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Effect of time and temperature of storage on the activity of the etanolic extracts of *Lantana camara* L. and *Heliotropium indicum* L. on *Colletotrichum gloeosporioides*

Efecto del tiempo y la temperatura de almacenamiento en la actividad de extractos etanólicos de *Lantana camara* L. y *Heliotropium indicum* L. sobre *Colletotrichum gloeosporioides*

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Abstract

The effect of time and temperature of storage of ethanolic extracts (EE) of *Lantana camara* and *Heliotropium indicum* on *Colletotrichum gloeosporioides* was investigated. The EE were stored at 8±2 and 26±2°C, during one year. Every 2 months they were diluted in potato dextrose agar (PDA) at 0; 0.5; 1 y 1.5% (v/v) concentrations, fungus disks were placed onto the media, mycelial growth (ICM) and sporulation (IE) inhibition were measured, as well as conidia germination. The EE stored at 8±2°C maintained their effectiveness on all variables during the 12 months storage, while those stored at 26±2°C started to lose their effectiveness at 6 to 8 months, ending up to 3% ICM, 4% IE and 18% conidia germination at 1.5% concentration. Results indicated that storage temperature and time are determinant for extracts effectiveness.

Key words: Natural extracts, secondary metabolites, biological control, anthracnose, stability.

Resumen

En el estudio se evalúo el efecto del tiempo y la temperatura de almacenamiento de extractos etanólicos (EE) de *Lantana camara* L. (Verbenacea) y *Heliotropium indicum* L. (Boraginaceae) sobre *Colletotrichum gloeosporioides*. Los EE se almacenaron durante 1 año a 8 ± 2 y 26 ± 2 °C. Cada 2 meses se diluyeron en agar papa dextrosa (PDA) a concentraciones de 0, 0.5, 1 y 1.5% (v/v) sobre las cuales se colocó el hongo para medir la inhibición del crecimiento micelial (ICM) y la esporulación (IE); y en microcultivos en PDA, la germinación de conidios. Los EE almacenados a 8 ± 2 °C conservaron su efectividad durante 12 meses en todas las variables evaluadas, mientras que aquellos a 1.5% de concentración a 26 ± 2°C comenzaron a perder su efectividad a los 6 y 8 meses después de preparados, llegando hasta 3% de ICM, 4% de IE y 18% de germinación. Los resultados indicaron que la temperatura y el tiempo de almacenamiento son determinantes para la efectividad de los EE.

Palabras clave: Antracnosis, control biológico, estabilidad, extractos naturales, metabolitos secundarios.

Introduction

The ethanol extracts of many wild plants have an important fungitoxic effect on many pathosystems (García and Pérez, 2009). Compounds derived from secondary metabolism have been considered for long time as waste substances without a defined physiological function. The fungitoxic effect is due to the involvement of secondary metabolites (MS) during the interactions between plants and biotic factors. As a result, there is an inhibition on the growth of plant organisms (allelopathy) and protection against infection and predators (Azcón-Bieto and Talon, 2000).

Research to develop natural antifungals, with stability over time, are scarce. Most of them are focus on the phytochemical effect exerted by MS pathosystems and not on those factors that affect their chemical stability (Montes, 2009). Sarapin (2000) suggests that taking into account the complex chemical composition of plant extracts (EV), analysis and control of the behavior of their components during storage should consider effective methods of conservation for the active ingredients they contain.

Colletotrichum gloeosporioides is a widely distributed plant pathogenic fungus, identified as causal agent of anthracnose in different crops of agricultural importance. The damage by this fungus is manifested by foliar necrosis with irregular spots. Those spots are slightly sunken on the leaf and the blade edges. In fruit the spots are round and dark (Agrios, 2005).

Lantana camara L. (Verbenaceae) and *Heliotropium indicum* L. (Boraginaceae) are known in Venezuela as white Cariaquito and scorpion tail, respectively. They have MS with antimicrobial properties that affect the development of different species of plant pathogenic fungi (Silva *et al.*, 1999; Rodriguez *et al.,* 2004). Due to the effective action of vegetal extracts on some plant pathogenic fungi, it is necessary to know the effect of time and storage temperature on the stability of the active ingredients of these extracts, defining the best conditions for preservation. The aim of this study was to evaluate the effect of time and storage temperature on ethanol extracts of *Lantana camara* L. and *Heliotropium indicum* L., and its action on *Colletotrichum gloeosporioides*.

