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Activity and mode of action of *Parastrephia lepidophylla* ethanolic extracts on phytopathogenic fungus strains of lemon fruit from Argentine Northwest

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ABSTRACT

Fungal infections of fresh ripe *Citrus limon* fruit are the main cause of economic losses during their commercialization. The aim of this work was to evaluate the activity of the ethanolic extract (EE) from *Parastrephia lepidophylla* (Wedd.) Cabrera, a plant species that grows in the Argentine Northwest, on phytopatogenic fungi of lemon. Also, attempts were made to elucidate the mode of action of EE on the growth inhibition of phytopathogenic isolates of *Penicillium digitatum* Sacc. and *Geotrichum citri-aurantii* (Ferraris) E.E. Butler. The effect of the polyphenolic extracts on the conidia germination, mycelium growth and integrity of the plasma membrane was evaluated. The EE was active against both pathogens (minimal inhibitory concentration and minimal fungicidal concentration values of 150 μg of gallic acid equivalent/ml and 350 μg GAE/ml, respectively for both species). The conidia swelling and germination and the subsequent germ tube elongation was more affected by EE (100% inhibition at 200 μg GAE/ml) than the vegetative body of the fungus (50% inhibion at 400 μg GAE/ml). The fungus cell walls would not constitute a target for the EE components while the mechanism of action of the phytocomplex would be plasma membranes disruption. *In vivo* tests showed that *P. leidophylla* EE (700 μg GAE/ml) decreases the incidence of green mold disease in artificially inoculated lemons.

1. Introduction

Lemon fruit (*Citrus limon* (L.) Burm. f.) contains many important chemical components, including phenolic compounds (mainly flavonoids and phenolic acids), vitamin C, folic acid, minerals, dietary fiber, pectin, essential oils and carotenoids (Balasundram et al., 2006; Ejaz et al., 2006), that account for their health-promoting effects (Vinson et al., 2001; Proteggente et al., 2002; Wilmsen et al., 2005). It was demonstrated that they have a role in blood lipid lowering and in the prevention of diseases, such as obesity, diabetes, cardiovascular diseases, and certain types of cancer (Chasquibol et al., 2003). Industrial uses of lemon include concentrated juice, essential oil, dried peel and frozen pulp. Losses caused by postharvest diseases are among the major problems of the citrus industry worldwide. Fungal infections are the main cause of postharvest rots of fresh lemon fruit during storage and transport, and cause significant economic losses in the commercialization phase. Citrus green mold and sour rot diseases, caused by *Penicillium digitatum* Sacc. and *Geotrichum citri-aurantii* (Ferraris) E.E. Butler respectively, are some of the most frequently encountered species (Eckert and Brown 1986; Eckert and Eaks 1989; Sommer et al., 2002). Synthetic fungicides have a major role in order to reduce postharvest losses due to phytopathogenic fungi, though problems resulting from their extensive use as well as their associated health and environmental risks have promoted the search for new and safer alternatives. Furthermore, frequent findings of resistant strains due to the continuous use of fungicides are a serious risk to the effectiveness of them. Many results have been reported on the antimicrobial properties of polyphenolic extracts obtained from plant species that grow within Argentinian area of Puna (Zampini et al., 2005, 2007, 2009a,b; D'Almeida et al., 2011, 2012). In a previous work we have reported the activity of aqueous extracts of three plant species from the Andean

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phytogeographic province against fungal species causing citrus postharvest diseases (Sayago et al., 2012). Furthermore, *in vivo* activity of *Parastrephia lepidophylla* extract against *P. digitatum* was as high as those obtained by *in vitro* assays. The phytocomplex has exhibited both preventive as well as curative activities against *P. digitatum* growth inoculated in laboratory assays on lemon fresh fruit (Sayago et al., 2012). The aim of the present work was to investigate the *in vitro* and *in vivo* antifungal activity of an ethanolic extract from *P. lepidophylla* and to elucidate the mode of action on the growth inhibition of *P. digitatum* and *G. citri-aurantii* isolates.

2. Materials and methods

2.1. Plant material

Specimens of *P. lepidophylla* (Wedd.) Cab. were collected in Laguna de Vilama, Jujuy (66°55′W, 22°30′S, 4500 m above see level), a mountainous area of Puna from Northwest Argentina (Andean phytogeographic province; Cabrera 1971). The botanical identification was done by botanist Ana Soledad Cuello (Universidad Nacional de Tucumán-CONICET). The voucher specimens are deposited in Fundación Miguel Lillo Herbarium (access number 68979/LIL), Tucumán, Argentina.

