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Method Article

Use of VOC Chambers to evaluate the impact of microbial volatile compounds on dry grain insect pests

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a b s t r a c t

Although increasing focus is being given to the control of insect pests with natural products, the study of volatile mediated microbe-insect interactions has been largely neglected so far. Currently, no standardized method is available for the *in vitro* evaluation of the effects produced on insects by Volatile Organic Compounds (VOCs) directly emitted by actively growing microbial strains. This manuscript presents a reliable protocol using VOC Chambers (Álvarez-García et al., 2021) to assess these effects and specifically those exerted on dry grain insect pests. The methodology also serves to evaluate the impact of these volatile interactions on the grain damage produced by the insects. It allows to vary the level of gas exchange with the environment, thus providing different experimental conditions to emulate diverse natural ones in which these interactions may occur.

Our method adapts the use of VOC Chambers for the evaluation of microbe-insect volatile interactions and the *in vitro* screening of microbial strains that produce potentially bioactive VOCs against insects.

It allows assessing the effects of these volatile interactions on the damage produced by insect pests on stored dry grains.

Sealed and unsealed setups can be used to determine the impact of ventilation and gas exchange on these interactions.

Specifications table

Subject Area:	Agricultural and Biological Sciences
More specific subject area:	Microbe-insect volatile interactions
Method name:	VOC Chamber setup to evaluate *in vitro* microbe-insect volatile interactions
Name and reference of original method:	VOC Chamber: Volatile Organic Compound Chamber for microbiological volatile interaction assays 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
Resource availability:	VOC Chambers will be sent free of charge to interested researchers upon request to the corresponding author of this manuscript (Samuel Álvarez-García; salvg@unileon.es), unless commercial agreements reached with third parties regarding the patent exploitation prohibit it in the future (in which case the VOC Chambers should be available in the market). A full report of the results obtained with this methodology can be found in the original research paper with which the present methodological one goes in co-submission [2].

Method details

Much of the conventional control of insect pests focuses on the application of chemically synthesized insecticides. However, the ban of many of these substances and the concern with regards to their effects on human health, the environment, and the arising of insect resistances have led to the search for new bioactive compounds. In this regard, biological interactions mediated by Volatile Organic Compounds (VOCs) and their use in biological control have been gaining attention during the last years. Although an increasing focus is currently given to the control of insect pests with natural products, the study of toxic effects of microbial VOCs on insects lacks specific, reliable, and standardized materials and protocols.

The following method is based on the one proposed by Álvarez-García et al. [1]. These authors developed the VOC Chamber, a novel technology and related protocols for the performance of microbial volatile interaction assays. In the present manuscript, we describe a modified method for the use of VOC Chambers to evaluate the effects of VOCs emitted by growing microbial strains on insects instead of against other microorganisms, as well as their impact on the damage these insects produce on dry grains. Thus, the main variations from the original protocol derive from the specific needs introduced by the use of insects and seeds.

This method is designed to evaluate effects exerted by VOCs produced by growing microbial strains and not by purified or isolated volatiles. It was developed to assess the following aspects: (I) The insect mortality produced by the microbial VOCs. (II) The variation in seed damage produced by the insects when exposed to the VOCs. (III) The effects that different experimental conditions concerning ventilation and gas exchange with the environment produce on the outcome of these interactions.

The method's effectiveness was assessed confronting *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchidae) against the VOCs produced by different *Trichoderma harzianum* strains and evaluating the damage caused on bean seeds by the insects.

The devices: VOC Chambers

VOC Chambers were first described by Álvarez-García et al. [1] and were originally intended to evaluate microbe-microbe volatile interactions. These devices are comprised of three main elements, a lower and an upper plate, equal to those of a standard Petri dish, and a perforated central piece that holds the chamber together and allows the flow of volatiles from the headspace of both plates through a 30mm hole on its center (Figures 1 and 2). This methodology is in turn an improvement from the protocol developed by Denis and Webster [4].

