Effect of phenolic compounds present in Argentinian plant extracts on mycelial growth of the plant pathogen *Botrytis cinerea* Pers.

Efecto de compuestos fenólicos presentes en extractos de plantas argentinas en el crecimiento micelial del fitopatógeno *Botrytis cinerea* Pers.

Hapon MV\(^1,2\), JJ Boiteux\(^1,2\), MA Fernández\(^1\), G Lucero\(^1,2\), MF Silva\(^1\), PH Pizzuolo\(^1,2\)

**Abstract.** The polyphagous plant pathogen *Botrytis cinerea* has the ability of growing fast under several conditions, and producing grey moulds in different plant tissues. Because of its adaptive plasticity, resistant strains have made difficult their chemical control. Therefore, new strategies of management and the use of natural compounds could be important control alternatives. The aim of this work was to study the potential value of four Argentine native plant extracts and some of its phenolic compounds as biodegugs on *B. cinerea*. Aqueous plant extracts of *Larrea divaricata*, *Prosopis strombulifera*, *Tessaria abvinthioides* and *Schinus molle* var. *areira*, were used. The capability to inhibit the mycelial growth was studied using either the crude plant extract or same its most important, detected phenolic compounds. Phenolic compounds were analyzed by Capillary Zone Electrophoresis (CZE) with UV detection, and then statistically analyzed by Principal Component Analysis to correlate them and their biological activity against the fungus. *Prosopis strombulifera* and *T. abvinthioides* extracts did not inhibit mycelial growth while *S. molle* stimulated it. *Larrea divaricata* demonstrated inhibition above 50% at plant extracts concentrations of 100 mg/mL.

**Keywords:** *Larrea divaricata*, Luteolin; Naringenin; Cinnamic acid, Capillary Zone Electrophoresis; Antifungal.

**Resumen.** El polífago fitopatógeno *Botrytis cinerea* tiene la habilidad de crecer rápidamente en diferentes condiciones y producir podredumbre gris en diferentes tejidos de plantas. Debido a su plasticidad adaptativa, la existencia de cepas resistentes ha dificultado su control químico. Es por esto que nuevas estrategias de manejo y el uso de compuestos naturales pueden ser importantes alternativas de control. El objetivo de este trabajo fue estudiar el valor potencial de cuatro extractos de plantas nativas de Argentina y alguno de sus compuestos fenólicos como biofármacos sobre *B. cinerea*. Se utilizaron extractos acuosos de *Larrea divaricata*, *Prosopis strombulifera*, *Tessaria abvinthioides* y *Schinus molle* var. *areira*, se estudió la capacidad de inhibir el crecimiento micelial usando tanto los extractos vegetales como sus más importantes compuestos fenólicos detectados. Los compuestos fenólicos fueron analizados por Electroforesis Capilar de Zona (CZE) con detección UV, y luego analizados estadísticamente por Análisis de Componentes Principales para correlacionar su actividad biológica contra el hongo. Los extractos de *P. strombulifera* y *T. abvinthioides* no inhibieron el crecimiento micelial mientras que *S. molle* estimuló. *Larrea divaricata* demostró una inhibición superior al 50% con concentraciones de 100 mg/mL de su extracto vegetal.

**Palabras clave:** *Larrea divaricata*, Luteolina; Naringenina; Ácido cinámico, Electroforesis Capilar de Zona; Antifúngico.
INTRODUCTION

Downy mildew, powdery mildew and bunch rots are among the main grapevine canopy diseases. Bunch rots could produce yield losses between 30 to 70%; Botrytis cinerea Pers. Fr. is the main microorganism involved in bunch rots (Lucero et al., 2009a). Typical symptoms of diseases produced by B. cinerea are soft rots on leaves and fruits followed by a grey mass of mycelia and conidia (Williamson et al., 2007). Factors that influence the occurrence of grey mould are high humidity, reduced light and moderate temperature (Williamson et al., 2007).

