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ECo-friendly postharvest protection: *Larrea cuneifolia*-nades extract against *Botrytis cinerea*

Control sustentable poscosecha: extractos de *Larrea cuneifolia* mediados por nades frente a *Botrytis cinerea*

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Abstract

Botrytis cinerea is a ubiquitous fungus causing gray mold, the main postharvest disease in fruit, which implies important economic losses in agriculture. With growing concern over health and environmental effects of pesticides, the search for eco-friendly alternatives is a clear priority. Plant extracts represent a rich source of biocompounds with attractive antimicrobial properties. In the last decade, Natural Deep Eutectic Solvents (NADES) has emerged as an auspicious green extraction media to achieve bioextract for a sustainable postharvest control. In the present study, a novel *L. cuneifolia* NADES-based bioextract was evaluated against *B. cinerea*. To this purpose, a NADES composed by lactic acid, glucose and water (LGH) was used as extracting agent and compared with traditional solvents in terms of antioxidant capacity and total phenolic content. Furthermore, the bioextract antifungal activity was tested *in vitro* and also *in vivo* on artificially inoculated grapes, in order to obtain preliminary data about the efficacy on gray mold development. The antimicrobial activity of the bioextract was assessed using agar diffusion method against *B. cinerea*, inhibition of 92% was achieved with the bioextract at 2%. Notably, *L. cuneifolia* bioextract showed an excellent performance for gray mold control on grapes, supporting their potential as alternative green fungicide.

Keywords
natural deep eutectic solvents • biocompounds • antimicrobial activity • medicinal plant • postharvest control

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Resumen

Botrytis cinerea es un hongo ubicuo que ocasiona la podredumbre gris, una de las principales enfermedades de fruta en poscosecha, siendo responsable de pérdidas económicas. Debido a la creciente preocupación por los efectos adversos del uso de pesticidas, la búsqueda de alternativas se presenta como una meta prioritaria. En este contexto, los extractos de plantas representan una rica fuente de biocompuestos con atractivas propiedades antimicrobianas. Recientemente, los solventes eutécticos naturales (NADES) se han propuesto como agentes extractantes sustentables de compuestos bioactivos a partir de plantas. En el presente estudio, se evaluó un bioextracto de *L. cuneifolia* basado en NADES hacia *B. cinerea*. Para este propósito, se usó un NADES compuesto por ácido láctico, glucosa y agua (LGH) como agente de extracción y se comparó con solventes tradicionales en términos de capacidad antioxidante y contenido fenólico total. Además, la actividad antimicrobiana del bioextracto se evaluó *in vitro* e *in vivo* en uvas inoculadas artificialmente. A una concentración del 2% el bioextracto fue capaz de inhibir el crecimiento miceliar de *B. cinerea* en un 92%. Interesantemente, *L. cuneifolia* mostró un excelente rendimiento para el control de la podredumbre gris en uvas, demostrando su potencial como alternativa sustentable a los fungicidas sintéticos.

Palabras claves

solventes eutécticos naturales • biocompuestos • actividad antimicrobiana • plantas medicinales • control poscosecha

Introduction

Botrytis cinerea (Pers. ex. Fr) is a ubiquitous fungus with a wide host range including vegetables, ornamental plants and fruits. This pathogen causes gray mold, the main postharvest disease in fruit, leading to important economic losses in agriculture (1, 2, 24). The chemical control of *B. cinerea* has been encumbered by the emergence of resistant strains. Moreover, the synthetic pesticides present high toxicity and low biodegradability. One of the greatest challenges that agriculture faces is the need of safer approaches for sustainable crop protection. In this sense, plant extracts with fungistatic or fungicidal activities have shown potential as effective alternatives for the control of several postharvest crop diseases (14, 16, 20, 38).