Materials and methods

To obtain the ethanol extract branches and leaves of wild adult plants of *L. camara* and *H. indicum* during the pre-flowering state were used. Samples were collected in the grounds of the Agronomy Graduate School of the Centro-Occidental Lisandro Alvarado University (UCLA), municipality Palavecino, Lara State, Venezuela. Branches and leaves were dried under shade and then crushed in an Oster blender. The resulting material was macerated and diluted in ethanol (96%) in a ratio of 800 g of dry matter in 3 liters of solvent. The liquid was filtered after 48 h through four layers of gauze. With the help of a Rotavaporador Brinkmann® the alcohol was separated to obtain the pure EE, which was transferred to vials of 1.5 ml covered with foil to prevent the incidence of light. Finally it was stored for 12 months at temperatures of 26 ± 2°C (ambient) and 8 ± 2°C (refrigerated).

Samples of EE were collected every 2 months to 12 months after preparations in order to perform in vitro evaluations. The samples were used to measure the effect on mycelial growth and sporulation of *C. gloeosporioides*, following the methodology of Araujo *et al.*, (2007). Besides, the effect on germination of conidia was also evaluated following the methodology of Ortiz (2010). A strain of the fungus, isolated from the mango plant (*Mangifera indica* L.), was used in both cases. The strain came from the collection of Postgraduate Mycology Laboratory of Plant Pathology at UCLA, which was previously cultured for 4 weeks in potato-dextrose-agar (PDA).

The EE were mixed in a previously autoclaved PDA at concentrations of 0, 0.5, 1 and 1.5% (v/v). The medium with EE was dispensed in Petri capsules. Once it solidified, the agar with mycelia were placed a disk of 0.5 cm diameter, then incubated at 26 ± 2°C. The diameter of the colony was measured every 2 days until the control colony (without EE) covered the capsule completely. A factorial design 6 x 2 x 3 with five replicates for each of the EE was used. Finally, the percentage of mycelial inhibition growth was calculated in each treatment using the formula:

$$\%ICM = \frac{CTT - CTE}{CTT} * 100$$

where: ICM = inhibition of mycelial growth, CTT = mycelial growth in the control treatment (0%); CTE = mycelial growth in assessed treatment.

From each treatment three discs of PDA with colony of *C. gloeosporioides* were taken with a punch of 0.5 cm diameter in order to measure the effect of EE on the fungus sporulation. The discs were placed in test tubes together with 10 ml of sterile distilled water, then they were stirred for 1 min. Using a hematocymeter the amount of conidia suspension was determined, allowing the calculation of the inhibition percentage of each treatment compared to the control (no EE) using the following equation:

$$\%IE = \frac{ETT - ETE}{ETT} * 100$$

where: IE = Inhibition of sporulation, ETT = sporulation in the control treatment (0%); ETE = sporulation of the assessed treatment.

To measure the effect of EE on the germination of *C. gloeosporioides* conidia, micro-cultures of PDA medium with EE stored in concentrations 0; 0.5; 1 and 1.5% (v/v) were prepared. The micro-cultures were applied on microscope slides, using three replicates per treatment. A suspension of 2.8 x 105 conidia/ml was prepared following the methodology of the above experiment besides an addition of a 20 µl aliquot on the microscope slides. The microscope slides were incubated in humid chambers at 26 ± 2°C. Random observations of the slides were performed after 12 hours in an optical microscope at 400X, counting the germinated conidia in each treatment. The germination percentage was calculated for the analysis of variance, using the Statistix® program, version 8.0, and the comparison of means by Tukey test (P <0.01).

