ISSN 0373-580 X
Bol. Soc. Argent. Bot. 48 (2): 201-209. 2013

Effect of aqueous and alcohol extracts of *Phytolacca tetramera* (Phytolaccaceae) leaves on *Colletotrichum gloeosporioides* (Ascomycota)

MARCELO HERNÁNDEZ[1], MÓNICA MURACE[2], JORGE RINGUELET[3], INÉS PETRI[4], DANIEL GALLO[4] and ANA ARAMBARRI[1]

Summary: *Phytolacca tetramera* Hauman "ombusillo" is an endemism of southeastern Buenos Aires province (Argentina). This species has fungicidal action against opportunistic pathogens of humans. In order to search natural alternatives for the control of diseases in plants caused by fungi, the objective was to evaluate the effects of aqueous and alcohol extracts of *P. tetramera* leaves on *Colletotrichum gloeosporioides* (Penz.) Sacc. This fungus has a wide distribution in different species with agricultural, forestry, and ornamental value. The antifungal activity of aqueous and ethanol leaf extracts was assessed *in vitro* against fungi. The fungus was subjected to two types of extracts already incorporated into the Potato Dextrose Agar (PDA) medium at 5-50% concentrations. The aqueous extract concentrations within the range 15-30% led to a decrease in the average diameter and speed of mycelium growth, while the range of 15-40% was the most effective in relation to a decrease in conidial production. Also, leaf alcohol extract inhibited the conidial production at concentrations of 5%, and had fungicidal action at concentrations of 15%. From "ombusillo" leaves a foam index of 250 was obtained. This high concentration of saponins would be at least one cause of the antifungal activity.

Key words: Antifungal activity *in vitro*, biological control, "ombusillo", Phytolaccaceae, saponins.

Resumen: Efecto de los extractos acuoso y alcohólico de la hoja de *Phytolacca tetramera* (Phytolaccaceae) sobre *Colletotrichum gloeosporioides* (Ascomycota). *Phytolacca tetramera* Hauman "ombusillo" es un endemismo del sudeste de la provincia de Buenos Aires (Argentina). Esta especie presenta acción fungicida contra patógenos oportunistas de humanos. Con el propósito de buscar alternativas naturales para el control de enfermedades en los vegetales, se planteó como objetivo evaluar el efecto de los extractos foliares acuoso y alcohólico de *P. tetramera* sobre el desarrollo del hongo *Colletotrichum gloeosporioides* (Penz.) Sacc., el cual tiene amplia distribución en especies de importancia agrícola, forestal y ornamental. El ensayo se realizó *in vitro.* El hongo fue cultivado en agar papa glucosado (APG), con aplicación del extracto en concentraciones del 5-50%. Las concentraciones del extracto acuoso del 15-30% produjeron una disminución del diámetro y velocidad media de crecimiento del micelio, mientras que las concentraciones del 15-40% fueron las más efectivas en el control de producción de conidios. El extracto alcohólico inhibió la producción de conidios con el 5% de concentración y con el 15% resultó fungicida. A partir de las hojas de "ombusillo" se obtuvo un índice de espuma de 250. Esta alta concentración de saponinas hace suponer que sería, al menos, una de las causas de la actividad antifúngica.

Palabras clave: Actividad antifúngica *in vitro,* control biológico, "ombusillo", Phytolaccaceae, saponinas.

Introduction

Diseases of cultivated crops is being considered as one important limitation to increase agricultural production. Therefore, protection of plants from pathogens remains a primary concern to agricultural scientists (Guleria & Kumar, 2006). Researchers have succeeded in controlling some devastating diseases since the very beginning of their appearance by using synthetic fungicides. On the other hand, the inappropriate use of such fungicides, expressed in terms of type, toxicity, number of applications and dosage have produced pollution that affects the agroecosystem, being the accumulation of waste potentially harmful to human and animal

[1]Departamento de Ciencias Biológicas.
[2]Departamento de Recursos Naturales y Medio Ambiente.
[3]Departamento de Ciencias Exactas, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata (UNLP), Calle 60 y 119, CC31 (1900) La Plata.
[4]Departamento de Biodiversidad, Fundación Biósfera. 16 Nº 1611 (1900) La Plata, Buenos Aires, Argentina.