Ethnobotanical studies support the use of several Argentinean autochthonous plants for antimicrobial purposes. Among these, the genus *Larrea* (Zygophyllaceae) is one of the most notable (3), being *L. ameghinoi, L. cuneifolia*, *L. divaricata,* and *L. nitida* the four species found in this country (32). *L. cuneifolia* extracts have been used as anti-inflammatory, antirheumatic, dysphoretic, amenagogic, antimicrobial and antioxidant agents (36). These properties have been attributed to the presence of bioactive compounds, being the phenolic compounds one of the most relevant group (21). Interestingly, certain classes of phenolics, such as hydroxybenzoic and hydroxycinnamic acid derivatives, flavonoids, and tannins have been explored for a long time as postharvest alternative control (22).

Extraction of plant phenolic compounds is traditionally performed with solvents such as methanol, ethanol, hexane, chloroform and diethyl ether and water (5, 11, 34). Even though these extracts are obtained from natural sources, their preparation using toxic organic solvents has many disadvantages for human health and for the environment. In this sense, the development of eco-friendly solvents is identified as a clear priority to achieve a sustainable extraction processes (28).

In the last decade, a new generation of solvents, called Natural Deep Eutectic Solvents (NADES), has been proposed as promising green extraction media (8, 17). NADES are mixtures consisting of natural metabolites that are naturally present in all types of cells and organisms such as sugars (glucose, sucrose, fructose, etc.); organic acids (lactic, malic, citric acids, etc.); urea and choline chloride (6, 9). NADES offer outstanding advantages including biodegradability, low toxicity, solute stabilization, sustainability and low cost (12, 27). It has to be pointed out that NADES are considered food grade solvents.

In the present study, a novel *L. cuneifolia* NADES-based bioextract was evaluated against *B. cinerea*. To this purpose, a NADES composed by lactic acid, glucose and water (LGH) was used as extracting agent and compared with traditional solvents in terms of antioxidant capacities and total phenolic contents. Furthermore, the bioextract antifungal activity was tested *in vitro* and also *in vivo* on artificially inoculated grapes, in order to obtain preliminary data about the efficacy on gray mold development.

Materials and methods

Chemicals and equipments

Compounds for LGH preparation including glucose anhydrous (≥ 99%), L (+) lactic acid (85-90%) were purchased from Biopack. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA) and Methanol (MeOH) was purchased from Baker (USA). 2,2'-azinobis(3-ethylbenzothiaziline-6- sulfonic acid (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent and gallic acid 99% (Gal) were obtained from Sigma Aldrich (St. Louis, MO,USA). Sodium carbonate anhydrous (Na^2CO^3) and Potato Dextrose Agar media (PDA) were obtained from Biopack. Sulfur dioxide generating pads as a commercial fungicide postharvest were purchased from PROPEL (Mendoza, Argentina).

Ultrasound Cleanson, Argentina, 200 W output power, 20 kHz frequency; Centrifuge Presvac DCS-16-RV and a Spectrophotometer Spectrum SP 2000 UV were used for extraction and determinations.

NADES preparation

LGH was prepared using a method previously described by Dai *et al.* (2013). The two-component mixture (lactic acid and dextrose; 5:1) with 15% of H_2O (v/v) was placed in a 20 mL amber glass vial. After, the mixture was heated in a magnetic stirrer with temperature control (Fisatom model 752A, Brasil) at 40°C for 60 min.

Plant material and extract preparation

L. cuneifolia plants were cultivated at a greenhouse and were identified by means of morphological, anatomical, and histochemical analyses. Leaves were harvested during flowering period and immediately frozen in liquid nitrogen, then lyophilized in darkness. Before the extraction, lyophilized material was grounded up to a fine powder with liquid nitrogen.

Extraction was performed according to Espino *et al.* (2018). Lyophilized plant material and extraction solvent (LGH, methanol or water) were placed in a 15 mL centrifuge tube (ratio plant- solvent of 75 mg mL^{-1}), homogenized by a vortex during 15 s and processed by ultrasound during 42 min at 40°C (±2°C). Then, the system was centrifuged for 30 min and the supernatant was filtered (0.45 µm). The extraction was performed in triplicate.

