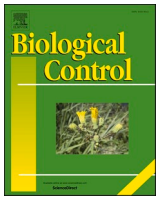
Biological Control 169 (2022) 104868

Contents lists available at ScienceDirect

Biological Control

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



Volatile-mediated interactions between *Trichoderma harzianum* and *Acanthoscelides obtectus*: A novel *in vitro* methodology to evaluate the impact of microbial volatile compounds on dry grain storage pests

Samuel Álvarez-García [a,*], Álvaro Rodríguez-González [a], Laura Zanfaño [a], Santiago Gutiérrez [b], Pedro A. Casquero [a]

[a] Grupo Universitario de Investigación en Ingeniería y Agricultura Sostenible (GUIIAS), Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad, Universidad de León, Avenida Portugal 41, 24071 León, Spain
[b] Grupo Universitario de Investigación en Ingeniería y Agricultura Sostenible (GUIIAS), Área de Microbiología, Escuela de Ingeniería Agraria y Forestal, Universidad de León, Campus de Ponferrada, Avenida Astorga s/n, 24401 Ponferrada, Spain

HIGHLIGHTS

- *Trichoderma harzianum* volatiles increase *Acanthoscelides obtectus* adult mortality.
- *T. harzianum* volatiles reduce *A. obtectus* emergence and bean damage.
- *erg1* (squalene epoxidase) silencing rises insect mortality, reducing adult emergence and bean damage.
- Ventilation plays a key role in microbe-insect volatile interactions.
- VOC Chambers are a reliable technology to test *in vitro* microbe-insect volatile interactions.

ARTICLE INFO

Keywords:
Trichoderma
Acanthoscelides
VOC
Trichodiene
Bean
VOC Chamber

ABSTRACT

Biological interactions mediated by Biogenic Volatile Organic Compounds (BVOCs) is a well-established field that has been researched for decades. Although extensive focus is currently given to the control of insect pests using natural molecules, the study of volatile interactions between microorganisms and insects has been largely neglected and has only begun to attract more attention in recent years. In this work, we developed a novel protocol to assess the effects of microbial BVOCs directly produced by growing microbial strains on dry grain insect pests and the seed damage they cause, using VOC Chambers to evaluate both sealed and unsealed conditions. Four *Trichoderma harzianum* strains were tested against *Acanthoscelides obtectus*, a wild type and three of its transformants. These had been previously obtained by introducing the *tri5* gene and thus overproducing the volatile trichodiene, or by silencing the *erg1* gene, which encodes for a squalene epoxidase, therefore reducing ergosterol levels and increasing squalene ones in the fungus. Results demonstrated that ventilation plays a key role in these interactions. All fungal strains significantly increased adult mortality in sealed conditions, while this effect was barely noticeable in unsealed ones. Nevertheless, subsequent insect emergence from bean seeds and bean damage were still significantly reduced in both conditions. The *erg1* silenced strains caused significantly higher levels of adult mortality than the rest in sealed conditions and lower insect emergence in both sealed and unsealed ones. Bean damage produced by insects was lower also when exposed to BVOCs from these strains in sealed conditions. Conversely, trichodiene overproduction did not show enhanced toxicity or significant reduction of insect emergence and bean damage in the tested conditions. Therefore, *T. harzianum* BVOCs, especially those from *erg1* silenced strains, should be further researched for their potential use in the biological control of *A. obtectus* infestation in dry grain storing facilities. VOC Chambers have shown themselves to be a

Abbreviations: BVOCs, Biogenic Volatile Organic Compounds; VOC Chamber, Volatile Organic Compound Chamber; BCAs, Biological Control Agents; FDP, Farnesyl diphosphate; PDA, Potato Dextrose Agar; PGI, Protected Geographical Indication; GLM, General Linear Model; ANOVA, one-way analysis of variance; LSD, Least Significant Difference.

* Corresponding author.
E-mail address: salvg@unileon.es (S. Álvarez-García).

https://doi.org/10.1016/j.biocontrol.2022.104868
Received 3 November 2021; Received in revised form 16 February 2022; Accepted 17 February 2022
Available online 28 February 2022

reliable method in the screening of *in vitro* volatile mediated interactions between growing microbial strains and insect pests.

1. Introduction

The bean weevil *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchidae) is one of the pests with a higher worldwide impact on stored legume seeds (Baier and Webster, 1992; Berger et al., 2017) which in turn poses a significant threat to food security as legumes play a pivotal role in human nutrition, especially regarding protein intake (Casquero et al., 2006; Sanon et al., 1998). This species, originated in Mesoamerica, has now spread worldwide (Berger et al., 2017), affecting both modern industrial storage facilities (Rodríguez-González et al., 2019) and traditional producers in low-income countries (Paul et al., 2009). The weevil attack can start in the fields or directly in storage, where the pest produces most of the damage (Baier and Webster, 1992). Female adults lay their eggs among the seeds, and the larvae bore holes to penetrate them, where they feed and develop until the new adult emerges (Berger et al., 2017). The damage caused to the seeds lowers the quality of the product, generates considerable loss of profit, and can even affect their ability to germinate, therefore jeopardizing further crop productivity (Paul et al., 2009).

Much of the conventional control strategy against this pest relies on the use of synthetic chemical insecticides (Rodríguez-González et al., 2018). Nevertheless, issues concerning health and environmental hazards, as well as the arising of pest resistance (Herrera et al., 2015), have led to Integrated Pest Management perspectives, including biological control, as an important way of addressing these challenges. In this regard, the role of Biogenic Volatile Organic Compounds (BVOCs) and their biocontrol capabilities have been studied for decades (Cordovez et al., 2015; Dukare et al., 2019; El Ariebi et al., 2016; Morath et al., 2012; Ponce et al., 2021; Schulz-Bohm et al., 2017). BVOCs are small molecules of volatile nature produced and emitted by living organisms (Herrman, 2010). They serve a multitude of purposes in inter-organismic relationships, including recognition, competition, repellence, or stimulation, and have been proven to mediate interactions between microorganisms, plants, and animals, including insect pests (Quintana-Rodriguez et al., 2015; Bueno et al., 2020; Álvarez-García et al., 2021; Taylor et al., 2021).

Numerous studies have been conducted confronting insects to plant volatile extracts and essential oils (Paul et al., 2009; Rojht et al., 2012a; Rodríguez-González et al., 2019; Gokturk et al., 2020; Palla et al., 2020). Regarding microbe-insect volatile interactions, research has been strongly focused on behavioral responses exerted by individual or mixed BVOCs on insects (Bueno et al., 2020; Gershow et al., 2012; Ponce et al., 2021). Both attraction and repellence have been extensively reported as effects produced by microbial BVOCs on stored-products arthropods (Rodríguez-González et al., 2018, 2019; Lozano-Soria et al., 2020; Ponce et al., 2021). In addition, microbial BVOCs also produce toxicity in insects, although research is less abundant. Herrera et al., (2015) demonstrated that isolated fungal BVOCs have insecticidal properties against *Sitophilus zeamais*. Inamdar et al. (2014)a,b and Zhao et al., (2017) reported toxic effects of fungal volatiles on *Drosophila melanogaster*. Furthermore, some studies have explored the physiological and molecular processes behind the effects produced by microbial BVOCs on insects (Inamdar and Bennett, 2014; Inamdar et al., 2014a,b).

Among those Biological Control Agents (BCAs) producing active BVOCs, *Trichoderma* stands out as one of the most studied and effective fungal genera (Guo et al., 2020; Macías-Rodríguez et al., 2020). Several investigations have demonstrated different effects produced by *Trichoderma* volatiles on plants (Cardoza et al., 2015; Hung et al., 2013; Lee et al., 2019, 2016), as well as a strong influence on microbial metabolism, growth, and development (Álvarez-García et al., 2021; Dukare et al., 2019; Mutawila et al., 2016; Taylor et al., 2021; Wang et al., 2018). Regarding their activity on insects, *Trichoderma* BVOCs have been assayed on *Drosophila* as a model organism, producing a variety of toxic effects, ranging from increased mortality to apoptosis and developmental issues (Inamdar et al., 2014a,b; Zhao et al., 2017).

