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RESEARCH

Endophytes induce volatile emission in olive trees with repellent activity against *Bactrocera oleae*

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Abstract

The olive fruit fly, *Bactrocera oleae* (Rossi), is a key pest of the olive tree worldwide. This study explores whether the endophytes *Alcaligenes faecalis*, *Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, and *Penicillium commune* can trigger the emission of repellent volatile organic compounds (VOCs) in olive trees, targeting *B. oleae*. Accordingly, olive trees were inoculated with each endophyte or with buffer (control), and after 3 months, both fruits and leaves were collected to perform olfactometer assays and to evaluate VOCs by HS-SPME and GC–MS. *Alcaligenes faecalis*, *A. pullulans*, and *B. amyloliquefaciens* were found to significantly repel *B. oleae* females, with each treatment inducing a distinct VOC profile. *Alcaligenes faecalis* and *B. amyloliquefaciens* were characterized by higher levels of alkenes, including o-cymene and d-limonene, while *P. commune* and *A. pullulans* induced the emission of alkanes. Six VOCs were found to be negatively correlated with fly entries in the olfactometer assays, with beta-myrcene being the most prominent, followed by d-limonene, o-cymene, and 5-octadecene (E). These repellent VOCs were positively correlated with each other, indicating that their combined emission may produce synergistic effects, potentially enhancing their repellency. In contrast, 1, 4-hexadiene, 5-methyl-3-(1-methyl ethylidene) was identified as an attractant, showing a strong positive correlation with fly entries, and appeared to be produced through different biosynthetic pathways than the repellent VOCs. Overall, this work identifies three endophytes and a combination of repellent VOCs as promising new strategies for sustainable management of the olive fruit fly.

Keywords *Olea europaea* · Olive fruit fly · Biocontrol · Alkenes · Olfactometer

Key message

- The olive fruit fly is a global pest of olive trees.
- Endophytes can induce volatile compounds in olive tree that repel the pest.
- *Alcaligenes faecalis*, *A. pullulans*, and *B. amyloliquefaciens* reduced fly attraction.
- Beta-myrcene, d-limonene and o-cymene were potentially linked to repellence.
- The study suggests new sustainable tools for pest biocontrol.

Introduction

Bactrocera oleae (Rossi) (Diptera: Tephritidae), known as the olive fruit fly, is a monophagous frugivorous since its larvae feed exclusively from fruits of trees of the genus *Olea* (Giunti et al. 2020). This species is the most damaging pest of cultivated olive tree (*Olea europaea* L. subsp. *europaea*) in all olive-growing areas, and in particular across the Mediterranean region (Ordano et al. 2015). It causes direct destruction of the fruit pulp through larval development, leads to premature drop of the infested fruits, and significantly reduces both the quality and quantity of olive oil, potentially causing a loss of up to 40% of the production (Malheiro et al. 2015). The southern European countries are the largest producers in the world, ensuring around 59% of the total olive oil production (International Olive Council

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[IOC], 2023). In these countries, *B. oleae* infestations can reach high levels, with up to 80% of infested fruits reported in Portugal, depending on the year, region, and cultivar (Malheiro et al. 2015). Given the socio-economic importance of olive cultivation in southern European countries, it is imperative to effectively manage this pest. Currently, chemical control through insecticides remains the main strategy for managing *B. oleae* in all olive-growing areas (Lantero et al. 2023). However, the emergence of insecticide-resistant insect strains (Chirgwin et al. 2024; Samanta et al. 2023), inconsistent effectiveness of chemical treatments (Chen et al. 2023), and increasing awareness of the adverse environmental side effects of pesticides (Maggi et al. 2023) highlight the urgent need for alternative, eco-friendly control measures.

A promising approach to meet this demand is the development of biological control tools based on the use of endophytes (Fuchs and Krauss 2019; Latz et al. 2018). Growing evidence suggests that this microbial community, living in internal plant tissues, can help host plants deal with biotic stresses, including insect pests (Jaber and Ownley 2018; Rabiey et al. 2019). This positive effect has been ascribed to the production of secondary metabolites by the endophyte within plant tissues, exhibiting insecticidal or repellent proprieties against insect pests (Aravinthraju et al. 2024; Panwar and Szczepaniec 2024). Among the various secondary metabolites produced during endophyte-plant interactions, volatile organic compounds (VOCs) stand out as one of the most promising for the management of insect pests (Makhlouf et al. 2024). Some studies have demonstrated the capacity of endophytes to alter the composition of plant VOCs, with an impact on insect pest control. Certain VOCs may repel pests, others may attract natural enemies, and some may even enhance plant defense responses (Makhlouf et al. 2024; Turlings and Erb 2025). VOCs might be particularly valuable for pest control due to their ability to diffuse through air and soil, enabling both long- and short-distance interactions (Makhlouf et al. 2024).

Both fungal and bacterial endo- and epiphytic communities of olive trees have been studied in several Portuguese cultivars (Gomes et al. 2019; Martins et al. 2021; Mina et al. 2020a). Interestingly, these studies identified specific microbial signatures associated with either the presence or the absence of disease in olive trees, suggesting their potential role in host plant health. Moreover, some of these endophytes, namely *Alcaligenes faecalis, Bacillus amyloliquefaciens* (Mina et al. 2020b), *Aureobasidium pullulans* (Sdiri et al. 2022), and *Penicillium commune* (Silva et al. 2023), showed the capacity to inhibit in vitro growth of various olive tree pathogens and reduce disease severity and incidence in *in planta* assays, making them good candidates for biocontrol. The protective effect caused by *A. pullulans* and *P. commune* was associated with their ability to induce in the host plant the release of a specific set of VOCs (Sdiri et al. 2022; Silva et al. 2023). Although these endophytes have shown promising results in controlling olive tree diseases, their effectiveness against olive pests has yet to be determined. Understanding this aspect could expand the use of these endophytes, enabling them to serve a dual role as biocontrol agents against both pests and diseases of the olive tree.

Therefore, the main aim of the present study is to evaluate the ability of the endophytes *A. faecalis*, *A. pullulans*, *B. amyloliquefaciens*, and *P. commune* to induce the production of VOCs in olive trees with repellent effects against *B. oleae*. Accordingly, olfactometer assays and volatile analyses of leaves and olives from olive trees inoculated with the endophytes were performed. Leaves were included in the assays as they are believed to contribute significantly to the VOC mixture influencing fly behavior, due to their larger surface area compared to fruits and their proximity surrounding the fruit. Specifically, this research aims to address the following questions: (i) Can the endophytes studied induce the production of VOCs in olive trees with repellent activity against *B. oleae*?; (ii) Do different endophytes vary in their effectiveness at inducing repellent VOC emissions?; (iii) Which VOCs could potentially be responsible for repelling *B. oleae*? Overall, this work identifies endophytes and VOCs that can be used as starting points for developing new and more sustainable tools for the management of the olive fruit fly. Their effectiveness needs to be assessed through further studies conducted under field conditions.