Total phenolic content

Total polyphenols were determined using Folin-Ciocalteu (FC) method described by Singleton and Rossi (1965) with modifications. For this determination, dilutions of the extracts were assessed at 5 % with LGH, MeOH or water. In a test tube, 50 µL of each extract dilution previously obtained, were mixed with the Folin-Ciocalteu reagent (200 µL) and, after 5 min, with an aqueous solution of Na_2CO_3 (1250 µL, 5 % w/v). Then, ultra-pure water was added to a final volume of 5000 µL. The mixture was incubated for 60 min in the dark, at room temperature, and the total phenol content was determined absortiometrically at 750 nm. Gallic acid calibration curve was prepared in the concentration range of 0-1000 µg mL^{-1} (R^2 = 0.9904) and results were expressed in µg of gallic acid per mL of extract. Each determination was performed in triplicate.

Antioxidant activity

DPPH (2,2´-diphenyl-1-picrylhydrazyl) assay*

The radical scavenging activity was measured in the extracts following the methodology described by Nuutila *et al.* (2003). The discoloration of the stable radical, 2,2'-diphenyl-1-picrylhydrazyl was tested. For this determination, dilutions of the extracts were

assessed at 2.5 % with LGH, MeOH or water. Then, 3.5 mL of DPPH* methanolic solution (0.045 mg mL^{-1}) was rapidly mixed with 250 µL of each extract dilution. After 5 min, the absorbance was measured at 515 nm (A_E). The decline in DPPH* concentration indicated the radical scavenging activity of the plant extracts. The initial absorbance of DPPH* solution was 1.375 (A_{DPPH}). The experiment was carried out in triplicate. Gallic acid solution (1000 µg mL^{-1}) was used as a reference (A_{REF}) and radical scavenging activity of the extracts were calculated as inhibition percentage (I %) as follows (Eq. 1) :

$$\%I_{DPPH*} = \frac{A_{DPPH*} - A_E}{A_{DPPH*} - A_{REF}} \quad (1)$$

ABTS (2,2´-azinobis(3-ethylbenzothiaziline-6- sulfonic acid) assay

Antioxidant activity was also measured following the method proposed by Re *et al.* (1999), using 2,2'-azinobis(3-ethylbenzothia-zoline-6-sulfonic acid) diammonium salt (ABTS). An ABTS ethanolic solution (2 mM) was added with potassium persulphate solution (2.45 mM) in order to produce radical cations (ABTS*). After 16 hours in dark, ABTS* solution was diluted with ethanol, to an absorbance of 0.70 (±0.02) at 734 nm (AABTS*). For this determination, dilutions of the extracts were assessed at 2.5% with LGH, MeOH or water. Each extract dilution (80 µL) was mixed with ABTS* ethanolic solution (3920 µL) and after 7 min the absorbance was measured (A_E). All the determinations were carried out three times and the absorbance sample was considered. A gallic acid solution (1000 µg mL^{-1}) was used as reference (A_{REF}) and results were calculated according to the following formula (Eq. 2):

$$\%I_{ABTS*} = \frac{A_{ABTS*} - A_E}{A_{ABTS*} - A_{REF}} \quad (2)$$

Antimicrobial activity

Microorganisms

The isolates of *B. cinerea* were obtained from the microorganism's collection of the Cátedra de Fitopatología (Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina).