Botrytis cinerea has the ability of surviving saprophytically or endophytically, and rapidly causes grey mould and other diseases (Lucero et al., 2009b). It has a wide range of hosts, and could colonize different plant parts; this is because of their action against a broad range of mono and dicotyledonous plants (Tenberge, 2007). This polyphagous pathogen has several modes of attacking hosts, and different sources of inoculum surviving in crop debris as mycelia and/or conidia and sclerotia (Williamson et al., 2007).

The control of this pathogen is mainly chemical and preventive. Despite existing various site-specific fungicides against gray mold (e.g., amilinopyrimidines, benzimidazoles, dicarboximides, hydroxanilides, quinone outside inhibitors), its effective control depends on fungicide resistance emergency (Grabke et al., 2012). The B. cinerea adaptive plasticity (Kumari et al., 2014) enables selection of resistant strains when mono or oligosite fungicides are excessively used. Moreover, inadequate use of agrochemicals results harmful to the environment and humans (Maia et al., 2014).

Worldwide cases of fungicide resistance have become a major problem, and B. cinerea is not the exception because it presents high both resistance factors and frequencies of mutant phenotypes in its population (Leroux et al., 2010). Some resistance cases in commercial strawberry fields have been disclosed with the use of fenhexamid (Grabke et al., 2012). The B. cinerea adaptive plasticity (Kumari et al., 2014) enables selection of resistant strains when mono or oligosite fungicides are excessively used. Moreover, inadequate use of agrochemicals results harmful to the environment and humans (Maia et al., 2014).

Fungal isolate and culture conditions. The B. cinerea strain used in the assays was isolated from naturally infected grape (Vitis vinifera L., cv Chardonnay) from Mendoza, Argentina. A monosporic colony obtained from a pure fungus culture was maintained on PDA (Potato Dextrose Agar) slant tube at 4 ± 1 °C, for long term conservation. When needed, colonies of the pathogen were subcultured on PDA at 22 ± 2 °C.

Chemicals and standards. Phenolic compounds (catechin, naringenin, cinamic acid, syringic acid, chlorogenic acid, vanillic acid, luteolin, quercetin and caffeic acid), constituents of BGE (30 mM boric acid buffer) and organic modifiers [acetoniitrite (ACN) and methanol (MeOH)] were purchased from Sigma (St. Louis, MO). Ultrapure water (resistivity 18.3 MΩ cm) obtained from Barnstead EASY pure® RF water system (Iowa) was used to prepare solutions, including the electrolyte solutions. Millex- HV Syringe Filters (0.22 µm pore size), Millipore® were also used.

Solvents and reagents. Standard stock solutions of the analytes were prepared by dissolving an appropriate amount of each pure substance in 10 mL of a HPLC-grade methanol to obtain a final concentration of 1000 mg/L. The resulting solutions were stored at 4°C in amber glasses. Working standard solutions at a 5 mg/L concentration were prepared on a daily basis by diluting appropriate aliquots of the previous standard stock solutions in methanol. Before use, all solutions were degassed by sonication for 5 min and filtered through 0.22 µm nylon filters.

Plant extracts and purification. Plant material of Argentine native botanical species [i.e., L. divaricata (jarilla),
**RESULTS AND DISCUSSION**

**Phenolic compounds profile of vegetal extracts.** All vegetal extracts showed a distinctive profile when analyzed by CZE (Table 1). Some phenolic compounds were detected only in one or a few extracts as rutin in \( P. \) strombulifera, naringenin in \( L. \) divaricata or syringic acid in \( S. \) molle and \( P. \) strombulifera. Only quercetin was found in all four extracts differing statistically on its concentration, except on \( S. \) molle and \( T. \) absinthioides. Moreover, \( P. \) strombulifera extracts have exhibited the greatest variety and the highest concentration of total phenolic compounds (635.8 mg/L). Meanwhile, \( L. \) divaricata showed the lowest amount of the studied phenolic compounds (231.0 mg/L). \( L. \) divaricata did not differ statistically from \( T. \) absinthioides.

