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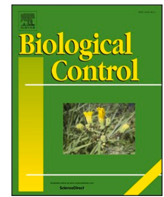

Potential of the endophyte *Penicillium commune* in the control of olive anthracnose via induction of antifungal volatiles in host plant

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HIGHLIGHTS

- Olive anthracnose (caused by *Colletotrichum* spp.) is a key fruit disease of olive crop.
- The endophyte *Penicillium commune* CIMO 14FM009 protects olive tree against *C. nymphaeae*.
- This effect was ascribed to the induction of plant volatile organic compounds (VOCs).
- Some VOCs groups reduce the growth and sporulation of *C. nymphaeae*.
- Both *P. commune* and VOCs could be promising tools to control olive anthracnose.

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ABSTRACT

Olive anthracnose, caused by several *Colletotrichum* species, is the most economically harmful fruit disease of the olive crop. This work aimed to evaluate the ability of the endophyte *Penicillium commune* CIMO 14FM009 to protect the olive tree against *Colletotrichum nymphaeae* via induction of plant volatile organic compounds (VOCs). Accordingly, olive tree branches were inoculated with the endophyte and one month later with the pathogen. After 0, 3, and 24 h of pathogen inoculation, the volatile composition of leaves and fruits was analyzed by HS-SPME-GC/MS, and compared with controls (branches inoculated with buffer, endophyte, or pathogen). The effect of plant-derived volatiles on *C. nymphaeae* was also evaluated. *Penicillium commune* induced the release of VOCs on the olive trees, with the capacity to reduce significantly the growth (up to 1.4-fold) and sporulation (up to 1.2-fold) of *C. nymphaeae*. This effect was most notorious on olives than on leaves, and occurred 3 h after pathogen-challenge, suggesting the need for a stressful stimulus for the production of antifungal VOCs. The observed inhibition was associated to a specific set of VOCs released from olives (mostly belonging to the alcohols and esters chemical classes) and leaves (mostly belonging to the alkenes). Curiously, a set of VOCs belonging to alkene, alkane and ester classes, were emitted exclusively in olive branches inoculated with *C. nymphaeae*. These findings provide new possibilities for controlling olive anthracnose using *P. commune* and/or volatiles, which efficacy should be tested in future works.

1. Introduction

The cultivated olive tree (*Olea europaea* L. subsp. *europaea*) is the most emblematic and important tree crop in the Mediterranean basin, where 98% of the world's olive oil is currently produced (Rallo et al., 2018). Portugal produces more than 3 % of the world's olive oil production (FAOSTAT, 2020), reaching exportations of 253 735 tonnes in the 2021/2022 crop year for an estimated value of €893.7 million (IOC, 2023). Alentejo, in the south of the country, is the leading production region, accounting for 72% of the olive oil production, followed by Trás-

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os-Montes (12% of olive oil production) (INE, 2022). Nonetheless, this crop is affected by several diseases that often lead to severe yield losses, with olive anthracnose being one of the most devastating (Talhinhas et al., 2018). This disease affects primarily fruits and often reaches epidemic levels causing total yield losses in the major olive oil-producing countries of the Mediterranean basin (Talhinhas et al., 2011; Cacciola et al., 2012; Talhinhas et al., 2018). In anthracnose endemic regions of Portugal, Spain and Italy, the disease can cause under wet or very humid conditions yield losses up to 80–100% (Talhinhas et al., 2011; Cacciola et al., 2012). Besides production losses, anthracnose is also responsible for the quality degradation of olive oils (Romero et al., 2022). Olive oil produced from anthracnose-infected fruits usually has high acidity values and peroxide content, and shows negative sensory characteristics (Peres et al., 2021; Romero et al., 2022). Such olive oils might lose the extra virgin or virgin oil qualification, having a great economic impact on the olive-oil industry (Romero et al., 2022). To date, several fungal species mainly belonging to the complexes *Colletotrichum acutatum* and *C. gloeosporioides* have been associated to olive anthracnose (Schena et al., 2017; Talhinhas et al., 2018; Moreira et al., 2021). *Colletotrichum nymphaeae*, from the *C. acutatum* complex, is the prevalent olive anthracnose pathogen in Portugal (Talhinhas et al., 2018). The management of this disease is mostly based on the use of copper-based pesticides, with limited efficacy and not compatible with sustainable production systems (Materatski et al., 2019). Therefore, there is a need to develop more effective and environmentally friendly tools to control olive anthracnose.

Recent findings provided evidence of the tremendous potential of plant-associated endophytes to improve plant growth and protection against pests and diseases (Bamisile et al., 2018; Grabka et al., 2022). This beneficial effect has been ascribed to the induction of host plant defense by the endophyte, via the induced systemic resistance (ISR) mechanism, allowing the plant to initiate a faster and stronger defense response when challenged by pathogens (Muthu Narayanan et al., 2022). Another mechanism that can protect host plants against pests is via the production of secondary metabolites either by the endophyte itself or by the host plant and induced by the endophyte (Li et al., 2022). Volatile organic compounds (VOCs) are, among the several secondary metabolites produced during endophyte-plant interactions, one of the most interesting chemical groups for future exploitation in biological control. Endophyte-derived VOCs are active in disease control as direct antimicrobial agents and as resistance inducers, preventing plant colonization by pathogens (Tilocca et al., 2020). Moreover, VOCs have the advantage over non-volatile metabolites, by spreading over large distances, thus having a long-distance range of action, and can be easily degraded (Tilocca et al., 2020).

Previous research in our group has provided a strong indication that the endophyte *Penicillium commune* (strain CIMO 14FM009), originally isolated from olive tree twigs of the cultivar Cobrançosa (which is moderately resistant to anthracnose), holds great promises as a biocontrol agent against olive anthracnose. Indeed, in dual-culture assays, this strain showed the ability to reduce significantly the growth (up to 40%), sporulation (up to 69%), and germination (up to 82%) of *Colletotrichum* spp. (Martins et al., 2013; Martins, 2020). Similarly, both the incidence and severity of anthracnose were significantly reduced by up to 70% by *P. commune* CIMO 14FM009 on bioassays with detached olives (Amaral, 2022). RNA-sequencing analysis revealed that *P. commune* CIMO 14FM009 induced in olive tree genes related to plant defense response, by producing secondary metabolites and reducing oxidative stress, in an early stage of infection (Amaral, 2022). The elucidation of the identity of these secondary metabolites is needed for more effective use of *P. commune* CIMO 14FM009 as a microbial control agent, due to their importance on plant defense response against pathogens.

Thus, this study aims to evaluate the involvement of VOCs in enhancing olive tree resistance towards anthracnose and in inhibiting *Colletotrichum nymphaeae*, provided by *P. commune* CIMO 14FM009, in field conditions.

2. Material and methods

2.1. Microbial isolates and preparation of inocula

The endophytic fungus *Penicillium commune* (strain CIMO 14FM009) and the pathogenic fungus *Colletotrichum nymphaeae* (strain CIMO 15FM003) belong to the fungal culture collection of the Mountain Research Center (CIMO-CC). The endophyte was originally isolated from symptomless twigs of anthracnose-tolerant cultivar Cobrançosa, collected in Mirandela, Trás-os-Montes region (northeast of Portugal) (Martins et al., 2016). This fungus was identified by sequencing of PCR products of the internal transcribed spacer (ITS) region (ITS1/ITS4 primers), β-tubulin (Bt2a/Bt2b primers) and calmodulin (cmd5/cmd6 primers) genes. *Colletotrichum nymphaeae* (strain CIMO 15FM003), which is one of the main causal agents of olive anthracnose in Portugal (Talhinhas et al., 2018), was previously isolated from the inner tissues of naturally infected olives (Martins et al., 2021). This strain was identified by sequencing the ITS region of rDNA by using ITS1/ITS4 primers and the degenerate primers Coll1F/Coll3Rb, as well as β-tubulin (Bt2a/Bt2b primers) and histone (CYLH3F/CYLH3R primers) genes.

Fungal inoculum used in the experiments was prepared from frozen stocks by transferring spores to Potato Dextrose Agar (PDA) medium. The fungi were grown at room temperature for up to 15 days, and the spores produced were then scraped from the agar plates with a sterile rod, and suspended on sterile 0.025% (v/v) Tween 80. The concentration of fungal spores was adjusted to $1x10^8$ conidia/mL with sterile 0.025% (v/v) Tween 80, in a Neübauer haemocytometer, under light microscope (Leica DM500), and further used as inoculum.