Antimicrobial activity of L. cuneifolia bioextract by solid agar assay

L. cuneifolia bioextract was filtered (0.2 µm) and added to sterile Potato Dextrose Agar (PDA) at different concentrations (0.05, 0.1, 0.25, 0.5, 1, 1.5, 2 % (v/v)) in Petri dishes (5.2 cm in diameter). A pathogen agar disk (diameter 4 mm), removed from an actively growing culture, was placed in the centre of each plate. A solvent control for the seven extract dilutions was included to confirm that LGH did not present antifungal effect. Three replicate plates for each concentration as well as control were prepared. The Petri plates were kept at 25 ± 2 °C for 4 days. After the incubation period the test was considered concluded. In order to evaluate the mycelial growth inhibition; the mean colony area was determined. These mean growth values were calculated as the inhibition percentage of mycelial growth related to the control treatment according to the following equation (Eq. 3):

$$\% \text{ mycelial growth inhibition} = ((c-t)100)/c \quad (3)$$

where:

c = control mean colony area

t = is treated mean colony area

Antimicrobial activity of L. cuneifolia bioextract in commercial grapes

Experiments were conducted with commercial grapes *cv* Red globe following the procedure proposed by Boiteux *et al.* (2015).

Fruits free from injuries and infections were selected. Grape bunch with similar shape and size, containing each of them between 10-13 grapes were selected. *B. cinerea* was cultured on PDA petri plates for three weeks at 25°C. Then, the spore suspension was prepared in sterile water at a concentration of 1×10^6 conidia mL^{-1}. In order to study the protective and curative activity of bioextract at 2 and 10% (v/v), grapes skin were sprayed with 3 mL of the bioextract 1 day before or after the pathogen inoculation. Two controls were performed, one using sterile water and the other with a postharvest commercial fungicide (SO_2 generating pads). Grapes were put in closed plastic boxes to maintain a relative humidity of approximately 90 % and incubated for 7 days at 22°C. The experiment was performed in triplicate. The efficacy of the bioextract was calculated according to the following formula (Eq. 4):

$$\% \text{ effectiveness} = ((C-T)/C) * 100 \quad (4)$$

where:

C= (number grapes affected with gray mould/number total grapes inoculated for control with commercial fungicide)*100

T= (number grapes affected with grey mould/number total grapes inoculated for each treatment) *100

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), and means were compared using Tukey test. All the analyses were done in triplicate. The results were significant at $p < 0.05$ unless specified otherwise. Statistical analyses were carried out using Statgraphics Centurion XVI.II and GraphPad Prism 5.0 Software.

Results and discussion

Total phenolic content and antioxidant capacity of extracts

Folin-Ciocalteau assay is widely applied to estimate the total phenol content (TPC) in plant extracts. Thus, this technique was used to compare the TPC in the methanolic, aqueous and LGH extracts of *L. cuneifolia*. As can be seen in figure 1, water extract showed the lowest TPC for all the solvents under study. Interestingly, LGH bioextract presented a satisfactory performance when compared with methanolic extract.

Different letters represent significant differences.

Letras distintas indican diferencias significativas.

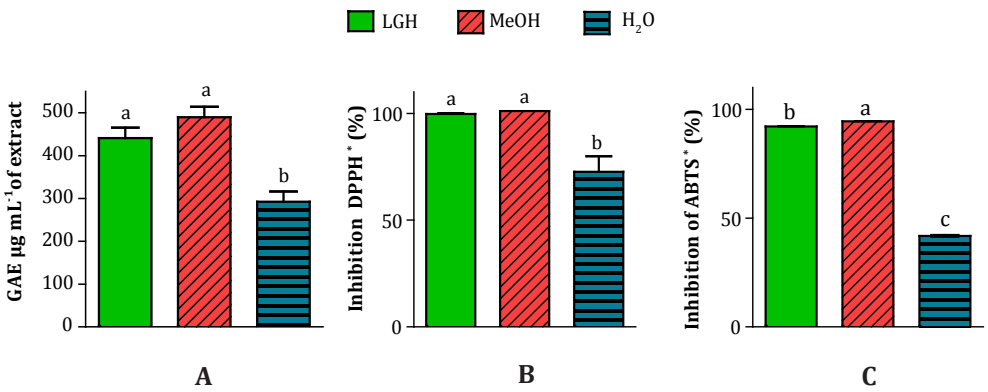


Figure 1. Total phenolic content (**A**) and antioxidant capacity determined by DDPH* (**B**) and ABTS* (**C**) methods of *L. cuneifolia* extracts obtained with different solvents (MeOH, H_2O and LGH).