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**P. strombulifera, S. molle var. areira (aguaribay) and T. absinthioides (pájaro bobo)** was harvested in the countryside area in the North of Mendoza province, Argentina. The harvest period was coincident with the flowering time of plants.

Crude plant extracts were obtained as proposed by Widmer & Laurent (2006), with some modifications. Briefly, 20 g of \( L. \) divaricata, \( P. \) strombulifera, \( T. \) absinthioides and \( S. \) molle var. areira leaves were taken and autoclaved on 1L Erlenmeyer flasks containing 200 mL of distilled water for 45 min at 121 °C. The supernatant was filtered and reduced by boiling in laminar flow-hood to 20 mL. The extract was then centrifuged at 2000 g for 15 min to remove solids. For sterilization, flasks were autoclaved for 20 min at 121 °C.

For the phenolic phase extraction, crude plant extract samples (1 g/mL) were thoroughly mixed with five parts (1:5, w/v) of acidic water until complete homogenization. An aliquot of 250 µL of each extract was used for extraction by the Solid Phase Extraction (SPE) method. Phenolic compound extractions were performed by a miniaturized SPE using a home-made column packed with suitable filtering material. C18 cartridges (50 mg) were made in 1 mL syringes using 5 mL of MeOH and 5 mL of acid water (water pH 2 with HCl). The samples were loaded onto the preconditioned column, leaving the sample on the solid phase under vacuum.

Then, the column was washed with 1 mL of acid water (water pH 2, with HCl). The phenolic fraction was eluted with MeOH (500 µL). The eluent was directly injected and analyzed by Capillary Zone Electrophoresis (CZE).

**Phenolic compounds in plant extracts by CZE.** Phenolic compounds were determined according with Boiteux et al. (2014). CZE separations were carried out using a Capel™ 105M apparatus equipped with a 67 cm full length, 50 cm effective length, 75 µm ID and 375 µm OD fused silica capillary. The running buffer was boric acid 30 mM; pH of the buffer was 9.5. The separation voltage was 25 kV and the capillary temperature was 25 °C. Samples were injected by hydrodynamic injection at 30 mbar for 2 s. Electropherograms were recorded at 290 nm.

**Effect of plant extracts on mycelial growth of B. cinerea.** Toxicity of crude plant extracts against the fungus was assayed using the amended media test (Soliman & Badeaa, 2002) on Potato Dextrose Agar (PDA). Extracts of \( L. \) divaricata, \( S. \) molle var areira, \( P. \) strombulifera and \( T. \) absinthioides were added to the culture medium at different concentrations (0.1 to 200 mg/mL). Sterile distilled water was used as a control. The culture medium with or without extracts was poured into 5.5 cm diameter Petri dishes. Subsequently, a 4 mm disk of solid culture medium, colonized with \( B. \) cinerea mycelia obtained from the edge of 15 day-old culture, was placed in the center of Petri dishes. The fungus colony area was daily measured using the software Axio Vision 4.8. The results were expressed as mycelial growth inhibition percentage (MGI) using the formula \([(C - T)/C]\) * 100, where C and T corresponded to the fungus colony area (cm²) of control and treatments, respectively.

**Individual effects of selected phenolic compounds on mycelial growth.** The effect of the pure phenolic compounds showing positive correlation with the antimicrobial activity against \( B. \) cinerea was evaluated. For this purpose, the solid agar bioassay described by Boiteux et al. (2014) was performed. In this case, a 4 mm disk of culture medium, colonized with \( B. \) cinerea mycelia was placed in the center of Petri dishes containing PDA amended with phenolic compounds at a concentration equivalent to that considered as bioactive in crude extract assays (9.5 mg/L of naringenin, 3.2 mg/L of cinnamic acid and 6.9 mg/L of luteolin). Phenolics compound were dissolved in MeOH. Control plates were run simultaneously, using the same growth medium plus the solvent and without phenolic compounds. Three replicate plates containing each phenolic compound including the control treatment were prepared. Petri dishes prepared as above were incubated at 22 ± 2 °C for 4 days, and colony growth was daily recorded. Mycelial growth inhibition was determined as described above.

