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Volatile organic compounds emitted by *Megaplatypus mutatus* associated fungi: chemical identification and temperature-modulated responses by the ambrosial beetle

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Abstract

Background In ambrosia and bark beetles–fungi interaction, volatile organic compounds (VOCs) play a central role in mediating various aspects of community dynamics of beetles and/or fungi. These functions include facilitating beetle habitat location, mate identification, and fungal partner differentiation. However, the understanding on this context remains limited, especially in the globally distributed subfamily Platypodinae, which comprises predominantly ambrosia beetles. There is a lack of chemical data on ambrosia fungi from native South American species. This study addresses this gap by characterizing VOCs from twelve fungal species associated with *Megaplatypus mutatus* and assessing species-specific behavioral responses during dispersal.

Methods Fungal VOCs were collected by gas chromatography–mass spectrometry combined with solid-phase microextraction and Y-olfactometry assays of males and females were performed at dispersal stage. Statistical analyses involved: non-metric multidimensional scaling multivariate plot and PERMANOVA test, a cluster analysis through unweighted pair group method with Jaccard index, and finally, a chi-square goodness-of-fit test for beetle behavioral assays.

Results We identified 72 VOCs from the fungal species isolated from *M. mutatus* galleries, exocuticle, and gut. The olfactory behavior of *M. mutatus* demonstrated its capacity to discriminate between volatile profiles, showing a preference for either the fungus or the control source. Our results also enhance the understanding in a chemotaxonomic context and in the behavioral responses of *M. mutatus* revealing the beetle's remarkable low temperature tolerance and its capability to maintain mobility and orientation toward volatile sources even after zero-degree Celsius exposure.

Conclusion This study presents a comprehensive insight into fungal VOC profiles, emphasizing the sources of isolation within pest associated fungi, as well as its symbiotic species from the *Raffaelea* genus. In conclusion, our findings suggest that *Megaplatypus mutatus* exhibits a general aversion to its fungal VOCs symbiont. However, a notable exception arises when the beetles are pre-exposed for 48 h to freezing conditions, highlighting the beetles' ability

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to withstand freezing conditions as adults and to exhibit altered responses to their fungal associates under these circumstances.

Keywords Ambrosia, *Raffaelea*, *Fusarium*, Volatiles, Platypodinae, *Megaplatypus*, Olfactory behavior, Chemotaxonomic, Freezing condition

Introduction

Fungal volatile organic compounds (VOCs) are increasingly recognized for their importance in interspecific interactions between fungi, plants, bacteria, nematodes and arthropods; as mediators of plant growth and defense, attractants of natural enemies, and biocontrol agents, they have been widely applied in integrated pest management programs (Hunt and Borden 1990; Bennett et al. 2012; Morath et al. 2012; Hung et al. 2015; Tobin et al. 2024).

Fungal VOCs play a critical role in the life cycle of the ambrosia bark beetle as part of the complex relationship between the beetles and their mycobiota. Previous studies have shown that certain fungal species emit a diverse range of VOCs that can either attract or repel beetles, even suggesting a convergent evolution of semiochemicals across kingdoms (Kandasamy et al. 2016; Martini et al. 2017; Zhao et al. 2019). These VOCs provide insight to beetles into the suitability of host substrates for brood production (Hulcr et al. 2011; Kuhns et al. 2014; Kandasamy et al. 2023).

Moreover, the integration of VOC-based chemotaxonomy has the potential to enrich traditional methods of fungal identification (Frisvad 2015), revealing that distinct VOC patterns can help predict trophic modes, non-symbiotic lifestyle, substrate-use and host-type of fungi, among other characteristics (Guo et al. 2021). This is particularly relevant in the context of ambrosia beetles–fungi interactions, where such studies are limited and absent for platypodine members.

Despite the important role of abiotic factors such as temperature, in these interactions, this aspect remains seldomly studied. Temperature, especially for poikilothermic symbionts, can have a significant impact on interaction dynamics and stability (Corbin et al. 2017; Lombardo et al. 2018), and these effects are subject to context-dependent shifts in evolving environments (Hofstetter et al. 2022). Previous studies have shown that the exposure to low temperatures can influence behavioral responses in bark beetles (Jenkins et al. 2001; Lombardo et al. 2018). This is demonstrated by the necessity of synchronized adult emergence, playing a pivotal role in overcoming tree defenses through mass attack and increasing the probability of survival in adverse climatic conditions (Logan and Bentz 1999).

The effects of rising temperatures on the growth performance of fungal associates have been well-addressed in few Scolytine species by Six and Bentz (2007) and Dysthe et al. (2015), among others; while the effects of lower temperatures on both the beetle and the fungus have been even studied less. This topic has been discussed by some authors in the context of invasive species: globally there are increasing reports of ambrosia and bark beetle interceptions during commercial transport of biological material such as logs or roundwood, posing a tangible threat to biodiversity, functional ecosystems and the economic productivity of forestry (Brockerhoff et al. 2006; Rabaglia et al. 2019; Pureswaran et al. 2022). International transport is mainly by sea and air, where biological products and species might be exposed to a wide range of conditions. Knowledge of the thermal limits of ambrosia beetle species and their ability to discriminate VOCs after exposure to extreme temperatures is a knowledge gap to be addressed for an increasing context of invasive species.

Megaplatypus mutatus (Chapuis) is the main native ambrosia forest pest in South America and is also an established forest pest in Italy (Griffo et al. 2012; Ceriani-Nakamurakare et al. 2022). The initiation of the new attack occurs as the male selects and colonizes a tree, followed by the emission of sex pheromones to attract females. They are monogamous and the parental couple dies within the gallery, the number of attacks can range from 1 to 15 per tree in poplar plantations [more detailed data on the biology of the pest and the host plant species attacked can be found in Ceriani-Nakamurakare et al. (2022)]. This species is present and frequently found in Argentina up to 42° SL where winter temperatures can easily reach − 15 °C or lower. Empirical data reveal minimal or absent expansion into new cold areas despite the presence of suitable breeding host plant species (reproductive hosts). During winter, both adults and larvae of this species may face exposure to cold temperatures that could potentially influence their host selection behavior in spring when beetles disperse. We prioritized a study scenario of zero degrees Celsius as the lowest plausible temperature within the tree and in the context of intercontinental log shipping. Therefore, this study constitutes an initial exploration to assess the potential impact of cold exposure on the insect's sensitivity to orientation, as well as its sensitivity to associated fungi and their volatile

compounds. Unlike most ambrosia beetles, this species typically initiates damage in vigorous trees that hold significance for forestry and fruit plantations; however, it is crucial to recognize that this process can lead to prolonged effects, potentially culminating in mortality.