Figura 1. Contenido de polifenoles totales (**A**) y capacidad antioxidante determinada mediante los métodos de DDPH* (**B**) y ABTS* (**C**) de los extractos de *L. cuneifolia* obtenidos con diferentes solventes (MeOH, H_2O and LGH).

Many studies have shown that plants rich in phenolic compounds also exhibit potent antioxidant activity. In general, the methods for determining the antioxidant capacity of plant extracts can deactivate radicals by two major mechanisms: assays based on the single electron transfer (SET) reaction and assays based on a hydrogen atom transfer (HAT). The DPPH test is SET-based method and ABTS used both HAT and SET mechanisms (18). Thus, in this work both assays were used to evaluate antioxidant activity of *L. cuneifolia* extracts (figure 1, page 431). The results demonstrated that for the two methods studied, LGH bioextract showed similar antioxidant activity than methanolic extract, whereas water extract presented the lowest activities.

According to our results, LGH reveals a great potential as green extraction media to obtain bioextracts rich in bioactive compounds in comparison with traditional solvents. Previous studies demonstrated that this green solvent has outstanding extractability for both polar and weak polar phenolic compounds compared to conventional solvents (15). We have developed in our lab a HPLC-DAD methodology for the determination of phenolic compounds in *Larrea* (13). The results of sample analysis validated the TPC values reported in the present study.

Recently NADES have been introduced as environmentally benign solvents for the bioextract preparation with antimicrobial properties (30). Therefore, LGH-bioextract was selected for evaluating the biological activity against *Botrytis cinerea.*

Antifungal activity of *L. cuneifolia* bioextract by solid agar bioassay

In order to evaluate the antifungal activity of *L. cuneifolia* bioextract, different concentrations (0.05, 0.1, 0.25, 0.5, 1, 1.5 and 2% (v/v)) on the mycelial growth of *B. cinerea* were tested (photo 1).

All the bioextract concentrations were able to inhibit the growth of *B. cinerea* in different percentages (figure 2, page 433). As can be seen, pathogen inhibition was observed even at low concentrations. The IC_{50} (concentration of the extract that inhibited 50 % the pathogen growth) was 0.67 % (0.5 g L^{-1}). At the maximum concentration tested (1.5 g L^{-1}), a 92 % of *B. cinerea* inhibition was achieved.