**Statistical analysis.** Statistical analysis was performed by ANOVA, and means were compared using Tukey’s test. All the analyses were done in triplicate. The results were significant at P≤0.05 unless specified otherwise. The principal component analysis (PCA) was used to find the relationships between phenolic compounds and percentage of \( B. \) cinerea mycelial growth inhibition. Statistical analyses were carried out using InfoStat-Statistical Software and Statgraphics Centurion XVI.II.
Table 1. Phenolic compounds (mg/L) found in plant extracts analyzed by Capillary Zone Electrophoresis.
Tabla 1. Compuestos fenólicos (mg/L) presentes en extractos de plantas analizados por Electroforesis capilar de zona.

<table>
<thead>
<tr>
<th>Phenolic compound (mg/L)</th>
<th>Plant extract</th>
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<tbody>
<tr>
<td></td>
<td><em>S. molle</em></td>
</tr>
<tr>
<td>Rutin</td>
<td>nd a</td>
</tr>
<tr>
<td>Catechin</td>
<td>97.37 ± 2.92 b</td>
</tr>
<tr>
<td>Naringenin</td>
<td>nd a</td>
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<tr>
<td>Cinnamic acid</td>
<td>5.10 ± 0.23 c</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>187.30 ± 4.16 c</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>4.27 ± 0.20 b</td>
</tr>
<tr>
<td>Luteolin</td>
<td>nd a</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>nd a</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>56.00 ± 1.21 d</td>
</tr>
<tr>
<td>TPC</td>
<td>361.73 ± 8.28 b</td>
</tr>
</tbody>
</table>

Data represent the mean values of three technical replicates ± standard error. “nd” (not detected). Mean values with different letters within the same row differs statistically according to Tukey’s test (P≤0.05).

Los datos representan la media de tres repeticiones técnicas ± error estándar. “nd” (no detectado). Los valores de media con letras diferentes en una misma fila difer en estadísticamente según el test de Tukey (P≤0,05).

Biological activity of plant extracts against *B. cinerea*.
The biological activity of the different plant extracts on mycelia of *B. cinerea* is shown in Figure 1. All extracts showed a diverse activity. Some of them exhibited a mycelia growth stimulation compared with the control like extracts belonging to *T. absinthioides* and *S. molle* var. *areira*. Conversely, *P. strombulifera* and *L. divaricata* extracts have exhibited inhibition of mycelial growth.

![Fig. 1. Mycelial growth inhibition (%) of *B. cinerea* cultured on PDA amended with increased concentrations of plant extract (mg/mL) during an incubation period of 4 days at 22 ± 2 °C. Bars “I” represent standard error.](image)

![Fig. 1. Inhibición del crecimiento micelial (%) de *B. cinerea* cultivado en PDA adicionado con concentraciones crecientes de extracto vegetal (mg/mL) durante un periodo de incubación de 4 días a 22 ± 2 °C. Barras “I” representan error estándar.](image)

Treatments with either *T. absinthioides* or *P. strombulifera* plant extract did not show statistical differences between their biological activities against the fungus at any of the concentrations analyzed.

The lowest stimulatory or inhibitory effects were observed between concentrations of 0.1 and 1.0 mg/L of plant extract. A dose-dependent mode of action was only observed in the inhibitory effect of the *L. divaricata* extract and in the stimulatory effect of the *S. molle* var. *areira* extract.

The most effective and interesting aqueous extract was that of *L. divaricata*, which produced the highest inhibition percentage (about 94%) at a concentration of 200 mg/mL. It was also able to hinder mycelial growth at the lowest concentration, i.e. 5 mg/mL. This extract did not stimulate the pathogen ever.

The variability of the biological activity could be related to the capability of *B. cinerea* of detoxifying compounds presents in plants (Pedras et al., 2011).

