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Larrea divaricata volatilome and antimicrobial activity against *Monilinia fructicola*

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ABSTRACT

Monilinia fructicola is the most destructive pre- and postharvest pathogen in stone fruit worldwide. A great attention has been paid to plant extracts, a rich source of bioactive chemicals, for the sustainable pest control. Volatile organic compounds from plant matrices have proved important antimicrobial activity. In the present work, an analytical procedure for the characterization of the volatile profile in *L. divaricata* extract by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography/mass spectrometry (GC–MS) was proposed. Moreover, the biological activity of *L. divaricata* extract was evaluated for their contact-phase and vapor-phase effects on *M. fructicola* mycelial growth. In order to improve the absorption of volatile compounds, the following experimental parameters were optimized: fiber coatings, time and temperature extraction. As a result of the optimization, CAR/PDMS fibers, 30 °C and 30 min were selected for the extraction volatile compounds from *L. divaricata* extract. A total of 79 VOCs were identified in the extract. Among them, aromadendrene was found at highest concentration. Other bioactive volatile compounds were detected such as eugenol, carvacrol, limonene and thymoquinone. The antimicrobial activity of *L. divaricata* extract on *M. fructicola* mycelial growth was evaluated under contact- and vapor-phase conditions. *L. divaricata* extract showed significant antimicrobial activity on *M. fructicola* mycelial growth in both conditions. The results reveal that *L. divaricata* is a promising antifungal agent which could be used as bio-fungicide in the protection of fruits and vegetables against postharvest infections.

1. Introduction

Monilinia fructicola (Winter) is the most destructive pre- and postharvest pathogen in stone fruit worldwide [1, 2]. This pathogen causes brown rot leading to important economic losses in food industry [3]. Current management of this disease still relies heavily on the use of synthetic chemical fungicides [4]. However, public concern over the potential impact of fungicides on the environment and human health, as well as the development of resistance strains, have promoted the search of novel methods for the control of *M. fructicola* [5]. Among the alternative treatments to chemical products, plant extracts have received growing attention due to their potential antimicrobial properties [6]. Several studies have demonstrated that plant extracts constitute a rich source of bioactive chemicals such as phytosterines, saponines, phenolic compounds, alkaloids, volatile compounds, glucosinolates, quinons, tannins, sterols [7, 8].

Larrea divaricata Cav. (Zygophyllacea) is a perennial woody shrub with widely distribution in Argentina. In folk medicine, different *L. divaricata* extracts have been used as anti-inflammatory, antirheumatic, dysphoretic, amenagogic and antimicrobial agents [9, 10]. Although phytochemical studies had reported the presence of secondary metabolites such as lignans, essential oils, phenolic compounds and glycosides [11, 12], the volatile profile of *L. divaricata* extract has not yet been reported.

The totality of volatile organic compounds (VOCs) synthesized by plants has been termed the volatilome (or volatome) [13], being terpenes, fatty acid derivatives, benzenoids, phenylpropanoids and amino acid derived metabolites, the most relevant groups. These compounds play an important role in plants; acting as signals in plant-plant communication, in the attraction of pollinators as well as in the defense against organisms pathogens [14, 15]. Thus, the volatile organic compounds in plant extracts could play an effective role in the control of postharvest diseases.

Analytical procedures for the analysis of volatile constituents from

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plants require sample preparation methods for the extraction and preconcentration of target compounds [16, 17]. Headspace-solid-phase microextraction (HS-SPME) is one of the most commonly used methods combining sampling, extraction, concentration and cleanup in a single step [15, 18]. This technique presents remarkable advantages for volatilome analysis, including high selectivity and sensitivity, possible automation of extraction, solvent-free process and small sample volume [14]. Extraction efficiency is affected by experimental factors [19, 20]. The fiber coating is a key factor in SPME, different coatings interact with analytes through different adsorption/absorption mechanisms [16, 21, 22].

In the present work, an analytical procedure for the characterization of the volatile profile in *L. divaricata* extract by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography/mass spectrometry (GC–MS) was proposed. Moreover, the biological activity of *L. divaricata* extract was evaluated for their contact-phase and vapor-phase effects on *M. fructicola* mycelial growth.

2. Materials and methods

2.1. Plant extract preparation

Larrea divaricata (10 plants) was cultivated at a greenhouse under natural radiation were identified by means of morphological, anatomical, and histochemical analyses. Leaves were harvested during flowering, immediately frozen in liquid nitrogen and then lyophilized in darkness. The extract was prepared from *L. divaricata* leaves (20 g) using the methodology proposed by Widmer and Laurent with modifications [23]. Then, the leaves were placed into Erlenmeyer flasks containing 200 mL of distilled water and autoclaved for 45 min at 121 °C at 1 atm. The liquid was filtered and the volume was reduced by boiling in laminar-flow hood to an approximate volume of 20 mL. Finally, the extract was centrifuged and the supernatant was autoclaved for 20 min at 121 °C at 1 atm. The extracts were stored at 4 °C until analysis.

