Microbiological Research 183 (2016) 100–108

Contents lists available at ScienceDirect

Microbiological Research

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

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

Miguel C. Landum[a], Maria do Rosário Félix[b], Joana Alho[a], Raquel Garcia[a], Maria João Cabrita[b], Fernando Rei[b], Carla M.R. Varanda[a,*]

[a] *Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Instituto de Investigação e Formação Avançada, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7006-554 Évora, Portugal*

[b] *Departamento de Fitotecnia, Escola de Ciências e Tecnologia, Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Núcleo da Mitra, Ap. 94, 7006-554 Évora, Portugal*

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form 1 December 2015
Accepted 4 December 2015
Available online 12 December 2015

Keywords:
Anthracnosis
Biocontrol
Olive
Volatiles

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-

* Corresponding author. Fax: +351 266760822.
E-mail address: carlavaranda@uevora.pt (C.M.R. Varanda).

http://dx.doi.org/10.1016/j.micres.2015.12.001
0944-5013/

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

the margins of actively growing colonies of fungal isolates and incubated for 12 days at 25 ± 2 °C. After that period solutions were filtered using filter papers 150 mm (Whatman) to remove spores and other fungal structures.

75 μl, 100 μl and 200 μl of the different culture filtrates were placed in a 1 cm circular hole made directly in the medium in the opposite side where *C. acutatum* was growing for two days in a 9 cm PDA plate. Three replicates for each filtered solution were used as well as a control where the hole was filled with 75 μl of PDB alone.

2.5. Extraction and chromatographic analysis of volatiles

Extractions were performed as described previously (Vilanova et al., 2012). Briefly, to a 10 ml tube, 8 ml of PDB wherein each fungus grew and a magnetic stir bar (22.2 mm × 4.8 mm) were added. 3-Octanol was used as internal standard. Extraction was done by stirring the sample with 500 μl of dichloromethane (Merck, Darmstadt, Germany) for 15 min. After cooling at 0 °C for 10 min, the magnetic stir bar was removed and the organic phase was obtained