2.2. Plant extracts preparation

Aerial parts of plant material were air-dried, ground and macerated in an alcoholic solution (50 g dry tissue/ 250 ml 60% ethanol) for 7 d at 30 °C with shaking (50 cycles/ min) in a water bath (Vicking, Dubnoff model). The extracts were filtered through two layers of gauze and centrifuged at 23,600 × *g* at 10 °C (Sorvall RC 5B) for 15 min. The supernatants were pooled and named *Crude Extract* (CE). The CE was concentrated at 40 °C at reduced pressure and then lyophilized. The Dry residue was first suspended in distilled water; after centrifuging (23,600 × *g*, 15 min) the supernatant was named *Aqueous Extract* (AE) and kept at −20 °C until use. The water insoluble material was then extracted with absolute ethanol (Sigma–Aldrich) and was named *Ethanolic Extract* (EE) and kept at −20 °C until use.

2.3. Chemical determinations

Total phenolic compound contents were determined by using the Folin–Ciocalteau colorimetric method (Singleton et al., 1999). Results were expressed as gallic acid equivalents (μg GAE/ml). Flavonoids were evaluated according to Woisky and Salatino (1998). Results were expressed as quercetin equivalents (QE μg/ml). Proteins were determined according to the Bradford (1976) method. The assays were performed using the micro-assay procedure of the Bio-Rad protein assay kit (Bio-Rad Laboratories). A solution of bovine serum albumin (1 mg/ml) was used as standard.

2.4. Fungal cultures

IEV 543 of *G. citri-aurantii* (Ferraris) E.E. Butler and IEV 544 of *P. digitatum* Sacc. strains were used (IEV: Culture collection of the Instituto de Estudios Vegetales, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina). The pathogenic fungi were isolated from lemon fruit with sour rot or green mold diseases. The identity of both fungal species was assessed according to Pitt and Hocking (1999). Stock cultures were maintained on Sabouraud Agar at 4 °C. Sabouraud Agar, Potato-Dextrose Agar and Glucose Broth (GB: 3% glucose, 1% casein peptone, pH 5.4–5.6) were used for routine fungus cultures. All reagents were purchased in Merck Chemicals Argentina, Buenos Aires.

2.5. Inoculum preparation

Conidia from 10 to 14 days cultures in PDA were suspended in sterile saline solution (0.9% NaCl) containing 0.05% Tween 80, vortexed and filtered through two layers of gauze. Conidia concentration was adjusted in a Neubauer chamber under light microscope.

2.6. Minimal inhibitory concentration and minimal fungicidal concentration determinations

Broth macrodilution tests were conducted to determine the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the EE.

Glucose Broth containing EE (50, 100, 150, 200, 250, 300, 350, 400, 450 or 500 μg GAE/ml) was inoculated with a *P. digitatum* or *G. citri-aurantii* conidium suspension to achieve a final concentration of 0.5×10^3 conidia/ml. The incubation was carried out at 28 ± 2 °C for 72 h. The growth of fungi was assessed by direct observation of cultures. MIC was defined as the lowest extract concentration necessary to inhibit fungal development. MFC determination was performed as follows: aliquots of 100 μl of suspensions from MIC determinations (72 h of incubation at 28 °C) were subcultured on extract-free SA medium and incubated for 72 h at 28 °C. MFC was defined as the lowest concentration of extract capable to produce the irreversible loss of viability of conidia in the assay conditions (Sayago et al., 2012).

2.7. Time-kill determination

Tubes containing different amounts of EE (350 or 700 μg GAE/ml) in a final volume of 1 ml of GB were inoculated with 0.5 × 103 conidia (*P. digitatum* or *G. citri-aurantii*) and incubated at 28 °C. Aliquots of 100 μl were transferred to extract-free SA every 6 h. The fungal growth was assessed up to 72 h of incubation at 28 °C.

2.8. Effect of EE on the spore germination

a) The effect of the EE concentration on spore germination. Tubes containing increasing concentrations of EE (0–500 μg GAE/ml) were inoculated with 1×10^6 conidia/ml and incubated for 12 h at 28 °C. The percentage of germination at each EE concentration was then evaluated under light microscope by counting the germinated conidia in Neubauer chamber. The results were expressed as% inhibition of germination, % Inh. = $[(C - T)/C] \times 100$, where *T* and *C* are the amount of germinated conidia in 1 μl of the suspension with and without EE, respectively.

