same. Values are means of five repetitions at 8 days after sowing.

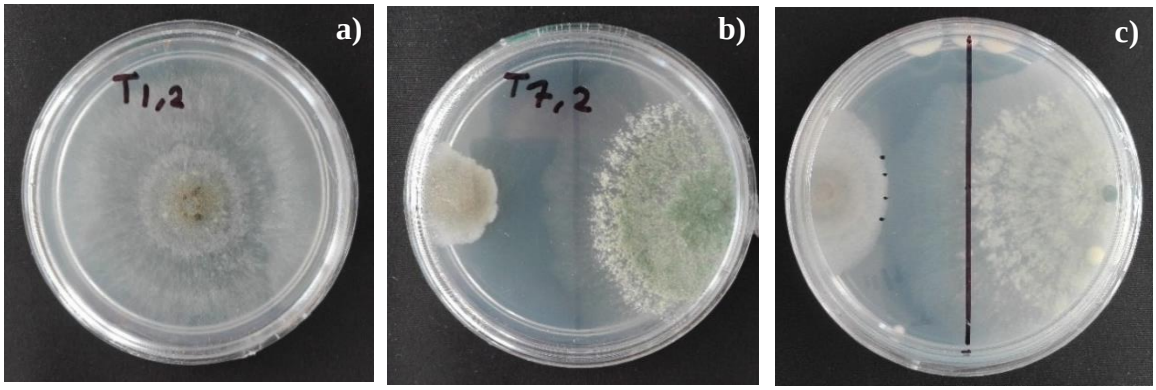

Figure 1. *T. harzianum* antagonism *vs Botrytis cinerea*. a) absolute control with *B. cinerea*; b) and c) *T. harzianum* (right) and *B. cinerea* (left).

Hyphal contact between the two pathogens occurred on the sixth day. *Trichoderma* had exponential growth from the second day, exceeding half of the Petri dish before *Botrytis*, stopping its growth and at the same time causing the death of hyphae in the zone of interaction with *B. cinerea* (Figures 1 B and C). The dual test with these fungi highlighted a type two antagonism, which is characterized because *T. harzianum* invaded 75% of the Petri dish, in addition to overgrowing and sporulating on the plant pathogen, *T. harzianum* gradually invaded the growth of *Botrytis* until it was completely covered.

Treatments with substances mixed in the culture medium influenced the response of *B. cinerea* and coefficients of variation of 2 to 31% were recorded (Table 3). Botrytis mycelium had 100% growth in PDA, the highest growth rate (VCD) (9.8 mm day^{-1}) and without mycelial inhibition; however, with the chemical fungicide the growth and the VCD were null, and the mycelial inhibition 100%. With *T. lemmonii* oil, from 24 h after inoculation of the fungus until the end of the experiment, the diameter of the mycelial inoculum was not modified, therefore, no VCD and 100% inhibition of the pathogen, a response similar to obtained with the fungicide product.

Table 3. Multiple comparison of means for the response variables of the bioassay.

Treatment	Dose	VCD	t(VCD)	INH	CREC
Control	0	9.8	3.28 a^{z}	0 e	100 a
Tagetes lemmonii	1 %	0	1 d	100 a	0 cd
Homeopathic arsenic	1 drop L^{-1}	8.56	3.08 ab	11.85 ed	88.15 a
Botrytis homeopathic	1 drop L^{-1}	6.76	2.77 b	31.24 cd	68.76 ab
Switch®	1 g L^{-1}	0	1 d	100 a	0 d
E. Governor	2 mL L^{-1}	2.82	1.95 c	65.8 b	34.2 cb
DMS	-	2.82	7.81	13.94	30.96
CV	-	2.01	0.33	14.03	43.22

VCD= diametral growth rate (mm day^{-1}); t(VCD)= VCD data transformation; INH= growth inhibition percentage; CREC= diametral growth percentage; DMS= minimal significant difference (Tukey, $p \leq 0.05$); CV= coefficient of variation; ^{Z}= averages with the same letter within the column are statistically equal.

With governor extract (*L. tridentata*) the fungus grew 34%, VCD of 2.82 mm day^{-1} and mycelial inhibition of 65.8%. With homeopathic arsenic, the VCD was 8.56 mm day^{-1} and 11.8% inhibition, response statistically the same as the absolute control. With *Botrytis* nosode, the VCD was 6.76 mm day^{-1} and 31.24% inhibition, statistically different from the control treatment. Both in the homeopathic substances and in the PDA control, *Botrytis* had the highest growth percentage (68 to 100%).

In general, the fungicide Switch® and the essential oil of *T. lemmonii* were inhibitory of the growth of the mycelium and in the case of the biological agent *T. harzianum* there was antagonism against *B. cinerea*, these treatments were of high antifungal effect compared to those of inhibition mean of the governor extract (65%) and those with low inhibition (11 and 31%) of homeopathic substances (Table 3; Figure 2).