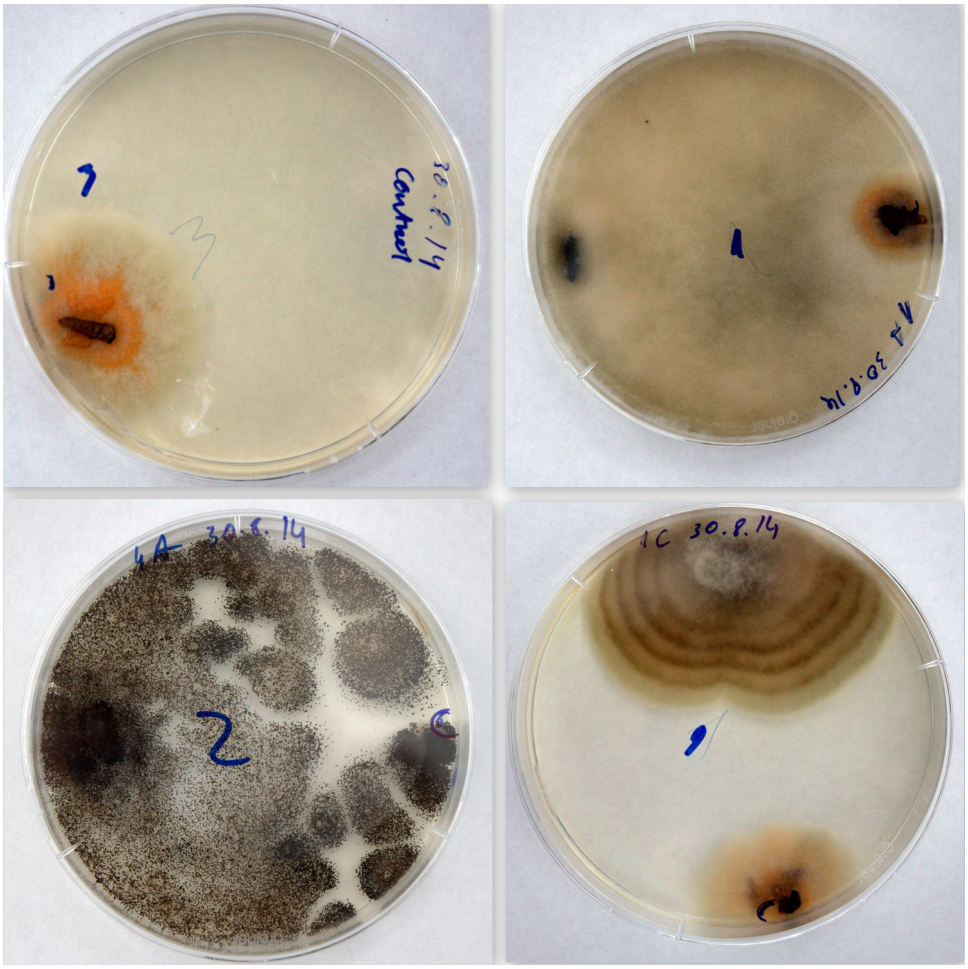


Fig. 1. Illustration of some of the fungi used in the direct inhibition test and the different type of interactions observed 7 days after inoculation. Top left: *C. acutatum* (control plate); top right: interaction type C shown between *Nigrospora oryzae* and *C. acutatum*; bottom left: interaction type A shown by *Aspergillus niger* and *C. acutatum*; bottom right: interaction type D shown by *Alternaria* sp. isolate 2 and *C. acutatum*.

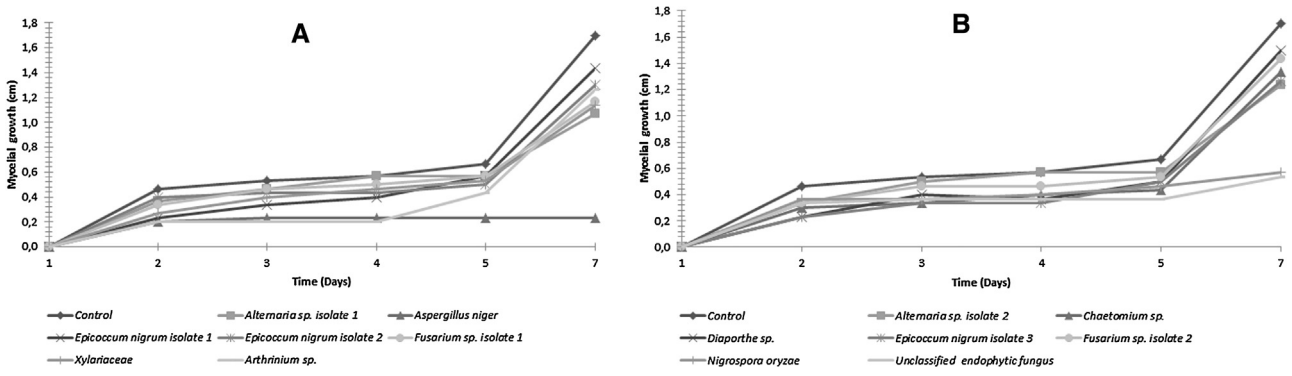


Fig. 2. *Colletotrichum acutatum* radial mycelial growth (cm) over time registered in direct inhibition tests using fungi isolated from (A) non-disinfected and (B) disinfected leaves.

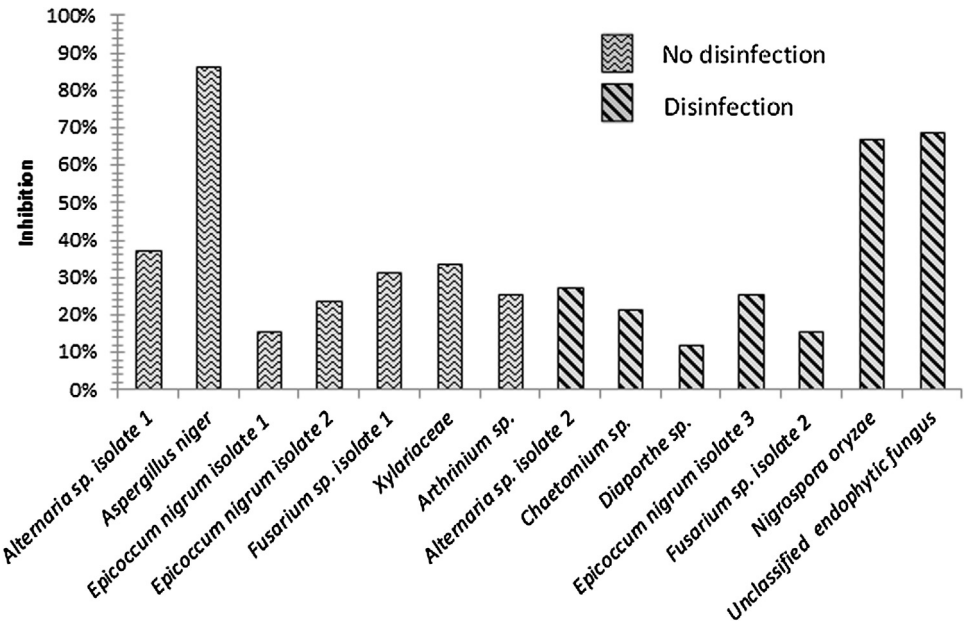


Fig. 3. Inhibition percentage values observed 7 days after inoculation of both fungi in PDA plates during direct inhibition test.