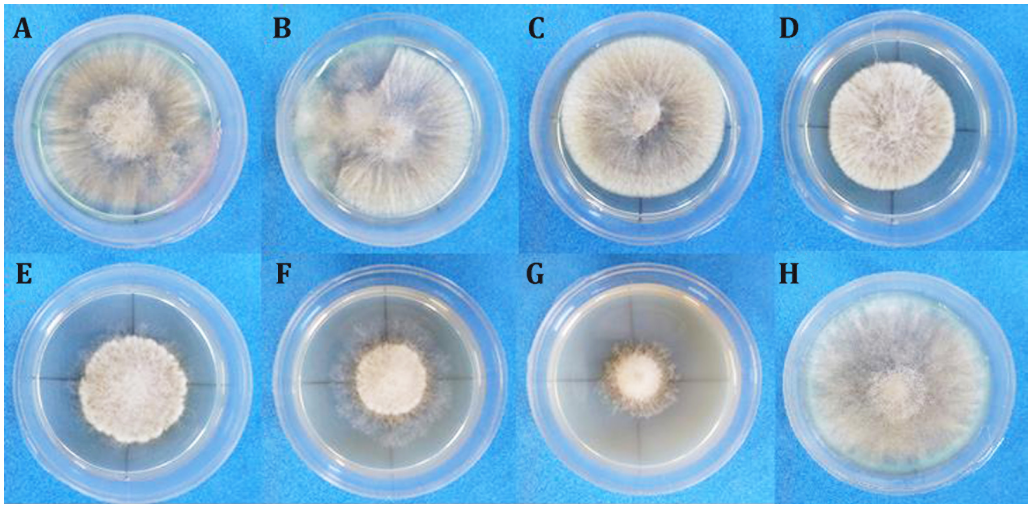


Photo 1. Mycelial growth of *B. cinerea* at different concentration of *L. cuneifolia* bioextract **A:** 0.05%; **B:** 0.1%; **C:** 0.25%; **D:** 0.5%; **E:** 1 %; **F:** 1.5%; **G:** 2% and **H:** control.

Foto 1. Crecimiento miceliar de *B. cinerea* a diferentes concentraciones del bioextracto de *L. cuneifolia* **A:** 0,05%; **B:** 0,1%; **C:** 0,25%; **D:** 0,5%; **E:** 1%; **F:** 1,5%; **G:** 2% y **H:** control.

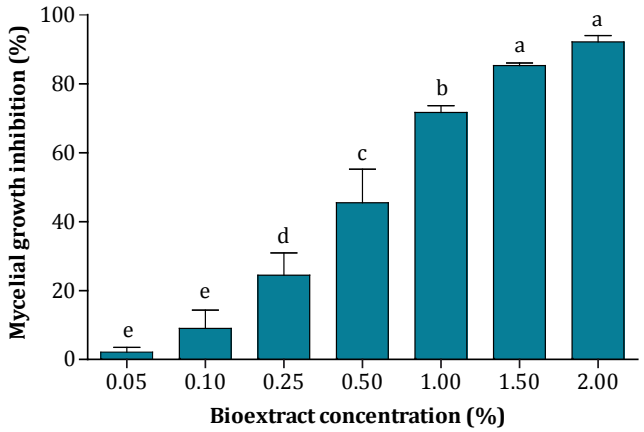


Figure 2. Percentage of *B. cinerea* mycelial growth inhibition at different concentrations of *L. cuneifolia* bioextract.

Figura 2. Porcentaje de inhibición del crecimiento miceliar de *B. cinerea* a diferentes concentraciones del bioextracto de *L. cuneifolia.*

Our results highlight that NADES-*Larrea cuneifolia* extracts shows outstanding activity against *B. cinerea*. Vast scientific knowledge supports the applications of the genus *Larrea* in antimicrobial assays. Alcoholic extracts of *L. divaricata* and *L. cuneifolia* showed considerably activity against filamentous fungi (*Lenzites elegans*, *Schizophyllum commune*, *Pycnoporus sanguineus*, *Ganoderma applanatum*, *Fusarium oxysporum*, *Penicillium notatum, Aspergillus niger* and *Trichoderma spp*) (29). Zampini *et al*. (2007) demonstrated the activity of *Larrea* ethanolic extracts against antibiotic-resistant bacteria.

Antimicrobial activity of our bioextract against *B. cinerea* mycelial growth was compared with plant extracts previously reported (table 1). NADES extract exhibits a much better pathogen inhibition efficiency than organic solvents extracts (7, 35). With regard to aqueous extracts, higher concentrations were required to achieve a similar *B. cinerea* inhibition to that obtained with the LGH extract (23). This could be explained by the great capacity of NADES to solubilize and stabilize bioactive compounds (10, 25, 37).

Table 1. Antimicrobial activity of plant extracts with different solvents against *B. cinerea*.

Tabla 1. Actividad antimicrobiana de extractos de plantas obtenidos con diferentes solventes hacia *B. cinerea*.