Materials and methods

Microbial isolates and inocula production

The microorganisms *Alcaligenes faecalis* (CIMO 19DM406)*, Aureobasidium pullulans* (CIMO 14FM023), *Bacillus amyloliquefaciens* (CIMO 19DM561), and *Penicillium commune* (CIMO 14FM009) were previously isolated from asymptomatic leaves or twigs of the olive tree (*Olea europaea* L.) as described in Martins et al. (2016) and Mina et al. (2020a,b). More information about the source and identification of these isolates is provided in Table S1. All these strains have been preserved in the microbial collection of the Mountain Research Center (CIMO-CC) in 30% (v/v) aqueous glycerol solution at − 80 °C.

The fungi and bacterial inocula used in the experiments were prepared from frozen stocks by transferring cells to Potato Dextrose Agar (PDA; VWR Chemicals) or Plate Count Agar (PCA; VWR Chemicals) medium, respectively. The microorganisms were grown at room temperature (25 ± 2 °C) for one week (*A. pullulans* and *P. commune*) or 24 h (*A. faecalis* and *B. amyloliquefaciens*).

Spores/cells produced were then scraped from the agar plates with a sterile rod and suspended in 250 mL of sterile phosphate-buffered saline (PBS) solution at pH 7.4 (137 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4). The number of suspended cell/spores of both *A. pullulans* and *P. commune* was counted under a Leica DM500 optical microscope using a Neubauer chamber. The optical density of the bacterial cell suspension was measured on a mySPEC Twin spectrophotometer (VWR) at a wavelength of 600 nm. The concentration of spores or cells was adjusted to 1×10^7 spores or cells/mL with sterile PBS and used as inoculum in the following experiments.

Plant inoculation and sampling

The influence of microorganism-induced volatiles in olive tree hosts on the behavior of the olive fruit fly was assessed in an olive orchard located in Mirandela (41°29′11.4"N 7°14′50.7"W), Northeast of Portugal. This orchard comprises olive trees of the cultivar *Cobrançosa*, over 10 years old, planted at a 7 × 7 m spacing and grown under rainfed conditions. It is managed according to integrated production guidelines (Malavolta and Perdikis 2018), and has not been subjected to soil mobilization over the last five years. A total of 25 olive trees were selected for the experiments. For each of the following treatments, *A. faecalis*, *A. pullulans*, *B. amyloliquefaciens*, *P. commune*, and PBS as a control, five trees were inoculated. The inoculation was performed in September 2021 by spraying a single branch in each of two canopy sides, namely the north and south sides, of each olive tree. Each selected branch was sprayed with 25 mL of sterile PBS (Control) or 25 mL of microorganism's suspension using a hand pump sprayer. The inoculated branches were covered with a plastic bag to provide a humid chamber for three weeks. The presence of the inoculated endophytes was confirmed two months after inoculation (November 2021) by assessing microbial growth from leaf explants. Accordingly, five leaves from each inoculated branch were collected, superficially sterilized following the procedure optimized by Martins et al. (2016), and cut into small pieces (ca. 5 × 5 mm). The leaf pieces were then placed on PDA and PCA media, and the Petri plates were incubated at 25 ± 2 °C in the dark. Microorganisms emerging from the explants were identified based on their morphological characteristics, confirming successful colonization of the inoculated species. Three months after the inoculation (December 2021), the inoculated branches of each treatment were collected, and their leaves and olives were used for *B. oleae* behavioral assays (using an olfactometer) and volatile analyses (using chromatographic analysis).

Insect collection and rearing

Bactrocera oleae adults used in the behavioral assays were obtained from field-collected olives in the Trás-os-Montes region (Portugal) in 2021, from November to December. Olives with signs of olive fly infestation were collected, transported to the laboratory, and further spread in humidified trays. The larvae and pupae emerging from the infested olives were collected daily and transferred to rearing cages (10 cm in diameter and 15 cm in height) for adult emergence and placed in an insectarium. Once hatched from pupae, adults were separated daily by gender and age into rearing cages. After 10 days of growth and sexual maturation, groups of females and males were transferred to a new rearing cage at a ratio of 1:1 and allowed to mate for 5 days to ensure the females became gravid. In all this process of rearing, larvae, pupae, and adults were maintained at a temperature of 25 ± 1 °C, relative humidity of 70 ± 10%, with a photoperiod of 16L:8D. Adults were fed ad libitum with an artificial diet (sucrose and yeast extract in a 4:1 ratio, w/w) and water. The diet was replaced every two days.

Olfactometry bioassays

The bioassays were performed in a multi-choice olfactometer without any artificial airflow. The multiple-choice olfactometer system consisted of a circular polyethylene terephthalate (PET) unit (4 cm high × 12 cm diameter) with a central hole at the top (3 cm diameter) covered with autoclaved cotton (Fig. S1). In the central unit (arena), 5 lateral holes (12 mm diameter) were made 1.5 cm above the base, to insert five lateral tubes of the same material and of equal length (7 cm long × 10 mm in diameter). Each tube was connected to a PET unit (lateral unit) identical to the central one but with only one side hole (12 mm diameter, 1.5 cm above the base). The top of these outer units lacked a central hole and was coated with trap glue (TAD ALL Weather—BIAGRO). These side arms were securely attached and sealed to the PET units using colorless and odorless hot glue.

Preliminary tests were carried out with gravid females to ensure that the olfactometer's dimensions did not impair their mobility and that visual cues were effectively eliminated, validating the appropriateness of the setup for the intended behavioral assays. These tests were performed without any odor or visual stimuli (blank vs blank), and no positional effects were detected. All bioassays were carried out in chambers lined with white plastic and illuminated from above with fluorescent tubes, with a photoperiod of 16L:8D. The temperature and the relative humidity were kept during the whole assay at 24 ± 1 °C and 40–50%, respectively. Two olives and three leaves from each of five different treatments (i.e., inoculated with *A. faecalis*, *A. pullulans*, *B. amyloliquefaciens*, *P. commune*, or PBS as a control) were placed in separate lateral PET units at the ends of lateral arms. In all olfactometer assays, one of the lateral PET units was left empty (blank). Due to the five-arm design of the olfactometer, the experiment was organized into eight rounds to accommodate all five treatments (including the control) and the blank. Each round consisted of five separate olfactometers running in parallel (Fig. S1). In each round, the blank was included in all five olfactometers while the five treatments were tested in four olfactometers as depicted in Fig. S1. Across the eight rounds, a rotation scheme was used to ensure that each treatment was tested in different arm positions to reduce positional bias. Each round was initiated daily at the same time (10 a.m.), for eight consecutive days. This design resulted in a total of 32 replicates for each treatment (8 rounds × 4 olfactometers).