Our previous studies on this pest have characterized the associated fungal community by studying the exocuticle and gut communities of males, females and larvae, as well as their galleries in *Populus deltoides* W. Bartram ex Marshall and *Casuarina cunninghamiana* Miq. (Ceriani-Nakamurakare et al. 2016, 2018, 2020). A total of 44 fungal taxa were recorded in these studies; the core fungal community is composed by members of *Fusarium solani* species complex (FSSC), *Candida insectalens* (D.B. Scott, Van der Walt & Klift) S.A. Mey. & Yarrow, *Graphium basitruncatum* (Matsush.) Seifert & G. Okada, and four putative species belonging to the genus *Raffaelea*. Functional roles and biological hypotheses have been proposed to understand the nature of this interaction. In summary, FSSC and *C. insectalens* were consistently abundant across diverse isolation sources. Taking into account Additional file 1, the putative roles for each species have been suggested: a potential phytopathogenic role for FSSC and a gut symbiotic association for *C. insectalens*. Meanwhile, *G. basitruncatum* has the capacity to contribute to the biosynthesis of a male sex pheromone component that attracts the female (Slodowicz et al. 2019) and it is the first fungal species to colonize new tree host tissues. Finally, *Raffaelea* species have been extensively studied among other ambrosia interactions and are considered a key nutritional symbiont in these interactions. Nevertheless, VOCs emitted by the core fungal community members have not been studied to date, nor the behavioral response elicited by them on *M. mutatus*.

In this study our aim was to characterize the volatile organic chemicals profiles from different fungal species associated with *M. mutatus* in order to contribute to a future VOC-based chemotaxonomy database for fungal identification. In addition, by mean of a two-way olfactometer, we analyzed the behavior of *M. mutatus* toward fungal VOCs. The beetles were either immediately exposed to VOCs at room temperature (22–24 °C) or subjected to cold (0 °C) or room temperature for 1, 12, and 48 h. Subsequently, beetles were assayed at room temperature. We propose that the behavioral response could be linked to the attraction of VOCs, with no discernible influence of the temperature treatment on the beetles. We hypothesize that these compounds might serve as either a nutritional source or cues for locating a pre-colonized host, a phenomenon documented in other ambrosia beetles (e.g. Ranger et al. 2021; Kendra et al. 2022).

Materials and methods

Collection of fungal volatiles organic compounds

The fungal species employed for analyzing fungal VOCs were selected from those recovered by culture methods in Ceriani-Nakamurakare et al. (2016, 2020). Priority was given to species with a putative role as symbionts; however, we also included two saprophyte species (Additional file 1: Section 1). This selection comprises a total of 12 strains: *Fusarium solani* species complex (FSSC), *Graphium basitruncatum* (GRAPH), *Raffaelea* aff. *arxii* D.B. Scott & J.W. du Toit (ARXII), *Raffaelea* sp. 1 and sp. 3 (RAFFA 1 and RAFFA 3); and the saprophyte species *Chaetomium* sp. (CHAET) and *Nigrospora sphaerica* (Sacc.) E.W. Mason (NIGRO), these seven species were isolated from gallery and/or beetle exocuticle; *Ambrosiozyma platypodis* (J.M. Baker & Kreger-van Rij) Van der Walt (AMBRO), *Candida insectalens* (CANDI), *Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki (MEYE), *Starmera* sp. (STAR) and *Debaryomyces* sp. (DEBA), these five species were isolated from gut. The strains belonging to *Raffaelea* used for these analyses comprise two unidentified species, RAFFA 1 and RAFFA 3, exclusively isolated from males, and a strain of ARXII isolated from galleries (Ceriani-Nakamurakare et al. 2016) (Additional file 1: Section 1).

Fungal strains were cultivated according to the conditions and culture media outlined by Slodowicz et al. (2019). In brief, fungal strains were grown in 50-ml glass vials on 10 ml of 2% solid malt extract agar (MEA) in the dark at 20 °C for 15 days. During solidification, the vials with agarized medium were placed at an angle of 45 degrees with respect to the horizontal direction to increase the surface occupied by the culture, and consequently, the concentration of its VOC emissions in the equilibrium vapor (Savel'eva et al. 2014). After the growth period of the fungal strains, the vials were sealed with Teflon caps using a crimp gripper and VOCs were accumulated for 2 days before analysis. Volatile compounds produced by the fungi were collected three-phase Carboxen/Divinylbenzene/Polydimethylsiloxane (50/30 µm) solid phase micro-extraction (SPME) fiber (Supelco, Bellefonte, PA, USA). This microfiber was previously conditioned at 240 °C in a gas chromatography–mass spectrometry Shimadzu GC-2020 injector (Shimadzu, Kyoto, Japan) for 30 min, and then inserted in the head space of 20-ml sealed vials containing the fungal strains thermostatized at 30 °C for 25 min. This process was repeated three times for each strain.

Fungal VOCs' identification

Subsequent analysis of the collected volatile compounds was performed using a gas chromatograph coupled to a quadrupole mass spectrometer (Shimadzu

QP-5050 GC–MS), operating in electron impact mode at 70 eV with total ion current registration within the mass range m/z 40/350. Samples were separated on both a DB-5MS (J&W Scientific, CA, USA), column (30 m × 0.25 mm × 0.25 μm) and a DB-WAX J&W Scientific column (30 m × 0.25 mm × 0.32 μm). Desorption of DB-5MS and DB-WAX columns was conducted following the method described by Slodowicz et al. (2019). To determine the chromatographic retention indices (RI) under the applied conditions, a mixture of normal alkanes ranging from C7 to C20 was injected. The retention times of hydrocarbons and volatile compounds from the samples were registered to calculate Kóvats indices (RI). These RI values were then compared with reference values on polar and non-polar columns. Identification of volatile organic compounds (VOCs) was performed by comparing spectra with the NIST-Wiley 8.0 library, ensuring a similarity of over 90% with compounds in the database (GCMS solution). Kóvats retention indices and co-elution with available standards were also used for identification. Furthermore, blank GC traces (2% MEA) were performed before the initial SPME extraction and periodically repeated to eliminate VOCs from VOC profiles.