Table 1
Chemical analysis of extracts of *P. lepidophylla*.

Formulation	Total amount (g)[a]		
	Phenolic compounds[b]	Flavonoids[c]	Proteins[d]
Crude extract	3.202 ± 0.129	1.969 ± 0.034	0.957 ± 0.034
Aqueous extract	0.636 ± 0.021	0.402 ± 0.012	0.690 ± 0.012
Ethanolic extract	2.496 ± 0. 021	1.225 ± 0.021	0.117 ± 0.010

Total amount of phenolic compounds, flavonoids and proteins obtained from 50 g of plant material.
[a] Data represent the average of three independent assays ± standard deviation.
[b] Phenolic compounds were determined according to Singleton et al. (1999) and expressed as gallic acid equivalents (GAE).
[c] Flavonoids were assayed by the method described by Woisky and Salatino (1998) and expressed as quercetin equivalents (QE).
[d] For protein determinations (Bradford, 1976) a standard of bovine serum albumin was used.

Table 2
Antifungal activity evaluation of *P. lepidophylla* extract by *in vitro* assays.

Assay	*P. digitatum*	*G. citri-aurantii*
MIC[a]	150	150
MIC (1.1 M sorbitol)[a]	150	150
MFC[a]	350	350
TK (350 μg GAE/ml)[b]	72	72
TK (700 μg GAE/ml)[b]	12	12

MIC, MIC sorbitol and MFC values were determined by broth macrodilution tests adding EE (50–00 μg GAE/ml) to the Glucose Broth. Time to kill assay (TK) was carried out by assessing the fungicidal activity of the EE every 6 h.

were then washed three times with sterile saline solution and incubated in GB at 28 °C for 12 h. The% of germinated cells was then estimated.

d) The effect of EE on the conidium swelling . The sizes of the conidia in presence (200 μg GAE/ml) and absence of EE were evaluated at time zero and after 12 h incubation at 28 °C. The observations were made under light microscope using a micrometer scale. One hundred conidia for each treatment were counted.