health (Bolivar *et al*., 2009). The use of plant origin pesticides has been suggested by some workers as natural alternatives to synthetic chemicals (Montes-Belmont, 2009). A series of recent studies have confirmed the efficacy of plant extracts in the control of fungal diseases (Farias Magalhães *et al*., 2003; Zapata *et al*., 2003; Sung Og *et al*., 2007; Bolivar *et al*., 2009; Pineda *et al*., 2010; Pérez *et al*., 2011). There is a history of the presence of active principles in the aqueous and alcohol extracts of the leaves and fruits of several species of the genus *Phytolacca* L., with analgesic, antiinflammatory, bactericidal, fungicidal, mitogenic and molluscicide action (Nickell, 1959; Parkhurst *et al*., 1973; Woo & Kang, 1975, 1976; Moreno & Rodríguez, 1981; Kang & Woo, 1987; Yang-Hua, 1989, 1990, 1992; Favel *et al*., 1994; Nielsen *et al*., 1995; Gattuso, 1996; Quiroga *et al*., 2001; Farias Magalhães *et al*., 2003; Delporte *et al*., 2009). Also, active principles have been found in fruit methanolic extracts of *P. tetramera* Hauman, which are a source of saponins with fungicidal action on opportunistic pathogens of humans by Escalante *et al.* (2002), Santecchia *et al.* (2002), and Zacchino (2004).

Phytolacca tetramera "ombusillo" is a shrub endemic of southeastern Buenos Aires, (Argentina). It grows in the districts of Magdalena (35º 05' lat. S-57º 31' long. O), Punta Indio (35º 16' lat. S-57º 13' long. O), Castelli (35° 55' 18.12" lat. S-57º 43' 16.19 " long. O), and Chascomús (35º 30' lat. S-58º 30' long. O) (Hauman, 1913; Cabrera, 1949; Cabrera & Zardini, 1978; Guaglianone, 1987; Delucchi, 2006; Galup, 2006; Hernández *et al*., 2009; Petri *et al*., 2010) (Fig. 1 A-C).

Colletotrichum gloeosporioides (Penz.) Sacc. is a cosmopolitan fungus recognized to cause damage to the organs of many cultivated agricultural, forest and ornamental species (e.g., *Carica papaya* L., *Cassia fistula* L., *Ceiba pentandra* (L.) Gaertn., *Ceiba speciosa* (A. St.-Hil.) Ravenna, *Codiaeum variegatum* (L.) Rumph. ex A. Juss., *Citrus* sp., *Liquidambar* sp., *Mangifera indica* L., *Olea europaea* L., *Populus* spp., *Quercus palustris* Münch.) (Callan, 1998; Deschamps & Wright, 2000; Benyahia *et al*., 2003; Cabrera *et al*., 2004; Meireles Barguil *et al*., 2008; Sergeeva *et al.*, 2008; Bolívar *et al.*, 2009; Farr & Rossman in nt.ars-grin.gov/fungaldatabases, consulted in 2011).

The control of diseases caused by different species of *Colletotrichum* depends on the use of healthy seeds, seed treated with hot water, the selection of resistant varieties, and the crop rotation. The products management during and after harvest, and the treatment of packing house and containers are also important, as well as the treatment of plants and seeds in different growth stages, with application of synthetic fungicides (Agrios, 1997; Bolivar *et al*., 2009).