The presence of the central piece allows to cover its hole with membranes or filters and makes it an ideal platform to place the insects and seeds on top. In addition, VOC Chambers come in vented and non-vented configurations (Figure 1, C and D), which allows to modify ventilation. Moreover, the
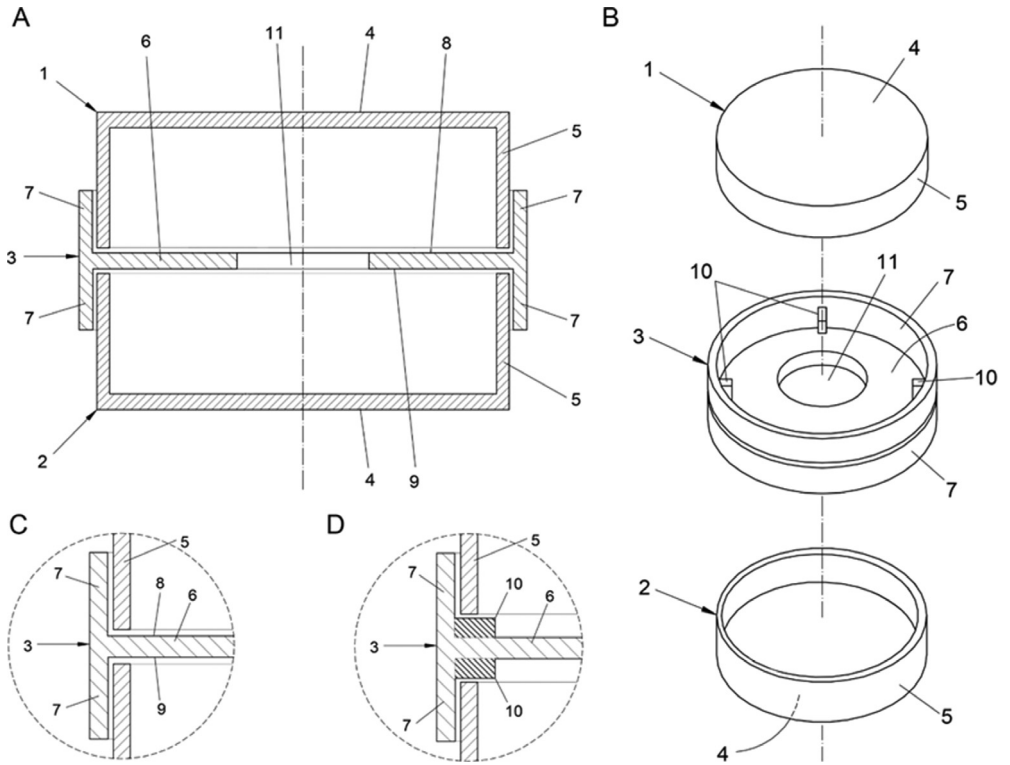
Fig. 1. (Provided by the authors from [1]). "(A) Frontal cross-section, non-vented VOC chamber. (B) Explosive view, vented VOC chamber. (C) Detail of the union between the plates and the central piece in the non-vented VOC chambers. (D) Detail of the union between the plates and the central piece with flanges in the vented VOC chambers. 1: upper plate, 2: lower plate, 3: central piece, 4: upper and lower walls (plates), 5: perimeter wall (plates), 6: intermediate wall (central piece), 7: lateral walls (central piece), 8: upper face (intermediate wall), 9: lower face (intermediate wall), 10: ventilation flanges (vented VOC chambers), 11: central hole.
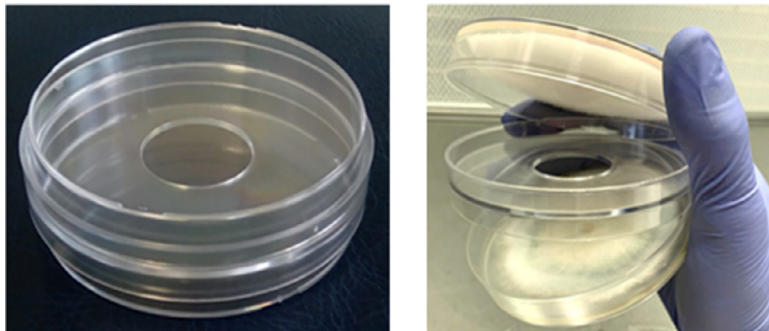

Fig. 2. (Provided by the authors from [1]) Prototypes like the ones used in the present work. (Left) Empty assembled VOC Chamber. (Right) Open VOC Chamber with fungal strains growing on both plates as in its original purpose for microbe-microbe volatile interactions.

chamber can be left unsealed, or the central piece can be sealed with parafilm to either one or both plates, providing further flexibility regarding gas exchange with the environment, in line with the methodological details reported by Kay et al., [6] regarding parafilm sealing in volatile interactions assays.