Insects and treatments

Adult beetles at the dispersal stage were collected daily from emergence traps deployed without bait and placed in cut trees from a *Populus deltoides* commercial plantation in Bragado, Buenos Aires, Argentina, following the methodology described in Ceriani-Nakamurakare et al. (2016, 2020). Beetles were collected in the early morning and promptly subjected to experimental conditions with an equal representation of both sexes. If necessary, individual beetles were briefly placed in collection tubes for less than 10 min at room temperature (RT, 22–24 °C) to ensure proper mobility (such as no missing legs or antennae, capability of roll-back movement, and normal walking), the beetles were carefully and promptly inspected before conducting the olfactometer bioassays. Insects were exposed to low temperature (pre-treatment) to study its effect on the orientation response of the beetles, recording individual responses of males and females to specific fungal species. Our experimental setup consisted of dispersal beetles at room temperature (RT, 22–24 °C) immediately used in the VOCs assay, along with two groups of specimens: one exposed to low temperature (0 °C), and another kept at RT. Each group had subsets exposed for periods of 1, 12, or 48 h. These exposures took place within sealed 2-ml collection tubes fitted with screw caps and were positioned in a temperature-controlled thermostatic bath. In addition, a temperature data logger was installed to continuously monitor the conditions inside a similar tube for control purposes. Scheduled experimental assays were conducted for both insect groups, after the specimens exposed to 0 °C fully recovered.

Y-olfactometer behavioral assays

The assays were conducted using a glass Y-tube olfactometer with similar characteristics and following the methods of setup outlined in Gonzalez-Audino et al. (2005) and Gatti Liguori et al. (2008). For each bioassay, the fungus odor source (FOS) was randomly assigned to one of the olfactometer arms, and the assignment was reversed after each replication. The FOS comprised 0.5 cm^2 portions of actively growing mycelia from six species grown in complete darkness for 7 to 10 days at 23 °C before their use in the experiments. *Fusarium solani* complex (BAFCcult 4502), *G. basitruncatum* (BAFCcult 4518), *Raffaelea arxii* (BAFCcult 4525), *Raffaelea* sp. 1 (BAFCcult 4523), *Raffaelea* sp. 3 (BAFCcult 4520), and *C. insectalens* (BAFCcult 4621) were the species chosen for the behavioral assays. The selection was made based on the proposed biological roles that these species play in ambrosia interactions, e.g. nutritional symbiont, phytopathogenic, microhabitat conditioning within the host plant, and in addition to be part of the core fungal community of *M. mutatus*. This significance is further elucidated in the studies of Ceriani-Nakamurakare et al. (2018, 20202016) through culture-dependent and independent approaches. Negative controls were carried out using MEA 2% culture medium of an age similar to that of the fungal cultures. A supplementary control was applied to the subset of females that were oriented to the control arm. Members of the group were randomly tested utilizing a positive control indicating (±)-sulcatol (Gonzalez-Audino et al. 2013) to ensure the integrity of their sensory abilities, thus validating the choice of control condition.

Statistical analysis

Data analysis among fungi based on volatile profile compositions was conducted through an ordination method using non-metric multidimensional scaling based on Gower's similarity coefficient (NMDS) with a permutational multivariate analysis of variance test (PERMANOVA), and a cluster analysis through unweighted pair group method with arithmetic mean using Jaccard index for presence/absence dataset (UPGMA). The UPGMA analysis was made for *Raffaelea* species and included retrieved data of VOCs from *R. lauricola* T.C. Harr., Fraedrich & Aghayeva isolated from *Xyleborus glabratus* Eichhoff (Cale et al. 2016) and *R. amasae* (Gebhardt) T.C. Harr., *R. canadensis* L.R. Batra, *R. montetyi* M. Morelet and *R. sulphurea* (L.R. Batra) T.C. Harr., species isolated from *Amasa concitatus* Wood & Bright,

Xyleborinus saxeseni Ratzeburg, *Platypus cylindrus* Fabricius, and *X. saxesenii*, respectively (Lehenberger 2022). In addition, a chi-square goodness-of-fit test (χ^2) was conducted for beetle behavioral assays. All analyses were carried out using base R functions and vegan package within R software (R Core Team 2020; Oksanen et al. 2023).

Results

Fungal VOCs' identification

The analysis of volatile profiles yielded the identification of a total of 72 VOCs (Table 1 and Additional file 1: Section S2). To enhance data interpretation due to the diversity of identified VOCs, a classification was employed based on their primary chemical groups including alcohols, alkenes, ketones, sulphurated compounds, terpenoids, amides, and carboxylic acids (Fig. 1). Among these, alcohols dominated the profiles of ARXII and RAFFA 1, accounting for approx. 50% of the total compounds; nevertheless, interspecific variations in proportions were observed within the *Raffaelea* genus. Ketones were significantly represented, comprising approx. 45% in FSSC and GRAPH, with RAFFA 3 recording a threefold higher proportion in this functional group compared to the other two *Raffaelea* species (~ 7%). A substantial proportion of esters was detected in CANDI, comprising around 60% of its VOC profile. Terpenoid compounds were notably absent in ARXII. On the other hand, FSSC and two *Raffaelea* species (1 and 3) exhibited terpenoid proportions of ~ 30% and GRAPH showcased the highest terpenoid presence among all organisms reaching up to 45%. Table 1 summarizes the volatile compounds detected from the fungi associated with *M. mutatus*.

All identified VOCs from each of the twelve fungal strains were included in the multivariate analyses. The VOC compositions clusters distinctly between fungi isolated from different sources, exoskeleton and gallery versus gut isolates, all from *M. mutatus* (Fig. 2; Stress value = 0.112, PERMANOVA $p = 0.004$ and $r^2 = 0.46$). Furthermore, among external sources, the VOC profile of GRAPH differs within the same isolation source group. A similar pattern is observed for MEYE from the gut source. In contrast, certain pairs of fungal strains, such as CANDI and DEBA, as well as the strains RAFFA 1 and RAFFA 3, exhibited more similar VOC profiles. Figure 2 displays the similarity in VOC profiles between the previous *Raffaelea* strains. Moreover, this similarity is reinforced by the UPGMA plot (Fig. 3a), where all *Raffaelea* species that were isolated from *M. mutatus* form a distinctive cluster. The Venn diagram effectively illustrates the VOC profile of *Raffaelea* species isolated from *M. mutatus* (Fig. 3b), where three VOCs are shared among these three species (12.5%): 3-methyl-1-butanol, 2,4-dithiopentane, and 1-undecene. *Raffaelea* spp. 1 and 3 share four VOCs (16.7%): 4-heptanone, 4-heptanol, camphor, and α-terpineol. Last, there is an overlap of two VOCs (8.3%) between RAFFA 1 and ARXII: 2-methyl-1-butanol and 4-methyl-1-pentanol.