Plant material	Solvent	Concentration	Mycelial growth inhibition	References
Larrea cuneifolia	LGH	1.5 g L^{-1} (2 % v/v)	92%	**Present study**
Fluorensia cernua	water	4 g L^{-1}	66%	(11)
Zanthoxylum rhoifolium	chloroform/ methanol	1 g L^{-1}	70%	(5)
Lippia origanoides	ethanol	0.5 g L^{-1}	44%	(34)
Thymus vulgaris	ethanol	0.5 g L^{-1}	37%	(34)
Calendula officinalis	ethanol cold water hot water	2.26 % (v/v) 3.23 % (v/v) 10 % (v/v)	≈40% 100% ≈75%	(23)
Dolichos kilimandscharicus	methanol	1 g L^{-1}	60-80%	(35)
Phytolacca dodecandra	methanol	1 g L^{-1}	40-50%	(35)
Maerua subcordata	methanol	1 g L^{-1}	30-40%	(35)
Ottonia martiana	ethanol	1 g L^{-1}	69%	(7)

NADES are recently introduced as environmentally benign solvents for the bioextract preparation with antimicrobial properties. Rajan *et al.* (2015) studied the antibacterial activity of the extract of ginger rhizome (*Zingiber officinale* Roscoe), prepared with different Natural Deep Eutectic Solvents. NADES extracts exhibited prominent antimicrobial activity against *Staphylococcus aureus*, *Streptococcus viridans, Salmonella typhi, Bacillus cereus, Klebseilla pneumonia, Vibreo cholera* and *Escherichia coli* using paper disc diffusion methods.

In order to assess the efficacy of *L. cuneifolia* bioextract on gray mold development, its antimicrobial activity was evaluated in commercial grapes.

Antimicrobial activity of *L. cuneifolia* bioextract in commercial grapes

The protective and curative activity of different concentrations of *L. cuneifolia* bioextract against *B. cinerea* were tested in grapes *cv* Red Globe (photo 2). Regarding that the extract at 2 % achieved the highest inhibition of the pathogen mycelial growth for in vitro assays, this concentration and 10% were chosen for in vivo tests.

Analyzing the obtained results (figure 3, page 435), a similar trend was observed on curative and protective application. Even though data showed no significant differences between the *L. cuneifolia* extracts at 10% and 2%. When comparing the two treatments, the protective assay presented the greatest effect against *B. cinerea*, showing an efficacy between 70 and 80 % in relation with chemical control. It has to be pointed out that the 2% (1.5 g L^{-1}) bioextract not only showed a high *B. cinerea* inhibition *in vitro*, but also an important effect for disease control in grapes.