The prototypes used to develop and validate the present method were non-vented VOC Chambers for which the base plates were obtained from commercial 90mm Petri dishes (J.D. Catalán S.L., Arganda del Rey, Madrid, Spain) and the central pieces produced with a plastic injection steel mold by J.D. Catalán S.L. (Arganda del Rey, Madrid, Spain) using polystyrene crystal (Figure 2).

Availability of the devices

As referred, VOC Chamber prototypes were produced by J.D. Catalán S.L. (Arganda del Rey, Madrid, Spain) under our request. At the time of writing this paper, VOC Chambers are not commercially available yet. VOC Chambers will be sent free of charge to interested researchers upon request to the corresponding author of this manuscript (Samuel Álvarez-García; salvg@unileon.es) unless commercial agreements reached with third parties regarding the patent exploitation prohibit it in the future (in which case the VOC Chambers should be available in the market).

Microbial strains, culture conditions, bean seeds, and insect rearing

Preliminary considerations

Please, note that the specific protocol described in the present manuscript is adapted to the particular insect species, microbial strains, and bean seeds used. Therefore, the application of this methodology using other organisms may require further adaptation to meet their needs and characteristics regarding manipulation, culture medium, storage, growth, etc.

Biological material

The method was developed and tested confronting *A. obtectus* adults to four *T. harzianum* strains, a wild type (*T.harzianum* CECT 24130, T34 from now onwards) and 3 of its transformants (E20, T34-5.27 and E20-5.7). E20 derives from T34 by silencing the squalene epoxidase-encoding gene *erg1* [3]. T34-5.27 and E20-5.7 derive respectively from T34 and E20 by expressing the *T. arundinaceum tri5* gene, which encodes for a terpene cyclase. These two transformant strains overproduce the volatile trichodiene ([7], [8]). Fungi were stored in spore suspension (glycerol 50%; -80°C) and were culture on PDA (Potato Dextrose Agar; Difco Becton Dickinson, Sparks, MD) at 25°C for reactivation.

Dry beans (*Phaseolus vulgaris* L.) belonging to the "Riñón Menudo" landrace (Protected Geographical Indication "Alubia de La Bañeza-León" (Spain) (EC Reg. n.256/2010; March 26th, 2010, OJEU L880/17) and collected in 2020 were used in the experiments. These seeds were chosen due to their small size and white color, which allowed to place a high number of them inside the VOC Chambers and helped to better observe the insects thanks to the contrast in color. Beans were left in storage for at least 30 days and later checked to ensure that they were not affected by any plague.

The starting *A. obtectus* population was built up by collecting infected beans from diverse storage facilities in the PGI "Alubia de La Bañeza-León" area. Insects were reproduced and kept in 4 l glass jars containing the referred seeds, which were periodically cleaned and replenished with new ones. The jars were kept in controlled chambers at 25±1°C, darkness, and relative humidity of 60±5%. All adults were removed 3 days prior to the performance of the assays to ensure an experimental population with a homogeneous age of no more than 3 days. This window period could be modified attending to both the specific cycle of the insects used and the number of individuals needed according to the experimental design.