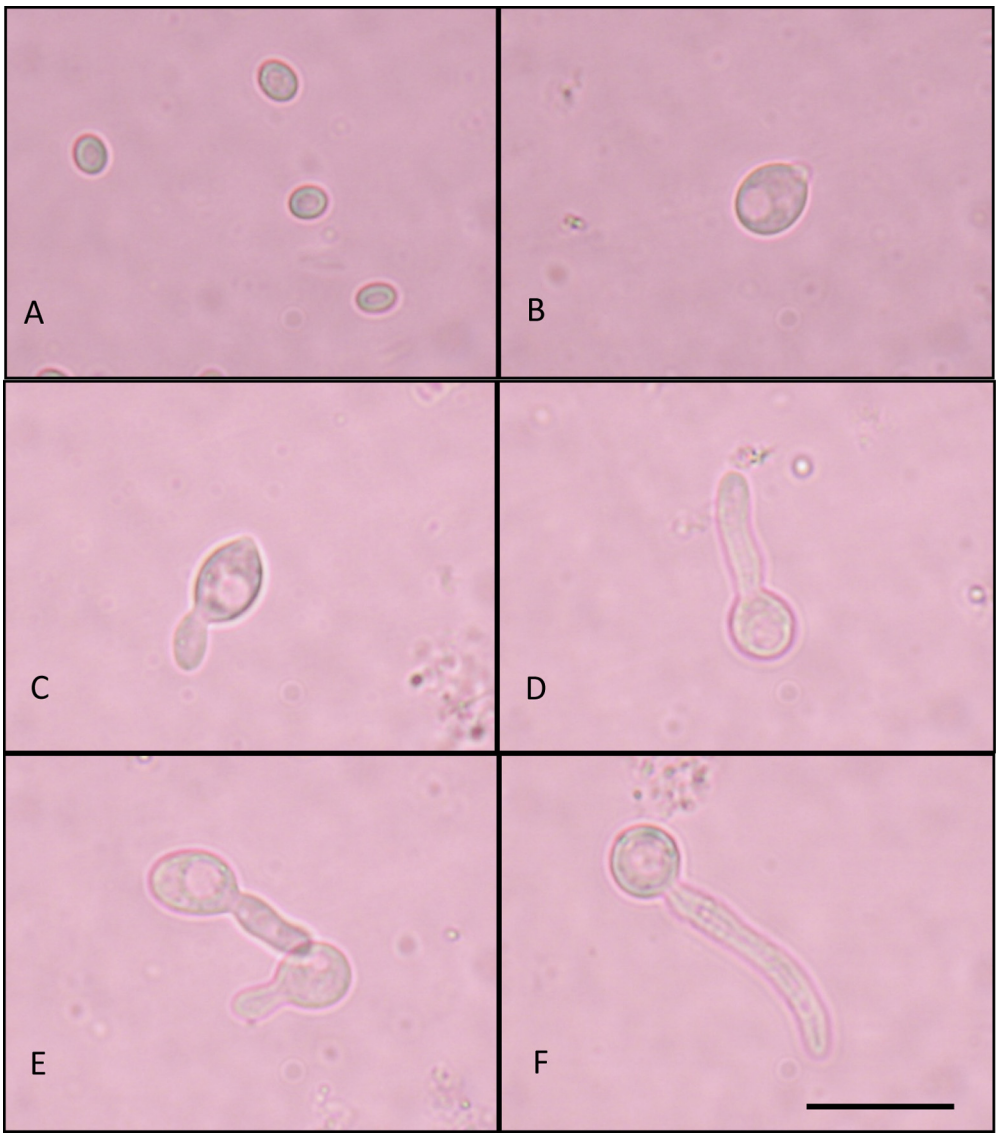

Fig. 1. Photomicrographs of *P. digitatum* conidia at different stages of germination. A: suspension of conidia without incubation. B–F: conidia in glucose broth free of ethanolic extract after 12 h of incubation. B: type 1 cells, with an incipient germ tube. C–F: type 2 cells, germ tube presenting different degrees of development. All figure parts are the same magnification (630×), scale bar = 20 μm.

b) The effect of the incubation time on the germination of conidia treated with EE. Conidial suspensions (1×10^6 conidia/ml) were treated with EE (200 μg GAE/ml) for 24 h at 28 °C. The percentage of germination was evaluated every 3 h from 12 h.
c) Recovery of viable conidia after treatment with EE. Conidial suspensions were exposed to increasing concentrations of EE (0–400 μg GAE/ml) and incubated for 12 h at 28 °C. Suspensions

2.9. Effect of EE on the mycelium growth of Penicillium digitatum

a) The growth kinetics of *P. digitatum* was carried out in 250 ml conical flasks containing 100 ml of GB and 100 μl of a spore suspension (1 ×106 conidia/ml). The flasks were incubated at 28 °C for different times up to 8 days. Then the mycelia were

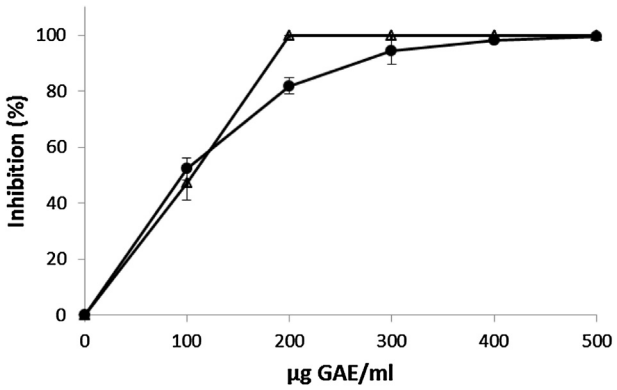

Fig. 2. Inhibitory effect of ethanolic extract on the germination of spores of *P. digitatum*. Cell types 1 and 2 (●) and type 2 (Δ) after 12 h of incubation (28 °C) of spore suspensions with different EE concentrations were counted in a Neubauer chamber. The results are expressed as % inhibition of germination ± SD of two independent assays.

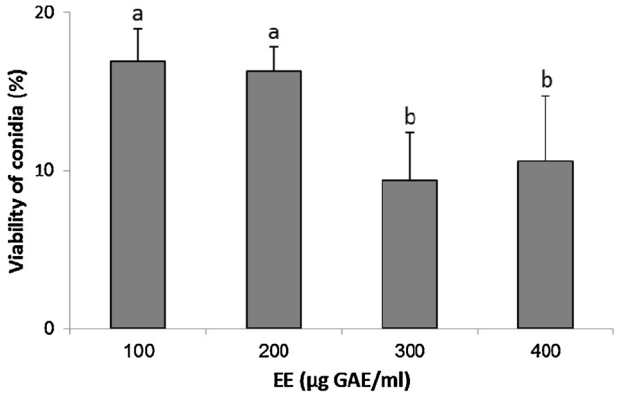

Fig. 3. Viability of *P. digitatum* conidia after the treatment with different ethanolic extract concentration during 12 h at 28 °C.

harvested and filtered through paper (Whatman N°1). The residues were dried in an oven at 60 °C up to constant weight and biomass estimated.

b) Five days cultures were treated with different amounts of EE (0–400 µg EAG/ml) and incubated at 28 °C for 72 h and then the biomass was estimated.

