Postharvest Biology and Technology 121 (2016) 1–8

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Effectiveness of essential oils for postharvest control of *Phyllosticta citricarpa* (citrus black spot) on citrus fruit

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form 22 June 2016
Accepted 2 July 2016
Available online 17 July 2016

Keywords:
Guignardia citricarpa
Quarantine disease
Orange
Lemon
Botanical fungicides

ABSTRACT

The *in vitro* and *in vivo* control of *Phyllosticta citricarpa* (citrus black spot, CBS) on citrus fruits was evaluated using 14 essential oils (EOs) extracted from Uruguayan native plants. *In vitro,* volatile components of *Chenopodium ambrosioides* completely inhibited fungal growth. The dilution agar test showed that fungus reproduction was completely inhibited by *Conyza bonaerensis* essential oil (EO). The other 12 EOs tested showed high inhibition after 10–15 d, presenting fungistatic effects. *In vivo,* assays were performed on fruit of lemon [*Citrus limon* (L.) Burm] and Valencia orange [*Citrus sinensis* (L.) Osbeck] in order to evaluate the effects of liquid and volatile EO phases on reproduction. Typical lesions of CBS without reproductive structures were exposed to *Ch. ambrosioides* and *C. bonaerensis* EOs for 20 d at 27 °C and cycles of 16 h light and 8 h dark. The volatile components of *Ch. ambrosioides* were able to control 100% of *P. citricarpa* reproduction in orange and lemon fruit. The growth of other fungi such, as *Colletotrichum gloeosporoides* and *Fusarium* sp., was also inhibited. Nevertheless, the liquid phase was not effective to control the pathogen. In another test, carton boxes with 20 fruits, each presenting typical CBS symptoms, but without the presence of reproductive structures, were exposed to 0.01 and 0.05% of *Ch. ambrosioides* EO (w/v). The fruits were stored at 5 °C for 20 d and then kept for 7 d at room temperature. A 40% reduction of reproduction was observed in citrus fruit exposed to 0.05% of EO. GC–MS analysis of EOs showed that *Ch. ambrosioides* EO was composed of 76% monoterpene compounds (49 and 27% of oxygenated and hydrocarbons, respectively), while *C. bonaerensis* EO presented 17% monoterpenes and 10% sequisterpenes.

1. Introduction

Citrus black spot (CBS) is considered a quarantine disease by the European and Mediterranean Plant Protection Organization (EPPO, 2009). The causal agent is *Phyllosticta citricarpa* (McAlpine) Van der Aa (synonym *Guignardia citricarpa* Kiely). Fruits are susceptible to CBS for 24 weeks after petalfall (Kotzé, 1981). However, *P. citricarpa* presents a long period of latency, and the disease symptoms develop on fruit which are close to or at the maturity stage (Kotzé, 2000). Under certain humidity and temperature conditions, the presence of pycnidia from *P. citricarpa* on fruit can induce spores release in a mucilaginous mass (Kotzé, 1981). The picnidiospores are then washed down by rainfall and infected fruit is considered as a low risk to long distance disease dispersal (Whiteside, 1967). Nevertheless, the European Union's regulations on plant protection refuse the admission of black spot affected citrus fruit, alleging that the diseased fruit is a risk to pathogen introduction into the European Union (EU) (Council directive 2000/29/EU). Therefore, detection of only one infected fruit in a shipment causes rejection of all containers, which results in significant economic losses for the exporting countries. CBS is present in most citrus fruit exporting Southern hemisphere countries (Sutton and Waterston, 1966). The control of CBS is based on a series of strategies such as the use of fungicides (Fialho et al., 2010; Kotzé, 2000). However, the most effective fungicides for CBS control have limitations, such as restricted maximum residue limits and development of fungicide resistance (Possiede et al., 2009). Consumers are increasingly demanding products free of chemicals because of their potential

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http://dx.doi.org/10.1016/j.postharvbio.2016.07.002
0925-5214/

effect on health and the environment (Morais, 2009a). Consequently, alternative disease control methods are starting to be considered (Fialho et al., 2010).

Plants synthesise secondary metabolites that they use for self-protection. These substances present natural selectiveness, low toxicity and are biodegradable (Antunes and Cavaco, 2010). EOs are bioactive secondary metabolites, complex mixtures of volatile compounds produced in specialised plant organs (Tabassum and Vidyasagar, 2013; Morais, 2009a). Certain EOs present antimicrobial, anti-inflammatory, anti-carcinogenic, antioxidant activities or stimulate the immune system (Bakkali et al., 2008). EOs are generally recognised as safe for the environment and human health (Hyldgaard et al., 2012; Adorjan and Buchbauer, 2010; Barbosa et al., 2008; Edris, 2007) and may play a pivotal role in postharvest CBS control since they are able to inhibit spore germination (Caccioni and Guizzardi, 1994). Uruguay's native flora has numerous species that contain EOs which already have a history of medicinal use (Alonso et al., 2008; Davies, 2004; Arrillaga de Maffei, 1969). The antifungal activity of EOs has started to be evaluated in the region (Jaramillo et al., 2012; Manzano Santana et al., 2011; Nascimento et al., 2011; dos Santos et al., 2010; Brun and Mossi, 2010; Stashenko, 2010; Viturro et al., 2010; Marin et al., 2008; Castañeda et al., 2007), but the EOs composition and quality may vary since it is associated with weather, soil, season, harvest age and phenological state, EO obtainment method and other factors (Cicció and Ocampo, 2010; Viturro et al., 2010; Morais, 2009b).

