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Screening water extracts and essential oils from Mediterranean plants against *Verticillium dahliae* in olive

A. Varo*, A. Mulero-Aparicio, M. Adem, L.F. Roca, M.C. Raya-Ortega, F.J. López-Escudero, A. Trapero

Departamento de Agronomía, Universidad de Córdoba-Campus de Excelencia Internacional Agroalimentario ceiA3, Edificio C4, Campus de Rabanales, Ctra. Madrid km 396, 14071, Córdoba, Spain

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ABSTRACT

Verticillium wilt in olive is considered the most serious biotic threat to this crop. Effective control of this disease relies on an integrated disease management strategy in which the biological control has an important role nowadays. This work describes the potential effect of 44 plant extracts and 20 essential oils against Verticillium dahliae. The results demonstrate the in vitro and in planta effectiveness of essential oil from Thymus, in particular Thymus sp. 04 (prepared in the laboratory), and the commercial product Thymus sp. 01, against Verticillium dahliae. The inhibition of mycelial growth and microsclerotia reached 100% in both treatments and achieved a disease reduction in olive plants by 65% and 42% for Thymus sp. 04 and sp. 01, respectively. These treatments showed the potential for essential oils use in the control of this pathogen in the frame of an integrated disease management strategy. This is the first report of the use of essential oils to control Verticillium wilt in olive plants. Further studies are warranted to identify the bioactive compounds in the essential oil that control V. dahliae and evaluate their potential use as natural fungicides.

1. Introduction

The extensive and intensive cultivation of olives in the Mediterranean and other regions throughout the world is threatened by the soilborne fungus *Verticillium dahliae*, which causes Verticillium wilt (VW) and limits production in these areas (Blanco López et al., 1984; López-Escudero and Mercado-Blanco, 2011). The incidence of this disease has increased over the past 30 years because of the establishment of orchards in fields previously cropped with susceptible hosts of the pathogen, the use of infected planting material (Blanco-López et al., 1984;Jiménez-Díaz et al., 2012) and the expansion of irrigation in the olive groves (Pérez-Rodríguez et al., 2015).

The pathogen can survive in soil for several years as microsclerotia (MS). The parasitic phase of the *V. dahliae* life cycle begins with the germination of MS in soil in response to root exudates (Schreiber and Green, 1963) and favorable soil environmental conditions. Germination gives rise to the formation of infective hyphae, which penetrate the plant roots and grow within the xylem vessels, producing mycelium and spores (Talboys, 1962). As a result of xylem colonization by the pathogen, water flow decreases, leading to water stress (Ayres, 1978). Populations of *V. dahliae* infecting olive plants are formed by two distinctive virulence groups called defoliating (D) and non-defoliating (ND) pathotypes. The D pathotype is highly virulent and the ND pathotype is moderately severe in olive plants (López-Escudero and Mercado-Blanco, 2011). Recently the ND and D have been characterized as race 1 and 2, respectively (Hu et al., 2015). Strategies for the management of Verticillium wilt should be focused on reducing the survival of these resting fungal structures or preventing their germination (Antonopoulos et al., 2008).

Due to the ineffectiveness of chemical controls, natural products including plant extracts (PEs) and essential oils (EOs) present many advantages in terms of sustainability, mode of action and toxicity within an integrated management strategy for the disease (Nega, 2014), where biological control arises as an alternative challenge. Moreover, interest in secondary metabolites from PEs and EOs, as potential antimicrobial agents for use in crop protection, has increased during recent decades (Isman, 2000; Burt, 2004).

Studies on the antifungal activity of PEs and EOs against plant

* Corresponding author.
E-mail address: angela.varo@uco.es (A. Varo).

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pathogens have been conducted *in vitro* conditions (Baruah et al., 1996; Carta et al., 1996; Bianchi et al., 1997; Wilson et al., 1997; Pina-Vaz et al., 2004). However, very few studies have focused on the antifungal activity of PEs and EOs against *V. dahliae* under *in vivo* conditions (Uppal et al., 2008).

The broad aims of this study were to investigate the antifungal effects of PEs and EOs obtained from Mediterranean plants against *V. dahliae* mycelial growth on Petri plates and on the viability of its MS in naturally infested soil. Additionally, research was extended to evaluate *in vivo* the potential biocontrol effect of PEs and EOs on VW disease in the susceptible olive cv. Picual.

2. Materials and methods

2.1. Pathogen isolates

Two *V. dahliae* isolates from the fungal collection of the Department of Agronomy at the University of Córdoba were used in this study: a mildly virulent strain ND pathotype V004 and a highly virulent D pathotype V117 (Blanco-López et al., 1989). The isolates were maintained on potato dextrose agar (PDA) slants at 4 °C. Plates of a 6-day-old single spore culture incubated on PDA at 24 °C in the dark were used as the pathogen inoculum source.