Notwithstanding the referred studies, the assessment of the effects produced by microbial BVOCs on insects and insect pests still faces a range of limitations derived from the volatile nature of the compounds and the shortage of reliable material and protocols to perform direct volatile-mediated interaction assays between growing microbial colonies and insects. Behavioral responses to volatiles are mostly assayed using olfactometers and similar devices (Ponce et al., 2021). These methodologies are usually designed to test attraction and repellence but not insecticidal activity. Additionally, wind tunnels, video tracking, Petri dishes setups, and vial assays have been employed to evaluate different volatile interactions (Gershow et al., 2012; Ponce et al., 2021; Rojht et al., 2012b). Most of these techniques could be adequate to assess purified volatiles but are ill-suited when evaluating the effects of directly produced BVOCs by growing microbes. In this regard, Inamdar et al. (2014)a,b developed a system to evaluate the effects of microbial volatiles on *D. melanogaster* by perforating the lids of Petri dishes. Although this study presented an interesting approach, it relies on non-specific material and handmade modifications that may compromise homogeneity and replicability. Moreover, these researchers only evaluated the effects of VOCs in sealed conditions. The importance of ventilation and gas exchange for inter-organismic volatile interactions has been pointed out and demonstrated by previous studies (Álvarez-García et al., 2021; Kai et al., 2016; Piechulla and Schnitzler, 2016). In this regard, specific VOC Chambers have been developed to perform standardized microbe-microbe volatile-mediated interaction assays (Álvarez-García et al., 2021).

Therefore, in this study, VOC Chambers (Álvarez-García et al., 2021) were used to expose *A. obtectus* adults to the BVOCs produced by four *T. harzianum* strains, a wild type (T34) and three of its transformants. Two of them (T34-5.27 and E20-5.7) overproduce the volatile trichodiene, while the other (E20) presents a reduction in ergosterol production and a subsequent accumulation of squalene (Cardoza et al., 2006; Malmierca et al., 2015a,b; Lindo et al., 2020), an intermediate compound in several important biosynthetic pathways although usually not present in volatile state. These strains have also been tested for their activity against phytopathogenic fungi (Álvarez-García et al., 2021; Taylor et al., 2021) and their effect on plant development and metabolism (Malmierca et al., 2015a,b; Lindo et al., 2020; Taylor et al., 2021). Besides, regarding microbe-insect interactions, Rodríguez-González et al., (2018, 2019) demonstrated that the squalene-overproducing strain E20 repelled *A. obtectus* adults of both sexes, and therefore reduced the damage they produced on beans. Moreover, all four strains significantly increased levels of insect mortality compared to the untreated controls. Nevertheless, in that study the fungal spores were sprayed over the beans and the insects entered in contact with them afterwards, making it difficult to evaluate whether the rise in mortality and the subsequent reduction of insect emergence and bean damage were directly caused by the microbial BVOCs or rather involved other interactions, like entomopathogenic activity or the production of toxic soluble metabolites.

We hypothesize that *T. harzianum* BVOCs exert biological effects on *A. obtectus* without the need for physical contact between insect and fungus and that ventilation plays a key role in these interactions. We also hypothesize that the genetic modifications of *T. harzianum* leading to trichodiene overproduction or altering squalene/ergosterol levels modify the outcome of these volatile interactions. Our final hypothesis was that VOC Chambers could be effectively used for the development of

a reliable protocol to evaluate microbe-insect volatile interactions. Therefore, our goals were: i) to determine the effects of *T. harzianum* BVOCs against *A. obtectus* in volatile-mediated interaction both in sealed and unsealed conditions, ii) to evaluate the subsequent influence of this volatile activity on the damage produced by *A. obtectus* on bean seeds, iii) to develop a reliable method for the assessment of microbe-insect volatile interactions using the VOC Chambers described by Álvarez-García et al., (2021), and iv) more specifically, to develop a protocol to assess the effects of BVOCs produced by growing microbial strains on dry grain storage pests and their impact.

2. Materials and methods

2.1. Microbial strains and culture conditions

Four *T. harzianum* strains were used to evaluate the effects of their BVOCs on *A. obtectus*. Among them, the parental strain *T. harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) (T34 from now onwards) is a well-characterized biocontrol agent (Cardoza et al., 2006; Malmierca et al., 2015b; Taylor et al., 2021). *T. harzianum* E20 derives from T34 by silencing the *erg1* gene, which encodes the squalene epoxidase. This modification resulted in the accumulation of squalene and the reduction in ergosterol levels (Cardoza et al., 2006). The other two transformants, T34-5.27 and E20-5.7, derive from T34 and E20, respectively, by expressing the *T. arundinaceum tri5* gene that encodes a terpene cyclase involved in the cyclization of farnesyl diphosphate (FDP) to trichodiene (Malmierca et al., 2015a, b). Therefore, trichodiene was overproduced by these transformants. This compound is the first specific intermediate in trichothecene biosynthesis and the only one of volatile nature in this pathway. Due to the accumulation of FDP in the E20 strain as a result of the silencing of *erg1*, and its subsequent channeling towards the formation of trichodiene, the production of this last metabolite in E20-5.7 has been demonstrated to be significantly higher than in T34-5.27 (Malmierca et al., 2015a, b).

All fungal strains were stored in 50% glycerol spore suspension at −80 °C in the "Pathogens and Antagonists Collection" at the "Pest and Diseases Diagnosis Laboratory" (PALDPD, University of León, León, Spain), and were activated by culturing on PDA (Potato Dextrose Agar; Difco Becton Dickinson, Sparks, MD) at 25 °C.

2.2. Bean seeds and insect rearing

The dry bean seeds (*P. vulgaris*) employed in this study belonged to the "Riñón Menudo" landrace and were collected during the year 2020 from the Protected Geographical Indication PGI "Alubia de La Bañeza-León" (EC Reg. n.256/2010; March 26th, 2010, OJEU L880/17).

The population of *A. obtectus* used in the present study was collected in successive years from several dry bean storage facilities belonging to the PGI "Alubia de La Bañeza-León". Insects were maintained and reproduced in 4 L glass jars containing the aforementioned bean seeds, and were kept in a controlled chamber with 25 ± 1 °C, relative humidity of 60 ± 5%, and darkness. All adults were removed from the jars 3 days before setting up the experiments, ensuring a homogeneous population of newly emerged 1 to 3 days-old insects to be introduced in the assays.

2.3. Effects produced by Trichoderma BVOCs on the mortality of A. Obtectus adults

Non-vented VOC Chambers (J.D. Catalán S.L.; Arganda del Rey, Madrid, Spain) as described by Álvarez-García et al., (2021) were used to perform the assays. These devices are composed of a lower and an upper Petri plate separated by a perforated intermediate piece that holds them in place facing one another, and thus forming a chamber that allows the flow of BVOCs from one plate to the other through a 30 mm central hole in the intermediate piece.

A novel protocol was designed and performed as follows (Fig. 1). Plugs (6 mm in diameter) from the fresh edge of 3 days-old colonies of the four *T. harzianum* strains were placed in the center of Petri dishes containing 18 ml of PDA. After two days of growth at 25 °C, the lids were removed, and the plates with the fungal culture were covered with an intermediate piece from the VOC Chambers, which was in turn covered with an autoclaved cellophane membrane and filter paper on top to allow fungal volatiles to pass through the hole while at the same time avoiding potential contamination by fungal spores and insects or bean

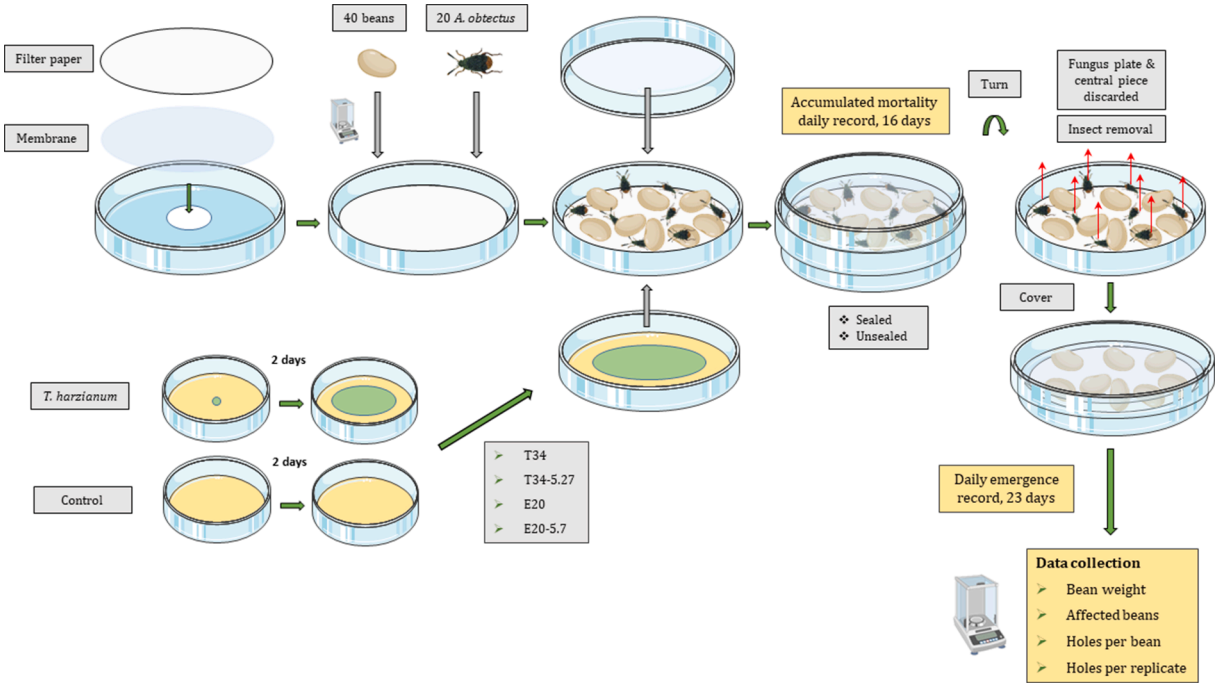


Fig. 1. Schematic representation of the protocol carried out using VOC Chambers (Álvarez-García et al., 2021) to evaluate the effects of *T. harzianum* BVOCs on *A. obtectus* adults and the damage they produce to bean seeds.

seeds falling onto the lower plate. Immediately afterwards, 20 unsexed 1 to 3 days-old *A. obtectus* adults and 40 undamaged bean seeds were placed over the filter paper and were finally covered with an upside-down Petri plate base to close the full VOC Chamber. The weight of the beans in each replicate was measured and recorded beforehand. Non-cultivated Petri plates with 18 ml of PDA were used as control treatment.

