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Antagonistic activity of fungi of *Olea europaea* L. against *Colletotrichum acutatum*

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ABSTRACT

Fungi naturally present in olive trees were identified and tested for their antagonistic potential against *Colletotrichum acutatum*. A total of 14 isolates were identified, 12 belonged to genera *Alternaria*, *Epicoccum*, *Fusarium*, *Aspergillus*, *Anthrinium*, *Chaetomium*, *Diaporthe*, *Nigrospora*, one to family Xylariaceae and one was unclassified. All fungal isolates showed some inhibitory action over the growth of *C. acutatum* during dual culture growth, however, when agar-diffusible tests were performed only five fungal isolates caused *C. acutatum* growth inhibition: *Alternaria* sp. isolate 2 (26.8%), the fungus from Xylariaceae family (14.3%), *Alternaria* sp. isolate 1 (10.7%); *Diaporthe* sp. (10.7%), *Nigrospora oryzae* (3.5%). Volatile substances produced by these isolates were identified through gas-chromatography techniques, as phenylethyl alcohol, 4-methylquinazoline, benzothiazole, benzyl alcohol, lilial, galaxolide, among others. These inhibitory volatiles could play a significant role in reduction of *C. acutatum* expansion in olive and their study as potential biocontrol agents should be further explored.

1. Introduction

Colletotrichum is a genus of Ascomycota fungi that contain some of the most successful plant pathogenic fungal species causing high economic losses to a wide range of woody and herbaceous crops, especially fruits, vegetables and ornamentals both in tropical and temperate regions worldwide (Bailey et al., 1992; Zivkovic et al., 2010; Baroncelli et al., 2014).

Due to their scientific and economic importance, species from the genus *Colletotrichum* have recently been ranked in the top ten fungal pathogens (Dean et al., 2012). One of the most pathogenic species of this genus is *Colletotrichum acutatum* Simmonds, which causes anthracnose and blight in important hosts such as olive, almond, peach, citrus, strawberry, among others (Förster and Adaskaveg, 1999; Martín and García-Figueres, 1999; Timmer and Brown, 2000; Zaitlin et al., 2000; Curry et al., 2002). *Colletotrichum acutatum* can affect most parts of the plant and symptoms range from shoot and leaf spots to fruit rot. Symptoms on fruits are extremely important due to the economic losses they cause, not only in the field (pre-harvest) but also during storage (post-harvest) (Bailey et al., 1992; Xia et al., 2011; Menezes et al., 2014; Zhong et al., 2014).

At present most effective control measures against *Colletotrichum* diseases rely on cultural control, using resistant cultivars, chemical control and/or biological control using antagonistic organisms. Measures of cultural control are extremely helpful to decrease inocula levels and to worsen fungi optimal development conditions; however, they are not sufficient and to combat *C. acutatum* they should be used as part of an integrated control. In addition, resistant/tolerant varieties are not available for all crops, costs associated with replacing an established crop with a resistant are very high and producers usually select cultivars based on other criteria than disease resistance. Due to this, the most common strategy to control *Colletotrichum* is the use of chemical fungicides. Although fungicides reduce the severity of the disease, eradication is difficult to attain and repeated applications are often necessary to maintain protection. In addition, the need to respect fungicide security periods before harvesting may result in an incomplete protection of the fruits in a developmental stage of very high susceptibility in diseases as olive anthracnose (Cacciola et al., 2012). Negative effects of the use of fungicides are evident; the abuse in the employment of chemical compounds has favored the deteri-

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oration of human health, environmental contamination and the development of pathogens resistant to fungicides (Prapagdee et al., 2008).

Biological control assumes therefore a great importance. In the last three decades there has been an increased interest in the use of biological agents for fungal plant pathogens control and numerous microorganisms have been identified as potential bio-control agents (Alabouvette et al., 2006). Plant hosts have been the best places to obtain good antagonists against pathogens attacking those hosts. Plants usually have several fungi present either on their surfaces (epiphytes) or inside tissues (endophytes) that do not cause any visible damage. These fungi are very diverse and their role lacks study. On the category of potential bio-control agents, endophytic microorganisms have gained a considerable importance. Some studies have shown that endophytic fungi produce compounds that inhibit the growth of other fungi both in the field and in storage (Mercier and Jiménez, 2004; Rodriguez et al., 2009; Wang et al., 2013).

The aim of this study focuses on assessing the ability of several fungi isolated from olive to inhibit the growth of the phytopathogenic fungus *C. acutatum* through their antagonistic activity and effect of secondary metabolites.

2. Materials and methods

2.1. Isolation of pathogen

The phytopathogenic fungus *C. acutatum* belongs to the collection of the Mycology Laboratory, Institute of Mediterranean Agricultural and Environmental Sciences (ICAAM), University of Évora, Portugal. It was originally isolated from fruits from an olive tree cv. *Galega vulgar* growing in Elvas, Portugal. Stocks of *C. acutatum* isolates were grown on potato dextrose agar (PDA) (Oxoid) plates, at room temperature (25–28 °C) and stored at 4 °C for later use.