Results and discussion

The results of analysis of variance showed differences (P < 0.01) between treatments for the variables of time, storage temperature, concentration and their interactions. The effect on the ICM did not change for extracts stored at 8 ± 2 °C when the mean (Table 1) was compared. After 12 months there was a 67% inhibition of the maximum concentrations of EE (1.5%) of *L. camara* and 62% of *H. indicum*. Storage at 26 ± 2 ° C resulted in a loss of effect of EE from 6 months in the case of *L. camera*, and 8 months for *H. indicum*. The lost after 12 months of storage was 4% for *L. camara* and 2% for *H. indicum*.

The analysis of variance also showed significant differences for sporulation (P < 0.01) between treatments for each variable and interactions. The average for the extract stored at

Table 1. Inhibition of mycelial growth (ICM) of *Colletotrichum gloeosporioides* treated with ethanolic extracts of *Lantana camara* and *Heliotropium indicum*, stored for 2, 4, 6, 8, 10 and 12 months at 8 ± 2°C and 26 ± 2°C.

Temp. (%)	Time (months)	*Lantana camara* Concentration (%)			*Heliotropium indicum* Concentration (%)		
		1.5	1	0.5	1.5	1	0.5
				Inhibition of mycelial growth (%)			
	2	68.36 a*	46.55 c	30.36 f	64.97 a	35.63 c	19.02 e
	4	68.34 a	46.24 c	30.23 f	64.69 a	35.61 c	18.06 e
8	6	68.25 a	46.17 c	29.77 f	64.09 a	35.27 c	17.88 e
	8	67.93 a	46.04 c	29.57 f	63.28 a	34.88 c	17.71 e
	10	67.67 a	46.01 c	29.45 f	62.96 a	34.71 c	17.51 e
	12	67.02 a	45.93 c	29.19 f	62.61 a	34.62 c	17.22 e
	2	68.24 a	46.05 c	29.60 f	64.53 a	35.43 c	18.16 e
	4	67.58 a	45.64 c	28.81 f	63.23 a	35.24 c	18.06 e
26	6	63.37 b	42.72 d	25.49 g	62.96 a	34.86 c	17.91 e
	8	37.91 e	21.41 h	8.85 j	40.72 b	31.39 d	14.02 f
	10	22.07 h	14.50 i	4.43 k	3.24 h	18.03 e	5.48 g
	12	4.24 k	4.38 k	4.14 k	2.87 h	3.60 h	2.82 h
CV(%)		12.71			22.76		
Signif.		P < 0.01			P < 0.01		

* The values in the same column with the same letter are not significantly different (P> 0.01) according to Tukey test.

8 ± 2 °C showed that EE maintains its effect on IE through the year of study (Table 2), with values of 78% for *L. camara* and 68% for *H. indicum*. The EE stored at 26 ± 2 ° C, however, reduced its effect from 6 months of storage in the case of *L. camara*, and 8 months in the case of *H. indicum*. Therefore, its effectiveness decreased drastically to 4% for IE at the end of 12 months.

The percentage of germination, like BMI and IE, varied significantly (P < 0.01) between treatments for each of the variables and their interactions (Table 3). The extracts preserved at 8 ± 2°C, for the two species, showed no differences (P > 0.05) within each concentration during the 12-month evaluation. The lowest percentages of germination at this temperature were obtained with the highest concentration

Table 2. Inhibition of sporulation (IE) of *Colletotrichum gloeosporioides* treated with ethanolic extracts of *Lantana camara* and *Heliotropium indicum*, stored for 2, 4, 6, 8, 10 and 12 months at 8 ± 2°C and 26 ± 2°C.

Temp. (%)	Time (months)	*Lantana camara* Concentration (%)			*Heliotropium indicum* Concentration (%)		
		1.5	1	0.5	1.5	1	0.5
		Inhibition of mycelial growth (%)					
8	2	80.64 a*	74.18 b	58.06 e	71.04 a	61.28 b	47.30 c
	4	80.16 a	74.09 b	57.98 e	70.96 a	60.20 b	46.70 c
	6	79.45 a	73.66 b	56.90 e	69.88 a	59.64 b	45.34 c
	8	79.19 a	73.11 b	55.34 e	68.30 a	59.44 b	45.06 c
	10	78.87 a	72.80 b	55.21 e	68.01 a	58.07 b	46.80 c
	12	78.44 a	72.03 b	54.06 e	67.82 a	57.20 b	46.21 c
26	2	79.48 a	73.06 b	56.98 e	72.88 a	59.98 b	46.23 c
	4	78.48 a	72.88 b	55.90 e	70.33 a	58.80 b	45.04 c
	6	67.74 c	60.21 d	44.79 f	69.88 a	57.24 b	44.32 c
	8	31.17 g	30.10 g	33.32 g	47.45 c	37.62 d	20.42 f
	10	8.59 h	9.66 h	7.97 h	26.49 e	7.52 h	11.82 g
	12	4.29 i	5.37 i	3.22 i	4.29 h	5.39 h	5.27 h
CV(%)		8.72			11.45		
Signif.		P< 0.01			P< 0.01		