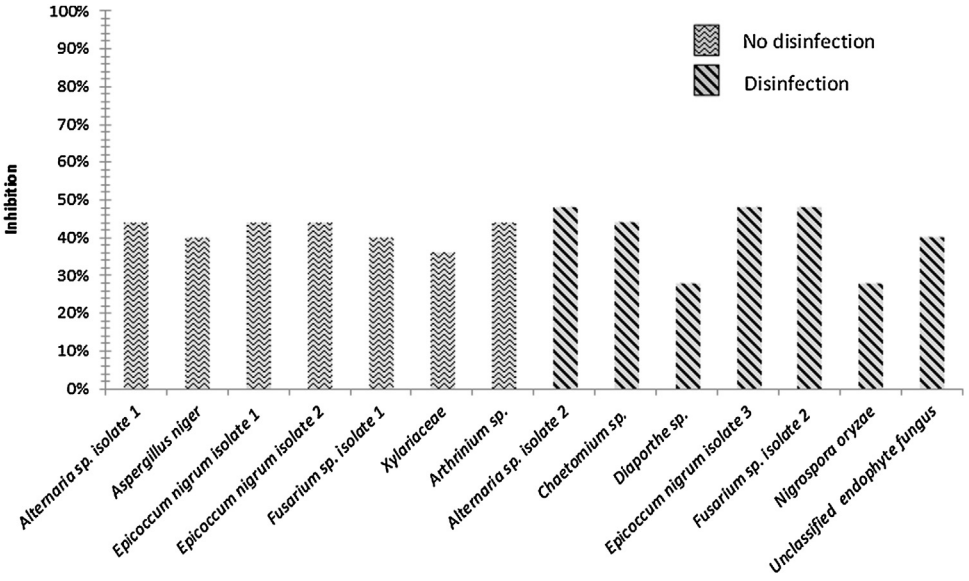


Fig. 4. Inhibition percentage values observed 7 days after inoculation of both fungi during volatile compound test.

Table 1
Classification of the interaction type between the antagonistic fungi and *Colletotrichum acutatum*.

Fungus	Type of interaction
Aspergillus niger; unclassified endophytic fungus	A
Nigrospora oryzae	C
Alternaria sp. isolate 1 and 2; *Arthrinium* sp.; *Chaetomium* sp.; *Diaporthe* sp.; *Epicoccum nigrum* isolate 1,2 and 3; *Fusarium* sp. isolate 1 and 2; Xylariaceae	D

by centrifugation (2948 × *g*, 5 min, 4 °C), with the extract being recovered in a vial using a Pasteur pipette. Then the aromatic extract was dried with anhydrous sodium sulfate (Merck, Darmstadt, Germany) and collected again in a new vial.

A Thermo Finnigan Trace GC gas chromatograph (Thermo Finnigan, Austin, TX), equipped with a Thermo Finnigan Polaris Q mass selective detector was used. Samples (1 μl) were injected in the splitless mode and volatiles were separated using a fused silica capillary column, ZB-Wax 30 m, 0.25 mm i.d and 0.25 μm film thickness (Zebron Capillary GC column; Phenomenex USA) under the following column temperature program: 40 °C (held 1 min) heated at 4 °C/min to 100 °C and then heated at 8 °C/min to 250 °C (held for 10 min); and a Rtx-5 (Crossbond 5% diphenil—95% dimetil polysiloxane) 30 m, 0.25 mm i.d and 0.25 μm film thickness (RESTEK, USA) using the following column temperature program: 40 °C (held 1 min) heated at 5 °C/min to 250 °C (held 5 min) and then heated at 5 °C/min to 300 °C (held 1 min).

Carrier gas was grade helium at a flow rate (constant flow) of 1.0 ml/min; the injection temperature was 250 °C, in splitless mode with a split flow of 50 ml/min. Detection was carried out by positive ion electron impact (EI) mass spectrometry in the full scan mode, using an ionization energy of 70 eV and a transfer line temperature of 250 °C.