2.2. Inoculation of olive trees and sampling

The involvement of VOCs in enhancing olive tree resistance towards anthracnose and provided by *P. commune* was assessed in an olive orchard of the School of Agriculture of the Polytechnic Institute of Bragança, located in Bragança (41°48′24″N, 6°43′52″W), Northeast of Portugal. This orchard comprises olive trees from the cultivar Cobrançosa with more than 50 years old, at a spacing of 7 × 7 m, and it is managed through integrated production guidelines (Malavolta and Perdikis, 2018). In this orchard, five trees were selected to perform the assay in September 2021. Accordingly, four branches bearing olive fruits of the same tree were selected and inoculated as followed: (a) branch 1 – *P. commune* and one month later with *C. nymphaeae*; (b) branch 2 – *P. commune*; (c) branch 3 – *C. nymphaeae*; (d) branch 4 – mock-inoculation with 0.025% (v/v) Tween 80. Inoculation was performed by spraying individual branches, previously covered with a plastic bag, with 20 mL of fungal spore suspension (10^8 spores/mL), with the aid of a hand pump sprayer. The spray was directed mainly to the leaves and fruits. After spraying, the plastic bag was sealed around the branch with a rubber band in order to avoid inoculum runoff from the branch to the other branches and to keep out moisture. After 0, 3 and 24 h of inoculation with *C. nymphaeae*, both olives and leaves were collected and immediately used to assessed the VOCs composition and their inhibitory effect against *C. nymphaeae*. Only asymptomatic olives and leaves at maturation stage 2 (epidermis shows red spots in less than half fruit) (Hermoso et al., 2001) were used in these assays.

2.3. Antifungal activity of endophyte against C. Nymphaeae

The capacity of the VOCs released by both olives and leaves collected from the branches of the four treatments (branch 1 – inoculated with *P. commune* and one month later with *C. nymphaeae*; branch 2 – inoculated with *P. commune*; branch 3 – inoculated with *C. nymphaeae*; branch 4 – mock-inoculation) was evaluated under *in vitro* by using the dual culture method. In these assays, the antifungal activity of the VOCs was

assessed by measuring the inhibition rates in mycelial growth, sporulation and spore germination of the pathogen *C. nymphaeae*. Accordingly, one leaf or olive was placed at the center of a petri plate (9 and 5 cm diameter, respectively), whereas an inoculum of the individual pathogen *C. nymphaeae* (10 μL of $1x10^8$ conidia/mL) was placed at the center of another plate, containing potato dextrose agar medium. The two plates were tightly sealed together with Parafilm, being the plate inoculated with the pathogen used as a lid. Plates without the olive/leaf but with *C. nymphaeae* were used as controls. The experiment was performed using fifteen replicates for each treatment, accounting a total of 60 petri plates (4 treatments × 15 replicates, n = 60). All the plates were incubated at 25 ± 2 °C, under a daylight regime. The colony radius growth of the pathogen was measured daily with a ruler, for three weeks, and used to estimate the radial growth rate (mm/day) of *C. nymphaeae*. At the end of the assay, the sporulation and viability of *C. nymphaeae* was also evaluated. For sporulation assessment, spore suspensions were prepared by transferring three mycelial plugs of *C. nymphaeae* into 1 mL of sterile 0.025% (v/v) Tween-80. After vortexing for 1–2 min, the concentration of *C. nymphaeae* spores on the suspension was estimated in a Neubauer counting chamber and results were expressed in spores/mL. Subsequently, germination, as a measure of viability, was evaluated by inoculating Petri dishes (5 cm diameter) containing water agar (15 g/L agar) with the same spore suspension used to quantify sporulation. After incubation, at 25 ± 2 °C for 16 h, the percentage of germination was evaluated in a Leica DM500 microscope by counting the number of germinated and non-germinated spores, from a total of 100 spores per petri dishes.

2.4. Analysis of the production of volatile compounds

The volatiles were analyzed, at each time sampling for the five replicates per treatment, by headspace solid-phase microextraction gas chromatography coupled to mass spectrometry (HS-SPME-GC/MS), following a similar procedure used by Malheiro et al. (2018). Briefly, around 7.0 and 0.5 -gram weight of fresh olives and leaves, respectively, was placed in 50 mL vials separately, containing 10 μL of 4-methyl 2-pentanol (10.65 ppm dissolved in methanol), which was used as an internal standard. The vials with olives or leaves were then sealed with a polypropylene cap with silicon septum and were placed in a water bath at 40 °C for 5 min to release volatile compounds. Then, under the same conditions of temperature and agitation, the SPME fiber (divinylbenzene/carbonex/polydimethylsiloxane) (DVB/CAR/PDMS 50/30 μm) (Supelco, Bellefonte, PA, USA) was exposed for 30 min for adsorption of the volatile compounds in the headspace. Volatile compounds were removed from the fiber by thermal desorption (220 °C) for 1 min in the chromatograph injection port. The fiber was kept in the injection port for 10 min for cleaning and conditioning for further analysis. The gas chromatograph used was a Shimadzu GC-2010 Plus equipped with a Shimadzu GC/MS-QP2010 SE mass spectrometer detector. A TRB-5MS column (30 m × 0.25 mm × 0.25 μm) (Teknokroma, Spain) was used. The injector was set at a temperature of 220 °C, and the manual injection was performed in splitless mode. The mobile phase consisted of helium 5.0 (Linde, Portugal), at a linear velocity of 30 cm/s and a 24.4 mL/min flow rate. The oven temperature was 40 °C for 1 min, followed by an increase of 2 °C/min until reaching 220 °C. The ionization source was maintained at 250 °C with an energy of 70 eV and a current of 0.1 kV. All mass spectra were obtained by electronic ionization in the *m/z* range of 35–500. Compounds were identified by comparing the mass spectra and through the Kovats index using databases such as NIST 69 (nist.gov)), PubChem (nih.gov)) and ChemSpider (ChemSpider | Search and share chemistry). Retention indices were obtained using a commercial n-alkane series, C7-C30 (Sigma-Aldrich, St. Louis, MS, USA), by direct splitless liquid injection (1 μL), while all further conditions of GC and MS were settled for the volatile analysis. Retention indices were calculated according to the Kovats index. The identified volatile compounds were expressed based on the areas determined by TIC (total ion chromatogram) integration. The default concentration of each volatile compound was calculated using the following formula: (peak area × 0.635)/(Area of the internal standard × Mass added in g). The results were expressed in ng per g of fresh weight (FW) of fruit or leaf tissue (ng/g of FW).

2.5. Data analysis

The results of the antifungal activity and of volatile compounds are presented as the mean of each parameter accompanied by its standard error (SE) or deviation (SD), respectively. To determine differences among the means, a one-way analysis of variance (ANOVA) with PAST v4.03 software was done, and the averages were compared using Mann-Whitney's test ($p < 0.05$). Before analysis, the normality of data was checked by using Shapiro-Wilk test. The same software was used to generate the graphs.

Principal component analysis (PCA) was performed to identify the volatile compounds that best discriminate the different treatments, in each sampling time (i.e., after 0, 3 and 24 h of inoculation with *C. nymphaeae*). This analysis was performed in R software v.3.5.1 205 (R Core Team, 2021) using the function *pca* from the "FactoMineR" package (Lê et al., 2008). Next, the biplot of the two first PCs was drawn using the fviz_pca_biplot function from the "factoextra" package (Kassambara et al., 2020). PCA arrows represent the contribution of each volatile compound to the two components (length of the arrow), and the specific gradient colour denotes their contribution to the explanation of the greatest variance in the dataset.

3. Results

3.1. Antifungal activity of VOCs against C. Nymphaeae

The volatiles emitted by both olives and leaves from the four treatments were screened after 0, 3 and 24 h of inoculation with *C. nymphaeae* for antifungal activity on the growth, sporulation and viability of *C. nymphaeae* (Fig. 1). The results showed that the inoculation of olives and leaves with *P. commune* was able to produce VOCs that inhibit the mycelial growth of *C. nymphaeae*, but to varying degrees depending on the time after pathogen inoculation. Indeed, the VOCs emitted by olives inoculated with *P. commune* or with *P. commune* + *C. nymphaeae* after 3 h of pathogen inoculation showed to inhibited significantly ($p < 0.05$) the growth of the pathogen up to 1.4-fold when compared to VOCs emitted by olives inoculated with *C. nymphaeae* or tween (control) (Fig. 1A). After 24 h post-inoculation with *C. nymphaeae*, VOCs emitted by olives inoculated with *C. nymphaeae* have a significantly ($p < 0.05$) greater inhibitory effect against pathogen growth than those emitted by olives inoculated with *P. commune* + *C. nymphaeae*. In comparison to olives, only after 3 h of pathogen inoculation was observed a reduction in *C. nymphaeae* growth due to VOCs emitted by leaves inoculated with *P. commune* or with *P. commune* + *C. nymphaeae* (Fig. 1B).