2.2. HS-SPME/GC–MS

2.2.1. Reagents and materials

The SPME tested fibers were Stable Flex™ polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), polydimethylsiloxane (PDMS, 100 μm), Stable Flex™ carboxen/polydimethylsiloxane (Car/PDMS, 85 μm), and Stable Flex™ divinylbenzene/carboxen/polydimethylsiloxane (Car/PDMS/DVB, 50/30 μm); SPME support and holder manual for SPME were purchased from Supelco (Bellefonte, PA, USA). Magnetic stirrer bar and 10 mL glass vial with PTFE-faced silicone septa were supplied by Varian (Lake Forest, CA, USA). Magnetic stirrer Ret Control Visc IKAMAG Safety Control (IKA, Wilmington, USA) was used. Fibers were conditioned following the instructions from manufacturers, and cleaned at 250 °C for 5 min. Ultrapure water was obtained from a RiO/Elix3-Sinergy185 purification system (Millipore, Sao Pablo, Brazil). The internal standard (IS) anisole was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Methanol chromatographic grade was purchased from Merck (Darmstadt, Germany).

2.2.2. HS-SPME conditions

In order to improve the absorption of volatile compounds, the following experimental parameters were optimized: four coating fibers (PDMS, CAR/PDMS, DVB/CAR/PDMS and PDMS/DVB); extraction temperature (25–40 °C); extraction time (10–60 min). As a result of the optimization, *L. divaricata* extract (2 mL) was spiked with an internal standard solution to obtain a final concentration of 1 μg mL^{-1}. Then, the extract was placed into a 10 mL glass screw-top vial with polytetrafluoroethylene/silicone septa. The sealed vial was placed in a thermoblock at 30 °C and stirrer at 1000 rpm. After thermal equilibration for 30 min, the SPME needle (CAR/PDMS fiber) was inserted through the septum and the fiber exposed in the HS (2 cm) for 30 min. After extraction, the SPME fiber was removed from the vial and inserted into the injection port of GC–MS. All the analyses were performed in triplicate.

2.2.3. GC–MS analyses

GC–MS analyses were performed on a Varian CP-3800 gas chromatograph with a Saturn 2200 Ion Trap Mass Spectrometric detector (Varian, Walnut Creek, CA, USA). The system was operated by Saturn GC–MS Workstation software Version 6.41. The column was a Factor Four TM capillary column VF-5MS (50 m × 0.25 mm I.D., with 0.25 μm film thickness; Varian, Lake Forest, CA, USA).

The oven temperature program was: 40 °C (2 min), 4 °C min^{-1} to 220 °C (5 min), 30 °C min^{-1} to 280 °C (10 min). The 1079 injector was equipped with a glass insert for SPME and the temperature was programmed isothermally. Desorption temperature was set at 220 °C based on previous studies carried out in our laboratory [24]. The injection was in split/splitless mode for 2 min, and then the split ratio was 1/50. The carrier gas flow rate (Helium 6.0, Linde, Buenos Aires, Argentina) was constant at 1 mL min^{-1}. The electron impact energy was 70 eV. Transfer line and ion trap temperature were 200 °C and manifold temperature was 40 °C. Mass spectrometry acquisition was carried out using the continuous scanning mode (5 μscan s^{-1}) from *m/z* 30 to *m/z* 300. Qualitative analysis was performed by comparison of mass spectra and retention times with standard compounds and/or by comparison of the mass spectrum with those of reference compounds in the NIST Mass Spectral Search Program (NIST Version 2.0). The relative amounts of volatile compounds were expressed with respect to IS (R2 = 0,998) as μg g^{-1} of plant extract. The volatile compounds were expressed as μg mL^{-1}. All data were reported as the mean ± SD for three replications and analyzed by Statgraphics Plus Version 5.0 program (Manugistic Inc., Rockville, MD, USA) [32].

2.3. Antimicrobial activity of Larrea divaricata extract on Monilinia fructicola mycelial growth

Monilinia fructicola was obtained from the microorganism's collection of the Cátedra de Fitopatología de la Facultad de Ciencias Agrarias de la Universidad Nacional de Cuyo (Mendoza, Argentina). The fungus was maintained on Potato Dextrose Agar (PDA). The effect of *Larrea divaricata* extract on *M. fructicola* mycelial growth was evaluated under contact- and vapor-phase conditions. The vapor phase was tested by using the technique of Ma et al. [25], in Petri plates (90 mm × 20 mm; 130 mL air space) containing Potato Dextrose Agar (PDA) [25]. Subsequently a 4 mm disk of *M. fructicola* obtained from the edge of 10 days old culture was placed in the center of Petri dishes. A filter paper disk (4 cm in diameter), impregnated with the extract at different concentrations (50 to 750 mg mL^{-1}), was placed on the inner surface of the Petri plate lid, in order to prevent direct contact of *M. fructicola* with the extract. Control was performed by replacing extract with sterile distilled water. Inoculated Petri plates were sealed immediately and were incubated at 21 ± 2 °C for 5 days. Each treatment contained three replicates and the assay was repeated three times. After incubation period, the *M. fructicola* colony area was measured using Axio Vision 4.8 software. The results were expressed as inhibition percentage of mycelial growth using the formula (1).

$$\text{Inhibition } (\%) = [(C - T)/C] * 100, \quad (1)$$

where C and T correspond to the fungus colony area (cm^2) of control and treatments (cm^2), respectively.

In the contact phase assay, the biological activity of *L. divaricata* extract against *M. fructicola* was tested using the solid agar bioassay [26]. In this case, a 4 mm disk of *M. fructicola* was placed in the center of Petri dishes containing Potato Dextrose Agar (PDA) amended with different concentrations of extract (50 to 750 mg mL^{-1}). Control plates were performed simultaneously, using the growth medium without