Effects of microbial VOCs on the mortality of insects in adult stage

Preliminary considerations

In the present work, we exposed *A. obtectus* adults to microbial VOCs and daily mortality was recorded. Preimaginal mortality (eggs, larvae) was not assessed, but it could be done using the same basic methodology and magnifying devices if needed for the observation of these stages. Additional parameters could be recorded as well during or after the assay.
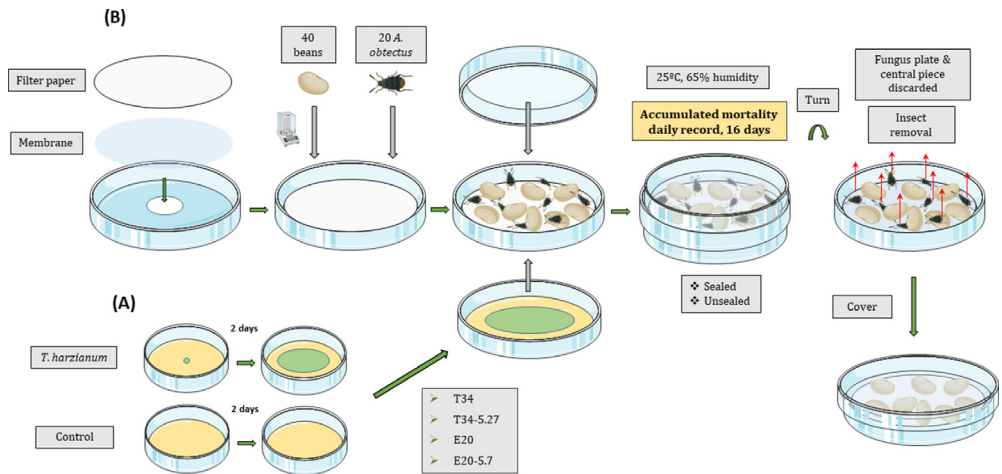
Fig. 3.. Schematic representation of the protocol carried out using VOC Chambers [1] to evaluate the effects of *T. harzianum* BVOCs on *A. obtectus* adult mortality. (A) Preparation of the treatments by culturing *T. harzianum* strains on PDA for two days or leaving uncultured ones for the control. (B) Setting up of the VOC Chambers for the confrontation assay as described in the text: covering of the central piece with cellophane membrane and filter paper; placement of 20 *A. obtectus* adults and 40 weighted beans in it; assembling of the chamber with the treatments in the lower plate and an empty one for the upper cover; daily record of mortality for the following 16 days; retirement of fungal culture and central piece; insect removal and storage; and covering of the beans with a new lid.

Materials and equipment

VOC Chambers, microbial culture media, cellophane membrane, filter paper, microbial strains, bean seeds, insects (adults), laminar flow cabinet, autoclave, precision scale, and controlled growth chamber for insects and microorganisms.

Procedure

Circles of cellophane membrane and filter paper were cut with a slightly smaller diameter than that of the central pieces of the VOC Chambers. They were wrapped in silver foil, autoclaved to ensure sterility, and left to dry in a stove (60℃). The cellophane used in these experiments was a Naturflex NP membrane (Novocel Flexibles, S.L., Colónia Prat, Puig-Reig, Barcelona, Spain) with a thickness of 24.3 μm and a permeability of 1.0 cc/m^2 * 24 hrs. at 23 °C and 0 % RH; and of 10 cc/m^2 * 24 hrs. at 23 °C and 50 % RH, according to the specifications provided by the manufacturer.

Petri dishes (90 mm in diameter) were filled with 18 ml of PDA medium. This ensures the same headspace volume for all VOC Chambers once assembled.

Plugs with 6 mm diameter were cut from the edge of three days-old growing microbial colonies and placed in the center of the aforementioned Petri plates with PDA. The fungi were thereafter left to grow for two days in a growth chamber at 25℃. Plates without microorganisms were set up in the same conditions for the control treatments (Figure 3, A). Please, note that the use of different microorganisms and experimental designs may require variations to these conditions.

Groups of 40 undamaged bean seeds were weighted (initial weight) and stored separately for each replicate. Right before the assembling of the VOC Chambers, 20 unsexed 1 to 3 days-old insect adults were selected for each replicate and placed inside falcon tubes.