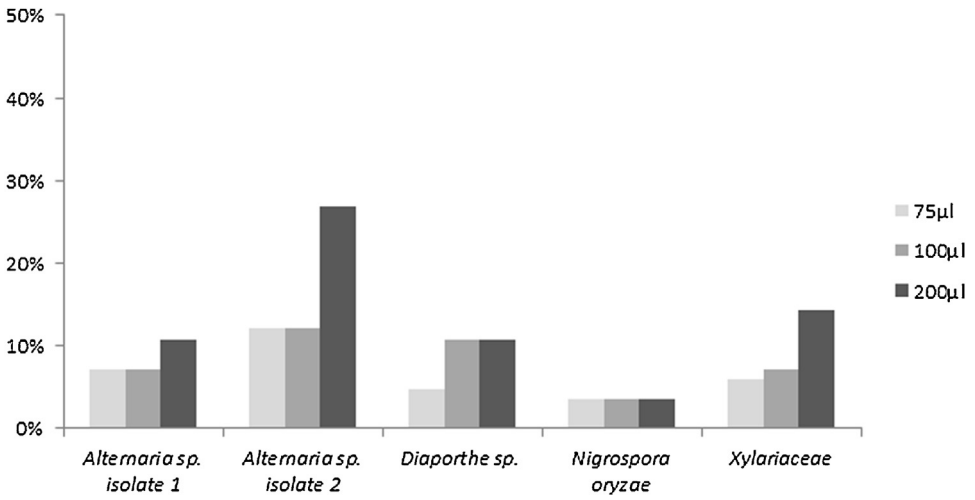
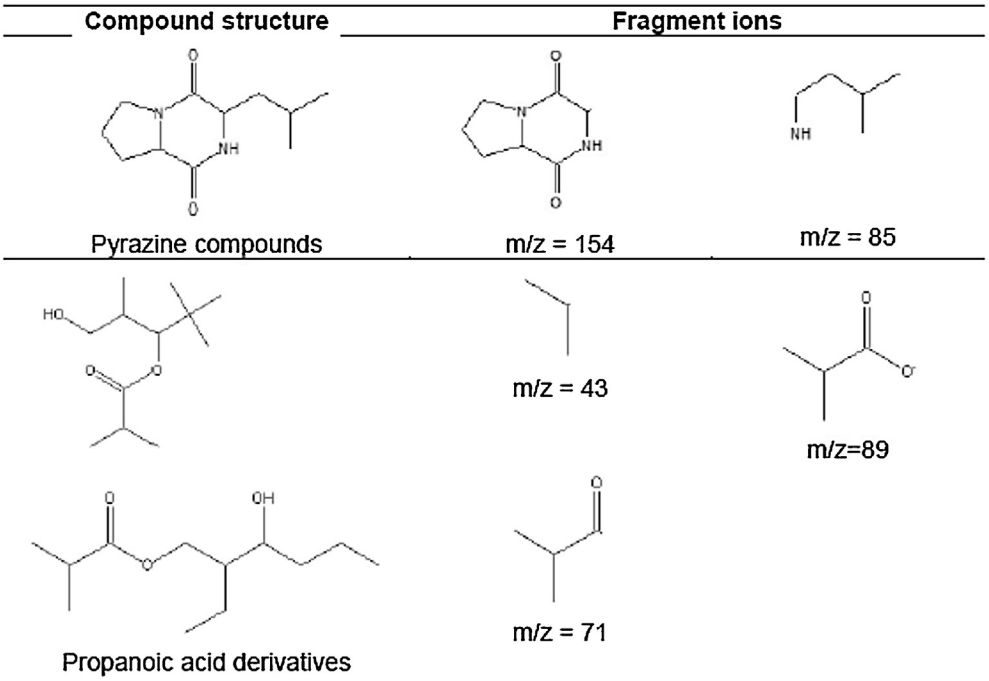


Fig. 5. Inhibition percentage values observed 7 days after inoculation during non-volatile compounds test.

Table 2
Compounds identified in at least one sample, and some compounds belonging to different chemical families.

Compounds	MW	MS ions	*N. oryzae*	*Alternaria* sp. isolate 2	*Alternaria* sp. isolate 1	Xylariaceae	*Diaporthe* sp.
Phenylethyl alcohol	122	91, 92, 122, 65	x	x	–	x	x
4-Methylquinazoline	144	144, 103, 117, 129	–	–	–	x	–
Benzothiazole	135	135, 108, 69, 82	–	–	–	x	–
Benzyl alcohol	108	79, 108, 107, 77	–	x	–	–	–
Lilial	204	189, 147, 131, 117	x	x	–	x	x
Galaxolide	258	243, 213, 244, 258	x	x	–	x	x
Chemical families							
Propanoic acid derivatives		71, 43, 89	x	x	x	x	x
Pyrazine compounds		154, 85	x	x	x	x	x
Benzaldehyde derivatives		92	x	–	–	–	–
Ketones compounds		55, 43, 83, 111, 126	x	–	–	–	–
Amines derivatives		72, 44	x	x	–	x	x
Phenol,bis-dimethylethyl		191, 57, 206	x	–	–	x	x

Table 3
Structure and the respective typical fragmentation pattern of some compounds belonging to a different chemical family that appeared in all fungal samples.