2.10. Effect of EE on cell wall

MIC values of EE in presence of 1.1M sorbitol were determined in order to evaluate its effect on cell wall of both fungal species (Pérez et al., 2007).

3. Effect of EE on cell membrane

Mycelia from cultures of 96 h at 28 °C in GB were suspended and washed in 2.5 ml of sterile saline solution. The suspensions were then centrifuged at 3000 rpm and the washing procedure was repeated twice more. Finally, the mycelia were suspended in GB containing EE concentrations equivalent to 1 × MFC or 4 × MFC. Negative (ethanol instead of EE) and positive (2% SDS) controls were also performed. All suspensions were incubated at 28 °C up to 48 h. At different times samples were taken and centrifuged at 3000 rpm. The supernatant absorbance at 260 nm was subsequently determined in order to evaluate the loss of cell components.

3.1. Preventive activity test

The assay was carried out according to Sayago et al. (2012). Fresh mature lemon fruit were provided by a local citrus processing plant. The experimental design was completely randomized with three replicates of 10 fruit per treatment. A fixed volume of *P. lepidophylla* formulation was mixed with wax used in industrial processing of lemon, in order to get 700 µg/L of GAE. Then, 1 ml of the mixture was applied to the lemon surface previously disinfected by immersion in ethanol 70% for 2 min. After 24 h, 1 ml of spore suspension (2.5×10^4 conidia/ml) of IEV 544 strain (*P. digitatum*) was applied on the whole fruit. The assay included a set of fruit without treatment, a set of fruit plus conidia suspension, a set with wax plus conidia suspension, and a set of fruit treated with wax containing extract plus conidia suspension. In all cases the treated fruit were maintained for 14 d at 25 ± 2 °C in closed boxes. Macroscopic evaluation of decay development was carried out every 48 h from 8 to 14 d. The index of incidence (*I*) was estimated as *I* = [number of infected fruits/*N*] × 100, where *N* = total number of fruits.

3.2. Statistical analysis

Data are presented as a mean ± standard deviation. Analysis of variance (one-way ANOVA; Minitab® 16.1.0) was performed using a probability level of less than 5% ($p < 0.05$), when appropriate. In the case of disease incidence assays, one-way ANOVA was applied to arcsine-transformed data.

4. Results

The fractionation of the CE of *P. lepidophylla* in AE and EE allowed the recovery of most phenolic compounds (PC) including flavonoids (F) in the EE (Table 1). However, a good yield of PC and F was also achieved in the aqueous solution; though proteins were poorly extracted by this procedure. The antifungal activity measurement was carried out by MIC determination. Our results indicated that EE was equally active against both fungal species with MIC values of 150 µg GAE/ml for both strains (Table 2). The MFC determination revealed that an extract concentration of

Table 3
Effect of EE on the swelling of *P. digitatum* and *G. citri-aurantii* spores.

Species	Spore size (microns)	2.5–5.0	5.1–7.5	7.6–10.0	10.1–12.5	>12.5
Cell distribution (%)						
P. digitatum	I	18.2 ± 2.5	63.5±4.0	18.3 ± 2.7	0	0
	II	10.1 ± 3.2	77.6 ± 2.5	12.3 ± 2.2	0	0
	III	0	10.3 ± 3.3	67.4 ± 5.2	22.3 ± 3.5	0
G. citri-aurantii	I	6.5 ± 3.3	20.9 ± 1.6	45.4 ± 3.3	28.1 ± 1.0	0
	II	0	34.6 ± 0.8	46.4 ± 2.6	19.0 ± 3.3	0
	III	0	0	9.8 ± 2.6	46.4 ± 2.6	19.0 ± 3.3

Conidium suspensions fresh (I), incubated 28 °C, 12 h with 200 µg GAE/ml (II) or incubated 28 °C, 12 h without EE (III) were submitted to an evaluation of cell sizes. The assay was performed in triplicate and the results expressed as % of total count of each treatment ± SD.