Two different sets of experiments were performed. A first one involved both Petri plates being sealed with the intermediate piece using 3 layers of Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid) to provide a more closed environment. This setup limits gas exchange with the exterior and allows for the build-up of higher BVOC concentration inside the chamber (sealed assay from now onwards). In the second assay, only the lower Petri plate was sealed with three layers of Parafilm®, leaving the upper plate unsealed. This disposition forces BVOCs to flow from the fungal culture to the upper plate, but at the same time provides a more open setup, increasing gas exchange with the exterior in the upper part of the chamber, and thus creates a lower BVOC concentration and higher oxygen availability for the insects (unsealed assay from now onwards). 5 replicates were performed per treatment.

Insects and beans were kept inside the VOC Chambers with the *Trichoderma* or the control plates for 16 days in a controlled chamber with 25 ± 1 °C and relative humidity of 60 ± 5%, while the mortality was daily recorded. After this period, the VOC Chambers were flipped over, and the intermediate pieces together with the plates with the fungal cultures were removed and discarded. *A. obtectus* adults (most of them dead by now) were removed from the experiment and stored at −80 °C for later microbial isolation assays. The remaining plates, containing only the bean seeds, were covered with a new lid after insect removal. The Petri plates containing the bean seeds were kept in the same conditions (25 ± 1 °C, relative humidity of 60 ± 5%) to allow a new generation of insects to develop inside them.

The fungal re-isolation was performed by placing 8 insect cadavers on Rose Bengal-Chloramphenicol Agar medium to verify Koch postulates and confirm that no *Trichoderma* spores had reached the insects through the central hole, thus ensuring that direct contact between fungi and insects was not interfering or jeopardizing the effects produced solely by volatile interactions. The absence of *Trichoderma* growth was checked after 7 days at 25 °C.

2.4. Effects produced by Trichoderma BVOCs on A. Obtectus reproduction, and the subsequent damage caused by the insects on dry bean seeds

The bean seeds coming from the previous section were checked every day for newly emerged insects. After the first insect appeared, the emerged adults were removed each day, and daily emergence was recorded for the following 23 days (Fig. 1). The count was stopped at this point to ensure that third-generation insects were not included in the results while considering that the emergence of new adults consistently ceased mostly around day 20.

After this emergence period, the 40 bean seeds from each replicate were weighted, and the results were compared with those obtained at the beginning of the assays. The percentage of weight loss (WL) was calculated as follows: WL (%) = (IW – FW) / IW × 100. Where IW represents the initial weight and FW the final weight. Besides, the number of affected bean seeds (showing at least one exit hole) per replicate, the total number of holes per replicate, and the number of holes per affected bean were counted and recorded.

2.5. Data treatment and statistical analyses

All assays were designed following a General Linear Model (GLM), with four fungal isolates plus the unexposed control. The cumulative percentage of mortality was calculated as Mortality (%) = number of dead insects / 20 × 100. The accumulated emergence was calculated by adding up the emerged insects on each replicate and day. Both percentage of mortality and accumulated emergence were transformed using the arcsine transformation formula (Gómez and Gómez, 1984) to perform the subsequent statistical analysis.

Data normality and equality of variances were assessed using Kolmogorov-Smirnovs and Levenés tests, respectively. A one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test ($p \leq 0.05$) were used to determine differences between treatments presenting normal and homoscedastic data. When these conditions were not met, a non-parametric Kruskal–Wallis H-test was performed, followed by a Mann-Whitney *U* test ($p \leq 0.05$). All statistical analyses were carried out using IBM SPSS Statistics 26.

3. Results and discussion

3.1. Effects produced by Trichoderma BVOCs on the mortality of A. Obtectus adults

The results presented in Fig. 2A show that the BVOCs produced by three of the four *T. harzianum* strains significantly increase the mortality of *A. obtectus* adults in sealed conditions compared to the control treatment. In this regard, T34-5.27 was the only one not showing statistically significant differences with the control. Additionally, this trichodiene-overproducing strain did not present differences with its parental strain T34 either. In turn, T34 showed significantly higher mortality than the control during the central days of the experiment (from day 4 to day 9). Finally, E20 and E20-5.7 significantly increased mortality, not only compared to the control but also to the other two fungal strains. These differences were more apparent during the first days of the assay, when adult mortality in the control treatment remained below 10%, and not higher than 30% for T34 and T34-5.27, while it rose to more than 60% for E20 and E20-5.7 on day 2, and around 80% on day 4 (Fig. 2A). These results point out an enhanced insecticidal activity in sealed conditions derived from the silencing of *erg1* and the resulting accumulation of squalene and reduction in ergosterol levels. Nevertheless, even though squalene can appear as a volatile compound and be emitted by organisms in some cases (Dutton et al., 2002; Jiang et al., 2015; Lanzón et al., 1994), it is better known for its biological role as an intermediate in several important biosynthetic pathways; for example, in the formation of hopanoids, and other triterpenoids (Do et al., 2009; Jiang et al., 2015; Spanova and Daum, 2011). Squalene is sometimes also accumulated as a final product inside the organism (Jahaniaval et al., 2000; Spanova and Daum, 2011). Therefore, it is unlikely that squalene itself directly accounts for the observed insecticidal effects.

Concerning trichodiene overproduction, T34-5.27 produced lower mortality than T34, although not statistically significant. This absence of significant differences in mortality between trichodiene overproducing strains and their parental ones seems to indicate that this compound does not possess significant insecticidal activity in the tested conditions.

Inorganic volatiles like CO_2, NH_3, or HCN cannot be completely ruled out for causing part of the basal activity in both the wild type and its transformants. pH changes derived from inorganic volatiles could also be involved. Besides, oxygen availability is likely to influence the results in these sealed conditions (Álvarez-García et al., 2021; Kai et al., 2016), especially with fast-growing microbes like *T. harzianum*. However, as clear differences are shown between similar strains, we can reasonably conclude that additional volatile traits other than the abovementioned ones must be involved in the observed insecticidal activity.

Regarding unsealed conditions (Fig. 2B), mortality was generally lower than in sealed ones for all treatments except the control and T34-5.27, which showed similar trends in both cases. Differences between treatments and the control were minimal in these unsealed conditions. Only T34 stood out with a slightly higher mortality rate than the control and E20, mainly during the first few days. Therefore, between sealed and unsealed conditions a major reduction in the insecticidal properties

