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# Antifungal activity of three Chilean plant extracts on *Botrytis cinerea*

Sofia Vio-Michaelis[1], Gastón Apablaza-Hidalgo, Miguel Gómez, Raúl Peña-Vera and Gloria Montenegro

Departamento de Ciencias Vegetales, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile
[1]Corresponding author: srvio@uc.cl

**Abstract:** The antifungal effect of the complete methanolic extract and a ethanolic fraction of three native Chilean plants (*Ephedra breana*, *Fabiana imbricata,* and *Nolana sedifolia*) were tested *in vitro* against *Botrytis cinerea* (the grey mold fungus) at 250 $\mu$g/mL for *E. breana* and *N. sedifolia* extracts and 400 $\mu$g/mL for *F. imbricata* extract. The results of this study showed that the ethanolic fractions of *E. breana* and *N. sedifolia* have a fungistatic effect during 14 days, while the fungus is exposed to the media with extracts. The complete methanolic fractions of the three studied plant species and the ethanolic fraction of *F. imbricada* did not show any fungicidal effect. The extracts and fractions were analyzed by high-performance liquid chromatography and the assayed compounds were: chlorogenic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillin, vanillic acid, rutin, caffeic acid, 3, 4-hydroxibenzoic acid (veratric acid), 3, 4-dimethoxycinnamic acid (caffeic acid dimethyl ester), and protocatechuic acid. Taking into account the antifungal activity of the ethanolic extracts of *E. breana* and *N. sedifolia* in *in vitro* assay, they may be an interesting alternative to control the phytopathogen *Botrytis cinerea*.
**Key words:** *Ephedra breana*, *Fabiana imbricata*, fungicidal, *Nolana sedifolia* phenolic compounds, plant extracts.

**Resumen:** Se estudiaron los posibles efectos antifúngico de los extractos metanólicos crudos y fracciones etanólicas de tres plantas nativas de Chile: *Ephedra breana*, *Fabiana imbricata* y *Nolana sedifolia* sobre el desarrollo micelial *in vitro* del hongo fitopatógeno *Botritis cinerea* (moho gris), a una concentración de 250 $\mu$g/mL de *E. breana* y *N. sedifolia*, y de 400 $\mu$g/mL para *F. imbricata*. Los resultados ponen en evidencia que las fracciones etanólicas de *E. breana* y *N. sedifolia* ejercieron un efecto inhibitorio del crecimiento micelial de *B. cinerea*, en condiciones *in vitro*. Este efecto demostró ser fungistático y se mantuvo durante los 14 días evaluados. Los extractos completos de ambas especies no mostraron efectos inhibitorios. Los extractos completos y las fracciones etanólicas fueron analizadas por cromatografía líquida de alta eficiencia y entre los compuestos encontrados fueron: ácido clorogénico, ácido cinámico, ácido *p*-cumárico, ácido ferúlico, vainillina, ácido vainíllico, rutina, ácido caféico, ácido 3,4-hidroxibenzoico (ácido verátrico), ácido caféico dimetil ester (ácido 3, 4 dimetoxicinámico) y ácido protocatéquico. Las fracciones etanólicas obtenidas pueden considerarse como una promisoria alternativa para el control de este fitopatógeno.
**Palabras claves:** compuestos fenólicos, *Ephedra breana*, extractos de plantas, *Fabiana imbricata*, fungistáticos, *Nolana sedifolia*.

Gray mold is an important disease that affects at least 230 plant species under field and postharvest conditions and it is caused by *Botrytis cinerea* Pers.Fr (González-Collado *et al*., 2006). In Chile, *B. cinerea* has usually been controlled by commercial fungicides. However, contamination problems due to the persistence of some of these chemicals both in soil and water results in important ecological problems. Therefore new control alternatives have been screened but little information is available in the literature on antifungal activity of natural products against *B. cinerea*. Some flavonoids, diterpenoids, sesquiterpenoids, monoterpenoids, stilbenes, steroidal glycoalkaloid, and triterpenoids have some effect against this fungus. Chemicals as captan, chlorotalonil, dichlofluanid, pirimetanil, or pirimidinamine have been traditionally used as chemical controls for *Botrytis* (Apablaza, 2000; Serey *et al*., 2007).The aim of the present study was to evaluate the fungitoxic effect of the extracts obtained from *Ephedra breana* Phil, *Fabiana imbricata* Ruiz et Pav., and *Nolana sedifolia* Poepp. against *B. cinerea*.