Besides growth, the VOCs emitted by olives and leaves inoculated with *P. commune* were able to inhibit the sporulation of *C. nymphaeae* (Fig. 1C and D). Indeed, after 3 h of pathogen inoculation was observed that VOCs produced by olives or leaves inoculated with *P. commune* + *C. nymphaeae* reduced significantly ($p < 0.05$) the sporulation (up to 1.2-fold) of *C. nymphaeae*, in relation to the control (tween). VOCs from olives inoculated with *P. commune* caused the highest reduction in pathogen sporulation, but this effect was not statistically significant from the other treatments. This result may be related to the large differences observed in pathogen sporulation (ranging from 0 to 10^6 spores/mL) within *P. commune* inoculated olives treatment. Curiously, at this time, the inoculation of olives with *C. nymphaeae* induced the production of VOCs with ability to increased significantly ($p < 0.05$) the sporulation of *C. nymphaeae* up to 1.0-fold when compared to control (olives inoculated with tween).

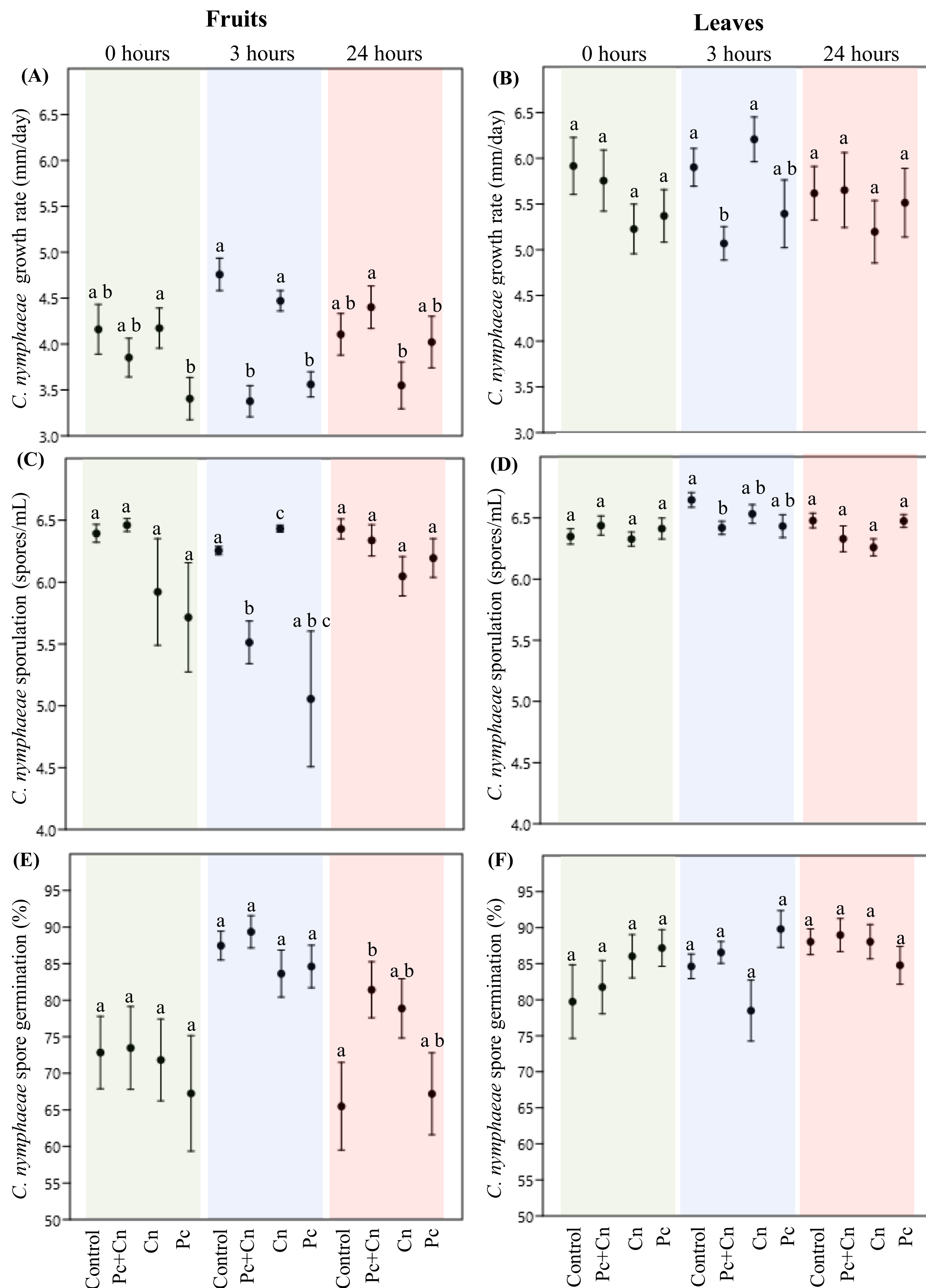

Fig. 1. Growth rate (**A**, **B**), sporulation (**C**, **D**) and germination (**E**, **F**) of *Colletotrichum nymphaeae* exposed to volatile compounds released by olives and leaves inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (Pc + Cn), *C. nymphaeae* (Cn) or *P. commune* (Pc), at 0, 3 and 24 h after pathogen inoculation. Results are expressed as mean ± SD (N = 15). Different letters denote a statistically significant difference ($p < 0.05$) between treatments, within each time of inoculation.

In contrast to growth and sporulation, *P. commune* was not able to induced the production of VOCs on olives and leaves with capacity to inhibit the viability of *C. nymphaeae* (Fig. 1E and F). Contrary to our expectations, the VOCs produced by olives treated with *P. commune* + *C. nymphaeae* (after 24 h of pathogen inoculation), showed to increased significantly ($p < 0.05$) the percentage of germination of *C. nymphaeae* up to 1.2-fold in relation to the control (olives inoculated with tween).

3.2. Volatile compounds emitted by olives and leaves

The production of VOCs by olives and leaves inoculated with *P. commune* + *C. nymphaeae* and corresponding controls (single inoculations with the endophyte, pathogen or tween) was assessed after 0, 3 and 24 h of inoculation with the pathogen. Overall, in the olives, 22 VOCs were identified, belonging to nine different chemical classes (Fig. 2A, Table 1, Table S1). Alcohols and esters were the most diversified chemical classes, with 6 different compounds each, being alkanes the most abundant, accounting from 26% to 77% of the total abundance. In the leaves, 52 VOCs were identified, belonging to nine different chemical classes (Fig. 2B, Table 2, Table S2). Esters, alkenes and alcohols, were the most diversified chemical classes, with 19, 11 and 8 different compounds, respectively, while alcohols and esters were the most abundant ones (accounting from 31% to 46% and 32% to 60%, respectively, of the total abundance).

3.2.1. Volatile profile in the different treatments

The VOCs emitted by the inoculated olives and leaves were quali- and semi-quantitatively different among the four treatments (Tables 1 and 2). The volatile profiles of the olives showed a greater number of compounds in treatments tween (17 volatiles), *P. commune* + *C. nymphaeae* and *C. nymphaeae* (each with 16 volatiles) than in *P. commune* (14 volatiles) (Table 1). Most of the identified compounds in olives were common to all four treatments (11 out of 19), being two exclusively identified in olives inoculated with *P. commune* + *C. nymphaeae*, namely acetic acid, hexyl ester and 3-octanol, 3,7-dimethyl-, and additionally one in olives inoculated with *P. commune* (dodecane, 2,6,11-trimethyl). In general, the leaves inoculated with tween, *C. nymphaeae* or *P. commune*, showed the highest number of volatiles (37, 42, and 37, respectively) (Table 2). In contrast, leaves inoculated with *P. commune* + *C. nymphaeae*, emitted the lowest number of volatiles (33). Half of the compounds identified in leaves were common to all four treatments (26 out of 52), being four exclusively identified in leaves inoculated solely with *P. commune* [2,4-hexadienal, (E, E)-;.beta.-ocimene; undecane and propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester] and one in leaves inoculated with *P. commune* + *C. nymphaeae* [naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1. alpha.,7.alpha.,8a.alpha.)]-]. 2-Pentene, 1-ethoxy-4-methyl-, (Z)-; octane, 1-ethoxy-; 1,4-hexadiene, 5-methyl-3-(1-methylethylidene)-; levomenthol; 4-*tert*-butylcyclohexyl acetate and decanoic acid, methyl ester, were uniquely emitted by leaves inoculated with *C. nymphaeae*.