Afterwards, the lids of the Petri dishes were removed inside a flow cabinet, and the plates were covered with a non-vented VOC Chamber central piece (without ventilation flanges), which was, in turn, covered first with a cellophane membrane and then with a filter paper (Figure 3, B and Figure 4, A, B, and C). This allows to place the insects and seeds on top (Figure 4 E, and F) and permits the flow of volatiles between the two sides while avoiding the pass of fungal spores and general cross-
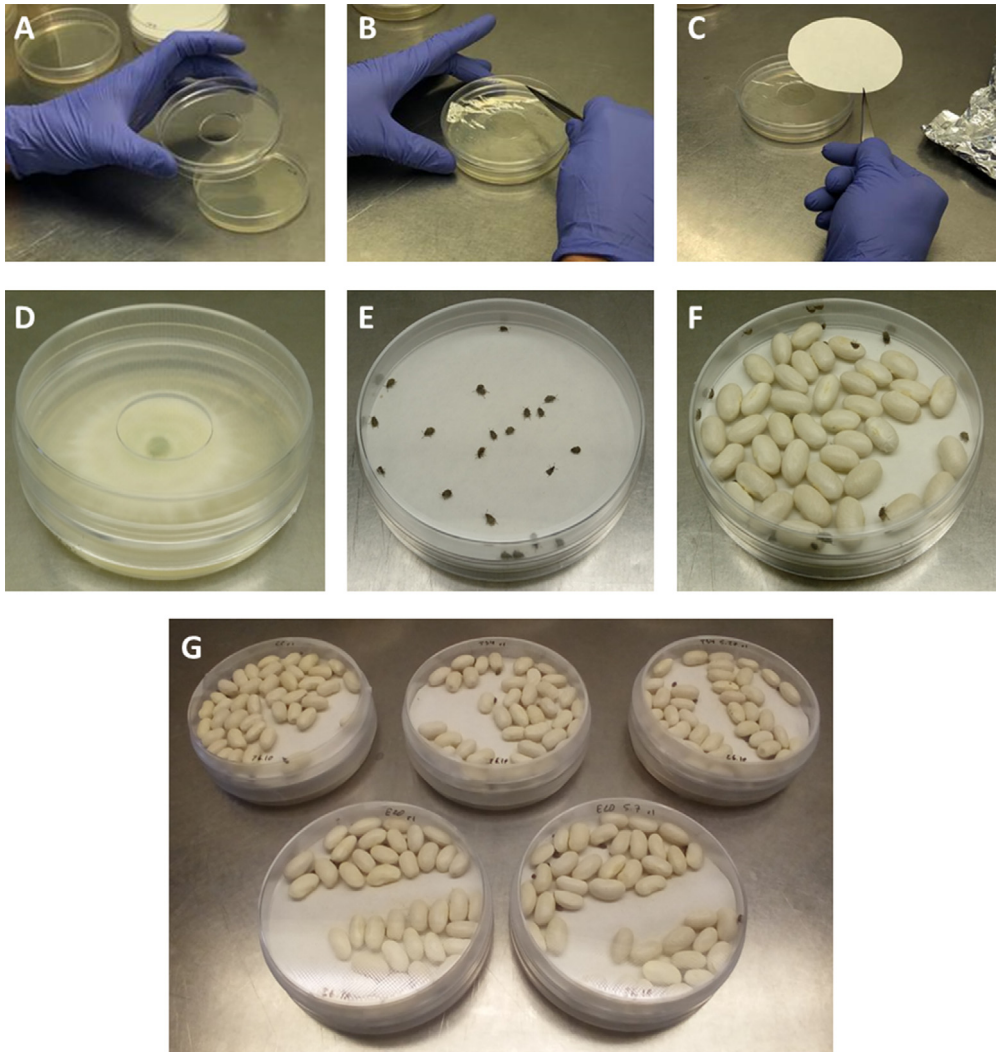
Fig. 4. Examples of some of the steps during the experimental procedure. (A) Placement of the central piece on top of a lower plate. (B) Covering of the central piece with a sterile cellophane membrane. (C) Covering with a sterile filter paper. (D) VOC Chamber before the placement of the membrane and filter paper showing a *T. harzianum* strain growing on PDA medium. (E) Assembled VOC Chamber with *A. obtectus* adults on top of the filter paper. (F) Assembled VOC Chamber with both *A. obtectus* adults and dry bean seeds on top of the filter paper. (G) An assembled replicate of each fungal treatment (*T. harzianum* T34, T34-5.27, E20, and E20-5.7) and the untreated control.

contamination through the central hole. Please, use a sterile clamp to manipulate the membrane and filter paper.