2.2. Plant material used for extracts and essential oils

Based on a literature survey, 29 commercial products and material from 35 botanical species (Table 1) were chosen for this study. Collection of wild species was assisted by Semillas Cantueso S.L. (Córdoba, Spain http://www.semillascantueso.com). that had previously identified the geographic place where different botanic populations grew in Andalucía (southern Spain). Surveys were conducted in diverse zones in the Sierra Morena and the Campus de Rabanales of the University of Cordoba. The plant material was processed in the laboratory. The leaves of *Oleae europaea* cultivars were collected from the World Olive Germplasm Bank of Córdoba (WOGB), and the *Brassicaceae* species were characterized and supplied by Dr. de Haro from CSIC-IAS at the growing stage. The freshly cut plant materials were sorted, dried with active ventilation at room temperature, ground to a fine powder in a hammer mill (Retsch GmbH and Co. KG, Haan, Germany), packed in paper bags and stored at 5 °C until use.

2.2.1. Plant extracts

The plant extracts (PEs) were obtained from several sources by steam distillation. A first group was purchased from the same companies mentioned below, and the purity was available for some of them. Another group consisted of PE obtained in the lab from different botanical species. Ground plant material samples (25 g of leaves and stems) of each plant were extracted with 100 ml of organic solvent (acetone) in a Soxhlet extractor. The mixture was boiled for 3 h, and the extract was concentrated by a distillation process to evaporate the acetone. The crude extracts obtained were then stored at −18 °C until further use. The extraction for each plant extract was run in duplicate.

Particularly, for *Allium* and *Melia*, the juices of both species were obtained according to the methodology of Curtis et al. (2004). Samples (100 g of leaves and stems) were chopped into small pieces

Table 1
Plant extracts and essential oils evaluated for their effectiveness against *Verticillium dahliae*.

Extract from	Origin (Purity)	Extract from	Origin (Purity)
Allium sativum	Lab preparation	*Lantana camara*	Lab preparation
Allium sp.	Bio 125®, Agromed S.L.	*Laurus nobilis*	Lab preparation
Atropa belladona	Fagron S.A. (3.45)	*Lepidium* sp.	Lab preparation
Azadirachta indica	Neem® Trabe S.A.	*Marrubium vulgare*	Fagron S.A.
Brassica napus	Lab preparation	*Melia azedarach*	Lab preparation
Brassica rapa	Lab preparation	*Mentha sativa*	Lab preparation
Castanea sativa	Fagron S.A.	*Nerium oleander*	Lab preparation
Cistus albidus	Lab preparation	*Olea europaea* cv. Lechín de Sevilla	Lab preparation (5.63)
Cistus ladanifer	Lab preparation	*Olea europea* cv. Arbequina	Lab preparation
Cistus laurifolius	Lab preparation	*Olea europea* cv. Cornicabra	Lab preparation
Citrus aurantium	Fagron S.A. (2.75)	*Olea europea* cv. Empeltre	Lab preparation
Citrus sp.	Bio 150, Agromed S.L.	*Olea europea* cv. Frantoio	Lab preparation (5.30)
Diplotaxis erucoides	Lab preparation	*Olea europea* cv. Picual	Lab preparation (7.30)
Diplotaxis virgata	Lab preparation	*Origanum vulgare*	Lab preparation
Eucalyptus camaldulensis	Lab preparation	*Papaver rhoeas*	Fagron S.A.
Ginkgo biloba	Fagron S.A. (11.00)	*Pinus pinea*	Lab preparation
Hammamelis virginiana	Fagron S.A.	*Pistacia lentiscus*	Lab preparation (2.90)
Hedera helix	Fagron S.A. (1.06)	*Rosmarinus officinalis*	Fagron S.A. (2.00)
Hirschfeldia incana	Lab preparation	*Salvia officinalis*	Fagron S.A. (1.25)
Inula viscosa	Lab preparation (6.00)	*Sambucus nigra*	Fagron S.A. (3.03)
Juglans regia	Fagron S.A. (3.73)	*Thymus vulgaris*	Lab preparation
Juniperus communis	Fagron S.A.	*Urtica* sp.	Lab preparation
Essential oil from	**Origin**	**Essential oil from**	**Origin**
Citric acid 01	Fagron S.A.	*Origanum vulgare*	Fagron S.A.
Citric acid 02	Fruitcare, Zoberbac S.L.	*Pinus* sp.	Fagron S.A.
Cymbopogon sp.	Fagron S.A.	*Rosmarinus officinalis*	Fagron S.A.
Eucaliptus sp.	Fagron S.A.	*Salvia officinalis*	Fagron S.A.
Illicium verum	Fagron S.A.	*Satureja* sp.	Fagron S.A.
Laurus nobilis	Fagron S.A.	*Thymus* sp. 01	Oleatbio, Trabe S.A.
Melaleuca alternifolia	Fagron S.A.	*Thymus* sp. 02	Biofungi, Fagron S.A.
Melaleuca cajeputi	Fagron S.A.	*Thymus* sp. 03	Bio 75, Agromed S.L.
Mentha sp.	Fagron S.A.	*Thymus* sp. 04	Lab preparation
Mirtus communis	Fagron S.A.	*Verbena officinalis*	Fagron S.A.

1 The purity, where available, of plant extracts indicates the percentage of dry matter, which was estimated by evaporating 1 ml of extract at 70 °C and weighing the dried residue.