The mass acquisition range was m/z 30–450 and the scanning rate 3 scan s^{-1}. Chromatographic peaks were identified by comparing their mass spectra with those reported in the literature and in commercial libraries NIST MS Search 2.0 and Wiley 7. The linear retention index values were calculated in both columns described. For each compound, the linear retention indexes calculated were compared with these reported by other authors and NIST library in order to have another tool to help on the identification of compounds. All samples were analyzed in duplicate.

2.6. Statistical analysis

Analysis of variance (ANOVA) was conducted regarding differences among antagonistic fungi in each period, using the IBM SPSS statistical package v.20. Multiple mean comparisons were made using Tukey HSD test when statistical differences were found between data sets ($P < 0.05$).

3. Results

A total of 14 different fungi isolates were recovered from disinfected and non-disinfected leaves of 50 trees of *Olea europaea* cv. *Galega vulgar* from an orchard located in Évora (Portugal).

Sequencing results confirmed the presence of seven distinct fungi in non-disinfected leaves: *Alternaria* sp. isolate 1; *Aspergillus niger*; *Epicoccum nigrum* isolate 1 and isolate 2; *Fusarium* sp. isolate 1; *Anthrinium* sp. and a fungus of the Xylariaceae family which was not possible to determinate the species; and seven species in disinfected leaves: *Alternaria* sp. isolate 2; *Chaetomium* sp.; *Diaporthe* sp.; *E. nigrum* isolate 3; *Fusarium* sp. isolate 2; *Nigrospora oryzae* and an undeterminated endophytic fungus.

Most predominant species were *E. nigrum*; *Alternaria* sp. and *Fusarium* sp., totalling seven of the 14 fungal species detected.

All 14 fungal isolates were screened for their antagonistic activity against the phytopatogenic fungus *C. acutatum*.

C. acutatum throughout the direct inhibition tests was characterized by a continuous increasing growth (Table 4) except in the presence of *A. niger*, *N. oryzae* and the unclassified endophytic fungus (Fig. 2A and B). *A. niger* (Fig. 1 bottom left) and the unclassified endophytic fungi inhibited the growth of *C. acutatum* through contact (interaction type A) whereas *N. oryzae* (Fig. 1 top right) inhibited the growth of *C. acutatum* with a space distance of less than 0.2 cm (interaction type C) (Table 1). All the other fungal isolates showed some mutual inhibition and their growth presented distances of more than 0.2 cm between antagonist and pathogenic fungi (interaction type D) (Fig. 1 bottom right).

The inhibition percentages calculated for fungal isolates ranged from 11.8% to 86.3% (Fig. 3), showing that all fungal isolates had some inhibitory action over the growth of *C. acutatum*. As expected from the interaction types found, *A. niger*, *N. oryzae* and the unclassified endophyte were those that demonstrated greater ability to inhibit the growth of *C. acutatum* in the direct inhibition test, with percentages of 86.3%, 66.7% and 68.6%, respectively.

Microscopic observations of the interactions between *C. acutatum* and the antagonistic fungi did not show any alterations in the pathogen hyphae (data not shown).

All the 14 fungal isolates tested produced volatiles that caused some inhibition on the growth of *C. acutatum*. Although differences between isolates were not significant, the most effective were *Alternaria* sp. isolate 2, *E. nigrum* isolate 3 and *Fusarium* sp. isolate 2 with an inhibition of growth of 48% and the less effective were *Diaporthe* sp. and *N. oryzae* presenting 28% (Fig. 4; Table 5).

As for the agar diffusible tests, only five fungal isolates, four of these isolated from disinfected leaves, produced substances capable of diminishing the radial growth of *C. acutatum* presenting

Table 4
Mean (±Std. error) of radial mycelial growth (cm) of *Colletotrichum acutatum* over time during the direct inhibition test.