3.2.2. Identification of VOC candidates for C. nymphaeae control

To identify which VOCs are characteristics of each treatment, a principal component analysis (PCA) was performed with olive and leaf VOCs analyzed after 0, 3, and 24 h of pathogen inoculation (Figs. 3 and 4). The results obtained from olives showed changes in the volatile profiles among treatments, for all the survey sampling times (Fig. 3). Indeed, the PCA for 0 h showed a clear separation of *P. commune* + *C. nymphaeae* from the other treatments due to the production of V4 (p-xylene), while the treatments *P. commune* or *C. nymphaeae* were clustered together due to the emission of V8 (1-hexanol, 2-ethyl-) and V15 (levomenthol). However, the largest differences among treatments was observed at 3 h after pathogen inoculation. At this time, the PCA showed that *P. commune* + *C. nymphaeae* treatment was distinct from the other treatments mostly due to the production of V10 (7-octen-2-ol, 2,6-dimethyl-), V11 (3-octanol, 3,7-dimethyl-) and V17 (propanoic acid, 2-methyl-, 3-methyl-2-butenyl ester), while *P. commune*, *C. nymphaeae* and control treatments were characterized due to the production of V22 (dodecane, 2,6,11-trimethyl-), V6 [3-hexen-1-ol, acetate, (Z)-] and V1 (trichloromethane), respectively. At 24 h, PCA grouped the *P. commune* + *C. nymphaeae* with *C. nymphaeae* treatments, indicating that they have

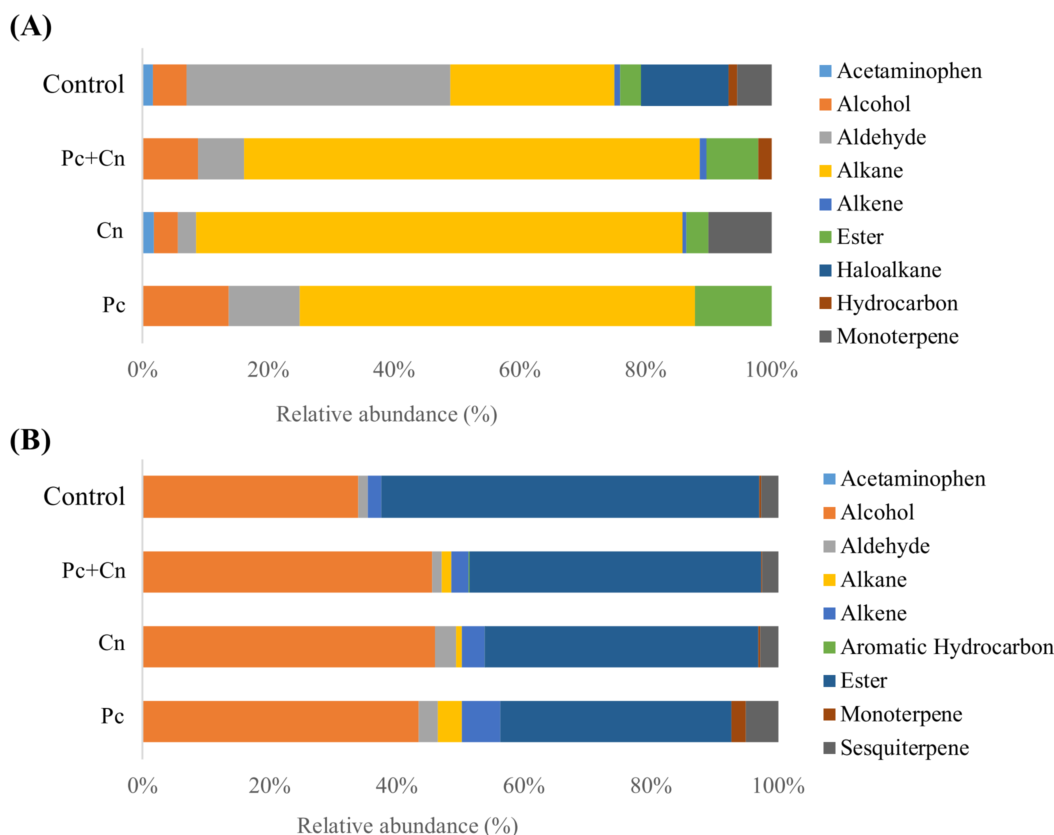

Fig. 2. Relative abundance (%) of volatile organic compounds per chemical classes identified in olives (**A**) or leaves (**B**) inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (Pc + Cn), *C. nymphaeae* (Cn) or *P. commune* (Pc), at 0, 3 and 24 h after pathogen inoculation.

Table 1
Volatile profile of olives inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (*P. commune* + *C. nymphaeae*), *C. nymphaeae* or *P. commune*, at 0, 3 and 24 h after pathogen inoculation. The results are expressed as nanograms per gram FW (mean ± SD, N = 5).

N°	Compound	Control			*P. commune* + *C. nymphaeae*			*C. nymphaeae*			*P. commune*		
		0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours
1	Trichloromethane	–	15.68 ± 3.25	–	–	–	–	–	–	–	–	–	–
2	Pentane, 2,2,4-trimethyl-	9.38 ± 4.54	44.94 ± 19.82	27.55 ± 7.25	135.96 ± 89.25	12.01 ± 0.54	10.70 ± 2.35	19.04 ± 7.37	304.34 ± 168.03	42.45 ± 24.53	36.53 ± 23.31	32.03 ± 8.00	17.22 ± 5.86
3	3-Hexen-1-ol, (Z)-	–	2.95 ± 0.59	2.85 ± 0.45	–	2.07 ± 0.35	–	–	2.46 ± 0.59	–	–	1.93 ± 0.19	1.08 ± 0.13
4	p-Xylene	0.85 ± 0.15	–	2.15 ± 0.32	1.99 ± 0.17	–	0.98 ± 0.19	–	–	–	–	–	–
5	Oxime-, methoxy-phenyl-	–	–	1.88 ± 0.23	–	–	–	2.79 ± 1.66	–	–	–	–	–
6	3-Hexen-1-ol, acetate, (Z)-	–	1.77 ± 0.52	1.49 ± 0.27	–	1.14 ± 0.11	0.64 ± 0.07	–	1.35 ± 0.36	0.87 ± 0.29	–	–	0.60 ± 0.11
7	Acetic acid, hexyl ester	–	–	–	–	–	0.68 ± 0.04	–	–	–	–	–	–
8	1-Hexanol, 2-ethyl-	–	1.14 ± 0.22	1.29 ± 0.17	–	1.34 ± 0.10	0.88 ± 0.17	1.45 ± 0.17	1.10 ± 0.03	0.93 ± 0.09	1.89 ± 0.79	–	–
9	.beta.-Ocimene	0.97 ± 0.26	10.67 ± 3.68	6.63 ± 2.73	–	–	–	11.41 ± 7.85	21.38 ± 14.79	14.71 ± 9.56	–	–	–
10	7-Octen-2-ol, 2,6-dimethyl-	–	1.32 ± 0.11	0.85 ± 0.08	1.17 ± 0.28	1.83 ± 0.10	1.01 ± 0.19	1.53 ± 0.25	1.48 ± 0.10	0.97 ± 0.19	1.41 ± 0.31	1.29 ± 0.17	–
11	3-Octanol, 3,7-dimethyl-	–	–	–	–	0.97 ± 0.29	–	–	–	–	–	–	–
12	Undecane	3.99 ± 1.63	–	0.50 ± 0.09	–	–	–	–	–	–	–	–	–
13	Nonanal	129.11 ± 92.26	5.11 ± 2.11	2.85 ± 0.21	3.26 ± 0.75	6.89 ± 3.01	2.86 ± 0.68	3.79 ± 0.79	3.90 ± 1.32	2.69 ± 0.45	3.61 ± 0.75	5.39 ± 2.48	3.35 ± 0.95
14	Furan, 3-(4-methyl-3-pentenyl)-	1.47 ± 0.58	–	0.71 ± 0.12	0.94 ± 0.27	0.79 ± 0.10	0.66 ± 0.13	0.92 ± 0.22	0.92 ± 0.31	0.94 ± 0.10	–	–	–
15	Levomenthol	–	1.00 ± 0.10	0.75 ± 0.10	–	1.00 ± 0.12	0.82 ± 0.21	0.93 ± 0.14	0.98 ± 0.13	0.71 ± 0.15	0.85 ± 0.24	–	–
16	Decanal	2.49 ± 0.75	1.11 ± 0.30	0.83 ± 0.13	0.95 ± 0.17	1.53 ± 0.45	0.83 ± 0.21	1.05 ± 0.14	1.03 ± 0.24	0.79 ± 0.10	1.01 ± 0.26	1.28 ± 0.44	0.90 ± 0.26
17	Propanoic acid, 2-methyl-, 3-methyl-2-butenyl ester	–	–	–	0.95 ± 0.20	0.78 ± 0.10	0.61 ± 0.06	0.91 ± 0.21	–	0.55 ± 0.07	0.64 ± 0.16	–	–
18	4-*tert*-Butylcyclohexyl acetate	–	0.77 ± 0.06	0.54 ± 0.10	0.64 ± 0.11	0.78 ± 0.03	0.54 ± 0.14	0.78 ± 0.17	0.78 ± 0.05	0.59 ± 0.14	0.73 ± 0.18	0.62 ± 0.06	–
19	Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	–	–	0.57 ± 0.11	2.18 ± 1.15	0.70 ± 0.14	0.65 ± 0.11	1.97 ± 1.10	0.73 ± 0.08	0.54 ± 0.04	1.43 ± 0.84	2.47 ± 1.50	0.48 ± 0.07
20	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	0.53 ± 0.08	1.13 ± 0.20	1.04 ± 0.30	3.60 ± 1.82	0.91 ± 0.04	0.92 ± 0.19	3.34 ± 1.75	1.31 ± 0.23	0.82 ± 0.09	2.14 ± 1.33	3.83 ± 2.26	0.71 ± 0.11
21	1-Dodecanol	–	–	–	–	–	–	–	–	0.29 ± 0.02	–	0.75 ± 0.30	–
22	Dodecane, 2,6,11-trimethyl-	–	–	–	–	–	–	–	–	–	–	0.52 ± 0.11	–