Ten mated females of *B. oleae,* 15 days post-emergence, were gently placed into the central PET unit through the hole and kept in the olfactometry for 23 h. All flies tested responded, with no females remaining in the central unit without selecting a side. The choice of odor source was recorded for each responsive female, defined as the fly either entering one of the side chambers and becoming glued to the top of the PET unit or remaining alive within the selected compartment. The mated females of *B. oleae* were used only once in the bioassay. Between each round, the olfactometers were thoroughly cleaned with warm water (25 °C) and neutral soap, followed by rinsing with distilled water.

Volatile characterization

The volatile profile of olives and leaves from the five different treatments (i.e., inoculated with *A. faecalis*, *A. pullulans*, *B. amyloliquefaciens*, *P. commune*, or PBS as a control), was evaluated through HS-SPME (headspace solid-phase microextraction) and GC/MS (gas chromatography with mass spectrometry detector). Accordingly, volatile compounds were first extracted by HS-SPME from samples comprising two olives and three leaves per olive tree. Three replicates were performed for each treatment. Each sample was placed in 50 ml individual vials, with all leaves being protected with aluminum in the petiole zone. Each vial was further sealed with a polypropylene cap with a silicon septum, followed by the injection of 5 µl of 4-metil-2-pentanol (0.127 mg/ml) (Sigma Aldrich, USA) with a syringe to be used as an internal standard. The volatiles were released by heating the samples in a water bath at 40 °C for 5 min. A fiber coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS 50/30 µm) (Supelco, Bellefonte, USA) was then exposed for 30 min to the headspace for volatile adsorption. Volatile analysis was performed using a Shimadzu GC-2010 Plus gas chromatograph equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. The volatile compounds were eluted from the fiber by thermal desorption in the injection port of the chromatography system for 1 min at 220 °C, using manual injections in splitless mode. The fiber was then kept in the injector port for another 10 min for cleaning and conditioning for further analyses. The volatile compounds were separated by using a TRB-5MS (30 m × 0.25 mm × 0.25 µm) column (Teknokroma, Spain). Helium (Praxair, Portugal) was used as the mobile phase at a linear velocity of 30 cm/s and a total flow of 24.4 ml/min. The oven temperatures were as follows: 40 °C (1 min); 2 °C/min until 220 °C (30 min). The ionization source was maintained at 250 °C with an ionization energy of 70 eV, and with an ionization current of 0.1 kV. Mass spectra were acquired by electron ionization in the m/z 35–500 range. The areas of the chromatographic peaks were determined by integrating the re-constructed chromatogram from the full scan chromatogram using the ion base (m/z intensity 100%) for each compound. For semi-quantitative purposes, the quantities of the identified volatile compounds were determined by comparing the base ion peak area of each compound to that of the internal standard. These ratios, without accounting for response factors, were then translated into mass equivalents using the mass of the internal standard. Compounds were identified by comparing their mass spectrometry (MS) fragmentation pattern with both reference standards (STD) and mass spectra databases, namely the NIST 69, PubChem, and ChemSpider. Compounds were identified by considering only those with fit and retrofit values above 80%. Retention indices calculated (RIcalc) were determined using a TRB-5MS capillary column, based on a reference series of n-alkanes ranging from C8 to C20. Literature-reported retention index values (RIlit) were from the TRB-5MS capillary column or equivalent (Table S2).

Data analysis

Statistical analyses of the olfactometer bioassay data were performed using Past software, version 4.03. Olive fruit fly preference for each treatment was assessed by calculating the average number of *B. oleae* females present in each compartment at the end of the bioassay, across replicates ($N = 32$). These mean values were then compared across treatments using the Kruskal–Wallis test, after confirming the non-normal distribution of data with the Shapiro–Wilk test. Pairwise comparisons between treatments were subsequently performed using Dunn's test, with statistical significance determined at p values below 0.05. The same statistical approach was applied to the volatile compounds detected in each treatment, with the individual compounds being presented as means ± standard deviations ($N = 3$).

Principal components analysis (PCA) was performed to identify which volatile compounds best distinguished the different treatments, by using RStudio version 2022.02.1 Build 461. The analysis included the identified volatile compounds and their concentrations for each treatment, namely *A. faecalis*, *A. pullulans*, *B. amyloliquefaciens*, and *P. commune*, as well as the control. This analysis was performed using the library devtools (pca function), with the number of principal components (PCs) being determined based on the cumulative proportion of variance explained. Visualization of the PCA results was performed using the ggbiplot library. The resulting biplot displays the first two principal components, with the arrows indicating the contribution of each volatile compound to the two principal components. Longer arrows indicate a stronger influence on these two components. Additionally, a color gradient was applied to the arrows to denote each compound's overall contribution to the total explained variance across all principal components.

To identify which volatile compounds in each treatment were most relevant in attracting or repelling gravid *B. oleae* females, a Random Forest (RF) analysis was conducted using the library randomForest. This analysis was performed separately for each treatment, namely control, *A. faecalis*, *A. pullulans, B. amyloliquefaciens*, and *P. commune*. The model incorporated 30 variables: 29 representing volatile compounds and one representing the total fly entry count. For each treatment, the RF model identified the compounds with the highest importance, evaluated based on the Mean Decreased Gini index. These index values were plotted in descending order to form an importance curve, with the cutoff set for values greater than 0.1. Volatile compounds above this cutoff point were considered to have the greatest importance in the model and were selected for subsequent data analyses.