The aim of this investigation, therefore, is to

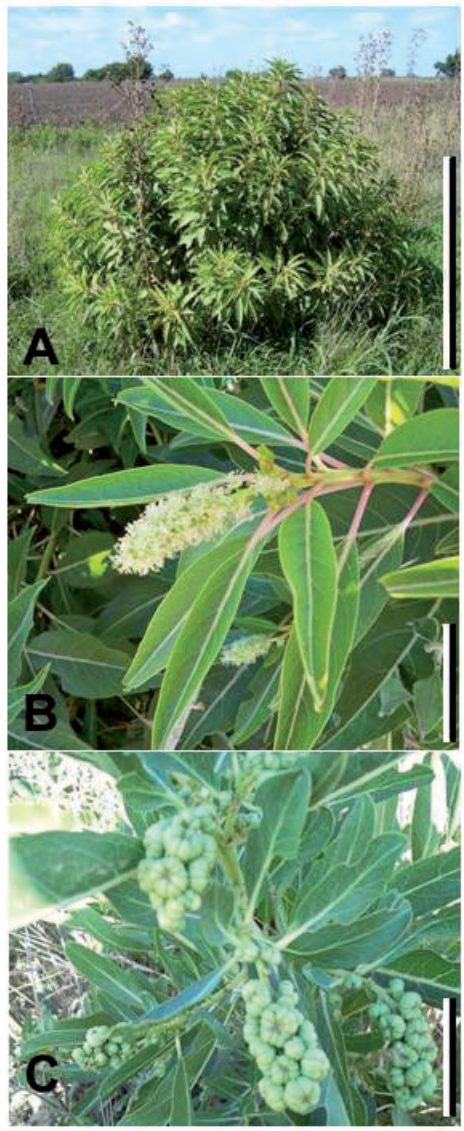

Fig. 1. A: *Phytolacca tetramera* plant. **B**: Leaves and inflorescence. **C**: Inflorescences with maturation fruits. Scales: A: 1 m. B, C: 5 cm. Photographs were taken by M. P. Hernández.

assess the *in vitro* antifungal activity of aqueous and alcohol (ethanol) extracts of *P. tetramera* leaves. This plant species will be tested as potential source of biologically active natural substances against *C. gloeosporioides*.

Materials and Methods

Plant material

Fresh and healthy branches with leaves of *Phytolacca tetramera* were collected. Leaves were separated and washed thoroughly to remove dry dirt covering surfaces and used to perform the assays. The reference material was deposited at herbarium of Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata under the data: Hernández, M. P. 60-67, 22-II-2011 (LPAG).

Fungal agent used

Pure culture of *Colletotrichum gloeosporioides* was obtained from the Laboratory of Protección Forestal, Facultad de Ciencias Agrarias y Forestales, at Universidad Nacional de La Plata. The isolate was obtained from diseased samples of *Ceiba speciosa*. It was isolated on 2% Potato Dextrose Agar (PDA), purified and maintained at 4°C until use.

Saponins assay

Determination of saponins was made according to Gallo (1979) methodology. In fact, one gram of fresh leaves with the addition of 100 mL of sterile distilled water was crushed. The resulting liquid of the filtered through cotton was placed to boil in water bath for 30 min. Once cold, it was completed to 100 mL with distilled water. Portions of 1 to 10 mL were taken by placing them in 16 cm x 16 mm test tubes. All were completed to 10 mL with distilled water (Table 1). The blocked tubes were waved in a longitudinal direction for 15 s. After 15 min the columns of foam were measured and the foam rate was obtained (maximum dilution of the sample, which maintains a 1 cm column of foam for more than 15 min).

Table 1. Dilution of aqueous extract (AE) with distilled water (DW).

Test tubes	I	II	III	IV	V	VI	VII	VIII	IX	X
AE (mL)	1	2	3	4	5	6	7	8	9	10
DW (mL)	9	8	7	6	5	4	3	2	1	0

Preparation of aqueous and alcohol extracts of P. tetramera leaves

One part of fresh leaf samples was exhaustively macerated with sterile distilled water and another identically part of leaves with 96% ethanol (1:1, w/v). The process was carried out at room temperature for 24 h according to Sharapin (2000). The mixtures were filtered and the solvent removed under vacuum in a rotary evaporator. Filtrates were preserved at 4ºC. To avoid any prospective chemical alterations, the extracts were used within 3-4 days.