2.2. Sample collection and isolation of fungal isolates

Samples of symptomless leaves were collected from 50 asymptomatic olive trees from cv. *Galega vulgar* from an orchard located in the South of Portugal (Évora). To search for epiphytes and endophytes, half of the collected leaves was not disinfected and the other half was surface sterilized by treatment with ethanol 70% (v/v) for 2 min, 3% sodium hypochlorite for 3 min, ethanol 70% (v/v) for 1 min, and a final wash repeated three times in sterile distilled water for 1 min each (Verma et al., 2007). Treated and untreated leaves were placed on Petri dishes (9 cm) containing PDA. Plates were incubated for seven days at room temperature (25 ± 3 °C).

Fungi were further purified by transferring the mycelia from the margin of the growing fungal colonies to individual Petri dishes (5 cm) containing fresh PDA and incubated at room temperature (25 ± 3 °C) for seven days.

2.3. Fungal identification

DNA was extracted from fungal cultures growing in PDA plates for seven days, using the DNeasy Plant Mini Kit (Qiagen), in accordance to manufacturer's instructions. The internal transcribed spacer (ITS) region of nuclear rDNA was PCR amplified from genomic DNA by using ITS1 and ITS4 primers (White et al., 1990).

The PCR reactions consisted of 30–80 ng of genomic DNA, 10 mM Tris–HCl (pH 8.6), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs (Fermentas), 1 μM of each primer and 2.5 U of DreamTaq DNA polymerase (Fermentas) in a total volume of 50 μL. Amplification was carried out in a Thermal Cycler (Bio-Rad) at 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 50 s, and 72 °C for 60 s and a final extension at 72 °C for 10 min.

PCR products were purified using GFX Gel DNA Purification Kit (GE Healthcare Biosciences) and sequenced in forward and reverse directions by Macrogen (The Netherlands). Sequence analysis of the ITS sequences was carried out using BioEdit Sequence Alignment Editor v.7.2.3 (Hall, 1999). The search for homologous sequences was done using Basic Local Alignment Search Tools at the National Center for Biotechnology Information and on Fungal Barcode website (http://www.fungalbarcoding.org).

2.4. Antagonistic tests

2.4.1. Direct inhibition test

Fungal isolates were tested in vitro for their antagonistic activity against *C. acutatum* using the direct opposition method (Dennis and Webster, 1971). Briefly, a 5 mm mycelia disc from the margin of actively growing colony of *C. acutatum* was placed at about 1 cm from the wall of a 9 cm PDA plate and at the opposite side, a similar sized disc of the fungal isolate was placed. Plates were incubated at 25 ± 2 °C and three replicates were used for each fungus tested. Control tests were also carried out using *C. acutatum* alone. For the estimation of the growth inhibition percentage, the radial growth of *C. acutatum* with each of the isolated fungi and control plates was measured with a verner caliper and recorded consecutively for five days and on the day seven, ten and 15. The inhibition percentage was calculated using the following formula (Royse and Ries, 1977):

$$I[\text{inhibition percentage}] = \left(\frac{\text{R1[colony radius in control]} - \text{R2[colony radius in test]}}{\text{R1}}\right) \times 100$$

The interaction type between the two fungi was assessed using a scale from *A* to *D* (Dharmaputra, 2003; Demici et al., 2012): *A* = growth inhibition of *C. acutatum* on contact with interacting fungus; *B* = mutual intermingling of *C. acutatum* and interacting fungus but both grow slowly and at different rate; *C* = mutual inhibition with a space distance <0.2 cm; *D* = mutual inhibition at distance >0.2 cm.

Microscopic observations were made to the margins of *C. acutatum* exposed to fungal isolates. These were mounted on microscopic slides and stained with lactophenol blue. A non-exposed *C. acutatum* was used as control. Slides were examined under a microscope (Olympus BX41).

2.4.2. Volatile compounds test

To evaluate the possible production of volatile compounds that could have some activity on the growth of *C. acutatum*, a simple trial was conducted (Rahmansyah and Rahmansyah, 2013).

Isolated fungi were incubated on PDA Petri dishes (9 cm) for five days. In small (5 cm) PDA plates *C. acutatum* incubated for 48 h. After those periods, the small plates containing the phytopatogenic fungus were placed inverted on top of each of the isolated fungus. The top was sealed with parafilm and adhesive tape to prevent diffusion of volatiles. The growth of *C. acutatum* in each of the plates containing a different fungus was compared with a control placed inverted in a plate containing only PDA medium. Three replicates were used.

After five days of incubation at 25 ± 3 °C, the diameters of the pathogen colonies were measured and the percentage of inhibition was calculated using the same formula as previously described.

2.4.3. Non-volatile compounds test

Erlenmeyer's flasks each containing 15 ml of potato dextrose broth (PDB) (Fluka) were inoculated with a 5 mm mycelia disc from