To examine the correlation between volatile compounds and the number of fly entries in the olfactometer bioassay, a Pearson correlation analysis was conducted. This analysis included seven volatile compound variables, previously identified as relevant for each treatment by the Random Forest analysis, along with a single variable representing the total fly entry count across all treatments. The analysis was performed using the Hmisc package (Pearson correlation function). For visualization, a correlogram was created using a combination of ggplot2, colorspace, corrplot, and RColorBrewer packages. In this correlogram, the color intensity and circle size are proportional to the correlation coefficients, which are also displayed numerically. The significance of the correlations was also indicated using asterisks, with $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

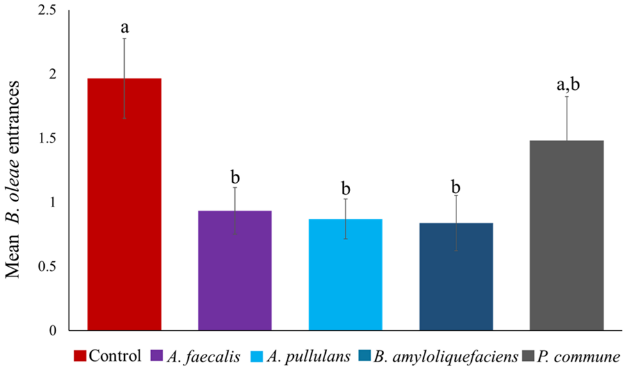


Fig. 1 Number (mean ± SE; N = 32) of gravid *Bactrocera oleae* females that chose odors emitted from non-inoculated control and microbial-inoculated (*Alcaligenes faecalis, Aureobasidium pullulans*, *Bacillus amyloliquefaciens,* and *Penicillium commune*) olives and leaves of the olive tree in a multi-choice olfactometer. Different letters indicate statistically significant differences ($p<0.05$) among treatments

Results

Olfactometry–preferences of *B. oleae* gravid females

Gravid females of *B. oleae* showed a significantly greater preference for the odors released by non-inoculated (control) olives and leaves compared to those inoculated with the different microorganisms, except for *P. commune* ($\chi^2=9.424$, df = 4, $p=0.135$) (Fig. 1). In contrast, the odors released by olives and leaves inoculated with *A. faecalis* ($\chi^2=9.424$, df = 4, $p=0.018$), *A. pullulans* ($\chi^2=9.424$, df = 4, $p=0.012$), and *B. amyloliquefaciens* ($\chi^2=9.424$, df = 4, $p=0.004$) caused significant repulsion compared to those of non-inoculated olives and leaves. Indeed, gravid *B. oleae* females exhibited a strong preference for non-inoculated olives/leaves, selecting them up to 2.5 times more frequently than the ones inoculated with any of these three microbial species. These three microbial species exhibited no significant differences in their effects, all demonstrating a similar repellent impact on the flies. Similarly, there were no significant differences in the preference of *B. oleae* between odors from olives and leaves inoculated with either these three microbial species versus tree inoculated with *P. commune* ($\chi^2=9.424$, df = 4, *A. faecalis*: $p=0.547$; *A. pullulans*: $p=0.596$; and *B. amyloliquefaciens*: $p=0.248$), despite a slight preference for *P. commune*.

Variation of volatile compounds emitted by the different treatments

A total of 29 volatile organic compounds (VOCs) were detected, with 28 putatively identified through comparison with the NIST mass spectral library (Table 1). The identified compounds belong to six different chemical classes (Table S2). Alkenes were the most diversified chemical class, comprising 16 different compounds. They were also the most prevalent, accounting for 50% of the total VOC abundance, followed by alkanes at 43% (Table 1 and Table S2).

The VOCs emitted by the olives and leaves of the various treatments were quali- and semi-quantitatively different (Fig. 2; Table 1). Samples inoculated with *P. commune* emitted the greatest number of VOCs (18), whereas those inoculated with *A. pullulans* emitted the fewest, with a total of 13 VOCs. Only six of the 29 detected compounds were common to all treatments. Olives and leaves from nearly all treatments emitted unique volatile compounds, except for those treated with *A. pullulans*. Humulene (alkene) was emitted solely by control samples, while (+)-4-carene and 2,4,4,6,6,8,8-heptamethyl-1-nonene (both alkenes) were emitted by samples treated with *A. faecalis*. Benzyl alcohol (alcohol), oxime-, methoxy-phenyl- (no chemical class), and p-xylene (alkene) were exclusively emitted by olives and leaves inoculated with *B. amyloliquefaciens*. Lastly, hexanoic acid (carboxylic acid) and trans-β-ocimene (alkene) were detected only in olives and leaves treated with *P. commune*.

In addition to these qualitative differences, variations in the abundance of VOCs were also observed among treatments (Fig. 2; Table 1). Olives and leaves from the control trees and those inoculated with *A. pullulans* and *P. commune* showed a higher relative abundance of alkanes (approximately 79%, 82%, and 52%, respectively, of the total abundance), with hexane, 2, 2-dimethyl- being the most abundant VOCs. This compound accounted for 82% of the total VOCs in the control treatment, 78% in the *A. faecalis* treatment, and 54% in the *P. commune* treatment. In contrast, olives and leaves from trees inoculated with *A. faecalis* and *B. amyloliquefaciens* exhibited a higher abundance of alkenes, accounting for up to 89% and 92% of the total VOCs, respectively. This enrichment was mainly driven by high levels of o-cymene and d-limonene, accounting together for up to 70% in the *A. faecalis* treatment and 58% in the *B. amyloliquefaciens* treatment. Interestingly, some treatments led to an increased emission of VOCs from specific chemical classes. For instance, samples treated with *A. faecalis* and *B. amyloliquefaciens* emitted a higher abundance of aldehydes, specifically nonanal, compared to other treatments. Likewise, olives and leaves inoculated with *P. commune* emitted a greater abundance of alcohols, notably 3-hexen-1-ol, (Z)-, than the other treatments.

Volatile compounds associated with each microbial treatment

To identify which VOCs are characteristic of each treatment, a principal component analysis (PCA) was performed (Fig. 3). The PCA showed the variance considering the two principal components (PC1 and PC2), highlighting differences in VOC profiles among treatments. Although the control, *P. commune*, and *A. pullulans* formed distinct groups, they clustered closely together, indicating similarities in their volatile compositions, albeit with some differences. Specifically, *A. pullulans* was characterized by a high emission of the alkane hexane, 2,2-dimethyl- (VOC 6), whereas *P. commune* was distinguished by the emission of the two alkenes 2,4,6-octatriene, 2,6-dimethyl-, (E,Z)- (VOC 16) and 1,4-hexadiene, 5-methyl-3-(1-methylethylidene)- (VOC 15). In contrast, *A. faecalis* and *B. amyloliquefaciens* were clearly separated from each other and from the other treatments, indicating a different volatile composition. The *A. faecalis* treatment was characterized by the emission of multiple VOCs, with the alkene o-cymene (VOC 19) and the carboxylic acid nonanoic acid (VOC 10) being key distinguishing compounds. The *B. amyloliquefaciens* treatment was distinguished by the emission of the alkene p-xylene (VOC 9) and the ester propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester (VOC 22). The clear separation of *A. faecalis* and *B. amyloliquefaciens* treatments from the control also suggests that these two endophytes trigger on the host olive tree the emission of a distinct volatile profile.