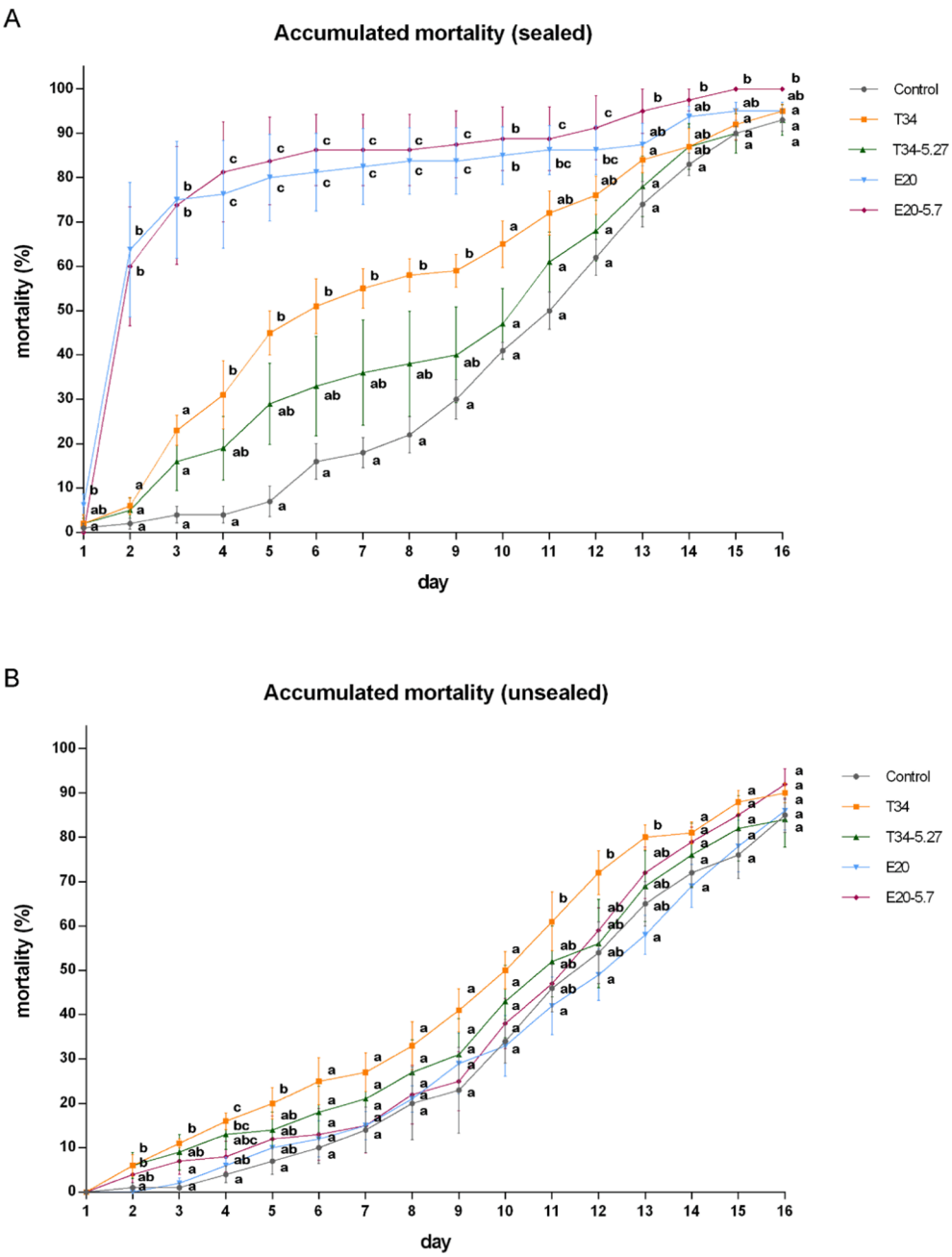


Fig. 2. Accumulated mortality (%) of *A. obtectus* adults in (A) sealed and (B) unsealed conditions exposed for 16 days to BVOCs produced by *T. harzianum* strains T34 (wild type, squares, orange); T34-5.27 (trichodiene overproducer, face-up triangles, green); E20 (*erg1* silenced, face-down triangles, blue); E20-5.7 (*erg1* silenced and trichodiene overproducer, diamonds, red); and untreated control (uncultured PDA medium, circles, grey). Data are represented as the accumulated percentage of dead insects (mean ± SE) from the initial 20 adults placed on each replicate. Different letters represent statistically significant differences between treatments for the same day, using one-way analysis of variance (ANOVA) followed by a Least Significant Difference (LSD) post hoc test ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of E20 and E20-5.7 BVOCs was observed (Fig. 2), suggesting that the previously described effects of the *erg1* silencing on insect mortality are only applicable in closed environments with limited gas exchange with the exterior. This, in turn, underlines the importance of ventilation and gas exchange in volatile-mediated interactions between insects and microorganisms, which had been already highlighted by previous studies for microbial and plant interactions (Kai et al., 2016; Piechulla and Schnitzler, 2016).

To confirm the absence of physical interaction between insects and fungi, eight insect cadavers were taken from each replicate and were cultured in Rose Bengal-Chloramphenicol Agar medium. *T. harzianum* did not grow from any of the fungal treatments or the control, thus confirming that the observed effects were the result of volatile-mediated interaction and were not influenced by direct contact.

Trichodiene has been described as the only non-phytotoxic intermediary in the trichothecenes' biosynthetic route (Desjardins et al., 2007). Nevertheless, trichodiene-overproducing strains have shown a significant plant-growth inhibitory effect (Malmierca et al., 2015a) as well as an increase in their antifungal activity (Álvarez-García et al., 2021, Taylor et al., 2021) compared to their non-trichodiene producing parental strains. These reported effects somehow contrast with the lower insect mortality obtained in the present study. Moreover, previous research showed that BVOCs emitted by the squalene overproducing strain E20 presented lower antimicrobial activity than the wildtype and the trichodiene overproducers, especially in vented conditions where this strain significantly promoted *Fusarium oxysporum* growth (Álvarez-García et al., 2021). Rodríguez-González et al., (2018, 2019), on the other hand, reported that the same trichodiene overproducers exerted an attractant influence on *A. obtectus* adults, opposed to that of the *erg1* silenced strains, which significantly repelled them. This, combined with the enhanced insecticidal effects we described for E20 and E20-5.7 volatiles, could mean that *A. obtectus* adults are able to detect the toxic BVOCs produced by these strains and then respond by moving away from their source. This ties in with previous works that show the

importance of olfaction in granivorous pests and the ability of the weevil to respond to BVOCs (Giunti et al., 2018; Khelfane-Goucem et al., 2014).

Rodríguez-González et al., (2018) reported that trichodiene-overproducing strains increased insect mortality compared to their parental strains (including E20) when spores were in contact with the adults, indicating that the effects produced by the same microbial strains vary greatly between volatile interactions and direct physical contact. In this regard, while BVOCs produced by E20 and E20-5.7 demonstrate important insecticidal properties here, these strains may present reduced infectious capability derived from the blocking of ergosterol production and/or squalene and FDP accumulation (Lindo et al., 2019). On the contrary, trichodiene-overproducers seem to present enhanced pathogenicity against *A. obtectus* adults but reduced volatile-mediated insecticidal activity.

3.2. Effects of Trichoderma BVOCs on A. Obtectus reproduction

Regarding adult emergence, day 1 was set for this assay when the first insect emerged, and so from then on emergence was daily recorded until day 23.

Accumulated emergence was lower in sealed than in unsealed conditions for all treatments, including the control (Fig. 3), indicating that oxygen limitation likely reduces the reproductive rate of the insects. Nevertheless, this reduction was sharper when insects were exposed to *T. harzianum*. In sealed conditions (Fig. 3A), the accumulated emergence was strongly reduced by all fungal strains compared to the control treatment. E20 and E20-5.7 produced lower emergence than T34 and T34-5.27, with statistically significant differences between E20 and T34-5.27. Furthermore, emergence was reduced to zero in more than half of the replicates exposed to E20 and E20-5.7. These results tie in well with those regarding adult mortality, as the higher mortality produced by the