## Materials and methods

*Plant material*. Blooming stems of all three species were collected. *Ephedra breana* and *Nolana sedifolia* were collected in the Region of Tarapaca, near Alto Patache on October 19th of 2008 and *Fabiana imbricata* was collected in the Metropolitan Region, El Yeso, Cajón del Maipo, December 2008. Species were identified by Miguel Gómez and reference samples were housed in the herbarium of Facultad de Agronomía at the Pontificia Universidad Catolica de Chile. The collected material was dried in darkness at room temperature. Then it was powdered and stored in a black plastic bags.

*Plants extracts*. To prepare the complete extracts of each species, 500 g of the dried and powdered material was macerated in 3 l of ethanol (96%) at room temperature and darkness for one week. Then the macerate was filtered to separate the residue plant material from the liquid (crude extract). To facilitate this process a Kitasato flask connected to a pump pressure was used. Afterwards samples of 400 ml of the crude extract were taken and evaporated under low pressure and temperature (Cakir *et al*., 2005) with a rotary evaporator R210 (Buchi) at 35 °C, to evaporate all the ethanolic alcohol from the sample (leaving a sticky residue). Then 400 ml of methanol for analysis by JT Baker were added to the rotoevaporation ball with the sticky residue and it was gently shaken for 3 min. The resulting liquid was placed in a bottle, discarding the debris sticking to the sides of the rotoevaporation ball. The methanol extract was again rotoevaporatorated at 35 °C, this time to eliminate all the methanol of the sample. Finally, 4 ml of distilled water were added to the rotoevaporation ball at room temperature and gently shaken for 3 min. The liquid was filtered using a disposable syringe filter of hydrophilic cellulose mixed ester with a porosity of 0.45 microns (Advantec DISMIC-25) and stored at -2 ºC in darkness.

To elaborate the fractions samples of 400 ml of the crude extract were rotoevaporated, resuspended in 400 ml methanol for analysis by JT Baker and again rotoevaporated following the method described above. Then a series of solvents sorted from lowest to highest polarity were added to the rotoevaporation ball. The first solvent added was n-hexane and it was stirred for 3 min. The liquid obtained was placed in a flask, then followed the same procedure with ethyl acetate, dichloromethane, chloroform, butanol, ethanol, methanol, and distilled water using the same rotoevaporation ball. Each of the fractions recovered was rotoevaporatorated to dryness under low pressure conditions and temperatures: n-hexane, ethyl acetate, and dichloromethane at 35-40 °C, butanol at 65 °C, ethanol 45 °C, methanol 45 °C, and water at 70 ºC (the chloroform fraction was not rotoevaporated to protect the equipment). Then the dry material deposited in the rotoevaporation ball was resuspended in 4 ml of distilled water at room temperature and stirred for 3 min. The liquid was filtered with a disposable filter hydrophilic cellulose mixed ester with a porosity of 0.45 microns (Advantec DISMIC-25) syringe and stored at -2 ºC in darkness.

*Total phenols assay using the Folin-Ciocalteu method*. The amount of phenols contained in the complete extract and in each fraction was determined using the Folin-Ciocalteu method, with gallic acid as the standard, and measured in a spectrophotometer at 765 nm after reaction for 10 min (Adapted from Dastmalchi *et al*., 2007).

*Determination of compound concentration using HPLC*. To determine de total content of water-soluble polyphenols of the complete extract and the ethanolic fraction, samples were sent to the Laboratorio de Biologia Molecular of the Facultad de Ciencias Biologicas of the Pontificia Universidad Catolica de Chile for high-performance liquid chromatography analysis. Determination of the polyphenols was performed by HPLC with diode array detection (DAD), coupled to an electrochemical detector.

*In vitro antifungal assays*. Inhibition of mycelial growth using the Food Poison Technique in PDA (Groover and Moore, 1962; Shahi *et al*, 1999). Potato Dextrose Agar (PDA, Dehydrated Culture Media from BD Difco™) was autoclaved and then maintained in a water bath at 40 °C. The extracts or fractions were added to sterile molten PDA to obtain final concentrations of 250 $\mu$g/mL for *E. breana* and *N. sedifolia* and 400 ug/mL for *F. imbricata*. The media were poured into disposable Petri dishes of 60 × 15 mm. After the solidification of media, disks (6 mm in diameter) were located in the middle of the dishes. The discs contained a seven day old mycelium of the pathogen. There were three replicates per experiment. Plates were incubated for 14 days at 23 °C and its growth was measured every 24 h until the first Petri dish was filled with mycelia (Groover and Moore, 1962; Feng and Zheng, 2007).