Volatile compounds potentially influencing *B. oleae* behavior

According to the results from multiple-choice olfactometry bioassays, gravid females of *B. oleae* were significantly repelled from olives and leaves inoculated with *A. faecalis*, *A. pullulans*, and *B. amyloliquefaciens*, contrasting with the control (non-inoculated trees). One of the main goals of this study was to determine whether this repellent effect was triggered by the microorganisms through the induction of volatile compounds in the host olive tree. To elucidate this, a random forest analysis was first conducted for each of the microbial treatments, including the control (non-inoculated), to identify the volatile compounds potentially having the greatest impact on the behavior of *B. oleae*. This analysis ranked the importance of the VOCs based on their Mean Decrease in Gini, with higher values indicating greater significance. Overall, this analysis identified seven VOCs as the most important in influencing *B. oleae* behavior, with Mean Decrease in Gini values exceeding 0.1 across all microbial-inoculated treatments (Fig. S2). These seven VOCs were further analyzed using Pearson correlations

Table 1 Volatile profile of combined olive and leaf samples from olive branches non-inoculated (control) and inoculated with *Alcaligenes faecalis*, *Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, and *Penicillium commune*. Results are expressed in relative percentage of the total chromatogram area (mean ± SD, N = 3). Different letters indicate statistically significant differences ($p < 0.05$) among treatments

Nº	Compound	Control	*Alcaligenes faecalis*	*Aureobasidium pullulans*	*Bacillus amyloliquefaciens*	*Penicillium commune*	Kruskal–Wallis test (df = 4)
1	Hexanoic acid	$-^{a}$	$-^{a}$	$-^{a}$	$-^{a}$	$0.33^{b}\pm0.12$	$\chi^2=3.857$, $p=0.037$
2	3-Hexen-1-ol, (Z)-	$0.84^{a}\pm0.26$	$-^{a}$	$-^{a}$	$-^{a}$	$3.00^{b}\pm0.73$	$\chi^2=3.857$, $p=0.049$
3	Heptanoic acid	$-^{a}$	$0.49^{a}\pm0.13$	$-^{a}$	$0.24^{a}\pm0.09$	$0.25^{a}\pm0.09$	$\chi^2=4.356$, $p=0.113$
4	Benzyl alcohol	$-^{a}$	$-^{a}$	$-^{a}$	$0.53^{b}\pm0.27$	$-^{a}$	$\chi^2=3.857$, $p=0.037$
5	Octanoic acid	$0.15^{ab}\pm0.09$	$0.52^{b}\pm0.15$	$-^{a}$	$0.54^{b}\pm0.27$	$-^{a}$	$\chi^2=8.744$, $p=0.031$
6	Hexane, 2,2-dimethyl-	$81.93^{a}\pm9.80$	$-^{b}$	$77.63^{a}\pm14.31$	$-^{b}$	$54.25^{ab}\pm16.03$	χ^2 8.744, $p=0.031$
7	2,2,4-Trimethyl-3-pentanol	$-^{a}$	$1.96^{b}\pm0.92$	$0.78^{ab}\pm0.63$	$2.27^{b}\pm0.61$	$-^{a}$	$\chi^2=8.744$, $p=0.031$
8	Oxime-, methoxy-phenyl-_	$-^{a}$	$-^{a}$	$-^{a}$	$2.74^{b}\pm1.41$	$-^{a}$	$\chi^2=3.857$, $p=0.037$
9	p-Xylene	$-^{a}$	$-^{a}$	$-^{a}$	$5.04^{b}\pm1.24$	$-^{a}$	$X^2=3.857$, $p=0.037$
10	Nonanoic acid	$-^{a}$	$0.35^{b}\pm0.21$	$0.07^{b}\pm0.03$	$-^{a}$	$-^{a}$	$\chi^2=7.448$, $p=0.024$
11	Nonanal	$1.18^{a}\pm1.03$	$4.26^{a}\pm1.11$	$1.68^{a}\pm1.51$	$3.53^{a}\pm0.88$	$2.27^{a}\pm1.49$	$\chi^2=6.533$, $p=0.163$
12	Octanoic acid, methyl ester	$-^{a}$	$-^{a}$	$0.14^{ab}\pm0.15$	$-^{a}$	$0.25^{b}\pm0.06$	$\chi^2=5.793$, $p=0.055$
13	.gamma.-Terpinene	$0.75^{a}\pm0.53$	$3.91^{b}\pm0.98$	$0.75^{a}\pm0.45$	$3.91^{b}\pm0.77$	$1.64^{c}\pm0.24$	$\chi^2=12.17$, $p=0.016$
14	1,3,7-Octatriene, 3,7-dimethyl-, (Z)-	$-^{a}$	$-^{a}$	$5.26\ ^{ab}\pm5.74$	$11.79^{b}\pm7.87$	$6.98^{b}\pm6.35$	$\chi^2=10.83$, $p=0.021$
15	1,4-Hexadiene, 5-methyl-3-(1-methylethylidene)-	$0.33^{b}\pm0.24$	$-^{a}$	$-^{a}$	$-^{a}$	$0.47^{b}\pm0.27$	$\chi^2=10.2$, $p=0.011$
16	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	$0.13^{a}\pm0.06$	$-^{a}$	$0.12^{a}\pm0.04$	$-^{a}$	$0.37^{b}\pm0.27$	$\chi^2=6.59$, $p=0.083$
17	(+)-4-Carene	$-^{a}$	$1.15^{b}\pm0.55$	$-^{a}$	$-^{a}$	$-^{a}$	$\chi^2=3.857$, $p=0.037$
18	.beta.-Myrcene	$0.47^{a}\pm0.25$	$3.11^{c}\pm0.98$	$0.61^{a}\pm0.38$	$2.50^{b}\pm$	$0.98^{ab}\pm0.25$	$\chi^2=11.63$, $p=0.020$
19	o-Cymene	$5.35^{a}\pm2.22$	$52.16^{b}\pm12.39$	$6.95^{a}\pm5.50$	$20.47^{ab}\pm8.94$	$10.79^{ab}\pm3.27$	$\chi^2=10.5$, $p=0.033$
20	D-Limonene	$5.32^{a}\pm3.11$	$18.02^{ab}\pm5.82$	$4.92^{a}\pm3.12$	$36.40^{b}\pm5.66$	$13.26^{ab}\pm11.08$	$\chi^2=10.43$, $p=0.034$
21	trans-.beta.-Ocimene	$-^{a}$	$-^{a}$	$-^{a}$	$-^{a}$	$0.48^{b}\pm0.43$	$\chi^2=3.857$, $p=0.037$
22	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	$0.04^{ab}\pm0.01$	$-^{a}$	$-^{a}$	$0.38^{b}\pm0.12$	$-^{a}$	$\chi^2=7.2$, $p=0.024$
23	.alpha.-Farnesene	$0.78^{ab}\pm0.55$	$-^{a}$	$-^{a}$	$1.23^{b}\pm1.16$	$1.04^{ab}\pm1.18$	$\chi^2=6.385$, $p=0.09$
24	Caryophyllene	$2.06^{ab}\pm1.60$	$6.35^{a}\pm4.90$	$0.88^{b}\pm0.70$	$6.74^{a}\pm4.08$	$2.28^{ab}\pm0.95$	$\chi^2=8.833$, $p=0.065$
25	Humulene	$0.12^{b}\pm0.11$	$-^{a}$	$-^{a}$	$-^{a}$	$-^{a}$	$\chi^2=3.857$, $p=0.037$