Antifungal bioassays

For each extract (aqueous and ethanol), eleven treatments were performed. Each treatment was replicated ten times. In fact, PDA medium was prepared and sterilized. The leaf extracts were thoroughly mixed with the medium, and poured in each sterilized Petri dishes of 9 cm diameter. Control (0), 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50% concentrations of leaf aqueous and alcohol extracts were used, respectively. After solidification, mycelial discs of 5 mm diameter were taken from 5-7 days old culture of *C. gloeosporioides*. They were placed in the center of each Petri dish. Dishes were incubated in an incubator at 25 ± 2ºC for 7 days. Aqueous and ethanol extract effects were evaluated by averaging measurements from each colony of the following parameters: colony average diameter growth (ADG), colony average speed growth (ASG), and conidia average production (CAP). The diameter of the mycelium growth rings were measured with a millimeter rule. The conidia count was made using a Nuebauer chamber by means of a Hokenn microscope. The photographs were taken with a Kodak easyshare C 653 digital camera (Fig. 8: A-C).

Statistical analysis

Treatments were arranged in a completely randomized design. All parameters evaluated (ADG, ASG, and CAP) were analyzed through ANOVA (to determine the statistical significance level) in statistical program (Statistica 7.0 for windows). To check significant differences between the levels of the main factor, Tukey comparison tests at 5% ($P < 0.05$), significance were applied (Figs. 2-7 and Tables 2-7).

Results

Saponins assays

The dilution of 4 mL of aqueous extract in 6 mL of distilled water led to obtain 1 cm column of foam for more than 15 min. Foam index = 250.

It was calculated:

100 mL - 1 g drug
4 mL x = 0.04 g drug

0.04 g drug - 10 mL
g y = 250 mL

Effect of aqueous extract on C. gloeosporioides

The aqueous extract of the *P. tretramera* leaves significantly decreased the average of the analyzed parameters (ADG, ASG and CAP) in relation to the control (Figs. 2, 3 and 4; Tables 2, 3 and 4).

Colony Average Diameter Growth (ADG). No significant differences were found between the mean values of ADG obtained with extract concentrations in the ranges 15-35% and 40-50%. Within the concentration range of 15-35% mycelium development was not observed. Significant differences were found at extract concentrations of 5%, 10% and respect to the ranges 15-35%, and 40-50%. Contrary to expectations, the extract concentration range of 15-35% exhibited means values significantly lower than those obtained within the range 40-50%. The highest average values of ADG were obtained at aqueous extract concentration of 5% and 10%, with significant differences between them, and with the rest of the treatments. The highest was at 5%. The lowest average value was obtained at extract concentration of 15%, proving to be the most effective concentration (Fig. 2; Table 2).

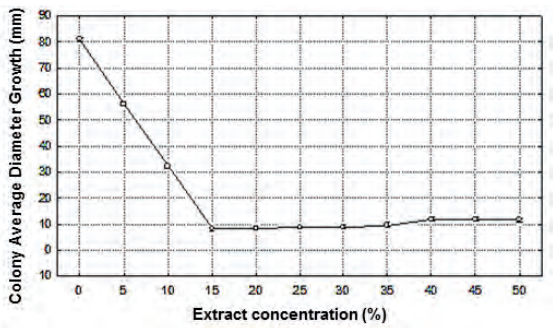

Fig. 2. Effect of aqueous extract of the *P. tetramera* leaves (%) on the colony average diameter growth (ADG) of *C. gloeosporioides*, in millimeters (mm).

Table 2. Multiple Range Test (Tukey, $p<0.05$) for colony average diameter growth (mm) per extract concentration (%).

Treatments	Average	Homogeneous groups				
15	8.175	X				
20	8.500	X				
25	8.925	X				
30	9.050	X				
35	9.500	X				
50	11.500		X			
40	11.850		X			
45	11.865		X			
10	33.19			X		
5	56.38				X	
(control) 0	81.050					X

Colony Average Speed Growth (ASG). No significant differences were found between the mean values of ASG obtained with extract concentrations in the range 15-35%. Contrary to expectations, there was a lower effect within the extract concentration range of 40-50% and no significant differences were found in the values obtained. As expected, the highest average values of ASG were obtained at aqueous extract concentrations of 5% and 10% with significant differences between them, and with the rest of the treatments. The highest was at 5%. The lowest average value was obtained at extract concentration of 20%, proving to be the most effective concentration (Fig. 3; Table 3).