"-"—not detected.

a similar volatile profile. The common feature for these two treatments was the production of V14 [furan, 3-(4-methyl-3-pentenyl)-], being *P. commune* + *C. nymphaeae* additionally distinguish from the other treatments due to the exclusive production of V7 (acetic acid, hexyl ester). These two treatments were, in turn, separated from *P. commune* and control.

The PCA score plots of leaves showed clear differences between the four treatments, only after 3 h of pathogen inoculation (Fig. 4). At this time, samples were grouped according to the treatment, with the exception of *P. commune* + *C. nymphaeae* and *P. commune* treatments that clustered together and thus seeming to have a similar VOCs profile. These two treatments were distinct from the control and *C. nymphaeae* treatment due to the exclusive production of V27 (1,5-heptadiene, 3,6-dimethyl-). The VOCs V42 [propanoic acid, 2-methyl-, 2, 2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester], solely produced in *P. commune* treatment, as well as V47 [1,6-octadiene, 2,5-dimethyl-, (E)-] and V51 [naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, 1S-(1. alpha.,7.alpha.,8a.alpha.)-], detected only in *P. commune* + *C. nymphaeae* treatment, have also a large contribution to distinguish these treatments from the others. In turn, leaves inoculated with *C. nymphaeae* were distinct from the other treatments mainly due to the production of V22 (octane, 1-ethoxy-), V31 (2,4,6-octatriene, 2,6-dimethyl-), V33 (levomenthol), V40 (4-*tert*-butylcyclohexyl acetate) and V41 (decanoic acid, methyl ester). Interestingly, most of these compounds (V22, V33, V40, and V41) were exclusively detected in this treatment. The control treatment was characterized mostly by VOCs V15 (1-hexanol, 2-ethyl-), V39 (n-valeric acid *cis*-3-hexenyl ester), and V46 (*trans*-3-hexen-1-ol, trifluoroacetate). In contrast to 3 h, at 0 and 24 h after pathogen inoculation, the volatile profile of the four treatments was almost similar. Despite this, a set of volatile compounds were found to be characteristic of a particular treatment. For instance, at 0 h, V27 (1,5-heptadiene, 3,6-dimethyl-) was characteristic of *P. commune* + *C. nymphaeae* treatment. Similarly, *C. nymphaeae* treatment was characterized mostly by V26 (phenylethyl alcohol) and V43 (propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl), while control treatment was characterized by the production of V12 (acetic acid, hexyl ester). In

Table 2
Volatile profile of leaves inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (*P. commune* + *C. nymphaeae*), *C. nymphaeae* or *P. commune*, at 0, 3 and 24 h after pathogen inoculation. The results are expressed as nanograms per gram FW (mean ± SD, N = 5).

N°	Compound	Control			*P. commune* + *C. nymphaeae*			*C. nymphaeae*			*P. commune*		
		0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours
1	Pentane, 2,2,4-trimethyl-	–	–	–	636.80 ± 347.42	1594.03 ± 1057.92	207.47 ± 54.11	390.99 ± 135.34	535.28 ± 234.43	278.28 ± 126.16	1509.99 ± 1225.10	1334.26 ± 943.25	545.48 ± 274.52
2	Butanoic acid, methyl ester	73.19 ± 12.45	–	158.96 ± 40.18	–	217.93 ± 133.40	–	–	–	92.42 ± 38.99	–	–	–
3	4-Pentenal, 2-methyl-	–	693.94 ± 275.00	101.38 ± 20.65	–	888.14 ± 496.95	350.26 ± 195.66	1233.51 ± 601.27	1843.66 ± 1036.00	117.37 ± 52.08	378.13 ± 134.99	1596.98 ± 926.12	276.59 ± 150.86
4	3-Hexen-1-ol, (Z)-	6567.96 ± 798.01	36457.79 ± 8519.06	8539.46 ± 1746.64	22230.36 ± 12171.04	34221.84 ± 12580.94	13096.58 ± 4972.98	21467.02 ± 9502.56	29427.48 ± 16786.06	6056.76 ± 2417.48	8937.20 ± 1908.82	24867.21 ± 14971.96	8278.02 ± 3331.33
5	1-Hexanol	326.38 ± 55.01	3920.10 ± 1312.85	452.89 ± 101.01	1336.03 ± 717.98	2190.61 ± 837.60	873.21 ± 416.04	1347.72 ± 535.08	1987.66 ± 1157.86	338.05 ± 192.79	464.54 ± 76.99	1347.09 ± 843.94	587.15 ± 313.68
6	Oxime-, methoxy-phenyl-_	47.31 ± 15.34	–		–	–	–	–	–	–	–	–	–
7	2,4-Hexadienal, (E,E)-	–	–	–	–	–	–	–	–	–	–	117.16 ± 63.89	–
8	Hexanoic acid, methyl ester	195.92 ± 37.83	936.21 ± 241.57	556.84 ± 209.38	553.00 ± 179.91	790.31 ± 261.71	362.35 ± 120.86	267.59 ± 65.58	553.02 ± 294.68	191.16 ± 95.42	200.42 ± 56.48	323.47 ± 172.72	–
9	3-Hexenoic acid, methyl ester, (E)-	3216.09 ± 776.60	15447.61 ± 4529.58	4140.11 ± 730.03	11378.13 ± 4952.44	11875.20 ± 5498.82	9186.31 ± 4042.54	6594.31 ± 2123.14	9181.51 ± 5840.11	2721.49 ± 1593.40	2744.41 ± 743.99	6023.31 ± 3259.21	4963.49 ± 2634.95
10	2-Hexenoic acid, methyl ester	63.61 ± 11.09	590.15 ± 174.43	93.81 ± 18.54	304.86 ± 176.02	275.49 ± 99.98	–	143.23 ± 46.06	283.00 ± 198.01	–	56.48 ± 14.02	139.31 ± 99.28	–
11	3-Hexen-1-ol, acetate, (Z)-	16020.99 ± 2637.01	33656.98 ± 4617.98	15644.35 ± 4869.91	9357.37 ± 787.59	16698.11 ± 1821.13	7367.97 ± 3037.81	16142.44 ± 5034.30	10275.89 ± 3202.10	6081.89 ± 2706.77	5949.72 ± 2954.37	4879.99 ± 3049.57	6558.97 ± 4630.89
12	Acetic acid, hexyl ester	135.28 ± 20.64	486.17 ± 180.62	197.27 ± 64.27	121.38 ± 40.55	228.91 ± 73.85	–	–	130.73 ± 40.34	81.65 ± 38.31	62.26 ± 15.53	113.22 ± 39.09	108.72 ± 62.67
13	4-Hexen-1-ol, (4E)-, acetate	70.03 ± 13.99	–	–	–	–	–	–	–	–	–	–	–
14	o-Cymene	–	–	–	–	–	38.37 ± 7.90	–	–	35.26 ± 12.54	–	–	–
15	1-Hexanol, 2-ethyl-	58.67 ± 4.95	112.92 ± 13.52	46.07 ± 6.03	–	–	–	50.21 ± 8.03	54.41 ± 16.65	–	–	–	30.98 ± 13.07
16	Benzyl alcohol	–	–	–	–	–	–	73.37 ± 23.68	65.20 ± 29.43	19.46 ± 5.95	21.43 ± 2.20	–	28.85 ± 11.97
17	trans-.beta.-Ocimene	77.70 ± 17.18	185.01 ± 80.88	–	–	–	66.34 ± 29.54	220.61 ± 111.82	100.99 ± 51.84	41.96 ± 3.85	59.38 ± 23.23	–	–
18	.beta.-Ocimene	–	–	–	–	–	–	–	–	–	700.13 ± 241.26	–	–
19	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	522.05 ± 127.44	1309.05 ± 611.30	317.74 ± 87.18	–	473.55 ± 267.71	506.05 ± 217.26	1789.47 ± 904.86	717.73 ± 184.64	356.36 ± 44.88	675.38 ± 247.53	543.70 ± 389.38	303.39 ± 232.44
20	2-Pentene, 1-ethoxy-4-methyl-, (Z)-	–	–	–	–	–	–	–	141.60 ± 77.33	–	–	–	–
21	1-Octanol	45.68 ± 10.62	136.92 ± 34.12	41.21 ± 8.02	42.99 ± 10.41	–	32.68 ± 8.21	48.23 ± 10.59	–	36.25 ± 16.42	22.88 ± 3.72	66.38 ± 25.45	–
22	Octane, 1-ethoxy-	–	–	–	–	–	–	–	48.36 ± 9.92	–	–	–	–
23	Undecane	–	–	–	–	–	–	–	–	–	–	152.48 ± 107.63	–
24	3-Hexen-1-ol, propanoate, (Z)-	158.66 ± 24.37	452.27 ± 66.67	207.54 ± 54.07	124.30 ± 18.13	349.85 ± 116.83	106.11 ± 31.16	162.82 ± 46.28	134.51 ± 41.70	71.30 ± 27.43	182.92 ± 98.37	–	–
25	Nonanal	149.03 ± 40.54	946.37 ± 577.23	192.56 ± 90.73	176.01 ± 63.62	214.28 ± 83.06	154.86 ± 73.63	265.98 ± 63.12	525.82 ± 243.85	109.77 ± 25.56	112.41 ± 29.03	186.33 ± 23.71	140.59 ± 50.66