Right away, the 20 previously selected insects and the 40 undamaged dry beans were placed over the paper of each replicate, which were finally covered with an upside-down base plate to assemble the full VOC Chamber (Figure 3, B and Figure 4 E, and F). Two different sets of experiments were performed. The sealed setup, in which both plates were sealed with 3 layers of Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid) to the central piece, and the unsealed setup, in which only the lower plate containing the microbial strains was sealed to the central piece with 3 layers of Parafilm®,
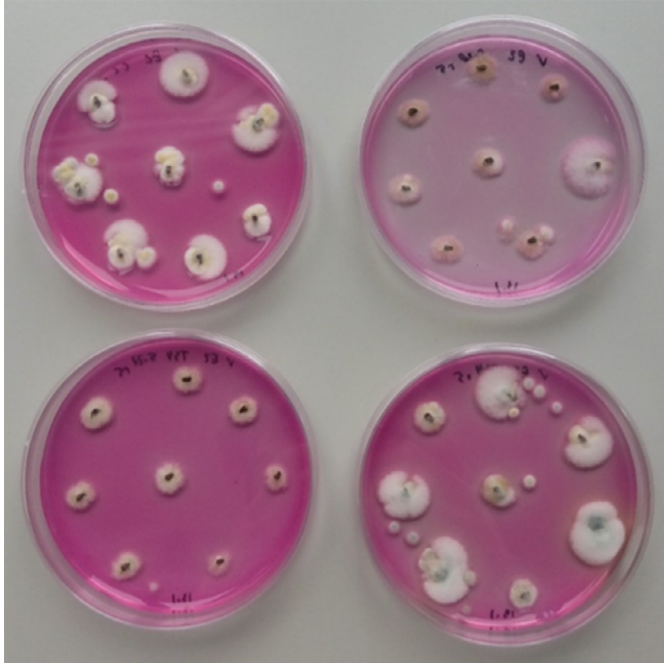
Fig. 5. Culture of *A. obtectus* cadavers on Rose Bengal-Chloramphenicol Agar medium to rule out the direct contact between *Trichoderma* spores and the insects (picture taken 7 days post-culturing). *Trichoderma* growth was absent from 100% of the samples, while natural occurring fungi present on the insects can be seen growing on the image, including in the control treatments.

while the upper one was left unsealed. The sealed assay is intended to simulate tightly closed environments with very limited gas exchange with the exterior, allowing for the buildup of high VOCs concentration inside the chamber. The unsealed assay, on the other hand, forces the VOCs to flow from the lower to the upper part of the chamber, but at the same time provides higher ventilation and gas exchange with the environment, reducing VOCs concentration and avoiding a potential lack of oxygen availability for the organisms. Five replicates were performed per treatment.

The assembled VOC Chambers were kept in darkness inside a controlled growth chambers at $25\pm1°C$ and a relative humidity of $60\pm5\%$. Adult mortality was visually recorded every day for the following 16 days without opening the VOC Chambers. After this period, the chambers were turned upside down and the fungal plates, central pieces, membranes and filter papers were discarded. *A. obtectus* adults, mostly already dead at this point, were removed and stored in sterile plastic bags at -80°C for a latter microbial isolation test to ensure that fungal spores had not contaminated the insects. The remaining dishes with the dry beans were covered with lids and introduced in a growth chamber at $25\pm1°C$ and relative humidity of $60\pm5\%$ to allow the development of a new generation of insects inside the seeds. They were checked every day until the first new adult emerged.

To confirm the absence of direct contact between the microbial strains and the insects, eighth randomly selected insect cadavers from each replicate were placed on Rose Bengal-Chloramphenicol Agar medium (Difco Becton Dickinson, Sparks, MD, USA) at 25°C. The absence of *Trichoderma* growing was visually assessed after 7 days (Figure 5 and Figure 6, A). The results demonstrated that no contact existed between the insects and fungal spores, which were not able to pass through the membrane and filter, ensuring that only volatile interactions were taking place inside the chamber.

Tips, errors, and proposed variations

Preliminary assays performed without introducing bean seeds in the system demonstrate that this part of the protocol can be used simply to evaluate the toxicity on adults without the following steps regarding seed damage.

Separate the insects in batches according to the number designed for each replicate before assembling the VOC Chambers. We did it by placing groups of 20 adults in separate Falcon tubes. Once the chamber is assembled, just transfer the whole group to it.

We found easier to assemble all the chambers at once with their corresponding microorganism (or control), membrane, and filter paper, and finally add the insects and seeds on the upper part.

We found more convenient to place the insects in the chamber before the beans to easily do a final count to ensure the number of adults on each replicate. Working with more or less mobile insects might require different manipulation, and highly active flying insects could demand extra care. Placing the insects inside a 4°C fridge for a few minutes may be useful to reduce temporarily their activity.

Although we used unsexed insects, they could be sexed to evaluate effects on male or female populations, minimize sex proportion influence in reproduction, etc.

A membrane, like the one we used, or a filter (for example a Whatman filter) are needed to avoid naturally occurring spores on the insects to drop from the upper part and contaminate the growth medium. Preliminary tests using only the filter paper resulted in widespread contamination of the lower plate, especially visible in the control treatment.