Conidia Average Production (CAP). A significant difference was found between the mean values of conidia production at extract concentration of 5% respect to the range of 10-50%. No significant differences were obtained at the extract concentration in the range 10-50%, however the most effective in

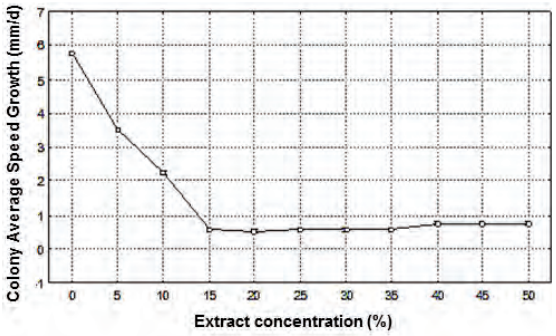

Fig. 3. Effect of aqueous extract of *P. tetramera* leaves (%) on the colony average speed growth (ASG) of the *C. gloeosporioides*, in millimeters per day (mm/d).

Table 3. Multiple Range Test (Tukey, p<0.05) for colony average speed growth per day (mm/d) per extract concentration (%).

Treatments	Average	Homogeneous groups
20	0.530	X
25	0.550	X
15	0.569	X
30	0.570	X
35	0.585	XX
50	0.715	XX
40	0.725	X
45	0.731	X
10	2.272	X
5	3.541	X
(control) 0	5.789	X

relation to a decrease in conidial production was the range 15-40%. The lowest average value was obtained at extract concentration of 15%, proving to be the most effective concentration (Fig. 4, Table 4).

Effect of alcohol extract on C. gloeosporioides

The alcohol extract of the *P. tretramera* leaves significantly decreased the average of the analyzed parameters (ADG, ASG and CAP) in relation to the control (Figs. 5, 6 and 7; Tables 5, 6 and 7).

Colony Average Diameter Growth (ADG). No significant differences were found between the mean values of ADG obtained with extract concentrations in the ranges 15-50%. Within the concentration range of 15-50% mycelium development was not observed. Significant differences were found at extract concentration of 5%, 10% and respect to the

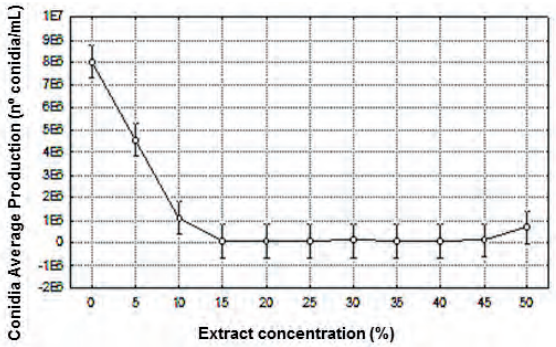

Fig. 4. Effect of aqueous extract of the *P. tetramera* leaves (%) on the conidia average production (CAP) expressed by number of conidia per milliliter (nº conidia/mL) of the *C. gloeosporioides.*

Table 4. Multiple Range Test (Tukey, p<0.05) for conidia average production (CAP) expressed in number of conidia per milliliter (nº conidia/mL) per extract concentration (%).

Treatments	Average	Homogeneous groups
40	50000.0	X
20	50000.0	X
25	50000.0	X
15	50000.0	X
35	50000.0	X
30	100000.0	X
45	112500.0	X
50	675000.0	X
10	1.1 E6	X
5	4.55 E6	X
(control) 0	8.0 E6	X

range 15-50%. The highest average values of ADG were obtained at alcohol extract concentrations of 5% and 10%, with significant differences between them, and with the rest of the treatments. The highest was at 5 %. The lowest average value was obtained at extract concentration of 15 %, proving to be the most effective concentration (Fig. 5; Table 5).