*Transfer experiments*. To determinate whether the controlling effect of the extract was fungicidal or fungistatic, transfer experiments were done. Mycelium discs that did not grow were

**Table 1.** Phenolic concentration of the complete extracts and ethanolic fractions of *Ephedra breana, Fabiana imbricata,* and *Nolana sedifolia* estimated with the Folin-Ciocalteu method and measured by spectrophotometry at 765 nm (adapted from Dastmalchi *et al.,* 2007).

| | Phenolic concentration (gallic acid equivalent units /mL) | | |
|---|---|---|---|
| Sample | Ephedra breana | Fabiana imbricata | Nolana sedifolia |
| Complete methanolic extract | 2,347 | 2,334 | 3,730 |
| Ethanolic fraction | 2,607 | 11,042 | 2,864 |

**Table 2.** Compounds assayed in complete extract and ethanolic fraction of *Ephedra breana*, *Nolana sedifolia,* and *Fabiana imbricata* and their concentration expressed in mg/100 Ml. Retention time is indicated in parentheses.

| Compound /Concentration | *Ephedra breana* | | *Fabiana imbricata* | | *Nolana sedifolia* | |
|---|---|---|---|---|---|---|
| | Complete | Ethanolic | Complete | Ethanolic | Complete | Ethanolic |
| 3, 4 dihydroxybenzoic | 0.12 **(13.92)** | 0.08 **(13.92)** | | | | |
| *p*-hidroxybenzoic acid | 0.54 **(16.61)** | 0.24 **(16.70)** | | | | |
| Caffeic acid | 0.23 **(17.73)** | 0.34 **(17.71)** | 1.42 **(17.68)** | | | |
| Vanillin | 0.20 **(18.61)** | - | | | 0.32 **(18.56)** | |
| Ferulic acid | 0.13 **(20.13)** | - | 0.84 **(20.16)** | | 0.59 **(20.16)** | 0.09 **(20.16)** |
| 3, 4-dimethoxybenzoic acid | 2.25 **(24.69)** | 0.62 **(24.69)** | | | 2.00 **(24.67)** | |
| Chlorogenic acid | | | 41.00 **(16.56)** | 9.95 **(16.56)** | 0.77 **(16.56)** | |
| *p*-coumaric acid | | | | | 0.15 **(19.73)** | 0.04 **(19.73)** |
| Rutin | | | 111.00 **(21.33)** | 25.00 **(21.33)** | 1.70 **(21.33)** | |
| 3, 4-dimethoxycinnamic acid | | | | | 0.95 **(22.87)** | 0.13 **(22.77)** |
| non identified flavonoid | | | 5.69 **(22.63)** | | | |
| Vanillic acid | | | | 1.14 **(17.55)** | | |
| Post alkaline hydrolysis assayed compounds | | | | | | |
| Caffeic acid | 0.33 | 0.72 | 24.60 | 5.49 | 0.97 | - |
| *p*-coumaric acid | 0.14 | - | 0.83 | 0.22 | 0.40 | 0.08 |
| Ferulic acid | 0.46 | - | 18.00 | 3.70 | 1.05 | 0.18 |

transferred to fresh PDA after 14 days of treatment and were incubated at 23 ºC. The mycelium growth was measured every 24 h for five days (Feng and Zheng, 2007).

*Statistical analyses*. Data from in vitro antifungal assays (obtained from the fourth day after inoculation) were subjected to an ANOVA analysis, followed by a pairwise comparison of means (Tukey). Statistical analyses were conducted with SAS/STAT Software for Windows.

## Results

*Total phenols assay using the Folin-Ciocalteu*. For the quantitative analysis of complete extracts and different fractions the Folin Ciocalteu method was used with gallic acid as a standard. The phenolic content of the complete extract and of the ethanol fraction of each plant studied are shown in the table 1, as gallic acid equivalent units per ml of water.

*Quantitative determination of phenolic compounds by using HPLC*. The compounds found by the HPLC method and their concentrations are shown in the table 2. The compounds detected were mainly phenolic acids: chlorogenic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillin, vanillic acid, cafeic acid, 3, 4-hydroxybenzoic, procatechuic acid, and rutin.