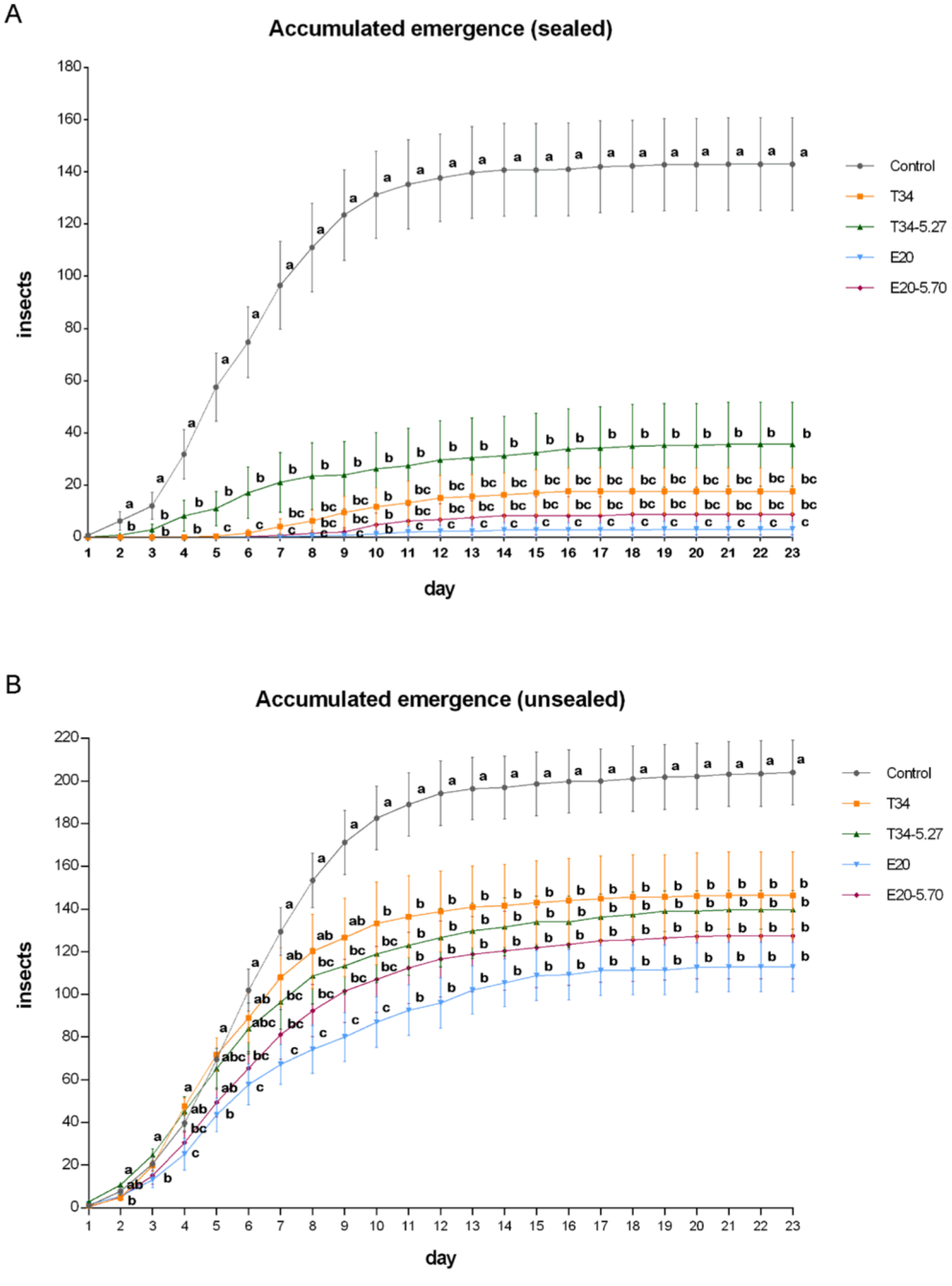


Fig. 3. Accumulated emergence of *A. obtectus* adults in (A) sealed and (B) unsealed conditions during 23 days after exposition to BVOCs produced by *T. harzianum* strains T34 (wild type, squares, orange); T34-5.27 (trichodiene overproducer, face-up triangles, green); E20 (*erg1* silenced, face-down triangles, blue); E20-5.7 (*erg1* silenced and trichodiene overproducer, diamonds, red); and untreated control (uncultured PDA medium, circles, grey). Data are represented as the total accumulated number of emerged insects on each replicate (mean ± SE) from day 1 onwards. Different letters represent statistically significant differences between treatments for the same day, using one-way analysis of variance (ANOVA) followed by a Least Significant Difference (LSD) post hoc test ($p \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

erg1-silenced strains correlates fine with the lower number of newly emerged insects on these treatments. In the same way, the control, with the lowest percentage of mortality, presented the highest accumulated emergence, followed by T34-5.27 and T34.

Insect emergence in unsealed conditions was also significantly reduced by all fungal treatments compared to the control (Fig. 3B). Nevertheless, this reduction was not as sharp as in sealed ones, suggesting that fungal BVOCs also affect *A. obtectus* reproduction in more open environments, though not as much as in closed ones. E20 and E20-5.7 produced the lowest accumulated emergence, being somehow statistically significant during the first days of the assay compared to the other fungal strains. Interestingly, in these unsealed conditions, the results seem not to correlate so well with the reported adult mortality. As described, very small differences were observed between treatments regarding adult mortality in unsealed conditions. Moreover, T34 and T34-5.27 were the treatments that presented higher insect mortality, albeit not statistically significant in many cases. However, E20 and E20-5.7 still produced a stronger reduction in insect emergence. These seemingly contradictory observations could be the result of increased mortality produced by E20 and E20-5.7 BVOCs in preimaginal stages of insect development (eggs, larvae) or derive from behavioral changes in exposed *A. obtectus* adults. In this regard, several studies have demonstrated that BVOCs exert toxic effects on preimaginal stages of insects (Zhao et al., 2017) and other organisms (Hummadi et al., 2021), as well as induce diverse behavioral responses in adults (Bueno et al., 2020; Hategekimana and Erler, 2020; Ponce et al., 2021). Interestingly, Rodríguez-González et al., (2018) described a deterrent effect of E20 towards *A. obtectus* females when spores were sprayed over bean seeds. Additional research should be conducted to clarify these aspects.

3.3. Effects of Trichoderma VOCs on the damage caused by A. Obtectus on dry bean seeds

After 23 days, the following parameters were recorded: percentage of affected beans; total number of holes per replicate; number of holes per affected bean; and the percentage of bean weigh loss.

In sealed conditions, all fungal treatments significantly reduced these parameters compared to the control (Table 1). Thus, 65% of the beans in the control were affected, while this percentage dropped to 24.5% in those exposed to T34-5.27 BVOCs, 16% with T34, 10% with E20-5.7, and 4.37% with E20 (Table 1A). The number of total holes followed a similar trend, with the control rendering a mean of 143.5 holes (significantly higher than all fungal treatments), while the treatments reduced it to 36.2, 17, 10, and 2.5 holes per replicate for T34-5.27, T34, E20-5.7, and E20 respectively (Table 1A). The number of holes per affected bean also differed between treatments, with the control showing the highest rate (5.49 holes/bean). Differences were statistically significant between the control and the treatments. This parameter is not presented for E20 and E20-5.7 in sealed conditions, as most of the replicates did not show affected beans at all (Table 1A), thus making it impossible to calculate. Finally, weight loss was significantly reduced by all treatments compared to the control, with a reduction of 14.56% in the control, 5.65% for T34-5.27, 3.57% for T34, 1.97% for E20-5.7, and 1.93% for E20. Differences were also statistically significant between these last two squalene-overproducing strains and T34-5.27 (Table 1A). The described results in sealed conditions indicate that the increase in adult mortality produced by the fungal strains substantially reduces the damage caused on bean seeds. As expected, this correlates in turn with the reduced emergence of new insects, as the strains causing higher mortality and lower emergence present lower bean damage as well.

Aluminum phosphide and phosphine have been used for decades as the main active compounds in the control of pests inside storage facilities, and their insecticidal activity and dynamics have been extensively tested against many insects, including *A. obtectus* (Arora et al., 2021; Arora and Srivastava, 2021; Hasan and Reichmuth, 2004; Hole et al., 1976). Nevertheless, rising concerns regarding pest resistance, residue persistence, safety, and toxicity advice in the search of more natural and less harmful compounds (Bogle et al., 2006; Murali et al., 2009; Nayak et al., 2020; Pérez Navero et al., 2009; Vardell et al., 1973). In this regard, BVOCs could be an adequate potential substitute, as phosphine acts in gaseous form (although mostly applied using tablets or granules) inside closed storing facilities. The application of these compounds could also be combined with controlled atmospheres.

In unsealed conditions, the effects on bean damage were less relevant (Table 1B). The percentage of affected beans was still significantly lower in all fungal treatments compared to the control, in which 67.5% of the beans were affected. T34 rendered the highest percentage of affected beans among the fungal treatments (58%), and T34-5.27 was the lowest (43.5%). In this case, no differences were shown between the *erg1*-silenced strains and the others. The number of total holes was higher for all treatments compared to that obtained in sealed conditions, as expected from the reported increase in accumulated emergence. However, all fungal treatments except the wild type T34 exerted a statistically significant reduction in this parameter compared to the control (200 holes/replicate), with E20 presenting the lowest total number of holes per replicate (116,6 holes/replicate) (Table 1B). Regarding the mean

Table 1
Percentage of affected beans; total holes per replicate (40 beans); number of holes per affected bean; and percentage of bean weight loss in (A) sealed and (B) unsealed conditions after 23 days of *A. obtectus* emergence from beans previously exposed to BVOCs produced by *T. harzianum* strains T34 (wild type); T34-5.27 (trichodiene overproducer); E20 (*erg1* silenced); E20-5.7 (*erg1* silenced and trichodiene overproducer); and CC: untreated control (uncultured PDA medium). Different letters represent statistically significant differences between treatments analysed by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test ($p \leq 0.05$), or non-parametric Kruskal–Wallis H-test followed by a Mann-Whitney *U* test ($p \leq 0.05$). NA: not applicable (this parameter could not be mathematically calculated for the corresponding treatment and conditions).