Even though an in vitro antifungal activity of *S. molle* was reported by Dikshit et al. (1986) against *Aspergillus* and *Penicillium* species, in our work *S. molle* plant extract showed a stimulatory effect against *B. cinerea*. It might be that they used an essential oil obtained from leaves instead of an aqueous extract. Nonetheless, Davicino et al. (2007) demonstrated that the aqueous extract had no activity against *P. notatum* and *A. niger*. Conversely, Rhouma et al. (2009) showed that leaf ethanolic, methanolic and aqueous extracts inhibited *F. solani* growth within values from 60% to 66%.

It is quite difficult to compare these results with those of other authors because of the composition of the plant extracts can vary depending upon the geographical region, the plant...
age or variety, and the extraction method (Al-Reza et al., 2010). Saks and Barkai (1995) demonstrated that *Aloe vera* L. aqueous extract inhibited the *B. cinerea* mycelial growth in a 68% at a concentration of 1 µL/L. Other plants extracts like those of *Quillaja saponaria* M. at 15% or *Pithecellobium dulce* at 25% inhibited the mycelial growth of *B. cinerea* in an either 45% or 60%, respectively (Baños et al., 2004; Fonseca Rivera, 2007).

This work, as well as those of Davicino et al. (2007) and Vogt et al. (2007) showed that *L. divaricata* extracts had an inhibitory activity against fungus. Davicino et al. (2007), however, found that ethanolic extracts of *L. divaricata* had a better inhibitory activity than aqueous extracts. In our work, the aqueous extract exhibited a good inhibitory effect against *B. cinerea*.

**Correlation between the analytical composition of the extracts and the in vitro antimicrobial activity.** Relationships between phenolic compounds and inhibition of *B. cinerea* mycelial growth were analyzed by PCA. From this study three principal components were obtained and contributed to 99.3% of the total variance (CP1 50.0; CP2 27.6 and CP3 21.7%) (Fig. 2). *Larrea divaricata* was characterized by a high concentration of naringenin, luteolin and cinnamic acid. These compounds could be associated with the *B. cinerea* mycelial growth inhibition. These results are in accordance with those of Boiteux et al. (2014). They reported their association with *Phytophthora* spp. mycelial growth inhibition. Conversely, the high concentration of chlorogenic acid present on *S. molle* var. *areira* extract could be associated with its low mycelial growth inhibition. Similar results were observed for such phenolic compound on different pathogen species such as *Acremonium* sp., *Beauveria* sp., *Fusarium solani*, *Microsporum canis*, *Scopulariopsis brevicaulis*, *Trichoderma* sp. (Sisti et al., 2008), and *Phytophthora* spp. (Boiteux et al., 2014). Other phenolic compounds like vanillic acid and syringic acid have been reported to have a low antifungal activity against *B. cinerea* (Mendoza et al., 2013).

Between the phenolic compounds that could be associated with *B. cinerea* MGI (Fig. 3), the most effective was luteolin (84.3%) followed by naringenin (75.7%) and cinnamic acid (67.8%). When the three selected phenolic compound were tested together, they showed an additive inhibitory activity (99.0%) (Fig. 4). It should be noted that the MGI of the *B. cinerea* strain studied was lower when it was treated with the *L. divaricata* crude extract than when it was treated with the pure phenolic compounds at the same quantities detected in the extract. This could be a consequence of the combined effects of different compounds present in a high complexity matrix like this plant extract. This bioassay provided sufficient evidence to confirm that the three compounds that the PCA analysis identified as those explaining the major growth inhibitory activity were effectively those.

The bioactivity of these compounds is probably interfering with the permeability of the fungus cell membrane altering its functionality. Some studies have reported that phenolic compounds could (1) dissipate the pH gradient and electrical potential components of the proton motive force, (2) interfere with the energy (ATP) generating and conservation system of the cell, (3) inhibit membrane-bound enzymes, and (4) prevent the substrate utilization for energy production (De
Fig. 3. Inhibition of mycelial growth of B. cinerea grown in PDA amended with selected phenolic compounds for 4 days at 22 ± 2 °C. (A) Control. (B) PDA amended with 9.5 mg/L of Naringenin; (C) PDA amended with 3.2 mg/L of Cinnamic Acid; (D) PDA amended with 6.9 mg/L of Luteolin, and (E) PDA amended with a mixture of 9.5 mg/L of Naringenin, 3.2 mg/L of Cinnamic Acid and 6.9 mg/L of Luteolin.