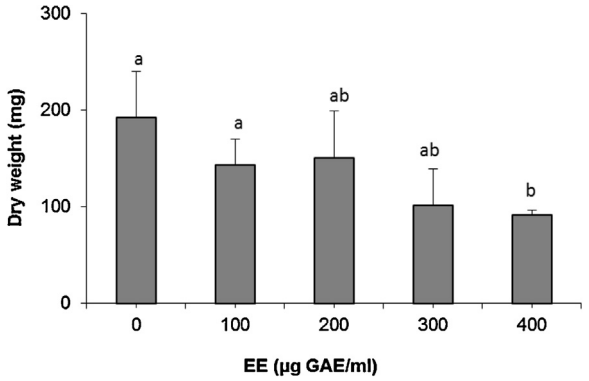

Fig. 4. Effect of different *P. lepidophylla* EE concentrations on the mycelial growth of *P. digitatum*. The % inhibition was estimated 3 days after adding the ethanolic extract.

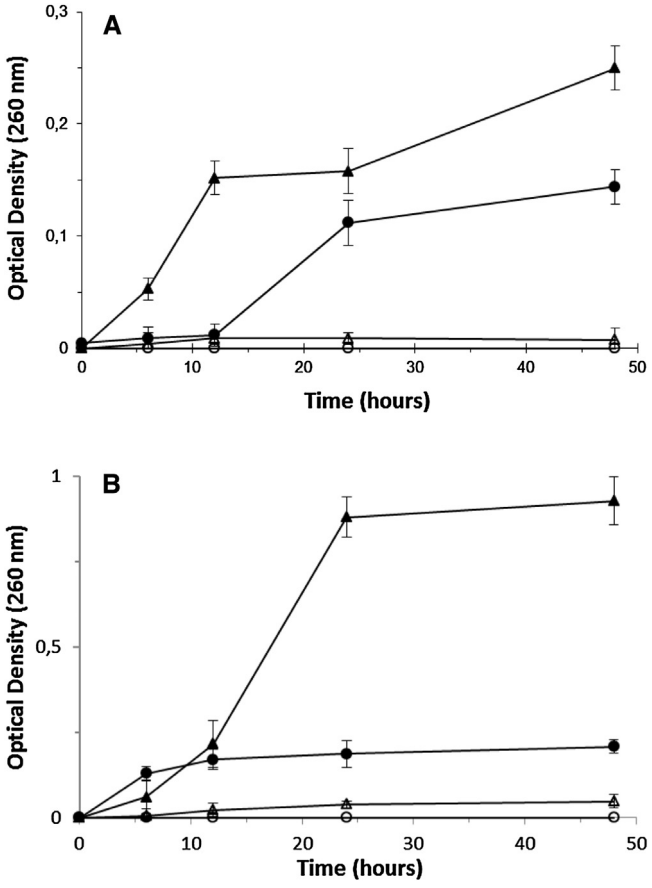

Fig. 5. Effect of ethanolic extract on the plasma membrane. The release of intracellular compounds at different times from suspensions of mycelium of *P. digitatum* (A) and *G. citri-aurantii* (B) incubated with EE was estimated by its absorbance at 260 nm. EE was assayed at 1 MFC (Δ) and 4 MFC (●). Also negative, without EE (○) and positive control with 2% SDS (▲) were processed. The assay was performed in duplicate.

350 μg GAE/ml is enough to cause irreversible inhibition of both species when the cultures were incubated for 72 h. However time-kill assays showed that increasing EE concentration up to 700 μg GAE/ml (2 × MFC) decreased the time required to produce irreversible inhibition of conidial viability to12 h (Table 2). When conidial suspensions were incubated for 12 h in an extract-free medium it was possible to observe cells with different germination stages: swelling conidia with a thinning cell wall or with an incipient germ tube (type 1 cells, Fig. 1B) or germinated conidia with a short germ tube (Fig. 1C) and conidia with longer germ tube (Fig. 1D–F), which were named type 2 cells. Only type 1 cells were found when the conidia were incubated within 200 μg of GAE/ml of EE, while no changes in size or shape of conidia were observed at 350 μg GAE/ml (Fig. 2). Furthermore, the EE (200 μg GAE/ml) inhibited the spore swelling of both fungal strains (Table 3).