*In vitro antifungal assays*. The inhibitory effects of the complete extract and ethanolic fraction of *Ephedra breana*, *Fabiana imbricata*, and *Nolana sedifolia* against *Botrytis cinerea* are shown in the table 3.

The statistical analysis was made with the data obtained at the fourth day after inoculation. Ethanolic fractions of *Ephedra breana* and *Nolana sedifolia* showed good inhibitory effects against *Botrytis cinerea* in *in vitro* assay at 250 $\mu$g/ml, but the ethanolic fraction of *Fabiana imbricata* showed no inhibitory effect. None of the complete extract showed inhibitory effects. Moreover, the complete extract of *N. sedifolia* enhanced the growth of *B. cinerea* showing statistical differences ($F = 1255.04$, d.f. = 7, 16, $P > 0.0001$; Table 3). As our knowledge, it is the first account for this effect in this solanaceous taxon.

*Transfer experiments*. Mycelium discs incubated in media

**Table 3.** Diameter for mycelial growth of *Botrytis cinerea* in PDA with all the treatments four days after inoculation. Lower-case letter indicates significant differences (Tukey, $P < 0.05$).

| Treatment | | *Ephedra breana* | | *Fabiana imbricata* | | *Nolana sedifolia* | |
|---|---|---|---|---|---|---|---|
| | Control (PDA with no extract) | Complete (250µg/mL) | Ethanolic (250µg/mL) | Complete (400µg/mL) | Ethanolic (400µg/mL) | Complete (250µg/mL) | Ethanolic (250µg/mL) |
| Diameter of mycelial growth (mm) | 44.2a | 45.5a | 0.0b | 45.5a | 45.7a | 46.0a | 0.0b |

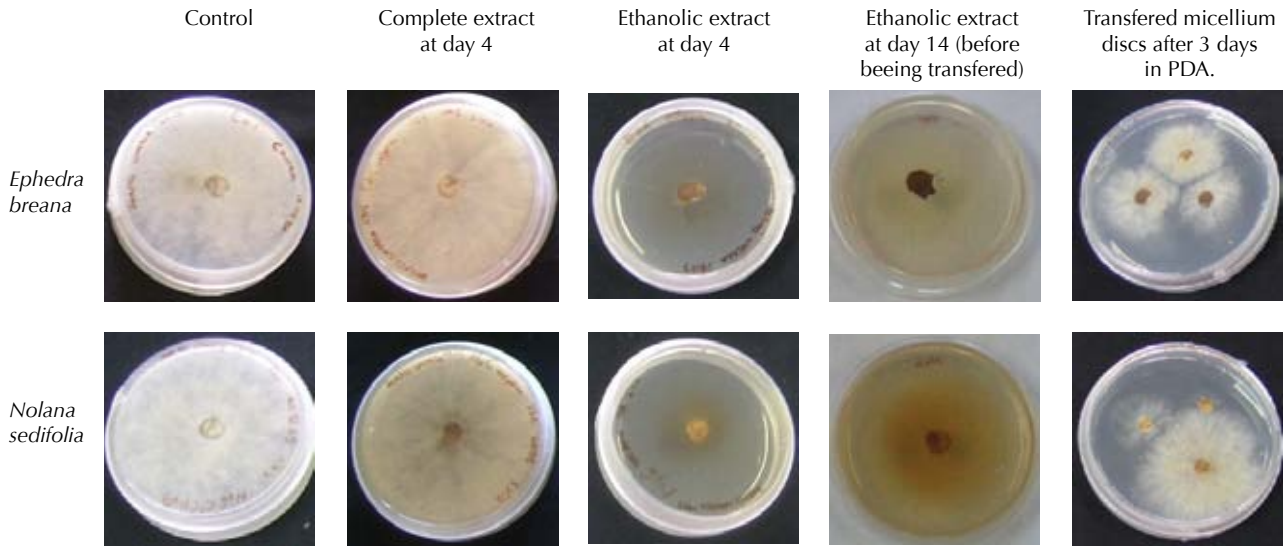


**Figure 1.** Mycelial growth of *Botrytis cinerea* in PDA (control sample) and PDA with complete extract or ethanolic extract of *Ephedra breana* and *Nolana sedifolia* at a concentration of 250 µg/mL four days after inoculation. The forth column shows the mycelium discs placed in PDA with ethanolic fraction of *E. breana* and *N. sedifolia* 14 days after inoculation before they were transferred into fresh made PDA. The fifth column shows the reactivation of growth of the mycelium discs after three days of been transferred into fresh PDA.

with ethanolic fraction of *Ephedra breana* and *Nolana sedifolia* did not show any growth 14 days after inoculation. These discs were transferred to fresh made PDA to determine if the controlling effect was fungicidal or fungistatic. In both cases the mycelial growth reactivated after been transferred, so the effect of these fractions at 250 $\mu$g/ml was fungistatic.