Colony Average Speed Growth (ASG). No significant differences were found between the mean values of ASG obtained with extract concentrations in the ranges 15-50%. Within the concentration range 15-50% mycelium development was not observed. Significant differences were found at concentrations of 5%, 10% and respect to the range 15-50%. The highest average values of ASG were

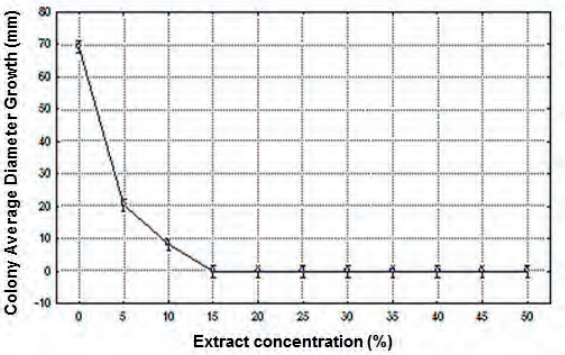

Fig. 5. Effect of ethanol extract of the *P. tetramera* leaves (%) on the colony average diameter growth (ADG) of *C. gloeosporioides*, in millimeters (mm).

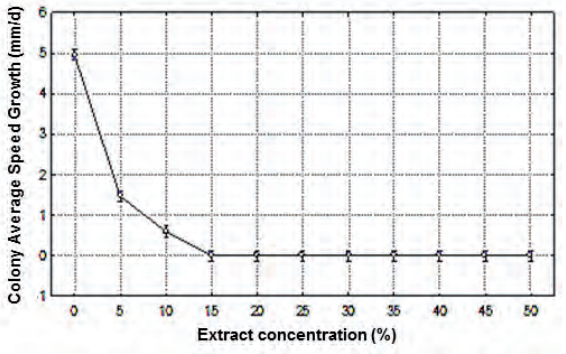

Fig. 6. Effect of ethanol extract of the *P. tetramera* leaves (%) on the colony average speed growth (ASG) of *C. gloeosporioides*, in millimeters per day (mm/d).

Table 5. Multiple Range Test (Tukey, p<0.05) for colony average diameter growth (mm) per extract concentration (%).

Treatments	Average	Homogeneous groups
25	0	X
45	0	X
40	0	X
15	0	X
20	0	X
50	0	X
30	0	X
35	0	X
10	8.375	X
5	20.5	X
(control) 0	83.75	X

Table 6. Multiple Range Test (Tukey, p<0.05) for colony average speed growth per day (mm/d) per extract concentration (%).

Treatments	Average	Homogeneous groups
25	0	X
45	0	X
40	0	X
15	0	X
20	0	X
50	0	X
30	0	X
35	0	X
10	0.598750	X
5	1.465.000	X
(control) 0	4.951.250	X

obtained at alcohol extract concentration of 5% and 10%, with significant differences between them, and with the rest of the treatments. The highest was at 5%. The lowest average value was obtained at extract concentration of 15%, proving to be the most effective concentration (Fig. 6; Table 6).

Conidia Average Production (CAP). Leaf alcohol extract concentration at 5% inhibited conidia production (Figure 3). A significant difference was found between the mean values of CAP within the concentration range of 5-50% in relation to the control. The average value obtained at extract concentration of 5% proved to be the most effective (Fig. 7, Table 7).

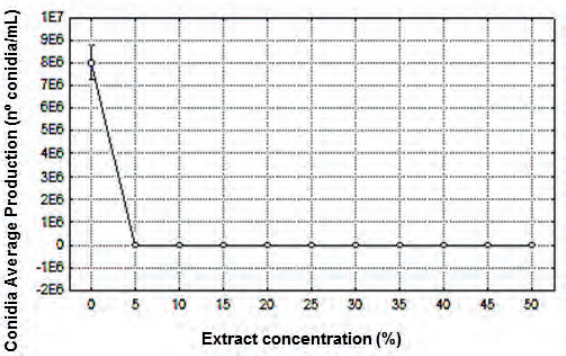

Fig. 7. Effect of ethanol extract of the *P. tetramera* leaves (%) on the conida average production (CAP) expressed by number of conidia per milliliter (nº conidia/mL) of the *C. gloeosporioides.*

Table 7. Multiple Range Test (Tukey, p<0.05) for conidia average production expressed in number of conidia per milliliter (nº conidia/mL) per extract concentration (%).