Table 1 (continued)

N°	Compound	Control	*Alcaligenes faecalis*	*Aureobasidium pullulans*	*Bacillus amyloliquefaciens*	*Penicillium commune*	Kruskal–Wallis test (df = 4)
26	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.alpha.)]-	$0.28^{ab} \pm 0.05$	$-^{a}$	$0.21^{ab} \pm 0.11$	$-^{a}$	$0.88^{b} \pm 0.76$	$\chi^2 = 9.462$, $p = 0.022$
27	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$-^{a}$	$3.66^{b} \pm 2.94$	$-^{a}$	$-^{a}$	$-^{a}$	$\chi^2 = 3.857$, $p = 0.036$
28	5-Octadecene, (E)-	$0.26^{ab} \pm 0.06$	$1.48^{b} \pm 0.63$	$-^{a}$	$1.67^{b} \pm 0.87$	$-^{a}$	$\chi^2 = 9.359$, $p = 0.023$
29	Sulfurous acid, cyclohexylmethyl pentadecyl ester	$-^{a}$	$2.59^{b} \pm 1.80$	$-^{a}$	$-^{a}$	$0.49^{b} \pm 0.36$	$\chi^2 = 5.422$, $p = 0.061$

"–" not detected

to assess their association with the number of *B. oleae* entries in the different compartments of the olfactometer. The results obtained showed that one VOC from the alkenes class, namely beta-myrcene, was significantly negatively correlated ($p < 0.05$, df = 22, correlation coefficient $r = -0.55$) with the number of fly entries, suggesting a potential repellent effect (Fig. 4). Other alkenes, in particular, 5-octadecene, (E)- (df = 22, $r = -0.47$), d-limonene (df = 22, $r = -0.47$), and o-cymene (df = 22, $r = -0.45$), were also found to have a moderate negative correlation with the flies' entries (all correlations above -0.45), though these correlations were not statistically significant ($p > 0.05$). In contrast, the alkene 1,4-hexadiene, 5-methyl-3-(1-methylethylidene), shows a strong positive correlation with fly entries (df = 22, correlation coefficient $r = 0.70$, $p < 0.01$), suggesting its potential role as an attractant. Interestingly, all the VOCs that showed negative correlations with the flies' entries also displayed positive correlations with each other. The strongest correlation (above $r > 0.6$, df = 22, $p < 0.01$) was observed between beta-myrcene and o-cymene, 5-octadecene, (E)-, or d-limonene. Additionally, significant positive correlations were found between 5-octadecene, (E)- and caryophyllene or d-limonene, and between d-limonene and oxime-, methoxy-phenyl-. In contrast, the VOC that was positively correlated with fly entries also showed a negative correlation with all the VOCs that were negatively correlated with fly entries.

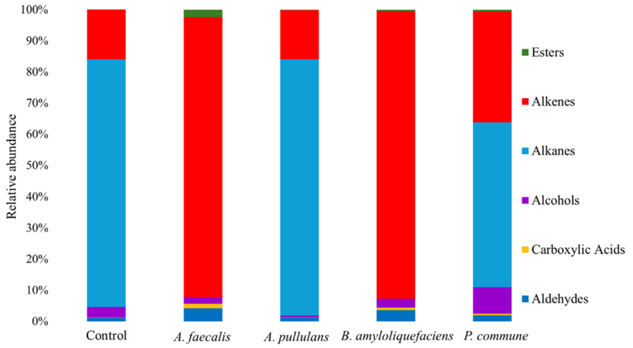


Fig. 2 Relative abundance (expressed in chromatographic area) of volatile organic compounds per chemical classes identified in olives and leaves non-inoculated (Control) and inoculated with *Alcaligenes faecalis*, *Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, and *Penicillium commune*. The results are presented for the total volatile compounds detected, regardless of the plant organ (olives and leaves)

Discussion

This work identified three endophytes, namely *A. faecalis*, *A. pullulans*, and *B. amyloliquefaciens*, as potential candidates for conferring olive tree protection against *B. oleae*. Inoculation of olive trees with any of these three microbial species showed to have a repellent effect, deterring gravid *B. oleae* females from approaching the fruits and leaves in olfactometer assays. To the best of our knowledge, this is the first study demonstrating the ability of *A. faecalis*, *A. pullulans*, and *B. amyloliquefaciens* to induce the release of repellent VOCs in the host plant against insect pests. Most previous work on these microorganisms has focused on their ability to suppress plant pathogens rather than to repel insect pests. For example, several strains of *A.*