Behavioral response of beetles to fungal VOCs

Megaplatypus mutatus behavioral responses to fungal volatiles were investigated using a Y-tube olfactometer setup with FSSC, RAFFA 1, RAFFA 3, ARXII, CANDI, and GRAPH. Dispersal-stage males and females only showed a positive orientation walking behavior in response to three fungal strains, belonging to FSSC, ARXII, and RAFFA 1. However, the percentage of response showed considerable variability, along with the influence of temperature treatments on these responses (Additional file 1: Section S3).

Figure 4 shows the response patterns of the beetles to the VOCs emitted by FSSC and RAFFA 1 at room temperature (RT) that were immediately subjected to experimental conditions, compared to zero degrees' Celsius group (0 °C). The analysis highlights the differences found at low temperature, where statistical differences emerged after the 48-h exposure treatment. Specifically, males showed a walking behavior toward RAFFA 1 volatiles ($\chi^2 = 4.17$, $p < 0.05$), while against FSSC all males walked toward the control treatment ($p < 0.05$; Additional file 1: Section S3). Female beetles, on the other hand, showed a significant orientation toward the volatiles emitted by FSSC ($\chi^2 = 8.16$, $p < 0.05$), suggesting that female individuals display a positive trend in which their responses to specific fungal VOCs increase with prolonged exposure to low temperatures.

Conversely, ARXII volatiles were associated with a low response, with approximately 30% of males and 15% of females only at room temperature, although these responses were not statistically significant (p value of 0.16 and 0.12, respectively; Additional file 1: Section S3). Notably, subsequent treatments did not induce an orientation to the fungal VOCs, in contrast to the response seen for RAFFA 1. For both male and female beetles, there was no orientation to the VOCs of the species GRAPH, RAFFA 3 or CANDI. Instead, the response to the control treatment reached 100% in all cases (Additional file 1: Section S3). This trend suggests a repellent effect of the emitted VOCS toward the beetles under the experimental conditions.

Discussion

Our current study reports 72 volatile organic compounds (VOCs) produced by 12 fungal species associated with the forest pest *M. mutatus*. The fungal strains evaluated were collected from galleries as well as from the

Table 1 Detection of VOCs from *M. mutatus*-associated fungi

VOC	VOC ID/Fungal ID	FSSC	ARXII	RAFFA 1	RAFFA 3	GRAPH	CANDI	MEYE	AMBRO	STAR	DEBA	CHAET	NIGRO
1	3-methyl-1-butanol	X	X	X	X	X	X	X	X	X	–	X	–
2	3-methyl-2-pentanone	X	–	–	–	X	–	X	–	–	–	X	–
3	4-methyl-3-hexanone	X	–	–	–	X	–	–	–	–	–	–	X
4	4-heptanone	X	–	X	X	–	–	–	–	–	–	–	X
5	2,4-dithiapentane	X	X	X	X	–	–	–	–	–	–	X	X
6	4-heptanol	X	–	X	X	–	–	–	–	–	–	–	X
7	alpha-pinene	X	–	–	–	–	–	–	–	–	–	–	–
8	1-undecene	X	X	X	X	X	–	–	–	–	X	X	X
9	camphor	X	–	X	X	–	–	–	–	–	–	–	–
10	alpha-terpineol	X	–	X	X	X	X	–	X	–	–	X	–
11	2-undecanone	X	–	–	X	X	–	X	–	–	–	X	X
12	2,2,4,4,6,8,8-heptamethyl nonane	X	–	–	–	–	–	–	–	–	–	–	–
13	(E,E)-1,3,5-heptatriene	–	–	–	–	X	–	–	–	–	–	–	–
14	4-methyl-2-hexanone	–	–	–	–	X	–	–	–	–	–	–	–
15	alpha-phellandrene	–	–	–	–	X	–	–	–	–	–	–	–
16	2-carene	–	–	–	–	X	–	–	–	–	–	–	–
17	beta-phellandrene	–	–	–	–	X	–	–	–	–	–	–	–
18	2-decanone	–	–	–	X	X	–	–	–	–	–	X	–
19	beta-elemene	–	–	–	–	X	–	–	–	–	–	–	–
20	9-aristolene	–	–	–	–	X	–	–	–	–	–	–	–
21	valencene	–	–	–	–	X	–	–	–	–	–	–	–
22	alpha-selinene	–	–	–	–	X	–	–	–	–	–	–	–
23	3-hydroxy-2-butanone	–	–	–	–	X	–	X	–	–	–	–	X
24	2-nonanone	–	X	–	–	X	–	X	–	X	–	X	–
25	azulene	–	–	X	X	X	–	–	X	–	–	–	X
26	2-methyl-1-butanol	–	X	X	–	–	X	X	X	X	–	X	–
27	1-pentanol	–	X	–	–	–	–	–	–	–	–	–	–
28	4-methyl-1-pentanol	–	X	X	X	–	–	–	–	–	–	X	–
29	3-methyl butanoic acid	–	X	–	–	–	–	–	X	X	–	–	–
30	1-hexanol	–	X	–	–	–	–	–	–	–	–	–	–
31	3-methyl butyl ethanoate	–	X	–	–	–	–	–	–	–	–	–	–
32	gamma-butyrolactone	–	X	–	–	–	–	–	X	–	–	–	–
33	1-heptanol	–	X	–	–	–	–	–	–	–	–	–	–
34	heptyl ethanoate	–	X	–	–	–	–	–	–	–	–	–	–
35	1-dodecene	–	X	–	–	–	–	–	–	–	X	X	X
36	dimethyl trisulfide	–	–	–	–	–	–	–	–	–	–	–	X
37	2-undecanol	–	X	–	–	–	–	X	–	–	–	X	–
38	3,4-dimethyl-1-pentanol	–	–	X	–	–	–	–	–	–	–	–	–
39	2-phenyl ethanol	–	–	X	X	–	X	X	X	X	X	X	X
40	3-methyl-2-pentanol	–	–	–	–	–	–	–	–	–	–	X	–
41	1-(methylthio) butane	–	–	–	–	–	–	–	–	–	–	X	–
42	2-(methylthio) ethanol	–	–	–	–	–	–	–	–	–	–	X	–
43	3-nonanol	–	–	–	–	–	–	–	–	–	–	X	–
44	2-octanol	–	–	–	–	–	–	X	–	–	–	X	–
45	3-octanol	–	–	–	–	–	–	–	–	–	–	X	–
46	2-hexanone	–	–	–	–	–	–	–	–	–	X	–	X
47	ethyl 2-methylpropanoate	–	–	–	–	–	X	–	X	X	–	–	–
48	ethyl 2-methylbutanoate	–	–	–	–	–	X	–	X	X	–	–	–
49	ethyl 3-methylbutanoate	–	–	–	–	–	X	–	X	–	–	–	–