The ethanolic fractions of *Ephedra breana* and *Nolana sedifolia* showed an inhibitory effect on the mycelial growth of *Botrytis cinerea* at a concentration of 250 $\mu$g/ml in in vitro assays. This effect was fungistatic and remained for 14 days. The complete extracts of *E. breana*, *Fabiana imbricata*, and *N. sedifolia*, and the ethanolic fraction of *F. imbricata* did not show any inhibitory effect in the *in vitro* assays at the same concentrations.

## Discussion

All the compounds determined with the HPLC method are reported in literature as antibiotic and fungistatic (Clarke, 1972; San Francisco and Cooper-Driver, 1984; Downum, 1992; Zadernowski *et al*., 2005; Ruelas *et al*., 2006; Vermerris and Nicholson, 2006; Feng and Zheng, 2007). For example, a combination of the following acids p-coumaric, ferulic, caffeic, and *p*-hydroxybenzoic at a concentration of 500 ppm inhibits the mycelial growth of *Botrytis cinerea* model, where the two first compounds had the more powerful antifungal effect (San Francisco and Cooper-Driver, 1984).

According to this HPLC analysis most of the compounds in the analyzed extracts and fractions were water-soluble phenols. The following compounds were found in the different samples analyzed: chlorogenic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillin, vanillic acid, rutin, caffeic acid, 3, 4-hydroxybenzoic acid (veratric acid), 3, 4-dimethoxycinnamic acid (caffeic acid dimethyl ester), and protocatechuic acid. All these compounds have been described as metabolic antimicrobial compounds (Clarke, 1972; Downum, 1992; Zadernowski *et al*., 2005; Ruelas *et al*., 2006; Vermerris and Nicholson, 2006; Feng and Zheng, 2007).

Although the mode of action of these compounds it is not completely explained it has been suggested that they act over cytoplasmic membranes, producing changes in their permeability causing the liberation of cell content (Veldhuizen *et al*., 2006; Feng and Zheng, 2007). It is also discussed that these compounds interfere in the electron transport, the nutrient absorption, the fatty acid synthesis, the adenosine triphosphatase activity, and other metabolic processes of the cell, probably due to the interaction with the enzymes responsible of those processes (Feng and Zheng, 2007). When interfering with the molecular compounds present in various microorganisms, these compounds have a large range of action, but in general a low level of toxicity (Downum, 1992; VanEtten *et al*., 1994).

Some authors suggest to call "phytoanticipins" the compounds present in host plant before any infection (VanEtten *et al*., 1994). These compounds are also called phototoxins, because they activate and become toxic with the exposition to UV radiation (Downum, 1992). There is evidence that there are some synergic and antagonistic interactions between phenolic compounds. These interactions can modify their antimicrobial effect (Deba *et al*., 2008), which can ex-

plain why the complete extracts of *Ephedra breana* y *Nolana sedifolia* did not showed any control effect, even though the ethanolic fractions of those plants can inhibit the mycelial growth. It is also interesting the presence of 3, 4-dimethoxycinnamic acid in the two fractions that show control of mycelial growth, and of 3, 4-dimethoxybenzoic acid in the ethanolic fraction of *E. breana*. 3, 4-dimethoxybenzoic acid has been reported to be a biocontrolling compound (Narasimhan *et al*., 2009).

There is also evidence that some fungi are insensitive to these defense metabolites, because they own the capability to inhibit the metabolic routes or they deviate them to the production of different products with less inhibitory effect (Clarke, 1972). In the case of *Botrytis cinerea* it is demonstrated that it produces enzymes able to catalyze the oxidation of the metabolites of the host (Dekker, 1972, cited en Levin, 1976; Pearson and Goheen, 1988). This fact may explain the appearance of a dark halo of the media coloration change present in the medium with extracts or fractions before the mycelial growth starts (Figure 1). The antifungal activity of the ethanolic extracts of *Ephedra breana* and *Nolana sedifolia* in *in vitro* assay can be an interesting alternative of control for *B. cinerea*. However, more studies should be done to determinate the *in vivo* effects.

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