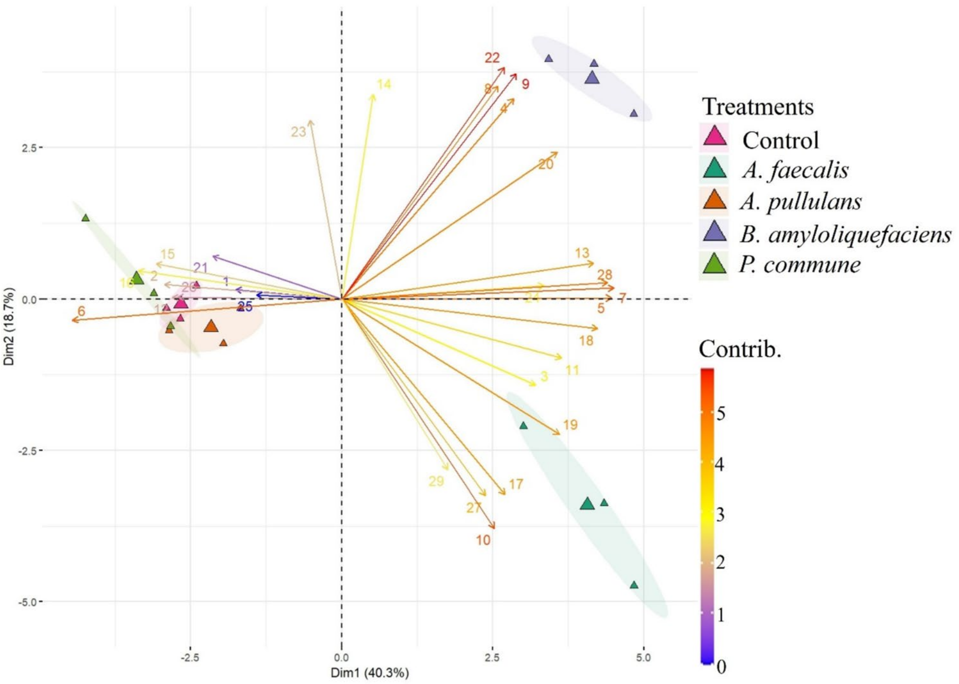
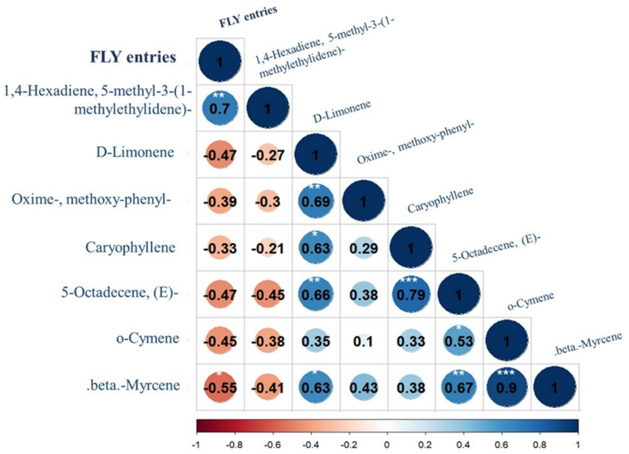


Fig. 3 Principal component analysis score plots obtained from the volatile profile of olives and leaves from olive trees non-inoculated (Control) and inoculated with *Alcaligenes faecalis*, *Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, and *Penicillium commune.* Each number corresponds to a particular volatile compound, as indicated in Table 1. The circles represent treatments, with the ones of greater size representing the average. The arrow length represents the strength of each volatile compound's influence on the first two principal components, while the color gradient indicates its overall contribution to the total explained variance

faecalis have shown significant protection against various fungal diseases, including clubroot in cabbage (He et al. 2022), root rot disease in olive (Legrifi et al. 2022), and brown rot in apples (Lahlali et al. 2020). Similarly, the yeast-like fungus *A. pullulans* showed promising results in reducing gray mold in strawberries (Iqbal et al. 2023) and grapes (Galli et al. 2021), as well as anthracnose in olives (Sdiri et al. 2022) and strawberries (Iqbal et al. 2023). Additionally, strains of the bacterium *B. amyloliquefaciens* have reduced several fungal diseases, such as brown rot in apples (Lahlali et al. 2020), early leaf spot in peanuts (Wang et al. 2023), verticillium wilt in cotton (Liu et al. 2023), as well as bacterial diseases, like olive knot disease (Mina et al. 2020b). The present study expands the known benefits of these microbial species, providing new evidence that their inoculation can also trigger host-mediated protection against insect pests.



	FLY entries	1,4-Hexadiene, 5-methyl-3-(1-methylethylidene)-	D-Limonene	Oxime-, methoxy-phenyl-	Caryophyllene	5-Octadecene, (E)-	o-Cymene	.beta.-Myrcene
FLY entries	1							
1,4-Hexadiene, 5-methyl-3-(1-methylethylidene)-	** 0.7	1						
D-Limonene	-0.47	-0.27	1					
Oxime-, methoxy-phenyl-	-0.39	-0.3	** 0.69	1				
Caryophyllene	-0.33	-0.21	* 0.63	0.29	1			
5-Octadecene, (E)-	-0.47	-0.45	** 0.66	0.38	*** 0.79	1		
o-Cymene	-0.45	-0.38	0.35	0.1	0.33	* 0.53	1	
.beta.-Myrcene	* -0.55	-0.41	* 0.63	0.43	0.38	** 0.67	*** 0.9	1

Fig. 4 Pearson correlation between the relevant volatile compounds by treatments (non-inoculated and microbial-inoculated olive trees), preselected by the Random Forest (Fig. S2) and the number of entries of gravid females of *Bactrocera oleae* per treatment in the multichoice olfactometer bioassays. The intensity of the color and the size of the circle are proportional to the correlation coefficients (r), with blue and red colors representing positive and negative correlations, respectively. The correlation coefficients are expressed numerically in the correlogram. Asterisks indicate statistically significant correlations at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