Table 1 (continued)

VOC	VOC ID/Fungal ID	FSSC	ARXII	RAFFA 1	RAFFA 3	GRAPH	CANDI	MEYE	AMBRO	STAR	DEBA	CHAET	NIGRO
50	2-heptanol	–	–	–	–	–	–	X	X	–	–	–	–
51	E)-3-nonadiene	–	–	–	–	–	–	–	X	–	–	–	–
52	(3-methylthio)-1-propanol	–	–	–	–	–	–	–	X	–	–	–	–
53	2-pentanol	–	–	–	–	–	–	–	X	–	–	–	–
54	2-heptanone	–	–	–	–	–	–	–	X	–	–	–	–
55	3-methyl-1,2-cyclopentanedione	–	–	–	–	–	–	–	X	–	–	–	–
56	ethyl butanoate	–	–	–	–	–	X	–	–	–	–	–	–
57	(3-methylbutyl) ethanoate	–	–	–	–	–	X	X	–	–	–	–	–
58	(2-methylbutyl) ethanoate	–	–	–	–	–	X	X	–	–	–	–	–
59	4-methyl-2-pentanol	–	–	–	–	–	–	X	–	–	–	–	–
60	5-methyl-2-hexanone	–	–	–	–	–	–	X	–	–	–	–	–
61	6-methyl-2-heptanone	–	–	–	–	–	–	X	–	–	X	–	–
62	5-methyl-2-heptanone	–	–	–	–	–	–	X	–	–	–	–	–
63	6-methyl-2-heptanol	–	–	–	–	–	–	X	–	–	–	–	–
64	methyl 3-methylbutanoate	–	–	–	–	–	–	–	–	X	–	–	–
65	1-octene	–	–	–	–	–	–	–	–	X	–	–	–
66	2-methylpropyl 3-methylbutanoate	–	–	–	–	–	–	–	–	X	–	–	–
67	3-methylbutyl 2-methylpropanoate	–	–	–	–	–	–	–	–	X	–	–	–
68	2-methylbutyl 2-methylbutanoate	–	–	–	–	–	–	–	–	X	–	–	–
69	pentyl 3-methylbutanoate	–	–	–	–	–	–	–	–	X	–	–	–
70	3-methylbutyl 3-methyl butanoate	–	–	–	–	–	–	–	–	X	–	–	–
71	*N*-(3-methylbutyl) acetamide	–	–	–	–	–	–	–	–	X	–	–	–
72	4-methyl-2-pentanone	–	–	–	–	–	–	–	–	–	X	–	–

FSSC *Fusarium solani* species complex, ARXII *Raffaelea arxii*, RAFFA 1 *Raffaelea* sp. 1, RAFFA 3 *Raffaelea* sp. 3, GRAPH *Graphium basitruncatum*, CANDI *Candida insectalens*, MEYE *Meyerozyma guilliermondii*, AMBRO *Ambrosiozyma platypodis*, STAR *Starmera* sp., DEBA *Debaryomyces* sp., CHAET *Chaetomium* sp., NIGRO *Nigrospora sphaerica*

exoskeleton or gut of males, females, and larvae. In addition, we investigated the orientation response of males and females to fungal VOCs using more than five hundred beetles in eighteen independent bioassays. Thus, providing a first comprehensive analysis of VOCs and exploring the potential role of different fungal species for the interaction with *M. mutatus.*

Alcohols dominated the profiles of *R. arxii*, *Raffaelea* sp. 1, *M. guilliermondii*, being 50% of the total compounds. Ketones exhibited a significant representation, approx. 45%, in *Fusarium solani* species complex, *G. basitruncatum* and *M. guilliermondii*. A similar analysis performed by Gugliuzzo et al. (2023) in ambrosia beetles' fungal communities showed that esters represented the largest functional group of compounds in the profile of *A. grosmanniae* but were less abundant in *Acremoniun* and almost absent in the volatile profile of *Cladosporium*. Monoterpenes were detected in emissions from the three fungal species studied, but sesquiterpenes were only detected from a species of *Acremonium* genus.

Among the recorded VOCs in the present work, five compounds were present in near 80% of the fungal species studied were 2-methyl-1-butanol, 2-phenylethanol, 3-methyl-1-butanol, α-terpineol, and 1-undecene. Four of these compounds are alcohols and have been previously reported as VOCs in several fungal species that interact with a wide range of insect orders, including Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, and Orthoptera (e.g. Davis et al. 2013; Zhou and Jander 2022). These VOCs have been associated with several ecological roles, ranging from being attractants, repellents, and/or fungistatics to potentially acting as pheromones that could inhibit reproductive development in insects (Nout and Bartelt 1998; Himuro et al. 2011; Cale et al. 2016). As for the alkene 1-undecene, it has been recorded as a fungal VOC (Ahearn et al. 1997) and it may be involved in repelling ants or other insects from entering breeding burrows of beetles (Geiselhardt et al. 2006), biocidal effect for nematodes (Popova et al. 2014), antifungal activity (Kong et al. 2020) and recently, it has been documented as an attractant for the first time in insects, with a particular focus on *Aedes* mosquitoes (Kashiwagi et al. 2022). However, to our knowledge, there are no

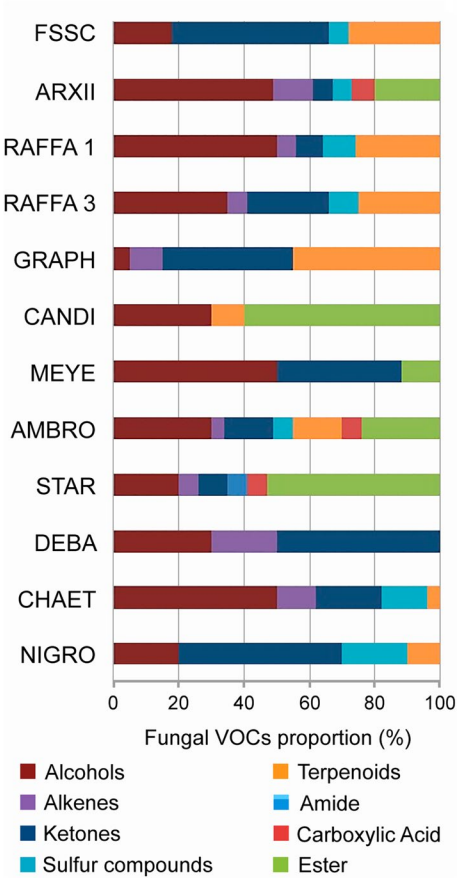

Fig. 1 Proportion of functional groups of volatile organic compounds emitted by fungi associated with *M. mutatus*. Acronyms are defined in the caption of Table 1

records of its detection in ambrosia interactions within platypodines.