(A) sealed

Treatment	Affected beans (%)	SE	Stat	Total holes	SE	Stat	Holes per bean	SE	Stat	Weight loss (%)	SE	Stat
CC	65.00	5.86	a	143.5	18.21	a	5.46	0.25	a	14.56	1.89	a
T34	16.00	4.65	bc	17.00	8.57	b	2.27	0.43	b	3.57	0.84	bc
T34-5.27	24.50	7.22	b	36.20	16.12	b	3.00	0.64	b	5.65	1.29	b
E20	4.38	4.38	c	2.50	2.50	b	NA	–	–	1.93	0.30	c
E20-5.7	10.00	7.71	bc	10	9.03	b	NA	–	–	1.97	1.04	c

(B) unsealed

Treatment	Affected beans (%)	SE	Stat	Total holes	SE	Stat	Holes per bean	SE	Stat	Weight loss (%)	SE	Stat
CC	67.50	3.26	a	200.0	15.49	a	7.39	0.35	ab	14.17	1.86	a
T34	58.00	5.33	b	153.0	23.55	ab	6.47	0.62	ab	10.75	2.10	a
T34-5.27	43.50	3.32	c	135.6	11.91	b	7.84	0.57	a	9.94	1.09	a
E20	48.00	3.10	bc	116.2	12.10	b	6.12	0.71	b	10.63	1.16	a
E20-5.7	47.50	7.37	bc	131.6	23.35	b	6.91	0.42	ab	9.83	1.37	a

number of holes per affected bean, no differences were observed between any of the treatments and the control, with statistically significant ones only between E20 (6.12 holes/bean) and T34-5.27 (7.84 holes/bean) (Table 1B). Finally, all treatments rendered a lower weight loss (around 10%) than the control (14.17%), but none of them was statistically significant. Interestingly, in all fungal treatments, these four parameters (percentage of affected beans, total number of holes, holes per affected bean, and weight loss) showed a sharp increase when comparing sealed with unsealed conditions (Table 1). Nevertheless, in the control, the percentage of affected beans and the weight loss were virtually the same in both conditions, even though the number of total holes (and thus the number of emerged insects) rose from 143.5 ± 18.21 in sealed conditions to 200 ± 15.49 in unsealed ones (Table 1B). This is likely due to a reduction in the size of newly emerged insects in unsealed conditions, where the increased number of holes per bean (meaning more insects growing inside each seed) arguably led to fewer resources available for each insect during its development, and thus in smaller adult size.

Therefore, the general reduction of insect mortality in unsealed conditions for the four *T. harzianum* treatments produced an expected increase in all parameters related to bean damage, especially regarding those of E20 and E20-5.7, which showed the highest levels of mortality and the lowest insect emergence in sealed conditions. Likewise the observed pattern of insect emergence, fungal treatments E20 and E20-5.7, even though they did not produce higher adult mortality than the control in unsealed conditions, did nevertheless show a statistically significant reduction in the percentage of affected beans, the total number of holes per replicate, and even on the number of holes per affected bean in the case of E20. This evidence indicates that fungal BVOCs are also able to reduce bean damage produced by *A. obtectus* in open environments, reinforcing the expressed hypothesis that processes other than toxicity to adults play a role on the described results.

Further research should be conducted, both in sealed and unsealed conditions, to determine the individual BVOCs or volatile mixtures that present the described properties, as well as their means of action. This could lead to de development of new strategies for the control of *A. obtectus* infestation in dry grain storing facilities. Additionally, new studies would be of great interest regarding the putative effects of these BVOCs on other insect pests as well as the full extent to which VOC Chambers could be of use in the field.

4. Conclusions

The VOC Chambers proved to be a reliable method to evaluate *in vitro* direct volatile-mediated interactions between growing microorganisms and insects. These devices represent a promising model for the screening of putative bioactive microbial BVOCs against dry grain storage pests, enabling both sealed and unsealed conditions to be tested. *T. harzianum* BVOCs increased *A. obtectus* mortality, reduced insect emergence from exposed beans, and reduced the overall damage produced by *A. obtectus* on bean seeds. These effects were stronger in sealed than in unsealed conditions, highlighting the importance of ventilation in microbe-insect volatile interactions. The *erg1*-silenced strains E20 and E20-5.7 significantly increased mortality in comparison to the wild-type T34 and its transformant T34-5.27 in sealed conditions, indicating that the accumulation of squalene and reduction of ergosterol levels enhance the insecticidal activity of *T. harzianum* BVOCs. These strains also lowered insect emergence and bean damage in both sealed and unsealed conditions. Contrary, trichodiene-overproducing strains did not show an increased activity compared to their parental ones, suggesting that trichodiene does not possess insecticidal properties in the tested conditions.

5. Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request. All microbiological strains used in this study will be made available to researchers upon reasonable request. VOC Chambers will be made available to researchers upon reasonable request, unless commercial agreements reached with third parties regarding the patent exploitation prohibit it (in which case the VOC Chambers should be available in the market).

Funding

This research was funded by the Junta de Castilla y León, Consejería de Educación throughout the project "Application of *Trichoderma* strains in sustainable quality bean production"; (LE251P18) and by the Fundación General de la Universidad de León y la Empresa (FGULEM) and the European Social Fund via a Proof of Concept (ULE-PoC) included in the Plan TCUE. The "Consejería de Educación de Castilla y León" and the European Social Fund financed the PhD grants of Samuel Álvarez García (ORDEN EDU/529/2017, June 26th).

7. Authors' contributions

S.A-G., P.A.C. developed the methodology; S.G. developed the microbial strains and designed the culture conditions; S.A-G. and A.R-G. planed the experimental design; S.A-G. and L.Z. performed the experiments; S.A-G. wrote the manuscript; A.R-G and S.A-G. performed data curation, data analysis and produced the figures; S.A-G., S.G., and P.A.C. revised and corrected the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The authors declare the existence of financial competing interests derived from the patent held by the University of León regarding the described technology (Oficina Española de Patentes y Marcas: **ES 2708899 B2**). Some of the authors of the present manuscript share part of the intellectual property as inventors: 55% Samuel Álvarez García, 7% Pedro A. Casquero, 7% Santiago Gutiérrez, and 5% Álvaro Rodríguez González].

References

Álvarez-García, S., Mayo-Prieto, S., Carro-Huerga, G., Rodríguez-González, Á., González-López, Ó., Gutiérrez, S., Casquero, P.A., 2021. Volatile Organic Compound Chamber: a novel technology for microbiological volatile interaction assays. J. Fungi 7, 248. https://doi.org/10.3390/jof7040248.

Arora, S., Srivastava, C., 2021. Locational dynamics of concentration and efficacy of phosphine against pulse beetle, *Callosobruchus maculatus* (Fab). Crop Prot. 143, 105475. https://doi.org/10.1016/j.cropro.2020.105475.

Arora, S., Stanley, J., Srivastava, C., 2021. Temporal dynamics of phosphine fumigation against insect pests in wheat storage. Crop Prot. 144, 105602. https://doi.org/10.1016/j.cropro.2021.105602.

Baier, A.H., Webster, B.D., 1992. Control of *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae) in *Phaseolus vulgaris* L. Seed stored on small farms-II. Germination and cooking time. J. Stored Prod. Res. 28 (4), 295–299. https://doi.org/10.1016/0022-474X(92)90012-F.

Berger, A., Degenkolb, T., Vilcinskas, A., Schöller, M., 2017. Evaluating the combination of a parasitoid and a predator for biological control of seed beetles (Chrysomelidae: Bruchinae) in stored beans. J. Stored Prod. Res. 74, 22–26. https://doi.org/10.1016/j.jspr.2017.08.009.

Bogle, R.G., Theron, P., Brooks, P., Dargan, P.I., Redhead, J., 2006. Aluminium phosphide poisoning. Emerg. Med. J. https://doi.org/10.1136/emj.2004.015941.

Bueno, E., Martin, K.R., Raguso, R.A., Mcmullen, J.G., Hesler, S.P., Loeb, G.M., Douglas, A.E., 2020. Response of wild spotted wing Drosophila (*Drosophila suzukii*) to microbial volatiles. J. Chem. Ecol. 46 (8), 688–698. https://doi.org/10.1007/s10886-019-01139-4.

Cardoza, R.E., McCormick, S.P., Malmierca, M.G., Olivera, E.R., Alexander, N.J., Monte, E., Gutiérrez, S., Cullen, D., 2015. Effects of trichothecene production on the plant defense response and fungal physiology: Overexpression of the *Trichoderma arundinaceum tri4* gene in *T. harzianum*. Appl. Environ. Microbiol. 81 (18), 6355–6366. https://doi.org/10.1128/AEM.01626-15.

Cardoza, R.E., Vizcaíno, J.A., Hermosa, M.R., Sousa, S., González, F.J., Llobell, A., Monte, E., Gutiérrez, S., 2006. Cloning and characterization of the *erg1* gene of

Trichoderma harzianum: Effect of the *erg1* silencing on ergosterol biosynthesis and resistance to terbinafine. Fungal Genet. Biol. 43 (3), 164–178. https://doi.org/10.1016/j.fgb.2005.11.002.