Day	Control	*Alternaria* sp. isolate 1	*Alternaria* sp. isolate 2	*Arthrinium* sp.	*Aspergillus*	*Chaetomium* sp.	*Diaporthe* sp.	*Epicoccum nigrum* isolate 1	*Epicoccum* isolate 2	*Epicoccum* isolate 3	*Fusarium* sp. isolate 1	*Fusarium* sp. isolate 2	*Nigrospora*	Unclassified endophytic fungus	Xylariaceae
1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.47 ± 0.03	0.37 ± 0.09	0.33 ± 0.07	0.20 ± 0.06	0.20 ± 0.00	0.30 ± 0.00	0.23 ± 0.07	0.23 ± 0.03	0.40 ± 0.00	0.23 ± 0.03	0.33 ± 0.07	0.33 ± 0.07	0.37 ± 0.03	0.33 ± 0.03	0.27 ± 0.03
3	0.53 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.20 ± 0.06	0.23 ± 0.03	0.33 ± 0.07	0.40 ± 0.00	0.33 ± 0.03	0.43 ± 0.03	0.33 ± 0.03	0.47 ± 0.03	0.47 ± 0.03	0.37 ± 0.03	0.37 ± 0.07	0.40 ± 0.06
4	0.57 ± 0.03	0.57 ± 0.03	0.57 ± 0.03	0.20 ± 0.06	0.23 ± 0.03	0.40 ± 0.00	0.37 ± 0.09	0.40 ± 0.00	0.43 ± 0.03	0.33 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.40 ± 0.00	0.37 ± 0.07	0.47 ± 0.03
5	0.67 ± 0.03	0.57 ± 0.03	0.57 ± 0.03	0.43 ± 0.03	0.23 ± 0.03	0.43 ± 0.03	0.50 ± 0.00	0.57 ± 0.03	0.50 ± 0.00	0.50 ± 0.00	0.57 ± 0.03	0.53 ± 0.03	0.47 ± 0.03	0.37 ± 0.07	0.53 ± 0.03
7	1.70 ± 0.00	1.07 ± 0.07	1.23 ± 0.09	1.27 ± 0.09	0.23 ± 0.03	1.33 ± 0.07	1.50 ± 0.06	1.43 ± 0.03	1.30 ± 0.15	1.27 ± 0.03	1.17 ± 0.12	1.43 ± 0.03	0.57 ± 0.03	0.37 ± 0.07	1.13 ± 0.03
10	2,07 ± 0.03	1.63 ± 0.09	1.50 ± 0.15	1.83 ± 0.09	0.23 ± 0.03	1.77 ± 0.18	1.67 ± 0.12	1.90 ± 0.10	1.77 ± 0.09	1.53 ± 0.03	1.90 ± 0.06	2,03 ± 0.07	0.57 ± 0.03	0.53 ± 0.03	1.93 ± 0.12
15	3,97 ± 0.03	1.63 ± 0.09	1.50 ± 0.15	1.83 ± 0.09	0.23 ± 0.03	1.77 ± 0.18	1.67 ± 0.12	1.90 ± 0.10	1.77 ± 0.09	1.53 ± 0.03	1.90 ± 0.06	2,03 ± 0.07	0.57 ± 0.03	0.53 ± 0.03	1.93 ± 0.12

Table 5
Mean (±Std. error) of radial mycelial growth (cm) of *Colletotrichum acutatum* over time during the volatile test.

Day	Control	*Alternaria* sp. isolate 1	*Alternaria* sp. isolate 2	*Arthrinium* sp.	*Aspergillus niger*	*Chaetomium* sp.	*Diaporthe* sp.	*Epicoccum* isolate 2	*Epicoccum* isolate 3	*Fusarium*sp. isolate 1	*Fusarium* sp. isolate 2	*Nigrospora oryzae*	Unclassified endophytic fungus	Xylariaceae
1	0.50 ± 0.00	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.40 ± 0.00	0.43 ± 0.03	0.40 ± 0.00	0.43 ± 0.03	0.50 ± 0.00	0.50 ± 0.00
2	0.63 ± 0.03	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.40 ± 0.00	0.43 ± 0.03	0.40 ± 0.00	0.47 ± 0.00	0.50 ± 0.00	0.50 ± 0.00
3	0.67 ± 0.03	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.40 ± 0.00	0.43 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.50 ± 0.00
4	0.67 ± 0.03	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.40 ± 0.00	0.43 ± 0.03	0.43 ± 0.03	0.50±0.03	0.50 ± 0.00	0.50 ± 0.00
5	0.73 ± 0.03	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.43 ± 0.03	0.50 ± 0.00	0.43 ± 0.03	0.60 ± 0.00	0.50 ± 0.00	0.53 ± 0.03
6	0.80 ± 0.00	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.43 ± 0.03	0.50 ± 0.00	0.43 ± 0.03	0.60 ± 0.00	0.50 ± 0.00	0.53 ± 0.03
7	0.83 ± 0.03	0.47 ± 0.03	0.43 ± 0.03	0.47 ± 0.03	0.50 ± 0.00	0.47 ± 0.03	0.60 ± 0.00	0.47 ± 0.03	0.43 ± 0.03	0.50 ± 0.00	0.43 ± 0.03	0.60 ± 0.00	0.50 ± 0.00	0.53 ± 0.03

some inhibition values: *Alternaria* sp. isolate 2 (26.8%), the fungus from Xylariaceae family (14.3%), *Alternaria* sp. isolate 1 (10.7%); *Diaporthe* sp. (10.7%), *N. oryzae* (3.5%) (Fig. 5).