This methodology could be adapted for other microbial strains, insects, or plant material. Different dry grains, as well as fruits, leaves or other plant parts could be assessed as long as they can fit inside the chambers. Furthermore, we don't see a reason to limit it to insects. Experiments using other arthropods, nematodes, etc. could be potentially performed. Experimental conditions must be adapted to the characteristics of each specific organism.

The placement of the insects in the lower plate and the microorganisms on the upper one could be explored if deemed more convenient for some reason.

Please, ensure that the Parafilm® is correctly placed and not broken, as variations in the sealing of the chambers can jeopardize the results. In addition, different dispositions regarding the sealing of the devices could be employed to modify the experimental conditions.

VOC Chambers can replace one or both plates for larger containers if more space is needed for the insects and or the plant material (seeds, fruits, leaves, etc.) [9]. Ensure that the mouth of the containers used fits correctly with the central piece of the VOC Chamber (having around 85-87 mm in diameter).

Assays could be also performed using Vented VOC Chambers (with ventilation flanges) to further increase the gas exchange rate with the environment.

Effects of microbial VOCs on the reproduction of the insects and the subsequent damage they caused on dry bean seeds

Preliminary considerations

Take into account the insect's developmental cycle when designing the experiments and check the presence of new adults periodically to ensure that daily insect emergence is recorded as soon as the first adults appear. Design the experiment to ensure that there is not an overlap of new generations. Other biological parameter on the insects apart from adult emergence could be further evaluated in this part of the protocol (i.e. morphology, size, behavior, sex, etc.). The same might be applied for the seeds. Moreover, physiological or molecular analyses could be carried out as well.

Materials and equipment

Controlled growth chamber for insects, precision scale

Procedure

The infected seeds coming from the previous section, kept in a growth chamber at 25±1°C and relative humidity of 60±5%, were monitored every day to control insect emergence. Once the first
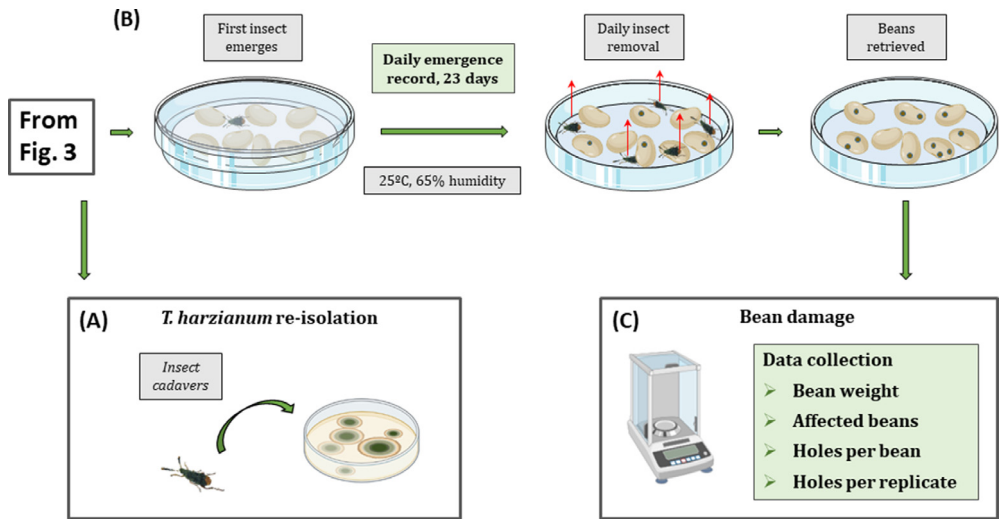
Fig. 6. This figure is the continuation of the process presented in Figure 3. Schematic representation of the protocol carried out using VOC Chambers [1] to evaluate the effects of *T. harzianum* BVOCs on the reproduction *A. obtectus* and the subsequent damage they caused on dry bean seeds. (A) Culture on PDA of *A. obtectus* cadavers from the previous experiments to ensure no contact was taking place between fungal spores and the insects. (B) Monitoring of the bean seeds until the first new *A. obtectus* adult emerged; record of daily emergence for the following 23 days alongside daily insect removal; retrieving of the tested beans for further evaluation. (C) Data collection of bean damage parameters for each replicate and treatment: final bean weight, number of affected beans (with at least one exit hole), number of holes per bean, and total number of holes per replicate.

new adult appeared, this was set up as the first day of emergence. Thereafter, daily emergence was recorded for the following 23 days. The count was finished at this point for two main reasons, first to ensure that third generation insects were not being recorded, and second because new emergences consistently ceased around day 20 in most cases. Adults were daily removed from the plates after being count (Figure 6).