The investigation of the capability of conidia to germinate after 12 h of incubation within an inhibitory concentration of EE (200 μg GAE/ml) showed that the germination inhibition of *P. digitatum* persisted until 24 h and identical results were obtained with *G. citri-aurantii* (data not shown). Furthermore, it was established that the viability of the conidia of *P. digitatum* isolate after treatment with EE for 24 h with its subsequent transfer, reduces the viability of the cells to percentages ranging from 17% (100 μg GAE/ml) to 10% (400 μg GAE/ml) (Fig. 3). Moreover, the addition of 400 μg GAE/ml of EE to the culture medium on the fifth day of incubation showed a biomass reduction of about 50% (Fig. 4) revealing that the extract exerted a moderate inhibitory effect on the growth of the mycelium. The growth kinetics of *P. digitatum* isolate had showed that the exponential phase occurred after six days of incubation at 28 °C (data not shown).

The addition of sorbitol to the culture media did not affect the MIC values for both fungal strains (Table 2), which indicated that the fungal cell walls would not constitute a target for the EE components. The test carried out to investigate whether EE causes any damage to plasma membrane of *P. digitatum* mycelium showed that when an EE concentration equal to 4 × MIC was used, the fungal cells released compounds that absorbed at UV in the same way as the SDS-treated cells, which was taken as positive control (Fig. 5A). The release of intracellular compounds increased from 12 to 48 h and was slightly detected at a concentration equal to MIC. Likewise, tests on *G. citri-aurantii* showed similar results (Fig. 5B).

The test of EE preventive activity against green mold showed no statistically significant differences between no inoculated fruits and fruits treated with wax containing EE plus conidia (Table 4). The result confirm the potentiality of *P. lepidophylla* EE to control the most common postharvest disease in many lemon producing countries even in Argentina.

5. Discussion

In this work it was established that the alcoholic fraction of the extract of *P. lepidophylla* is active against *P. digitatum* and *G. citri-aurantii* isolates. The assessment of *in vitro* antifungal activity of EE revealed to be greater than that of plant aqueous extracts against the same fungal isolates. MIC and MFC values of AE against *P. digitatum* were 400 and 600 μg GAE/ml, respectively (Sayago et al., 2012), while the values determined for the EE resulted to be 150 μg GAE/ml (MIC) and 350 μg GAE/ml (MFC) in the same experimental conditions. In the case of *G. citri-aurantii* the EE also proved to be more active than the AE. MIC and MFC values found for AE were 400 μg GAE/ml (Sayago et al., 2012), whereas 150 (MIC) and 350 μg GAE/ml (MFC) were determined for the EE. Thus, while the MIC

Table 4
Preventive effect of *P. lepidophylla* extract against green mold disease of lemon fruit.

Fruit treatment	Index of incidence (%)
No treatment	16.7 a
Conidia suspension	86.7 b
Wax plus conidia suspension	80.0 b
Wax containing extract plus conidia	23.3 a

Fruit were separated in sets of 10 lemons in cardboard boxes. Values are an average of 3 replicates. Values with different letters are significantly different according to one-way ANOVA applied to arcsine transformed data ($p < 0.05$).

values determined for AE are in a range corresponding to plant extracts considered active against susceptible isolates, values obtained from the EE are comparable to those expected for isolated natural compounds (Escalante et al., 2002; Feresin et al., 2003; Fenner et al., 2005; Svetaz et al., 2010). Since the antifungal activity of plant extracts could be attributed to flavonoids and coumarins (Ortuño et al., 2006) or to phenolic compounds (Gatto et al., 2011), these results would be explained in part by the greater richness of the EE in phenolic compounds. Further, according to dos Santos Oliveira and Badiale Furlong (2008) who worked with phenolic compounds, the type of phenolic structure is more important than the concentration.

Tests to investigate whether vegetative thallus or conidia of *P. digitatum* are more important for the antimicrobial effect of EE, showed that the mycelium of the fungus is poorly affected by the activity of the extract compared to that of spore swelling and germination and the subsequent germ tube elongation. Indeed, the inhibition of the biomass formation due to the action of 400 μg GAE/ml of EE for 72 h is of about 50%, while the spore germination is affected to a greater extent at a lower EE concentration applied for shorter times. Thereby a clear inhibition of cell swelling was observed with 200 μg of GAE/ml, applied during 12 h. Also, the germ tube elongation is completely inhibited, although about 20% of swollen cells with an incipient germ tube are still found. Moreover when EE concentration is increased to 350 μg GAE/ml an inhibition of cell swelling and germ tube occurrence of about 95% is reached. These results are similar to those recently reported by Gatto et al. (2011) who worked with phenolic extracts obtained from various wild edible plants and found that the extracts showed greater ability to inhibit germ tube elongation than conidia germination.