(*continued on next page*)

Table 2 (*continued*)

N°	Compound	Control			*P. commune* + *C. nymphaeae*			*C. nymphaeae*			*P. commune*		
		0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours
26	Phenylethyl Alcohol	–	136.38 ± 48.82	–	–	83.56 ± 35.53	–	82.80 ± 35.53	47.33 ± 27.88	16.87 ± 9.26	18.81 ± 3.97	104.29 ± 71.57	19.94 ± 8.06
27	1,5-Heptadiene, 3,6-dimethyl-	–	–	–	207.18 ± 68.42	1164.90 ± 790.92	–	–	–	–	–	714.34 ± 561.65	–
28	Furan, 3-(4-methyl-3-pentenyl)-	406.94 ± 213.88	244.13 ± 42.02	237.57 ± 92.56	–	–	–	306.68 ± 76.13	237.44 ± 62.70	134.48 ± 43.01	1131.92 ± 751.74	–	393.76 ± 274.36
29	Octanoic acid, methyl ester	63.45 ± 7.11	152.52 ± 21.39	74.80 ± 8.43	135.45 ± 63.56	109.62 ± 30.65	57.23 ± 14.23	83.28 ± 15.65	75.80 ± 27.90	36.24 ± 13.25	65.41 ± 13.57	73.32 ± 31.33	62.70 ± 34.68
30	1,4-Hexadiene, 5-methyl-3-(1-methylethylidene)-	–	–	–	–	–	–	136.82 ± 63.09	74.75 ± 24.79	–	–	–	–
31	2,4,6-Octatriene, 2,6-dimethyl-	36.04 ± 9.06	–	–	–	–	29.03 ± 12.47	83.82 ± 40.54	46.34 ± 10.34	20.75 ± 2.86	33.16 ± 7.92	–	–
32	Butanoic acid, 3-hexenyl ester, (Z)-	60.94 ± 6.89	292.37 ± 48.51	118.63 ± 35.90	54.62 ± 8.96	147.07 ± 33.76	52.59 ± 21.14	81.06 ± 31.30	84.80 ± 28.29	39.81 ± 17.09	71.80 ± 42.91	102.23 ± 77.96	74.36 ± 55.85
33	Levomenthol	–	–	–	–	–	–	–	45.78 ± 14.55	28.23 ± 12.76	–	–	–
34	Not identified	477.67 ± 93.71	2601.35 ± 452.82	972.47 ± 283.41	673.25 ± 272.02	1994.47 ± 881.80	291.40 ± 98.55	710.99 ± 217.21	820.55 ± 229.14	334.18 ± 122.42	781.08 ± 429.09	1290.75 ± 960.42	701.07 ± 486.65
35	Decanal	36.84 ± 11.44	148.66 ± 65.11	42.67 ± 18.73	47.60 ± 17.26	–	43.32 ± 19.70	63.70 ± 16.02	111.32 ± 53.66	29.87 ± 8.58	28.66 ± 8.24	54.97 ± 24.08	–
36	2-Dodecene, (E)-	–	–	34.39 ± 7.23	–	–	17.10 ± 2.72	30.66 ± 5.70	–	–	31.37 ± 7.10	29.29 ± 14.24	22.59 ± 7.76
37	Nonanoic acid, methyl ester	–	50.40 ± 11.08	24.53 ± 6.11	71.56 ± 48.78	–	15.26 ± 3.66	70.48 ± 23.57	40.37 ± 9.42	16.15 ± 4.14	31.75 ± 4.95	41.83 ± 7.75	23.01 ± 7.92
38	*cis*-3-Hexenyl-.alpha.-methylbutyrate	–	973.68 ± 213.71	514.94 ± 173.00	247.65 ± 52.72	630.49 ± 185.51	213.89 ± 81.45	327.78 ± 135.15	304.43 ± 80.46	150.41 ± 62.68	365.92 ± 237.17	344.08 ± 262.69	305.10 ± 233.33
39	n-Valeric acid *cis*-3-hexenyl ester	277.99 ± 57.93	85.93 ± 30.81	–	–	–	–	–	–	–	–	–	–
40	4-*tert*-Butylcyclohexyl acetate	–	–	–	–	–	–	–	25.35 ± 4.74	19.78 ± 8.33	–	–	–
41	Decanoic acid, methyl ester	–	–	–	–	–	–	48.47 ± 20.19	20.12 ± 6.81	–	–	–	–
42	Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	–	–	–	–	–	–	–	–	–	–	41.20 ± 15.72	–
43	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl	–	49.60 ± 5.06	–	–	–	–	60.25 ± 28.27	23.72 ± 7.89	15.44 ± 5.84	31.87 ± 11.51	63.69 ± 25.71	63.59 ± 28.17
44	Copaene	24.69 ± 1.85	94.88 ± 22.93	26.03 ± 5.14	37.51 ± 17.56	–	16.36 ± 3.85	30.26 ± 6.67	21.27 ± 6.85	12.52 ± 3.89	17.52 ± 3.30	16.02 ± 5.68	14.20 ± 2.73
45	Hexanoic acid, 3-hexenyl ester, (Z)-	–	65.99 ± 19.99	–	–	–	–	–	24.55 ± 11.78	–	–	–	–
46	*trans*-3-Hexen-1-ol, trifluoroacetate	–	158.43 ± 23.67	–	–	–	–	45.70 ± 13.51	65.78 ± 35.21	–	–	–	–
47	1,6-Octadiene, 2,5-dimethyl-, (E)-	–	–	37.92 ± 8.77	–	124.08 ± 41.37	–	–	–	–	–	–	–
48	Caryophyllene	535.72 ± 246.20	2242.60 ± 605.95	498.57 ± 67.13	893.46 ± 348.33	1704.17 ± 833.10	343.37 ± 113.41	1053.70 ± 302.86	949.70 ± 112.79	289.36 ± 91.56	1423.09 ± 614.48	1193.81 ± 797.11	651.40 ± 304.58
49	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	45.02 ± 11.55	117.08 ± 25.50	28.32 ± 3.51	45.28 ± 13.76	93.89 ± 41.62	22.93 ± 7.94	–	49.48 ± 4.52	–	70.00 ± 29.57	58.86 ± 37.45	33.87 ± 15.45
50	Humulene	97.66 ± 38.84	276.58 ± 92.06	56.18 ± 5.15	103.34 ± 36.54	238.36 ± 131.33	38.07 ± 13.67	118.47 ± 32.74	106.94 ± 22.12	30.91 ± 10.25	199.29 ± 100.46	177.63 ± 125.82	84.52 ± 41.33
51	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-	–	–	–	–	63.00 ± 22.20	–	–	–	–	–	–	–