Casquero, P.A., Lema, M., Santalla, M., De Ron, A.M., 2006. Performance of common bean (*Phaseolus vulgaris* L.) landraces from Spain in the Atlantic and Mediterranean environments. Genet. Resour. Crop Evol. 53 (5), 1021–1032. https://doi.org/10.1007/s10722-004-7794-1.

Cordovez, V., Carrion, V.J., Etalo, D.W., Mumm, R., Zhu, H., van Wezel, G.P., Raaijmakers, J.M., 2015. Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil. Front. Microbiol. 6 https://doi.org/10.3389/fmicb.2015.01081.

Desjardins, A.E., McCormick, S.P., Appell, M., 2007. Structure-activity relationships of trichothecene toxins in an *Arabidopsis thaliana* leaf assay. J. Agric. Food Chem. 55 (16), 6487–6492. https://doi.org/10.1021/jf0709193.

Do, R., Kiss, R.S., Gaudet, D., Engert, J.C., 2009. Squalene synthase: A critical enzyme in the cholesterol biosynthesis pathway. Clin. Genet. 75 (1), 19–29. https://doi.org/10.1111/j.1399-0004.2008.01099.x.

Dukare, A.S., Paul, S., Nambi, V.E., Gupta, R.K., Singh, R., Sharma, K., Vishwakarma, R. K., 2019. Exploitation of microbial antagonists for the control of postharvest diseases of fruits: a review. Crit. Rev. Food Sci. Nutr. 59 (9), 1498–1513. https://doi.org/10.1080/10408398.2017.1417235.

Dutton, A., Mattiacci, L., Amadò, R., Dorn, S., 2002. A novel function of the triterpene squalene in a tritrophic system. J. Chem. Ecol. 28, 103–116. https://doi.org/10.1023/A:1013514903036.

El Ariebi, N., Hiscox, J., Scriven, S.A., Müller, C.T., Boddy, L., 2016. Production and effects of volatile organic compounds during interspecific interactions. Fungal Ecol. 20, 144–154. https://doi.org/10.1016/j.funeco.2015.12.013.

Gershow, M., Berck, M., Mathew, D., Luo, L., Kane, E.A., Carlson, J.R., Samuel, A.D.T., 2012. Controlling airborne cues to study small animal navigation. Nat. Methods 9 (3), 290–296. https://doi.org/10.1038/nmeth.1853.

Giunti, G., Palmeri, V., Algeri, G.M., Campolo, O., 2018. VOC emissions influence intra- and interspecific interactions among stored-product Coleoptera in paddy rice. Sci. Rep. 8 (1) https://doi.org/10.1038/s41598-018-20420-2.

Gokturk, T., Kordali, S., Ak, K., Kesdek, M., Bozhuyuk, A.U., 2020. Insecticidal effects of some essential oils against *Tribolium confusum* (du Val.) and *Acanthoscelides obtectus* (Say), (Coleoptera: Tenebrionidae and Bruchidae) adults. Int. J. Trop Insect Sci. 40 (3), 637–643. https://doi.org/10.1007/s42690-020-00113-y.

Gómez, K.A., Gómez, A.A., 1984. Statistical procedures for agricultural research, second ed. Biometrics. John Wiley & Sons, New York.

Guo, Y., Jud, W., Ghirardo, A., Antritter, F., Benz, J.P., Schnitzler, J.-P., Rosenkranz, M., 2020. Sniffing fungi – phenotyping of volatile chemical diversity in *Trichoderma* species. New Phytol. 227 (1), 244–259. https://doi.org/10.1111/nph.16530.

Hasan, M.M., Reichmuth, C., 2004. Relative toxicity of phosphine against the bean bruchid *Acanthoscelides obtectus* (Say) (Col., Bruchidae). J. Appl. Entomol. 128 (5), 332–336. https://doi.org/10.1111/j.1439-0418.2004.00850.x.

Hategekimana, A., Erler, F., 2020. Fecundity and fertility inhibition effects of some plant essential oils and their major components against *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). J. Plant Dis. Prot. 127 (5), 615–623. https://doi.org/10.1007/s41348-020-00311-3.

Herrera, J.M., Pizzolitto, R.P., Zunino, M.P., Dambolena, J.S., Zygadlo, J.A., 2015. Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize. J. Stored Prod. Res. 62, 74–80. https://doi.org/10.1016/J.JSPR.2015.04.006.

Herrman, A., 2010. The chemistry and Biology of volatiles. John Wiley & Sons, Ltd, Chichester. https://doi.org/https://doi. org/10.1002/9780470669532.

Hole, B.D., Bell, C.H., Mills, K.A., Goodship, G., 1976. The toxicity of phosphine to all developmental stages of thirteen species of stored product beetles. J. Stored Prod. Res. 12 (4), 235–244. https://doi.org/10.1016/0022-474X(76)90039-4.

Hummadi, E.H., Dearden, A., Generalovic, T., Clunie, B., Harrott, A., Cetin, Y., Demirbek, M., Khoja, S., Eastwood, D., Dudley, E.d., Hazir, S., Touray, M., Ulug, D., Hazal Gulsen, S., Cimen, H., Butt, T., 2021. Volatile organic compounds of *Metarhizium brunneum* influence the efficacy of entomopathogenic nematodes in insect control. Biol. Control 155, 104527. https://doi.org/10.1016/j.biocontrol.2020.104527.

Hung, R., Lee, S., Bennett, J.W., 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. Fungal Ecol. 6 (1), 19–26. https://doi.org/10.1016/j.funeco.2012.09.005.

Inamdar, A.A., Bennett, J.W., 2015. A common fungal volatile organic compound induces a nitric oxide mediated inflammatory response in *Drosophila melanogaster*. Sci. Rep. 4 (1) https://doi.org/10.1038/srep03833.

Inamdar, A.A., Masurekar, P., Hossain, M., Richardson, J.R., Bennett, J.W., 2014a. Signaling pathways involved in 1-octen-3-ol-mediated neurotoxicity in *Drosophila melanogaster*: Implication in parkinson's disease. Neurotox. Res. 25 (2), 183–191. https://doi.org/10.1007/s12640-013-9418-z.

Inamdar, A.A., Zaman, T., Morath, S.U., Pu, D.C., Bennett, J.W., 2014b. *Drosophila melanogaster* as a model to characterize fungal volatile organic compounds. Environ. Toxicol. 29 (7), 829–836. https://doi.org/10.1002/tox.21825.

Jahaniaval, F., Kakuda, Y., Marcone, M.F., 2000. Fatty acid and triacylglycerol compositions of seed oils of five *Amaranthus* accessions and their comparison to other oils. JAOCS. J. Am. Oil Chem. Soc. 77, 847–852. https://doi.org/10.1007/s11746-000-0135-0.

Jiang, Y., Chen, H., Chen, X., Köllner, T.G., Jia, Q., Wymore, T.W., Wang, F., Chen, F., 2015. Volatile squalene from a nonseed plant *Selaginella moellendorffii*: Emission and biosynthesis. Plant Physiol. Biochem. 96, 1–8. https://doi.org/10.1016/j.plaphy.2015.07.010.

Kai, M., Effmert, U., Piechulla, B., 2016. Bacterial-plant-interactions: Approaches to unravel the biological function of bacterial volatiles in the rhizosphere. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00108.

Khelfane-Goucem, K., Medjdoub-Bensaad, F., Leppik, E., Frérot, B., 2014. Dry bean volatile organic compounds mediating host choice in *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae: Bruchinae). Ann. la Soc. Entomol. Fr. 50 (2), 167–176. https://doi.org/10.1080/00379271.2014.938547.

Lanzón, A., Albi, T., Cert, A., Gracián, J., 1994. The hydrocarbon fraction of virgin olive oil and changes resulting from refining. J. Am. Oil Chem. Soc. 71 (3), 285–291. https://doi.org/10.1007/BF02638054.

Lee, S., Behringer, G., Hung, R., Bennett, J., 2019. Effects of fungal volatile organic compounds on *Arabidopsis thaliana* growth and gene expression. Fungal Ecol. 37, 1–9. https://doi.org/10.1016/j.funeco.2018.08.004.

Lee, S., Yap, M., Behringer, G., Hung, R., Bennett, J.W., 2016. Volatile organic compounds emitted by trichoderma species mediate plant growth. Fungal Biol. Biotechnol. 3, 1–14. https://doi.org/10.1186/s40694-016-0025-7.

Lindo, L., Cardoza, R.E., Lorenzana, A., Casquero, P.A., Gutiérrez, S., 2020. Identification of plant genes putatively involved in the perception of fungal ergosterol-squalene. J. Integr. Plant Biol. 62 (7), 927–947. https://doi.org/10.1111/jipb.12862.