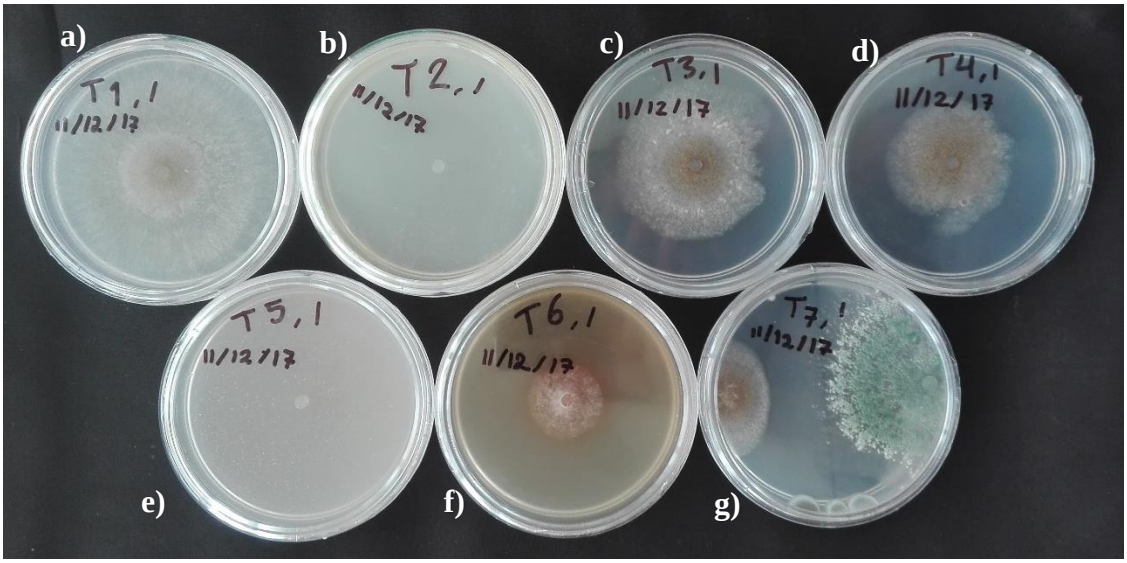

Figure 2. End of the 8-day bioassay when the absolute control completely covered the Petri dish, a) absolute control; b) *T. lemmonii*; c) homeopathic arsenic; d) *Botrytis* homeopathic; e) Switch®; f) governor; and g) antagonic *Trichoderma harzianum*.

Considering the diametral growth rate (using the transformation) on the eighth day, the high growth rate of the mycelium in PDA (9.8 mm day^{-1}) and in homeopathic arsenic (8.56 mm day^{-1}) stood out; the response was intermediate with *Botrytis* nosode (6.76 mm day^{-1}), low speed with the governor extract (2.82 mm day^{-1}) and null with *T. lemmonii* or Switch® (Table 3).

The dynamics of the growth rate of the mycelium in contact with the organic and homeopathic substances evaluated during the eight days (Figure 3) presented particular trends. Until the second day, all treatments had a similar response (10 mm day^{-1}), but on the third day the growth rate of the fungus in the homeopathic and PDA control treatments increased from 19 to 31 mm day^{-1} on the 5th day. With the *L. tridentata* extract the growth of the fungus remained stable from day 2 to 4 (10 mm day^{-1}), but on the 5th day it increased slightly (14 mm day^{-1}) and continued to increase with a slow trend during the three subsequent days (19, 21 and 26 mm day^{-1}).

In the control and in homeopathic arsenic and nosode, on day five it was observed that the mycelium acquired a different growth rate (35, 33 and 31 mm day^{-1}, respectively), a trend more defined on days 6 (46, 42 and 38 mm day^{-1}, respectively), 7 (59, 53 and 45 mm day^{-1}, respectively) and 8 (76, 66, 54 mm day^{-1}, respectively), separating the *Botrytis* nosode further in those last four days, by reducing the growth rate of the mycelium with respect to the control (decrease in VCD of 4, 8, 14 and 22 points, respectively) and homeopathic arsenic (decrease in VCD of 2, 4, 8 and 12 points, respectively).

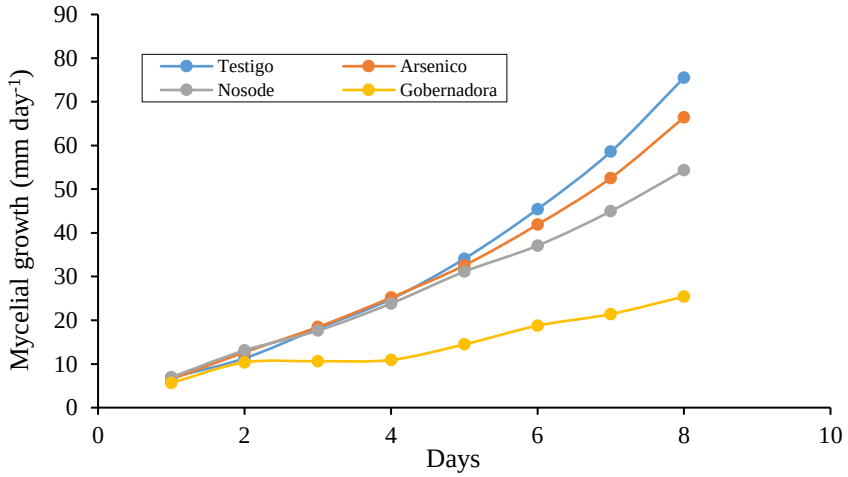

Figure 3. Diametrical growth of the mycelium (mm·day^{-1}) of *Botrytis cinerea* during the *in vitro* evaluation period of the treatments: control (PDA), governor extract, homeopathic arsenic and *B. cinerea* nosode. The substances *T. lemmonii* and Switch® do not appear because the fungus did not show growth.