Fig. 3. Inhibición del crecimiento miceliar de B. cinerea cultivado en PDA adicionado con compuestos fenólicos seleccionados por 4 días a 22 ± 2 °C. (A) Control. (B) PDA adicionado con 9,5 mg/L de Naringenina; (C) PDA adicionado con 3,2 mg/L de Ácido Cinámico; (D) PDA adicionado con 6,9 mg/L de Luteolina y (E) PDA adicionado con una mezcla de 9,5 mg/L de Naringenina más 3,2 mg/L de Ácido Cinámico más 6,9 mg/L de Luteolina.

Fig. 4. Inhibition of mycelial growth of B. cinerea (%) grown in PDA amended with selected phenolic compound for 4 days at 22 ± 2 °C. Treatments: NAR: 9.5 mg/L of Naringenin; CIN: 3.2 mg/L of Cinnamic acid; LUT: 6.9 mg/L of Luteolin and CIN+LUT+NAR: 9.5 mg/L of Naringenin; 3.2 mg/L of Cinnamic acid and 6.9 mg/L of Luteolin.

Fig. 4. Inhibición del crecimiento miceliar de B. cinerea (%) cultivado en PDA adicionado con compuestos fenólicos seleccionados por 4 días a 22 ± 2 ºC. Tratamientos: NAR: 9,5 mg/L de Naringenina; CIN: 3,2 mg/L de Ácido Cinámico; LUT: 6,9 mg/L de Luteolina y CIN+LUT+NAR: 9,5 mg/L de Naringenina más 3,2 mg/L de Ácido Cinámico más 6,9 mg/L de Luteolina.

Oliveira et al., 2011; El-Mogy and Alsanius, 2012; Pusztahelyi et al., 2015.

These are known to alter microbial cell permeability and interact with membrane proteins, causing deformation in the structure and functionality of these proteins. These disadvantageous changes may lead to dysfunction and subsequent disruption of membranes.

As we noticed for the L. divaricata extract, other Larrea species like L. tridentata have demonstrated inhibition activity against microorganisms (Martins et al., 2013). Other compounds present on some extracts obtained from Larrea species [e.g., nordihydroguaiaretic acid (NDGA)] have shown inhibitory activity against pathogenic fungi like A. flavus and A. parasiticus (Vargas-Arispuro et al., 2005). Regardless that NDGA was detected in some L. divaricata extracts, Davicino et al. (2011) noticed that its concentration was higher in ethanolic than aqueous extraction. The same consideration could be made for the chloroformic extract, which had a good antifungal activity, containing a considerable amount of NDGA (Vogt et al., 2007; Vogt et al., 2013). This is an important point because of NDGA is a lignan with nephrotoxic and hepatotoxic effects. Consequently, extracts without NDGA or with a low concentration of it could be more acceptable and considered safer (Stege et al., 2011).

This work indicated that other phenolic compounds, like naringenin, cinnamic acid and luteolin, might also have inhibitory activity against the plant pathogen B. cinerea.

CONCLUSIONS

Results support that L. divaricata aqueous extracts are potent inhibitors of B. cinerea growth. A large part of this bioactivity was explained by the presence of phenolic substances such as naringenin, cinnamic acid and luteolin. Although the other studied plant species have, between their constituents, some of the previous mentioned bioactive substances, their concentration was likely not high enough to inhibit the pathogen. Therefore, the aqueous extracts of L. divaricata could represent a potentially valid control alternative. This will be known after verifying its effectiveness in vivo, and adjusting the most suitable concentration and formulation for its use.

Our findings are not only of fundamental interest, but also have practical implications for crop protection, food safety, and animal and human health. Further toxicological and pharmacological studies will be useful to confirm the use of bioagrochemicals, using phytochemicals like those from L. divaricata leaves.
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