Lindo, L., McCormick, S.P., Cardoza, R.E., Kim, H.S., Brown, D.W., Alexander, N.J., Proctor, R.H., Gutiérrez, S., 2019. Role of *Trichoderma arundinaceum tri10* in regulation of terpene biosynthetic genes and in control of metabolic flux. Fungal Genet. Biol. 122, 31–46. https://doi.org/10.1016/j.fgb.2018.11.001.

Lozano-Soria, A., Picciotti, U., Lopez-Moya, F., Lopez-Cepero, J., Porcelli, F., Lopez-Llorca, L.V., 2020. Volatile organic compounds from entomopathogenic and nematophagous fungi, repel banana black weevil (*Cosmopolites sordidus*). Insects 11, 1–19. https://doi.org/10.3390/insects11080509.

Macías-Rodríguez, L., Contreras-Cornejo, H.A., Adame-Garnica, S.G., del-Val, E.k., Larsen, J., 2020. The interactions of *Trichoderma* at multiple trophic levels: inter-kingdom communication. Microbiol. Res. 240, 126552. https://doi.org/10.1016/j.micres.2020.126552.

Malmierca, M.G., McCormick, S.P., Cardoza, R.E., Alexander, N.J., Monte, E., Gutiérrez, S., 2015a. Production of trichodiene by *Trichoderma harzianum* alters the perception of this biocontrol strain by plants and antagonized fungi. Environ. Microbiol. 17 (8), 2628–2646. https://doi.org/10.1111/1462-2920.12506.

Malmierca, M.G., McCormick, S.P., Cardoza, R.E., Monte, E., Alexander, N.J., Gutiérrez, S., 2015b. Trichodiene production in a *Trichoderma harzianum* erg1-silenced strain provides evidence of the importance of the sterol biosynthetic pathway in inducing plant defense-related gene expression. Mol. Plant-Microbe Interact. 28 (11), 1181–1197. https://doi.org/10.1094/MPMI-06-15-0127-R.

Morath, S.U., Hung, R., Bennett, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biol. Rev. 26 (2-3), 73–83. https://doi.org/10.1016/j.fbr.2012.07.001.

Murali, R., Bhalla, A., Singh, D., Singh, S., 2009. Acute pesticide poisoning: 15 years experience of a large North-West Indian hospital. Clin. Toxicol. 47 (1), 35–38. https://doi.org/10.1080/15563650701885807.

Mutawila, C., Vinale, F., Halleen, F., Lorito, M., Mostert, L., 2016. Isolation, production and in vitro effects of the major secondary metabolite produced by *Trichoderma* species used for the control of grapevine trunk diseases. Plant Pathol. 65, 104–113. https://doi.org/10.1111/ppa.12385.

Nayak, M.K., Daglish, G.J., Phillips, T.W., Ebert, P.R., 2020. Resistance to the fumigant phosphine and its management in insect pests of stored products: A global perspective. Annu. Rev. Entomol. 65 (1), 333–350. https://doi.org/10.1146/annurev-ento-011019-025047.

Palla, F., Bruno, M., Mercurio, F., Tantillo, A., Rotolo, V., 2020. Essential oils as natural biocides in conservation of cultural heritage. Molecules 25, 1–11. https://doi.org/10.3390/molecules25030730.

Paul, U.V., Lossini, J.S., Edwards, P.J., Hilbeck, A., 2009. Effectiveness of products from four locally grown plants for the management of *Acanthoscelides obtectus* (Say) and *Zabrotes subfasciatus* (Boheman) (both Coleoptera: Bruchidae) in stored beans under laboratory and farm conditions in Northern Tanzania. J. Stored Prod. Res. 45 (2), 97–107. https://doi.org/10.1016/j.jspr.2008.09.006.

Pérez Navero, J.L., Ibarra de la Rosa, I., Frías Pérez, M.A., Arroyo Marín, M.J., Pérez Jorge, P., 2009. Intoxicación letal por inhalación accidental de fosfuro alumínico. An. Pediatr. 71 (5), 427–431. https://doi.org/10.1016/j.anpedi.2009.07.025.

Piechulla, B., Schnitzler, J.-P., 2016. Circumvent CO_2 effects in volatile- based microbe – plant interactions control set-ups. Trends Plant Sci. 21 (7), 541–543. https://doi.org/10.1016/j.tplants.2016.05.001.

Ponce, M.A., Kim, T.N., Morrison III, W.R., 2021. A systematic review of the behavioral responses by stored-product arthropods to individual or blends of microbially produced volatile cues. Insects 12 (5), 391. https://doi.org/10.3390/insects12050391.

Quintana-Rodriguez, E., Morales-Vargas, A.T., Molina-Torres, J., Ádame-Alvarez, R.M., Acosta-Gallegos, J.A., Heil, M., Flynn, D., 2015. Plant volatiles cause direct, induced and associational resistance in common bean to the fungal pathogen *Colletotrichum lindemuthianum*. J. Ecol. 103 (1), 250–260. https://doi.org/10.1111/1365-2745.12340.

Rodríguez-González, Á., Álvarez-García, S., González-López, Ó., Da Silva, F., Casquero, P. A., 2019. Insecticidal Properties of *Ocimum basilicum* and *Cymbopogon winterianus* against *Acanthoscelides obtectus*, Insect Pest of the Common Bean (*Phaseolus vulgaris*, L.). Insects 10 (5), 151. https://doi.org/10.3390/insects10050151.

Rodríguez-González, A., Casquero, P.A., Cardoza, R.E., Gutiérrez, S., 2019. Effect of trichodiene synthase encoding gene expression in *Trichoderma* strains on their effectiveness in the control of *Acanthoscelides obtectus*. J. Stored Prod. Res. 83, 275–280. https://doi.org/10.1016/j.jspr.2019.07.006.

Rodríguez-González, Á., Casquero, P.A., Suárez-Villanueva, V., Carro-Huerga, G., Álvarez-García, S., Mayo-Prieto, S., Lorenzana, A., Cardoza, R.E., Gutiérrez, S., 2018. Effect of trichodiene production by *Trichoderma harzianum* on *Acanthoscelides obtectus*. J. Stored Prod. Res. 77, 231–239. https://doi.org/10.1016/j.jspr.2018.05.001.

Rojht, H., Košir, I.J., Trdan, S., 2012. Chemical analysis of three herbal extracts and observation of their activity against adults of *Acanthoscelides obtectus* and *Leptinotarsa decemlineata* using a video tracking system. J. Plant Dis. Protect. 119 (2), 59–67.

Sanon, A., Ouedraogo, A.P., Tricault, Y., Credland, P.F., Huignard, J., 1998. Biological control of Bruchids in cowpea stores by release of *Dinarmus basalis* (Hymenoptera: Pteromalidae) Adults. Environ. Entomol. 27, 717–725. https://doi.org/10.1093/ee/27.3.717.

Schulz-Bohm, K., Martín-Sánchez, L., Garbeva, P., 2017. Microbial volatiles: Small molecules with an important role in intra- and inter-kingdom interactions. Front. Microbiol. 8, 1–10. https://doi.org/10.3389/fmicb.2017.02484.

Spanova, M., Daum, G., 2011. Squalene - biochemistry, molecular biology, process biotechnology, and applications. Eur. J. Lipid Sci. Technol. 113 (11), 1299–1320. https://doi.org/10.1002/ejlt.201100203.

Taylor, L., Gutierrez, S., McCormick, S.P., Bakker, M.G., Proctor, R.H., Teresi, J., Kurtzman, B., Hao, G., Vaughan, M.M., 2022. Use of the volatile trichodiene to reduce *Fusarium* head blight and trichothecene contamination in wheat. Microb. Biotechnol. 15 (2), 513–527. https://doi.org/10.1111/1751-7915.13742.

Vardell, H.H., Cagle, A., Cooper, E., 1973. Phosphine residues on soybeans fumigated with aluminum phosphide. J. Econ. Entomol. 66, 800–801. https://doi.org/10.1093/jee/66.3.800.

Wang, W., Kang, S., Li, N., Liu, X., Nourollahi, K., Islam, M., Alfiky, A., 2018. Volatile compound-mediated recognition and inhibition between *Trichoderma* biocontrol agents and *Fusarium oxysporum*. Front. Microbiol. 9, 1–16. https://doi.org/10.3389/fmicb.2018.02614.

Zhao, G., Yin, G., Inamdar, A.A., Luo, J., Zhang, N., Yang, I., Buckley, B., Bennett, J.W., 2017. Volatile organic compounds emitted by filamentous fungi isolated from flooded homes after Hurricane Sandy show toxicity in a *Drosophila* bioassay. Indoor Air 27 (3), 518–528. https://doi.org/10.1111/ina.12350.