The objective of the present study was to evaluate *in vitro* and *in vivo* antifungal activity of EOs of Uruguayan native plants against *P. citricarpa* and to identify their main chemical components.

2. Material and methods

2.1. Plant material

Aerial parts of the aromatic plants were collected in rural zones of Salto Department (31°22′S57°56′O) in Uruguay (Table 1). Species identification was performed by the Departmento de Botánica, Universidad de la República (Uruguay), and a sample of each species was deposited in the herbarium of the Botanical Garden museum "Prof. Atilio Lombardo", Montevideo, Uruguay.

2.2. Essential oil extraction

The essential oils were obtained by hydrodistillation using a clevenger type apparatus (Minteguiaga et al., 2015). Dry plant material was grinded and weighted before distillation, and the extraction times were measured for every plant species. The EOs were stored in glass bottles at 4 °C until used in the bioassays or analysed by GC–MS.

2.3. Plant pathogen

Phyllosticta citricarpa was isolated from Valencia [*Citrus sinensis* (L.) Osbeck] orange fruit showing typical symptoms of the disease (Kotzé, 2000). Its identity was confirmed through morphological, cultural and biological characterisation of the pathogen in Oatmeal Agar (OA) and Potato Dextrose Agar (PDA) culture media (Difco®, France) following the protocols previously reported (Hidalgo Góngora and Pérez Vicente, 2010; EPPO, 2009; Baayen et al., 2002). The fungus was kept in PDA culture medium at 4 °C. Subcultures were prepared 15 d before each assay.

2.4. GC–MS analysis

GC–MS analyses were carried out using a QP 5050 (Shimadzu, Kyoto, Japan) apparatus under the experimental conditions previously reported by Minteguiaga et al. (2015).

2.5. In vitro bioassays

2.5.1. Volatile phase test

The bioactivity of the oil volatile compounds was evaluated using the inverted petri dish technique (Bocher, 1938). Petri dishes (9 cm diameter) containing 15 mL of sterile PDA medium were inoculated with a 6 mm diameter disc of 15 d old *P. Citricarpa* mycelium. The EOs, 1 mg L^{-1} (0.1% w/v), were applied on a glass slide on the inside of the lid, with the dish inverted. As control, the same procedure was carried out, but without the addition of EO. The Petri dishes were immediately sealed with Parafilm® and incubated upside down at 25 °C in darkness. The assay was performed in triplicate and repeated twice. The colony growth diameters were measured every 5 d for 40 d.

2.5.2. Agar dilution test

The agar dilution test (Rahman et al., 2011; adapted from Grover and Moore, 1962) was used to evaluate the EOs contact inhibitory effect on *P. citricarpa* growth. The EOs were homogenised in dimethyl sulfoxide (DMSO) (9:1 v/v) for 10 s at 1300 rpm using an Ultra-Turrax T25 macerator (Janke & Kunkel, Staufen, Germany) and incorporated to PDA at 1 g L^{-1}. The same procedure was carried out for the control treatment, without EOs. Each plate was inoculated with a 6 mm diameter disc of 15 d old fungus mycelium. The petri dishes were immediately sealed with Parafilm® and incubated at 25 °C in darkness. The diameters of the colonies were measured every 5 d until no more growth was observed in the

Table 1
Selected native species for essential oil extraction, sample location and phenological stage at the moment of harvest.

Plant species	Common name	Sample location	Phenological stage
Schinus molle L.	"Anacahuita" American pepper	(31°16′ S 57°54′ W)	fruiting
Achyrocline flaccida (Weinm) DC	"Marcela"	(31°22′ S 57°44′ W)	flowering
Baccharis dracunculifolia DC	"Chilca blanca"	(31°25′S 57°59′ W)	flowering
Baccharis trimera (Less.) DC	"Carqueja"	(31°23′ S 57°43′ W)	flowering
Conyza bonariensis (L.) Cronquist	"Yerba carnicera" Flax-leaf fleabane	(31°23′ S 57°43′ W)	flowering
Pluchea sagittalis (Lam.) Cabrera	"Yerba lucera" Wingstem camphorweed	(31°19′ S 57°58′ W)	flowering
Chenopodium ambrosioides L.	"Paico macho" Jesuit's tea, wormseed	(31°23′ S 57°48′ W)	flowering
Ocimum selloi Benth.	"Albahaca de campo" Pepper basil	(31°23′ S 57°42′ W)	flowering
Blepharocalyx salicifolius Berg	"Arrayán"	(31°23′ S 57°42′ W)	flowering
Acca sellowiana (Berg) Burret	"Guayabo del país" Pineapple guava	(31°22′ S 57°42′ W)	fruiting
Eugenia uniflora L.	"Pitanga" Surinam cherry	(31°23′ S 57°42′ W)	vegetative
Psidium cattleianum Sab	"Arazá rojo" Cherry guava	(31°23′ S 57°42′ W)	fruiting
Aloysia gratissima (Gillies & Hook) Tronc	"Cedrón del monte" Whitebrush	(31°16′ S 57°53′ W)	flowering
Lippia alba (Mill.) N.E.Br.	"Salvia trepadora" Bushy lippia	(31°23′ S 57°57′ W)	flowering