Researchers have recently been exploring the relationship between VOC profiles and the biological context in which they arise. Farh and Jeon (2020) showed the correlation between the various lifestyles of filamentous fungi and their distinct emissions. Subsequently, Guo et al. (2021) determined that the VOC profiles of fungal species could be related to their trophic modes, substrate preferences, and engagement in symbiotic or non-symbiotic relationships. Our multivariate analyses support the hypothesis and provide evidence of a differentiation between fungal species isolated from two sources, e.g. the gut of *M. mutatus* on one side and the exoskeleton and gallery on the other (Fig. 2). This differentiation might be linked to the taxonomic affiliations of the gut species, which are mostly members of the Saccharomycetales order. On the other hand, Nordstrom et al. (2022) showed that *Fusarium* species with high morphological and genetic similarities can be distinguished by their VOC profile by SPME with GC–MS with a significant degree of accuracy, even at different inoculation time points.

Our findings provide evidence for the potential usefulness of VOCs profiles in the chemotaxonomy of *Raffaelea* species. Previous research has suggested that the fungal strains under study belong to three distinct species (Ceriani-Nakamurakare et al. 2016). The current results demonstrate that VOC profiles exhibit discriminatory potential among the examined *Raffaelea* strains. This is particularly noteworthy considering the similarity in habitat, and the observed similarities in VOC profiles are likely attributable to the taxonomic proximity of these three species. Nevertheless, it is important to acknowledge the limitation of the analysis due to the scarce availability of previous VOC studies within this genus. This context highlights the potential of VOC analysis as a new technique for identifying fungal guilds, lifestyles, and species, providing a complementary or alternative approach to current morphological or DNA-based approaches.

In terms of ambrosia interactions, *M. mutatus* represents the only member of Platypodinae with five recorded *Raffaelea* species. In example, Guerrero (1966) reported *Raffaelea santoroi*, and Ceriani-Nakamurakare et al. (2016, 2018, 2020) reported four *Raffaelea* species, including *Raffaelea* aff. *arxii* and three additional unidentified species named *Raffaelea* spp. 1, 2, and 3. These findings suggest that the relationship between *M. mutatus* and the *Raffaelea* genus is characterized by specific associations with one or more of the recorded species. The low fidelity among *Raffaelea* species could be interpreted as a sign of specificity for the genus. However, this does not preclude the possibility that *M. mutatus* could develop novel microorganism associations when exposed to new fungi, either due to range expansion of the fungi or the beetle (e.g. Wingfield et al. 2016; Morales-Rodriguez et al. 2021). Our current results showed varying behavioral responses from *M. mutatus* when exposed to VOCs from three *Raffaelea* species; for most species, the response was more congruent with a repellent action. Previous studies have shown that the interaction between ambrosia-bark beetles and their associated fungi may be driven, in part, by VOCs acting as cues that facilitate the detection of specific symbionts, inoculum hotspots inside the gallery, and aiding in the short-range detection of host plants (Hulcr et al. 2011; Ranger et al. 2021; Kendra et al. 2022; Gugliuzzo et al. 2023). Our bioassay results suggest that the blend of VOCs emitted by the fungi associated with *M. mutatus* may function as a repellent cue for identifying already colonized trees or trunk areas that have been attacked and the associated fungi is established. Similar results were reported by Luna et al. (2014) where the authors observed that *Xyleborinus saxeseni*

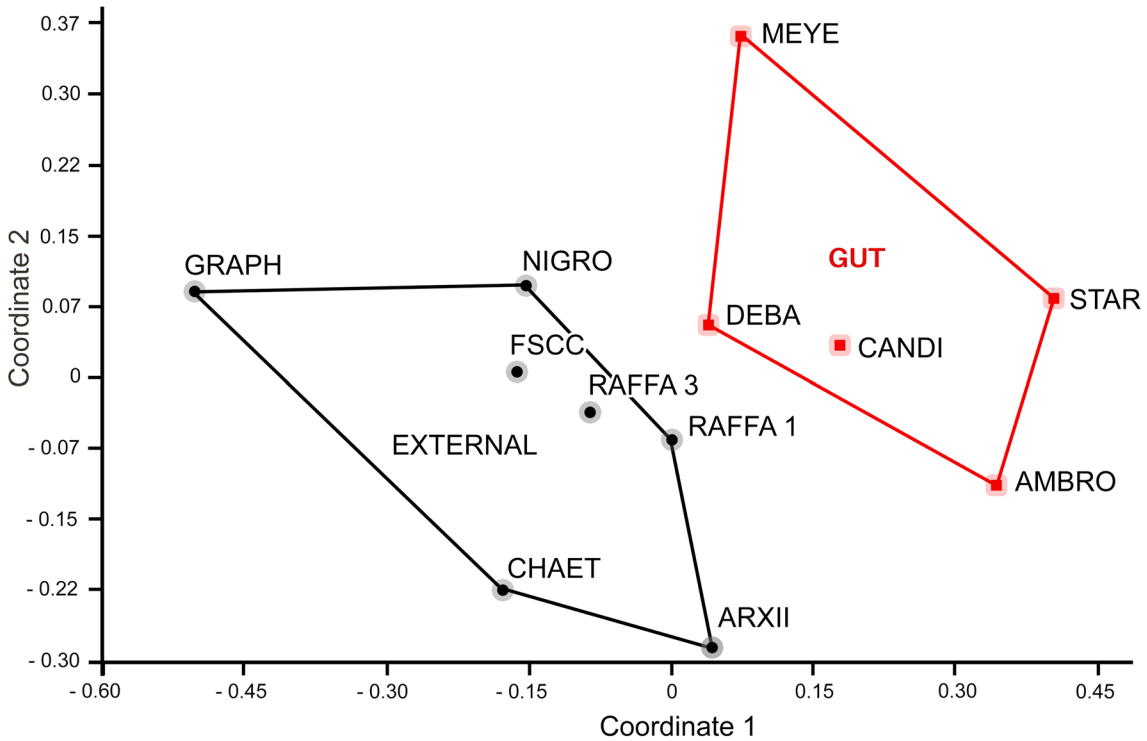

Fig. 2 NMDS ordination plot depicting fungal species differentiation based on VOC composition. Species isolated from *M. mutatus*' gut are represented by red squares, isolation from exoskeleton and gallery are indicated by black circles. Acronyms are defined in the caption of Table 1. Stress = 0.112; PERMANOVA $p = 0.004$ and $r^2 = 0.46$