* The values in the same column with the same letter are not significantly different (P> 0.01) according to Tukey test.

Table 3. Conidia germination of *Colletotrichum gloeosporioides* treated with ethanolic extracts of *Lantana camara* and *Heliotropium indicum*, stored for 2, 4, 6, 8, 10 and 12 months at 8 ± 2°C and 26 ± 2°C.

Temp. (%)	Time (months)	*Lantana camara* Concentration (%)			*Heliotropium indicum* Concentration (%)		
		1.5	1	0.5	1.5	1	0.5
		Inhibition of mycelial growth (%)					
8	2	67.33e	73.73d	82.66c	16.53f	49.66e	66.60d
	4	67.73e	74.08d	82.77c	17.20f	51.33e	67.66d
	6	67.86e	74.88d	82.80c	19.33f	52.93e	68.00d
	8	69.73e	75.06d	83.46c	19.96f	53.00e	68.26d
	10	69.86e	75.40d	83.93c	20.31f	53.36e	68.97d
	12	70.05e	75.80d	84.15c	20.72f	53.80e	69.21d
26	2	66.23e	73.20d	81.73c	16.26f	49.60e	66.20d
	4	67.96e	74.53d	82.40c	17.66f	50.01e	67.06d
	6	87.46b	80.54c	87.06b	18.27f	52.13e	67.33d
	8	88.40b	86.40b	90.80b	52.53e	54.06e	80.24c
	10	90.91b	90.53b	91.33b	79.86c	86.41b	85.46b
	12	94.13a	92.40b	94.40a	90.93a	92.40a	89.46a
CV(%)		3.83			15.28		
Signif.		P< 0.01			P< 0.01		

* The values in the same column with the same letter are not significantly different (P> 0.01) according to Tukey test.

(1.5%) of EE, especially with *H. indicum*. This species reached a value of 20.7% after 12 months of storage, while *L. camara* achieved 70% germination at the same concentration. The EE conserved at 26 ± 2°C showed an increasing loss of effectiveness after 6 months in the case of L. camera, and 8 months in the case of *H. indicum*. Germination of conidia at all concentrations of EE varied between 89.4 and 94.4% at 12 months.

The results showed that temperature has a direct effect on the efficacy of the plant extracts, which can be related to the thermolabile character MS (Sarapin, 2000). Bayer *et al.,* (1987) explained that MS, especially phenols, can be decomposed completely or partially at room temperature due to the breakdown of the benzene rings, which are the structural basis for many of them. Moreover, Rosas (2004) indicated that low temperatures favour the conservation of the chemical characteristics of the EV, explaining the losses of effectiveness at 8 °C for the extracts.

The literature does not report effectiveness in time, nor over the different temperatures of EE compared to plant pathogenic fungi. However, the results of this study indicated that there is a direct relationship between the two factors; indicating that the storage time depends on the temperature conditions.

Conclusions

The EE of *H. indicum* and *L. camara*, stored at 8 ± 2°C, did not lose their effectiveness under the control of *C. gloeosporioides* during 12 months of storage. However, storage at 26 ± 2°C decreased their effectiveness on mycelial growth, sporulation and germination of conidia of the fungus. In the case of *L. camara* the effectiveness decreased after 6 months of preparation, while *H. indicum* was after 8 months.

EE of *H. indicum* was more effective on the germination of conidia, while *L. camara* was better on mycelial growth and sporulation.

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