(*continued on next page*)

Table 2 (*continued*)

N°	Compound	Control			*P. commune* + *C. nymphaeae*			*C. nymphaeae*			*P. commune*		
		0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours	0 Hours	3 Hours	24 Hours
	methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-												
52	.alpha.-Farnesene	–	350.59 ± 108.64	93.25 ± 27.66	183.54 ± 100.77	449.79 ± 242.50	72.18 ± 25.10	475.06 ± 211.20	282.78 ± 91.53	–	837.47 ± 595.60	427.36 ± 329.73	257.37 ± 196.22

"-"—not detected.

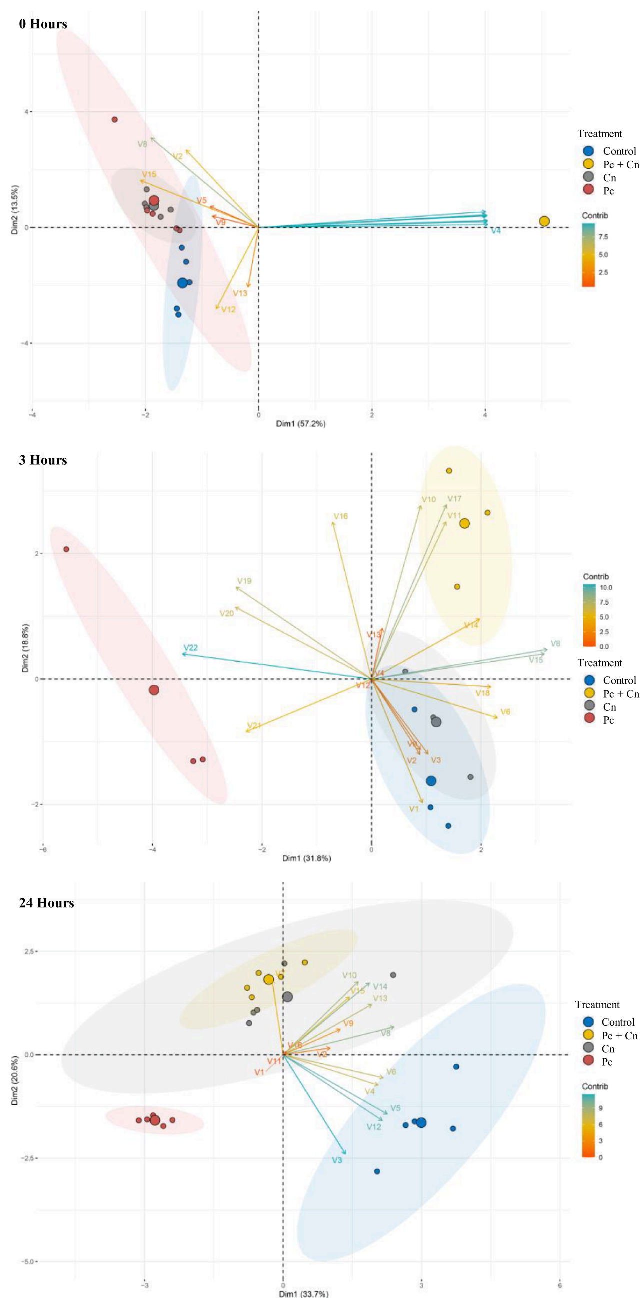

Fig. 3. Principal Component Analysis score plots obtained from the volatile profile of olives inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (Pc + Cn), *C. nymphaeae* (Cn) or *P. commune* (Pc), at 0, 3 and 24 h after pathogen inoculation. Each V-number corresponds to a particular volatile compound, as indicated in Table 1. Gradient color represents the contribution of each variable (i.e., volatile compounds) to the explanation of most variance in the data set. Each circle represents treatments, being the ones with the greater size representing the average.

turn, at 24 h, both *P. commune* + *C. nymphaeae* and *C. nymphaeae* were characterized by the production of V14 (o-cymene), V17 (trans-.beta.-ocimene), and V31 (2,4,6-octatriene, 2,6-dimethyl-), while *P. commune* and control were characterized by the emission of V43 (propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl), and V36 [2-dodecene, (E)-], respectively.

4. Discussion

The own endophyte strain *P. commune* (CIMO 14FM009) has

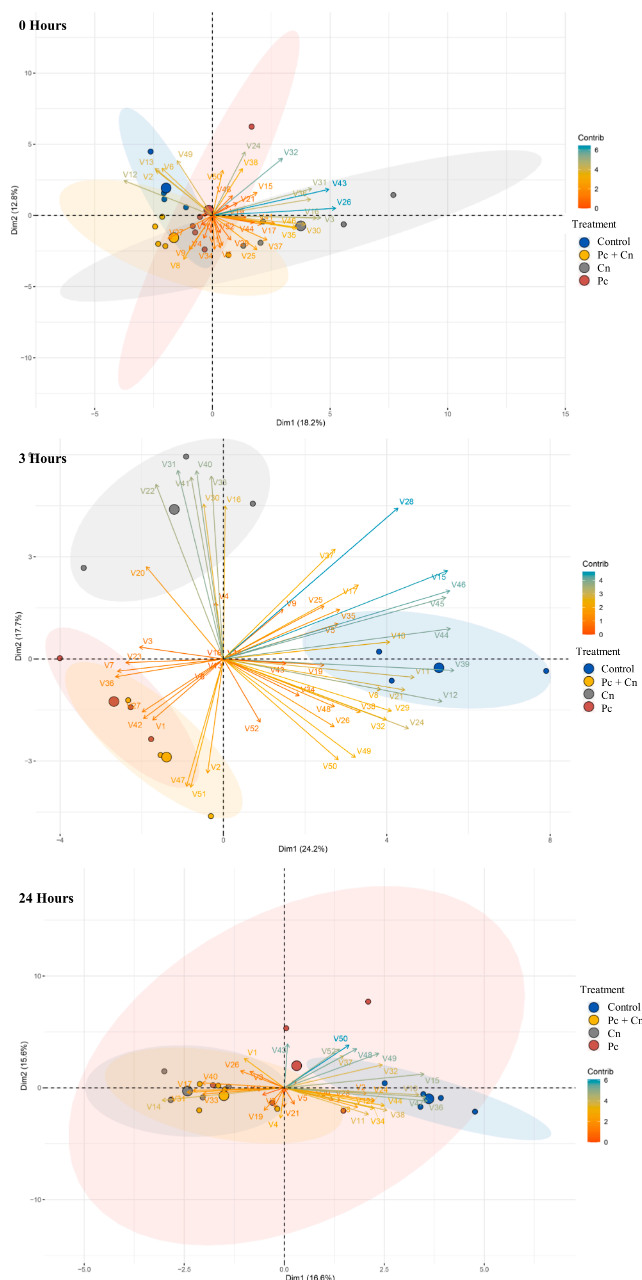

Fig. 4. Principal Component Analysis score plots obtained from the volatile profile of leaves inoculated with Tween (Control), *Penicillium commune* and *Colletotrichum nymphaeae* (Pc + Cn), *C. nymphaeae* (Cn) or *P. commune* (Pc), at 0, 3 and 24 h after pathogen inoculation. Each V-number corresponds to a particular volatile compound, as indicated in Table 2. Gradient color represents the contribution of each variable (i.e., volatile compounds) to the explanation of most variance in the data set. Each circle represents treatments, being the ones with the greater size representing the average.