Considering the stages of conidial germination identified by Wendland (2001) consisting of: (1) activation, (2) isotropic growth, (3) emergency polarized growth of the germ tube and (4) elongation of the germ tube, it can be stated that *P. lepidopylla* EE in a concentration slightly higher than the MIC is capable of produce a high-level inhibition of at least the steps (2), (3) and (4), while a concentration slightly higher than the MFC produces the almost complete inhibition of germination. Additionally, the germination inhibition of the two species remained at 100% until at least 24 h of incubation. Instead, activity of Aureobasidina A, a cyclic depsipeptide antibiotic with antifungal properties against phytopathogenic fungi causing postharvest diseases produced an inhibition of germination of *P. digitatum* conidia that fell from 100% to 80%, between 8 and 24 h of treatment, while other fungal species (*P. italicum, P. expansum, B. cinerea* and *M. fruticose*) showed greater declining percentages (Liu et al., 2007).

Phenolic extracts from edible plants provoke germ tube defects, the cell wall disruption and loss of cellular components of some fungi (Gatto et al., 2011; Adebayo and Aderiye 2011) suggesting that the slightly lipophilic nature of some phenolic compounds contribute to its progressive accumulation in the fungal cell membrane altering its permeability and affecting some transport mechanisms (Kanaani and Ginsburg 1992). According to da Cruz Cabral et al. (2013) who reviewed the application of plant compounds in order to control fungal spoilage in foods, one of the aspects of the inhibitory action of active compounds in plant extracts is related with changes in permeability of cell membranes or with interactions with membrane enzymes, resulting in the loss of rigidity and integrity of the hypha cell wall. The present work established that the cell wall of the conidia would not be involved in the mode of action of the *P. lepidophylla* phytocomplex. However, the release of intracellular components due to EE (4 × MFC) demonstrated a potential action of the phytocomplex on the mycelium plasma membrane of the two species of fungi. The transfer of the EE from the culture media containing ungerminated conidia caused a poor recovery of the cell viability in 12 h treatment μg GAE/ml, suggesting an irreversible inhibition with the minor concentration used. A total loss of cell viability was not observed up to 12 h of treatment.

From the above findings, we conclude that the main effect of EE on *P. digitatum* would be to alter the mechanisms that lead to the swelling of the conidia and subsequent germ tube elongation, which occur during germination, and that the moderate effect of the extract on the development of mycelium once formed, would also contribute to the observed fungicidal effect. In this regard, Liu et al. (2007) who measured morphological alterations of both germinating conidia and mycelium of *P. digitatum* showed that the conidia had a higher sensitivity to a natural-Aureobasidina A than that shown by the mycelium of the fungus. Many events, like pH gradient and electrical potential, interference with ATP generation, enzyme inhibition (Coutinho de Oliveira et al., 2011; El-Mogy and Alsanius 2012) culminate with inhibition of germination, suppression of mycelial growth, and germ tube elongation, suggesting that the action of plant compounds might be related to the perception/transduction of signals involved in the switch from vegetative to reproductive development (da Cruz Cabral et al., 2013).

Fungal growth involves conidia germination and hyphal extension, eventually forming visible mycelium. A product, like lemon fruit, will be spoiled shortly after conidia are germinated (Dao et al., 2008); according to these authors prevention of germination would prevent fungal growth and subsequent spoilage being highly desirable to reduce the initial load of viable conidia by applying products that inactivate them. From this point of view the *P. lepidophylla* ethanolic extract would be a potential alternative to control decay in citrus. Finally the *in vivo* assays confirm the potentiality of the extract to be used as a safer alternative to synthetic fungicides.

6. Conclusions

This work is the first report on the activity of alcoholic extracts of *P. lepidophylla* against phytopathogenic fungi and about the probable mode of action of the phytocomplex. The extract affects mainly spore swelling and germination. The fungal cell walls would not constitute a target for the EE constituents while the mechanism of action of the phytocomplex would be concerned with plasma membranes of fungal strains. The extract is able to reduce the incidence of green mold in lemons with artificially provoked disease.

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