The repellence effectiveness observed in the olfactometer assays varied among endophytic species, with *P. commune* showing a lower capacity to induce the emission of repellent compounds in the host plant compared to the other endophytes tested. The observed variations among microbial treatments may be explained by differences in the timing of volatile production and release, thereby affecting their effectiveness in repelling *B. oleae*. For instance, *P. commune* may trigger the production of repellent volatiles in the olive tree at a different time frame compared to the other three microorganisms, potentially releasing them before or after the three-month assay period. Indeed, a recent study demonstrated that the same *P. commune* strain used in this work induced the emission of volatile compounds in both olives and olive tree leaves one month after inoculation, exhibiting antifungal properties by significantly reducing the growth and sporulation of the pathogen *Colletotrichum nymphaeae* (Silva et al. 2023). Another explanation is that each endophyte induces distinct volatile profiles in the host plant, which could, in turn, influence *B. oleae* behavior differently. For example, olive trees inoculated with *A. faecalis* and *B. amyloliquefaciens* released higher amounts of alkenes, a chemical class that includes several compounds known for their repellent properties (Baker and Cardé, 1979). Specifically, both treatments were characterized by the high emission of o-cymene and d-limonene, both of which are well-known for their repellent and toxic effects on various harmful insects (Feng et al. 2021; Lin et al. 2024). Besides these, each endophyte also promoted the release of unique VOCs. Some of them, like benzyl alcohol in *B. amyloliquefaciens*-treated plants (Aboelhadid et al. 2023) and (+)-4-carene in *A. faecalis*-treated plants (Kasrati et al. 2024), were previously shown to have repellent and insecticidal effects. Other unique compounds identified in these two treatments, while not directly linked to insect repellence, have been detected in plant crude extracts, which showed insecticidal activity (2,4,4,6,6,8,8-heptamethyl-1-nonene; Ravi et al. 2018) and antimicrobial properties (oxime-, methoxy-phenyl-; Burranboina et al. 2022). *P*-xylene, another VOC uniquely identified in *B. amyloliquefaciens*-treated plants, was previously found to have a weak effect on olive fruit fly oviposition, and a limited role in influencing its overall behavior (Scarpati et al. 1993). However, toxic effects of *p*-xylene and related compounds (xylene) have been reported against insects (Maliszewska and Tęgowska 2018), suggesting that it may have an effect on insects. Beyond these unique compounds, the PCA further identified several VOCs that were highly associated with particular endophytes, reinforcing the distinct volatile signatures induced by each microbial treatment. A key distinctive VOC in *B. amyloliquefaciens*-treated plants was propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester. This compound was identified exclusively in oviposition host plants of *Gasterophilus pecorum*, suggesting it may influence the insect's oviposition behavior (Zhou et al. 2020). Therefore, it is hypothesized that this compound may also influence oviposition behavior in *B. oleae*. For *A. faecalis*, a key distinctive VOC was nonanoic acid, a compound previously reported to have significant insecticidal and repellent activity against several insect species (Temeyer et al. 2024; Farag et al. 2021). Finally, *A. pullulans* was distinguished by the emission of hexane, 2,2-dimethyl-. Although specific research on its effects on insects is limited, several studies have shown that plant extracts and essential oils containing this compound exhibit insecticidal properties (Bayas et al. 2019; Karamaouna et al. 2013).

A major strength of this study is the identification of beta-myrcene as the key compound potentially responsible for repelling *B. oleae*, supported by its significant negative correlation with fly entries in olfactometer assays. Multiple studies have demonstrated notable insecticidal and repellent properties for this compound. When formulated in chitosan nanoparticles, beta-myrcene has shown effectiveness against *Aedes aegypti,* achieving 100% larval mortality (Costa et al. 2024). Additionally, beta-myrcene-loaded hydrogels exhibited a 57% deterrence rate against *A. aegypti* mosquitoes (Duarte et al. 2024). Five other VOCs (D-limonene, oxime-, methoxy-phenyl-, caryophyllene, 5-octadecene (E), and o-cymene) also showed a negative correlation with *B. oleae* entries in olfactometer assays, though the results were not statistically significant. An interesting finding of this study was the strong positive correlation among all VOCs with potential repellent properties against *B. oleae*. This suggests that these compounds may be emitted together, possibly due to shared biosynthetic pathways. Given their potential repellent effects, it is hypothesized that combining these compounds could enhance their efficacy. Based on the study's results, a promising combination for *B. oleae* repellency includes beta-myrcene, o-cymene, 5-octadecene (E), and d-limonene. As previously mentioned, beta-myrcene, o-cymene, and d-limonene are well-known for their insecticidal and/or repellent properties, often found in essential oils used for pest management (Feng et al. 2021; Costa et al. 2024; Duarte et al. 2024; Lin et al. 2024). Similarly, 5-octadecene (E) exhibits multiple bioactivities against insects, including mating disruption (Kanno 1978), insecticidal effects (Aboaba et al. 2019), and larvicidal properties (Hepsibha et al. 2022). Such multifaceted effects make this combination a promising tool for the management of *B. oleae*. Moreover, the relatively low toxicity of some of these compounds, particularly d-limonene, to beneficial organisms (Lin et al. 2024), along with its current use in pest control formulations (Isman, 2020), further supports their integration into integrated pest management (IPM) strategies. In contrast, the volatile 1,4-hexadiene, 5-methyl-3-(1-methyl ethylidene)- showed a strong positive correlation with fly entries, indicating an attractive effect on *B. oleae*. This compound also had negative correlations with all the VOCs that repelled *B. olea* flies, suggesting that the attractive compound is produced under different conditions or by different biosynthetic pathways than the repellent compounds.

Overall, the results suggest that some VOCs and/or their blends, such as beta-myrcene, o-cymene, 5-octadecene (E), and d-limonene, could be a valuable tool for incorporation into IPM strategies to manage *B. oleae* in olive groves. Moreover, these compounds can be strategically combined with the VOC with potential attractive effect to simultaneously deter *B. oleae* from olive fruits and guide them toward traps, forming a classic push–pull strategy. Such an approach is likely to improve overall control efficacy against the olive fruit fly. Nevertheless, additional research is still needed to assess the repellent or attractive effects of these compounds, both individually and in blends, under field conditions, to support their development as effective tools for integrated pest management of the olive fruit fly. In addition to confirming the role of these VOCs, other behavioral parameters, such as residence time, movement patterns, or antennal responses, should be evaluated in olfactometer assays to strengthen evidence for their effect on fly behavior.

Conclusions

Overall, this study indicates that the endophytes *A. faecalis*, *A. pullulans*, and *B. amyloliquefaciens* are potential candidates for conferring olive tree protection against *B. oleae* by inducing the release of repellent volatile compounds (VOCs) in the host plant. Among these VOCs, beta-myrcene, o-cymene, 5-octadecene (E), and d-limonene are the most promising repellent compounds. Their positive correlations with each other suggest that they might be produced together, potentially creating a synergistic effect that deters flies. The effectiveness of these VOCs against *B. oleae* should be further tested under field conditions, by evaluating VOC blends in concentrations similar to those emitted by olive trees. These findings may provide opportunities to develop new tools for integrated management of *B. oleae*, through the exploitation of these endophytes and/or VOCs.

Author contributions

P.B. and J.A.P. designed the experiments, supervised the study and revised the manuscript. A.E.C. performed most of the experiments, analyzed the data and drafted the manuscript. P.C. assisted with data analysis and revised the manuscript. N.R. assisted with the analysis of volatile compounds. All authors have read and agreed to the published version of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors declare no competing interests.

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