previously revealed potential to act as biocontrol agent against *Colletotrichum* spp. under *in vitro* conditions, either using dual cultures (Martins et al., 2013; Martins, 2020) or bioassays with detached olives (Amaral, 2022). In this study, the effectiveness of this endophyte to protect olive tree against olive anthracnose was tested under field conditions. Overall, the results showed that *P. commune* (strain CIMO 14FM009) protects the olive tree from anthracnose by inducing the release of volatile organic compounds in the host plant, with the capacity to reduce both the growth and sporulation of the pathogen *C. nymphaeae*. The antimicrobial potential of endophytic strains of *P. commune* was previously reported against a number of phytopathogens, such as *Sclerotinia* sp. (Katoch and Pull, 2017), *Botrytis cinerea* (Miles et al., 2012) and *Pyricularia oryzae* (Hosseyni et al., 2013), under *in vitro* conditions. In our study, the VOCs with the greatest antifungal activity against *C. nymphaeae* were released from olives, suggesting that *P. commune* had a greater protective effect on fruits than on leaves. This is particularly important for olive anthracnose, since it affects mainly fruits during ripening (Talhinhas et al., 2011; Cacciola et al., 2012; Talhinhas et al., 2018). Also, the induction of antifungal VOCs by *P. commune* was observed particularly on pathogen-exposed plants and at 3 h after the pathogen challenge. Thus, the presence of a stressful stimulus seems to be an essential requisite for the production of these antifungal VOCs. Nevertheless, although the inconsistency of the results, *P. commune* seems also to be able to induce the release of antifungal VOCs on olives without the need for biotic stimulus.

As indicated by the principal component analysis, the protective effect conferred by *P. commune* against *C. nymphaeae* was ascribed to the release of specific VOCs by the host plant over time following pathogen inoculation (0, 3 and 24 h). Indeed, the observed inhibitory effect of VOCs released from olives inoculated with *P. commune* or with *P. commune* + *C. nymphaeae* towards *C. nymphaeae* growth at 0 h, was associated particularly with 1-hexanol, 2-ethyl- and p-xylene, respectively (Fig. 3). 1-Hexanol, 2-ethyl- is a very well-known toxic compound (Wakayama et al., 2019) and belongs to the alcohol chemical class, which is one of the main groups of VOCs derived from biological control microbes (Zhao et al., 2022). Similarly, p-xylene is a VOC belonging to the aromatic hydrocarbon class with recognized antimicrobial properties (Ajilogba and Babalola, 2019). However, the greatest differences in volatile profile among the different treatments were found after 3 h of pathogen inoculation, which was coincident with the release of antifungal VOCs from olives and leaves inoculated with *P. commune*. In particular, *P. commune* + *C. nymphaeae* treatment was characterized by the release of a set of VOCs [V10, V11, and V17 from olives (Table 1), and V27, V47, and V51 from leaves (Table 2)], which may play an essential role in defending fruits/leaves from pathogen colonization and invasion. Indeed, these VOCs belong to chemical classes, such as alcohols (V10 and V11), esters (V17) and alkenes (V27, V47, and V51), which are known antimicrobial agents (Di Francesco et al., 2015; Poojary et al., 2015). In particular, the VOCs V11 (3-octanol, 3,7-dimethyl-) and V51 [naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, 1S-(1.alpha.,7.alpha.,8a.alpha.)-], have been reported to be potentially involved in the inhibition of microbial growth (Utegenova et al., 2018; Yuyama et al., 2018; Peng and Ng, 2022) or in the induction of plant defense (Nahid et al., 2012; Rosenstiel et al., 2012; Peng and Ng, 2022). Thus, their fast production upon pathogen infection, suggests that these VOCs can not only directly suppress *C. nymphaeae* growth and sporulation, but can also activate the host immune response of host plant in response to pathogen infection, as previously suggested for other beneficial microorganisms (Hammerbacher et al., 2019). Curiously, at 24 h post-inoculation, olives inoculated with *P. commune* + *C. nymphaeae* or *C. nymphaeae* showed a similar volatile profile, suggesting a greater contribution of the pathogen for the emitted VOCs when compared to the endophyte. This blend of VOCs showed to stimulate the germination of *C. nymphaeae*, which may represent a mechanism of the pathogen to increase its survival under adverse conditions, like plant defense responses. In fact, the induction of spore germination is a common survival mechanism reported for fungal pathogens in response to the host-generated oxidative burst (Singh et al., 2021). The characteristics VOCs associated to olives inoculated with *P. commune* + *C. nymphaeae* or *C. nymphaeae* included some alkenes [furan, 3-(4-methyl-3-pentenyl)- and 2,4,6-octatriene, 2,6-dimethyl-], monoterpenes (o-cymene and trans-.beta.-ocimene) and esters (acetic acid, hexyl ester). These VOCs are likely to act as inducers of spore germination of *C. nymphaeae*. Some VOCs known to be involved in the plant's defense against pathogens, have been shown to play an essential role in pathogen development. For example, aldehydes and esters were

reported to stimulate spore germination of *Uromyces appendiculatus* (French et al., 1993).

Our results also showed that both olives and leaves emitted exclusive blends of volatiles in response to *P. commune* inoculation. Some of these VOCs emitted solely in plants inoculated with the endophyte have antimicrobial properties, such as dodecane, 2,6,11-trimethyl-(Nahid et al., 2012; Harikrishnan et al., 2021) and 1-dodecnol (Togashi et al., 2007). Hence, they are likely to play essential roles in inhibiting pathogen growth and promoting resistance upon plant pathogen attack, as previously suggested for other plant-endophyte interactions (Navarro-Meléndez and Heil, 2014).

On branches inoculated with *C. nymphaeae*, was similarly detected the emission of a specific set of VOCs belonging to the alkene, alkane and ester classes, which may act as disease biomarkers. These VOCs, which are emitted solely upon *C. nymphaeae* infection, are possibly mediating the pathogen's growth/pathogenicity, or host's response to infection, as previously suggested for other pathosystems (Stergiopoulos et al., 2013; Quintana-Rodriguez et al., 2015; Cellini et al., 2018). Curiously, some of the VOCs exclusively detected in *C. nymphaeae* treatment belong to esters, and this group is reported to be produced on plants as a defense response to pathogen infection (López-Gresa et al., 2018).

The observed changes in volatile profiles over time following pathogen inoculation in the several treatments suggest that these compounds may represent signs of ongoing metabolism derived from the microbial-plant interaction. Based on our experiments it is difficult to determine the source of these VOCs. In fact, they can be derived from *P. commune*, which in turn will be mixed with the VOCs produced by the plant, or can be a plant-derived metabolite whose production was induced by *P. commune* by changing the biosynthesis of plant metabolites. Previous studies have similarly reported the difficulty in identifying the real contribution of endophytes to host chemical composition, particularly in field experiments (Pang et al., 2021).

5. Conclusion

In conclusion, this work identified *P. commune* as a potential candidate to confer protection to olive trees against anthracnose by means of induction of VOCs production in the host plant. These VOCs showed to be effective in suppressing mycelial growth and sporulation of *C. nymphaeae* under *in vitro* conditions. Such inhibitory effect is likely due to the release of a set of VOCs belonging mostly to classes of alcohols (7-octen-2-ol, 2,6-dimethyl- and 3-octanol, 3,7-dimethyl-), esters (propanoic acid, 2-methyl-, 3-methyl-2-butenyl) and alkenes [1,5-heptadiene, 3,6-dimethyl-; 1,6-octadiene, 2,5-dimethyl-, (E)-; and naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, 1S-(1. alpha.,7. alpha.,8a.alpha.)-9]. Therefore, besides *P. commune* also these VOCs have great potential for use in olive anthracnose management. However, the sensitivity of VOCs to evaporation or to reaction with other compounds requires the development of effective formulations for their commercial use. Moreover, more studies are needed to evaluate the efficacy of these VOCs against the pathogen *C. nymphaeae*, either *in vitro* or *in planta* conditions.

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CRediT authorship contribution statement

Sofia Silva: Investigation, Formal analysis, Writing – original draft. **Helgeneusa da Costa:** . **Teresa Lopes:** Investigation, Data curation. **Vitor Ramos:** Investigation, Data curation. **Nuno Rodrigues:** Investigation, Data curation. **José Alberto Pereira:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Teresa Lino-Neto:** Writing – review & editing. **Paula Baptista:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105373.

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