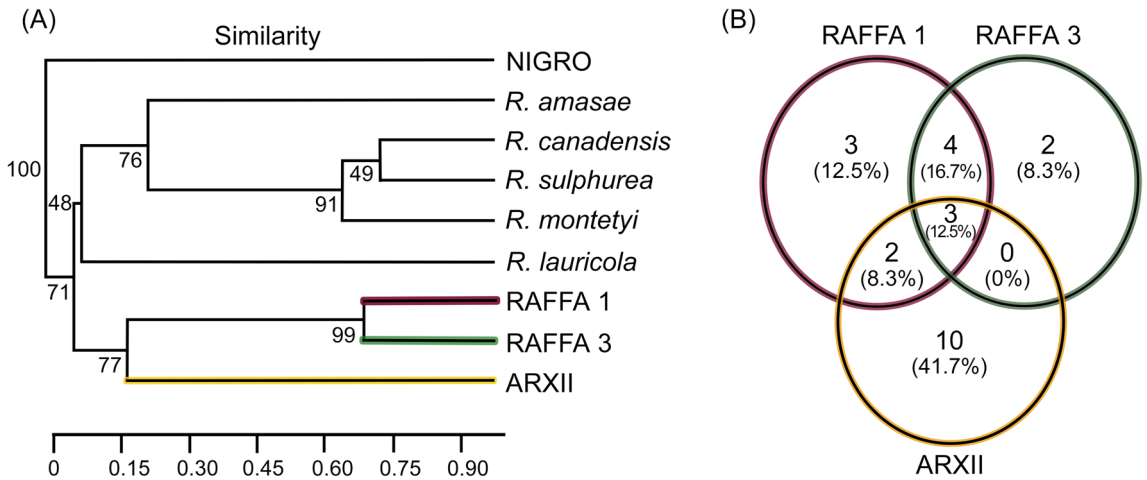

Fig. 3 *Raffaelea* species regarding VOC profile composition. **A** UPGMA illustrating the interrelationships among *Raffaelea* species, Jaccard index and 1000 bootstrap replications. **B** Venn diagram depicting strains isolated from *M. mutatus*, showing the shared presence of three compounds (12.5%): 3-methyl-1-butanol, 2,4-dithiapentane, and 1-undecene. *Raffaelea* species isolated from *M. mutatus* are indicated by colored lines. Acronyms are defined in the caption of Table 1

avoid volatiles from its associated ambrosia fungus *Geosmithia morbida* M. Kolařík, Freeland, C. Utley & Tisserat. Furthermore, taking into account prior research findings that highlight that both females and males are capable of carrying key components of the core fungal community during dispersal (Ceriani-Nakamurakare et al. 2016, 2018, 2020), the obtained results support the above-mentioned findings, thus showing that, in the

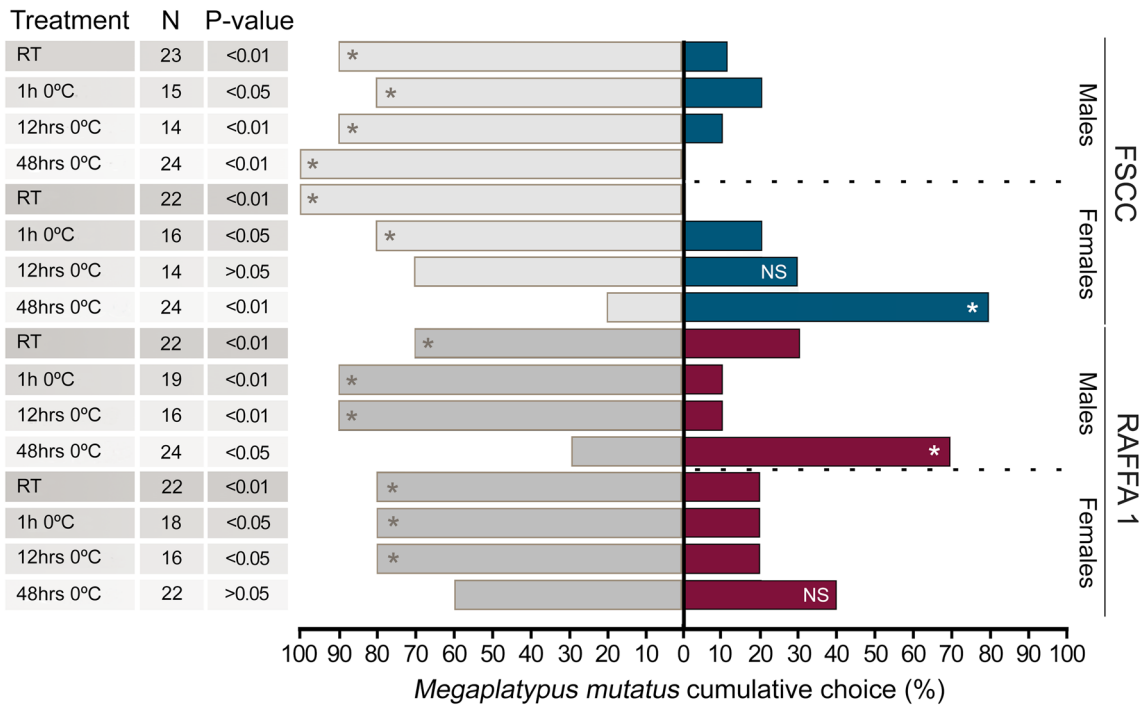

Fig. 4 Behavioral response of *M. mutatus* toward fungal volatiles emitted by strains of FSSC and RAFFA 1, the two key species that induce orientation behavior in males and females at the dispersal stage. Colors denote FSSC (blue), RAFFA 1 (red), and control (gray). Acronyms are defined in the caption of Table 1. Asterisks indicate statistically significant differences while NS for non-statistical differences. *N*: number of insects used, RT: room temperature for immediately VOCs assays (see Additional file 1: Section S3)