Although only a few volatile compounds were identified, the five samples under study presented some different characteristics (Table 2). Remarkably, the sample of *Alternaria* sp. isolate 1 was the poorest one in terms of volatile compounds. Some compounds seem to be characteristic of some samples, namely Xylariaceae (4-methylquinazoline and benzothiazole) and *Alternaria* sp. isolate 2 (benzyl alcohol). Phenylethyl alcohol is present in all samples, except in sample of *Alternaria* sp. isolate 1, although in different concentrations. In samples *Diaporthe* sp. and *N. oryzae*, it was the largest peak while in Xylariaceae sample the peak area is very small. Despite *Alternaria* sp. isolate 1 peak has a similar relative area to that presented in *Diaporthe* sp. and *N. oryzae*, there is a huge peak that shows a fragmentation pattern that can be ascribed as an ester of an organic acid, probably containing a fluor atom.

Pyrazine compounds and propanoic acid derivatives, two chemical families, were detected in all the fungal samples tested (Table 3). Compounds presenting fragmentation ions typical of pyrazine compounds, m/z 154 and m/z 85, appear several times in the chromatogram, which seems to indicate the presence of at least four pyrazine derivatives. Regarding propanoic acid derivatives all samples seems to have two different compounds belonging to this family.

4. Discussion

Plants are often colonized by many fungi that do not cause any disease symptoms. Many of these may have beneficial effects on plant growth by providing essential nutrients to the plant (Harrison, 2005), indirectly making it less susceptible to pathogens, or directly protecting them through an antagonistic effect on pathogens (Wang et al., 2013).

In this study, 14 fungal isolates were obtained from 50 olive trees. Five of these isolates were identified to species level, seven to genus level, one at family level and one was unidentified. Most predominant fungi were isolates of *E. nigrum*, *Alternaria* sp. and *Fusarium* sp. totalling half of the fungi isolated. Isolates belonging to these species/genera were found in both disinfected and non-disinfected leaves. Fungal isolates obtained from disinfected leaves are endophytes and fungal isolates obtained in non-disinfected leaves may be either endophytes or epiphytes. However, all fungal isolates obtained in this study are commonly found as endophytes in a wide range of plants and not exclusive of olive.

Results of direct inhibition tests revealed that all fungal isolates inhibited mycelial growth of *C. acutatum*. The highest pathogen growth inhibition percentages 86.3%, 66.7% and 68.6%, were produced by *A. niger*, *N. oryzae* and an unclassified endophyte fungus, respectively.

Although initially all fungi were placed on opposite sides of the Petri dishes, from day ten forward, antagonist mycelial growth led to a direct contact of the pathogen with *A. niger* or to a distance of less than 0.2 cm with *N. oryzae* and the unidentified endophyte fungus. This could be an indication of the importance that the physical proximity between fungi could have on their antagonistic capability, since competition for space and food can be an important limiting factor for the establishment of any fungus, fact well established for other antagonists (Alabouvette et al., 2006; Howell, 2003).

On the other hand, the pathogen growth inhibition starting from day two in some isolates, especially *Anthrinium* sp., *A. niger*, *Diaporthe* sp.; *E. nigrum* isolates 1 and 3 and the unidentified Xylariaceae member, would suggest that they might reduce *C. acutatum* growth by producing certain metabolites rather than by

competition or parasitism. In fact, the antagonistic properties of microorganisms that act as biocontrol agents are based on the activation of multiple mechanisms. They can control pathogens by competing for nutrients and space, by mycoparasitism, antibiosis or metabolite production (Heydari and Pessarakli, 2010) either by affecting the pathogen of by promoting plant growth and defence mechanisms.

All fungal isolates identified in this study are ascomycetes, which are known to be active producers of antimicrobial compounds (Zhi-Lin et al., 2012). Some species of *Aspergillus* and *Fusarium*, have already been profiled for their organic compounds and some antifungal components were isolated (Morath et al., 2012; Siddiquee et al., 2015). *Alternaria* sp., *A. niger*, *Epicoccum* sp. and members of the Xylariaceae family have also shown to produce compounds mostly unidentified (Newcombe et al., 2009; Wani et al., 2010; Zhi-Lin et al., 2012). The volatile compounds test showed that all isolates produced volatiles that, at some level, diminished the growth of *C. acutatum*. Differences in growth were significant from day five onwards for all fungal isolates and slightly more accentuated in *Alternaria* sp. isolate 2, *E. nigrum* isolate 3, *Fusarium* sp. isolate 2 (Table 5).