Discussion

The antagonistic effect of *T. harzianum* against strawberry gray mold in the *in vitro* study model confirms other antecedents in this regard (Calvo, 2012). Probably the temperature of 18 °C did not favor a higher growth rate of *Trichoderma* in the first days in relation to that reached by *Botrytis* in this regard, it is indicated that the optimal temperature for Trichoderma is 25 °C (Merchan, 2014). The result of the *Trichoderma-Botrytis* antagonism confirms the direct use of the biological agent in the fruiting and post-harvest stages of strawberry (Merchan, 2014).

However, since this result is favorable to decrease or replace fungicide products, the production or purchase of *Trichoderma* represents an investment cost, perhaps less than the purchase of the fungicide itself. For its part, the agrochemical had an immediate effect, reported both *in vitro* and *in vivo* in strawberry (Mercier *et al*., 2010); however, the negative implication to the human and the environment are food for thought. As for organic substances, the inhibitory effect of the governor extract coincides with that observed in other studies where this plant substance is attributed the property of fungistasis in *Botrytis* (Lira, 2003).

Regarding the outstanding antifungal effect of *T. lemmonni* oil, similar to that produced by the product Switch (Table 3), it had already been reported against *Fusarium oxyosporum* (Jarquin, 2016), but not with the same intensity with which it occurred against *Botrytis*, a result that is reported for the first time; the presence of dihydrotagetone, (E) tagetone and (E) ocimenone in the essential oil (Tucker and Marciarello, 1996) are possibly the substances responsible for the described biological activity. Due to the similarity of the response of the mycelium submitted to Switch® and to *T. lemmonni* oil, it is suggested that the essential oil of this species of *Tagetes* has a biocidal property. An ongoing investigation aims to explore other concentrations and establish the inhibition concentration, in addition to its toxicological evaluation *in vivo*.

The result obtained on the effect of mycelial inhibition of *B. cinerea* caused by the nosode of the same pathogen (Table 3; Figure 1) is of special importance because it would be the seventh experience that shows direct effect of homeopathic substances on the growth of phytopathogenic fungi (Gama *et al*., 2015; Rissato *et al*., 2016; Toledo *et al*., 2016; Hanif and Dewar, 2017; Oliveira *et al*., 2017; Serrato *et al*., 2018).

In vitro bioassays of application by fumigation with nosode directly on mycelium or spores could solidly confirm some published results and also those of the present work. On the other hand, *Botrytis* 7 CH nosode was better than 6 CH homeopathic arsenic (Figure 1), when the latter in that same potency, *in vitro* conditions inhibits the germination of *Alternaria* spores (Trebbi *et al*., 2016) This comparison suggests specificity of the homeopathic according to the dynamisation or specificity by biological origin of the homeopathic substance.

The successful results related to the application of homeopathic substances against fungal problems in plants indicate that the fungus does not establish in the plant (Hanif and Dewar, 2015) or, it grows in a restricted way without damage to the plant (Rissato *et al*., 2016 ; Hanif and Dewar, 2017; Oliveira *et al*., 2017) furthermore, it is not clear whether nosode or other homeopathic substances have the same inhibitory effect, nor is it established whether their action is direct against the pathogenic organism. Therefore, the *in vitro* model has favored partially answering the previous questions.

In this case, it was evident that the fungus is influenced in its growth by the homeopathic product. Taking into account published works on the action of homeopathic substances applied to the foliage of plants to control fungal diseases, it follows that the homeopathic stimulus, either by substances derived from the pathogenic organism or from any other origin, trigger plant response mechanisms to your protection. It has recently been shown that nanoparticles with greater biological activity than with macromolecules are generated with the dynamization and succussion process of substances (Rajendran, 2017).

From an ecological perspective, organic, biological and homeopathic treatments offer advantages compared to synthetic fungicide. The possibility of reducing the concentration of essential oil of *T. lemmonii* is considered important to strengthen the threshold of use of this substance, especially in a preventive strategy, additionally its economic viability would be valued. Rudilla oil is remedial in action, while Governor's extract, *Trichoderma*, and homeopathic substances are slow and may be preventative in their best performance.

Especially the low cost of *Botrytis* nosode represents a promising advantage that stimulates research to continue exploring different dynamizations from those tested or to test other homeopathic substances, whenever there is accompanying *in vivo* work, that in general they are few in the subject of agrohomeopathy.

Conclusions

In vitro conditions in which responses of *B. cinerea* mycelium were evaluated, antagonistic effect of *T. harzianum*, biocidal effect of the agrochemical and essential oil of *T. lemmonii*, and fungistatic effect of *L. tridentata* extract and nosode 7 CH were evidenced.

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