dispersal phase, adult beetles actively transport the requisite fungal community for host plant colonization. This underscores an intentional and purposeful role in the vectoring process. Outside the gallery, beetles usually exhibit a repellent behavior, suggesting a reduced likelihood of encountering the fungal partners. The absence of proactive searching for fungal inoculum may also indicate that dispersal beetles, in most conditions, lack a behavioral tendency to regain inoculum. In terms of the host finding process of *M. mutatus*, the current results show that adults are usually not attracted to the VOC cues that might be involved in the detection of conspecifics on the same tree (attacks), supporting the hypothesis and according field observations, that this species usually does not accumulate a high number of attacks on the host poplar (ranging from 1 to 15) and tends to select vigorous trees. Consequently, in *M. mutatus* chemical signals conveyed through VOCs appear to be more related to substrate occupation than, for example, reproduction. Among abiotic factors, temperature plays a multiple role in the dynamic interaction between semiochemicals and insect behavior in ecological systems. As ectotherms, beetles are sensitive to temperature changes, which can disrupt their physiological processes, thereby modulating their ability to perceive and respond to chemical signals, including VOCs (Jenkins et al. 2001; Overgaard and MacMillan 2017). Our assays enabled us to assess the impact of pre-exposure to low temperatures on the beetles' orientation responses, as the exposure time to low temperatures increased, both males and females also increased their tendency to orient toward the VOCs of *Raffaelea* sp. 1. It is worth noting that this species was only detected in adult beetles (Ceriani-Nakamurakare et al. 2016). Males showed a significant response to this species, after pre-exposition to low temperature, which could indicate that low temperature trigger behaviors related to congener detection. These results may partially explain the increased incidence of attacks on host plants with concurrent infestations following cold winters (data not shown). On the other hand, we observed that females showed an orientation toward the VOCs of FSSC after being exposed to low temperatures. This taxon was recorded in significant abundance in females, males and galleries (Ceriani-Nakamurakare et al. 2016). Consistent with its putative functional role, dispersing females may actively seek as a cue for conspecific detection or to retrieve the inoculum after exposure to low temperatures, considering it as a potential nutritional source and its nematicidal properties (Ceriani-Nakamurakare et al. 2016, 2020).

From the perspective of the functional roles of the partners in the association, it becomes evident that these results are challenging to interpret mainly due to the experimental set-up, the limited number of insects and its novelty in relation to *M. mutatus,* and to the best of our knowledge, in the context of the subfamilies Platypodinae and Scolytinae. Our novel results suggest that VOCs should be considered as a complementary approach to enrich fungal species systematics, and that volatile compound-mediated interactions are a fundamental component likely driving ambrosia beetle–fungus interactions. Therefore, future work with deeper and more comprehensive research will explore potential variability, mainly by fulfilling the study with *G. basitrumcatum* and *C. insectalens* (0 °C), heterospecific fungi growing together and using their natural substrate, as well as additional bioassays with the beetle that collectively represent what happens in nature and thus provide insightful data to fill the knowledge gaps.

Implementation of integrated pest management is closely linked to a thorough understanding of the pest's biology and the factors contributing to its establishment and spread. Subsequent studies will investigate whether the recorded VOCs play a role in shaping the fungal community by acting as selective agents for organisms with elevated tolerance thresholds to specific VOCs and/or through the interaction of emitted compounds. In addition, these studies will evaluate whether blends of multispecies VOCs are capable of driving behavior that may be relevant to *M. mutatus* control strategies. Meanwhile, further bioassays will be made to gain a better understanding of how temperature influences the behavioral responses of the forest pest to fungal volatiles, as well as their thermal limits. This continuous research will greatly advance the understanding of dynamics within the ambrosial interactions.

Conclusions and perspectives

Our research on *M. mutatus* provides insights that contribute to a comprehensive understanding of the ambrosial beetle–fungi interaction and novel observations in the behavior of *M. mutatus*, which displays an ability to discern between volatiles profiles and orientating its choice to the fungus or control source. These findings also shed light on the behavioral alterations that arise when *M. mutatus* is pre-exposed to different periods of zero degree Celsius, revealing the beetle's remarkable capacity to tolerate such conditions while retaining its ability to walk and orientation toward volatile sources. Our findings suggest an unexplored area within platypodine members that could enhance actual pest management or serve as a starting point for alternative control strategies.

Abbreviations

FOS	Fungus odor source
VOCs	Volatile organic compounds
GC-MS	Gas chromatography-mass spectrometry
MEA	Malt extract agar
RT	Room temperature
SPME	Solid-phase micro extraction
FSSC	*Fusarium solani* species complex
ARXII	*Raffaelea arxii*
RAFFA 1	*Raffaelea* sp. 1
RAFFA 3	*Raffaelea* sp. 3
GRAPH	*Graphium basitruncatum*
CANDI	*Candida insectalens*
MEYE	*Meyerozyma guilliermondii*
AMBRO	*Ambrosiozyma platypodis*
STAR	*Starmera* sp.
DEBA	*Debaryomyces* sp.
CHAET	*Chaetomium* sp.
NIGRO	*Nigrospora sphaerica*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13717-024-00490-z.

Additional file 1: Section S1. Metadata of the fungal species employed in this study, isolated from the ambrosia beetle *Megaplatypus mutatus*. **Section S2**. Kóvats retention indexes calculated on DB-5 and DB-WAX columns and identification methods of all the volatile organic compounds found in fungal species associated with the ambrosia beetle *Megaplatypus mutatus*. Identification methods included (RI = retention index by Kóvats, S = coelution of standards, and MS: mass spectra by comparing with the NIST-Wiley 8.0 database). **Section S3**. Behavioral response of *Megaplatypus mutatus* toward volatiles emitted by fungal strains. Assays were conducted at room temperature (RT, 22–24 °C) with the number of insects (N) used in a 1:1 sex ratio.

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Author contributions
ECN, CC, and PGA conceptualized the experiments; MS and PGA analyzed and identified the volatile profiles; ECN and CC isolate, identified, and provided the fungal strains; ECN did the field work, the behavioral assays, and the data analyses; MS and ECN wrote the first draft of the manuscript. All authors read and contributed to the further versions of the manuscript and approved the submission.

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Availability of data and materials
The original contributions presented in the study are included in the article. The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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