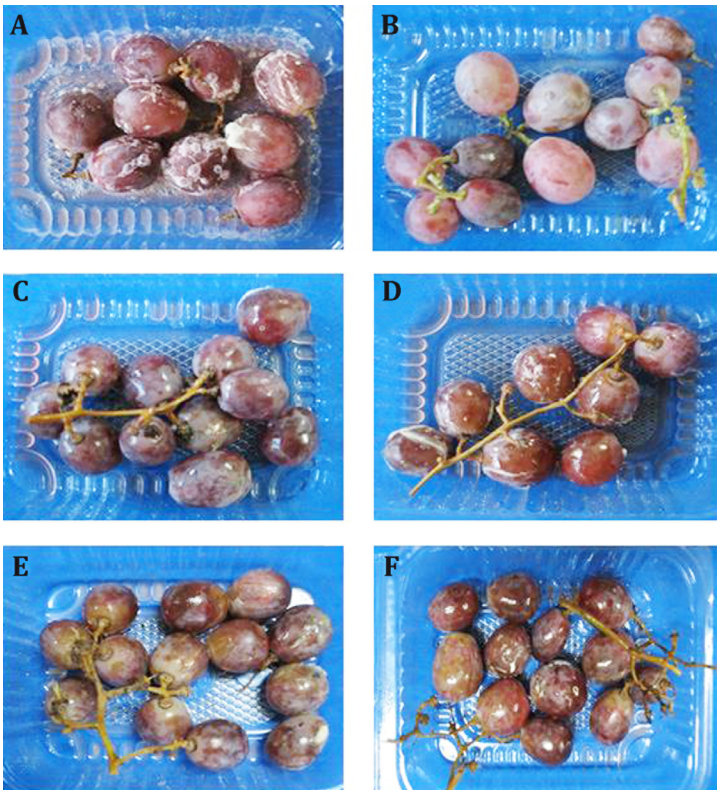
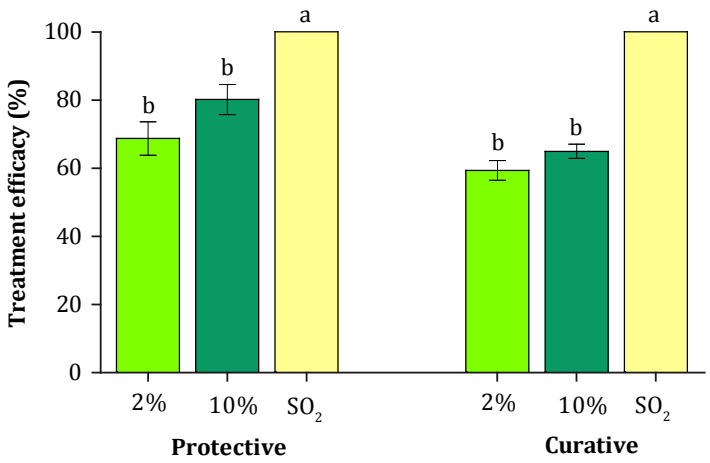


Photo 2. *In vivo* antimicrobial assay in *B. cinerea*-inoculated grapes: (**A**) sterile water, (**B**) postharvest commercial fungicide (SO_2 generator), (**C**) curative 2%, (**D**) curative 10%, (**E**) protective 2%, (**F**) protective 10%.

Foto 2. Racimos *cv* Red Globe inoculadas artificialmente con *B. cinerea* sometidos a diferentes tratamientos: (**A**) agua estéril, (**B**) fungicida comercial (generador de SO_2), (**C**) tratamiento curativo 2%, (**D**) tratamiento curativo 10%, (**E**) tratamiento preventivo 2%, (**F**) tratamiento preventivo 10%.



Different letters indicate significant differences.

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Figure 3. *In vivo* antimicrobial efficacy (%) of *L. cuneifolia* bioextract (2 and 10%) in protective and curative test over *B. cinerea*-inoculated grapes after 7 days at room conditions.

Figura3. Porcentaje de eficiencia del bioextracto de *L. cuneifolia* (2 and 10%) para el control de *B. cinerea* en racimos de uva inoculados artificialmente luego de 7 días de incubación.

Previous reports carried out on table grapes had demonstrated that plant extracts have remarkable potential as biopesticides; Kanetis *et al.* (2017) demonstrated that the acetonic extract of *Salvia fruticosa* was effective for the control of *B. cinerea* on this fruit. Also, the extracts of *Borago officinalis, Orobanche crenata, Plantago coronopus, P. lanceolata, Sanguisorba minor, Silene vulgaris, Sonchus asper, Sonchus oleraceus,* and *Taraxacum officinale* induced a significant reduction of grey mould disease (22).

Conclusions

This work highlights the ability of NADES as solubilisation vehicles for plant derived postharvest protection agents. LGH reveals a great potential as green extraction media to obtain bioextracts rich in total phenolic content and similar antioxidant activity when compared with traditional solvents. The bioextract obtained presented an effective antimicrobial activity against *Botrytis cinerea*. Notably, *L. cuneifolia* bioextract on grapes showed an excellent performance for gray mold control in protective assay, supporting their potential as alternative green fungicide. Further researches are needed for the applicability of this bioextract in commercial processes.

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