Treatments	Average	Homogeneous groups
25	0	X
5	0	X
10	0	X
15	0	X
20	0	X
50	0	X
30	0	X
35	0	X
40	0	X
45	0	X
(control) 0	8.5 E6	X

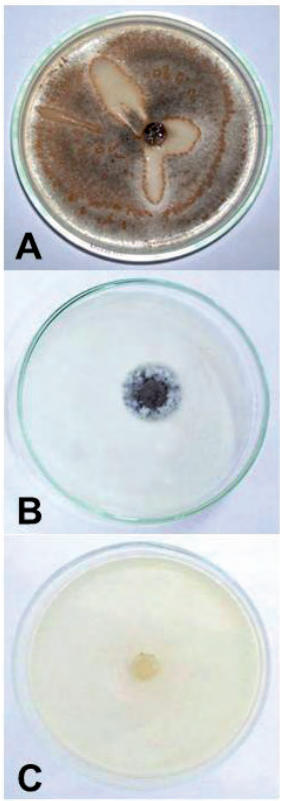

Fig. 8: *Colletotrichum gloeosporioides* mycelium growth. **A**: Control. **B**: Treatment at alcohol extract concentration of 5%. **C**: Treatment at alcohol extract concentration of 15%.

Discussion

There is an ample interest to develop environment-friendly alternatives to synthetic fungicides for the control of fungal plant diseases. In fact, there are a large number of contributions about this theme. In this study we compared the *in vitro* antifungal activities of aqueous and alcohol *P. tetramera* leaf extracts. Our results demonstrated alcohol extract provided the best fungus control. These results are in agreement with previous references on antifungal activities of different species and substance tested, such as, from essential oils extracted from different plants (Sung Og *et al.*, 2007), saponins from Fabaceae (Farias Magalhaes *et al*., 2003) to fungicide property of propolis (Pineda *et al*., 2010). Also, accord with similar determination on *Colletotrichum gloeosporioides* using ethanol extracts of leaf from different species, which were rich in essential oils (Bolivar *et al*., 2009). Our test of persistent foam in dilute aqueous solution proved that *P. tetramera* leaves are rich in saponins. It is knowledge that the presence of saponins in the genus *Phytolacca* was determined by the first time by Dominguez (1928) in *P. dioica* L. Later, many saponins were isolated from different species of *Phytolacca* (Woo & Kang, 1975, 1976; Kang & Woo, 1987; Yang-Hua, 1989, 1990, 1992; Nielsen *et al.*, 1995; Santecchia *et al.*, 2002). In 1996, Gattuso (1996) referred the presence of the triterpenoid saponins given them a chemotaxonomic significance to the subfamily Phytolaccoideae. On the other hand, it is known that saponins have antifungal activity (Moreno & Rodríguez, 1981; Farias Magalhaes *et al*., 2003), because their capacity to form complexes with membrane sterols and producing membrane disintegration (Glauert *et al*., 1962; Montes-Belmont, 2009). Also, the inhibitory effect against human pathogenic fungi activities of saponins isolated from *P. tetramera* fruits were reported by Escalante *et al*. (2002), and Zacchino (2004). In this paper, we agree with previous authors and according to their and our results we attributed to this chemical compound the antifungal activity of aqueous and alcohol extracts of *P. tetramera* leaves.

Conclusion

Aqueous and alcohol extract of the *P. tretramera* leaves significantly decreased the average of the

analyzed parameters (colony average diameter growth, colony average speed growth, and conidia average production) in relation to the control. However, the alcohol extract was more effective because with a concentration of 5% reduced the mycelium growth of *Colletotrichum gloeosporioides* and inhibited conidia production, and at a concentration of 15% had fungicidal action. A foam index of 250 was found in *P. tetramera* leaves. In fact, we attributed to this chemical compound the effect against *C. gloeosporioides.*

Acknowledgements

We are indebted to Maria Alejandra Migoya who helped us to prepare figures, and Romina Ywatany for valuable suggestions upon the English text. We thank to the reviewers because their suggestions helped to improve the manuscript.

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Recibido el 10 de mayo de 2012, aceptado el 3 de julio de 2012.