As for the agar diffusible compounds test, greater differences were observed, from the 14 isolates tested, only five isolates produced compounds capable of inhibiting the growth of *C. acutatum*. Among these are the two isolates of *Alternaria* sp. that reached inhibition percentages of 26.8% and 10.7% when the highest solution volume (200 μl) was used. This observation is in line with recent studies that highlight the ability of some *Alternaria* sp. isolates to produce bioactive metabolites with antifungal activities (Lou et al., 2013). Other isolates that produced compounds capable of reducing *C. acutatum* growth, included *Diaporthe* sp., *N. oryzae* and the unidentified Xylariaceae. The lack of results on the rest of the isolates tested may be due to lack of production of substances with antifungal activity in general or in particular against *C. acutatum* or in insufficient concentrations to produce a positive inhibitory result. Agar diffusible substances produced by these isolates were identified through gas chromatography techniques. The highest variety of compounds (nine) was detected in *N. oryzae* and in the Xylariaceae member and the lowest (two) in *Alternaria* sp. isolate 1.

All compounds detected are known to have some antifungal properties (Carter et al., 1958; Morath et al., 2012; Yadav et al., 2011). The benzyl alcohol, detected only in *Alternaria* sp. isolate 2, may be responsible for the highest inhibition of *C. acutatum* growth caused by this isolate, either by itself or in association with the other compounds. The compounds 4-methylquinazoline and benzothiazole, only found in the Xylariaceae member, may be responsible for the second highest inhibition of *C. acutatum*.

Two families of compounds, pyrazine and propanoic acid derivatives, already known as substances with antifungal properties (Morath et al., 2012), were detected in all the fungi tested.

Identical compounds were detected in samples from *N. oryzae* and *Diaporthe* sp. with the exception of benzaldehyde derivatives and ketones compounds that were only detected in *N. oryzae*. These benzaldehyde derivatives and ketones compounds however do not seem to enhance *N. oryzae* antifungal activity against *C. acutatum*.

It is of great interest to test these fungal compounds individually, namely benzyl alcohol produced by *Alternaria* sp. isolate 2, or combined and at higher doses to determine their possible use as control substances of *C. acutatum*.

Interestingly, fungi that produced more antagonistic compounds against *C. acutatum* usually presented the lowest inhibition percentage values in the direct inhibition test, suggesting that, although fungi may have different competition strategies they may have a predominant one, which may depend on the conditions and/or pathogen. For example, some isolates of *A. niger* have shown to produce inhibitory compounds against a variety of fungi (Zhi-Lin et al., 2012), however the isolate used in this study did not stand out in the production of compounds capable of inhibiting *C. acutatum* growth, and *A. niger* was more successful in inhibiting *C. acutatum* growth through a rapid growth and competition for space/nutrients.

In agriculture, there is an increased interest in the use of biological control agents. Many species of organisms have been studied as antagonists as a wide range of pathogens, however the study of antagonists against *C. acutatum*, is limited. The outcome of this study is particularly useful in the identification of likely fungal isolates, or substances, candidates for *C. acutatum* biocontrol as well in understanding the mechanisms they use to reduce pathogen growth.

This study increases the knowledge of fungal communities and the substances they produce that may be used in biological control of *C. acutatum*, the causal agent of the most important fungal disease of olive fruits worldwide, olive anthracnose. Current control measures are based on preventive use of chemical treatments, selection of resistant cultivars and early harvesting (Cacciola et al., 2012). This study contributes to the development and implementation of environmental friendly management strategies by reducing the excess of fungicide on crop plants, which is one of the main concerns of public health.

Acknowledgments

This work was supported by Inalentejo ALENT-07-0324-FEDER-001747, OPERAÇÃO: Gestão Integrada da Proteção do Olival Alentejano. Contributos para o seu desenvolvimento e implementação. Carla Marisa R. Varanda is recipient of a PhD fellowship from Fundação para a Ciência e a Tecnologia (FCT), SFRH/BPD/76194/2011, financed by QREN – POPH – Typology 4.1—co-financed by MES national funding and the European Social Fund. This work has been supported by FEDER and National funds, through the Programa Operacional Regional do Alentejo (InAlentejo) Operation ALENT-07-0262-FEDER-001871/Laboratório de Biotecnologia Aplicada e Tecnologias Agro-Ambientais.

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