After the emergence period, bean seed from each replicate were retrieved and weighted with a precision scale (final weight), and these results were compared to those obtained before setting up the assay (initial weight). The percentage of weight loss (WL) was calculated using the following equation: WL (%) = (IW – FW) / IW x 100. In which IW is the initial weight and FW the final weight. Additionally, three other parameters were recorded: the number of affected beans per replicate (those with at least one exit hole), the number of holes per affected bean, and the total number of holes per replicate (Figure 6).

Tips, errors and proposed variations:

Emergence could be recorded other than daily for biological or methodological reasons.

The period of time in which emergence should be recorded may vary between insect species.

Note that the variation in weight can be caused not only by the insects but also by other environmental conditions.

The number of holes per affected bean cannot be calculated in those replicates with 0 emerged insects, as, by definition of affected bean (those with at least one exit hole), the value of this parameter cannot be below 1. Moreover, 0/0 would render a mathematically indeterminate result.

As previously stated, feel free to expand the parameters assessed in the study, both for the insects and the plant material.

This experiment could be followed up by a germination or plant development assay.

Data treatment and statistical analyses

Assays were designed following a General Linear Model (GLM), using four fungal isolates plus the unexposed control. All results were recorded, treated, and represented separately for sealed and unsealed configurations. Regarding adult mortality, two parameter were calculated, the cumulative percentage of mortality was calculated as Mortality (%) = number of dead insects / 20 × 100. As for the insect emergence, we calculated the accumulated emergence by adding up the insects emerged on each replicate and day. Both parameters, percentage of mortality and accumulated emergence, were transformed using the arcsine transformation formula [5] before carrying out the subsequent statistical analyses. Alternative transformation methods could be of use or more convenient attending to the characteristics of the obtained results.

Data normality and equality of variances were assessed using Kolmogorov-Smirnovś and Leveneś tests, respectively. To determine differences between treatments with normal and homoscedastic data, a one-way analysis of variance (ANOVA) followed by a Least Significant Difference (LSD) post hoc test ($p \leq 0.05$) were performed. When normality or homoscedasticity were not met, a non-parametric Kruskal–Wallis H-test was used, followed up by a Mann-Whitney U-test ($p \leq 0.05$). Statistical analyses were carried out using IBM SPSS Statistics 26.

Method validation and final considerations

As referred, the method was validated confronting *A. obtectus* adults in sealed and unsealed conditions to the VOCs emitted by four *T. harzianum* strains: T34, E20, T34-527, and E20-5.7.

In sealed conditions, all treatments except T34-5.27 produced a significant increase in adult mortality in comparison to the control, with E20 and E20-5.7 significantly higher than the rest of the treatments. In contrast, when this parameter was evaluated in unsealed conditions, differences were negligible among all strains and the control, demonstrating that the VOCs produced by the fungal strains reached a toxic concentration for adults inside the sealed chambers but not in the unsealed ones ([2]. Accepted after English editing in Biological Control: BCON-D-21-00691R1).

Regarding the accumulated emergence, this parameter was significantly reduced by all fungal strains in sealed conditions, especially by E20 and E20-5.7, which is in accordance with the mortality results. Interestingly, emergence was also significantly reduced by all fungal strains in unsealed conditions, being again E20 and E20-5.7 the most active ones. As adult mortality was not affected in this case, the results suggest that the microbial VOCs exert other effects on the insect, either causing toxicity against preimaginal stages or modifying behavioral aspects that affect *A. obtectus* reproduction. Bean damage followed a similar trend, being generally reduced by the fungal treatments, especially E20 and E20-5.7, and with higher effect in sealed conditions ([2]. Accepted after English editing in Biological Control: BCON-D-21-00691R1).

These results demonstrate that VOC Chambers are a reliable and simple methodology to evaluate microbe-insect volatile interactions, especially for the screening of actively growing microbial strains with interesting volatile traits against insect pests. This method proved to be able to discriminate the activity exerted by VOCs emitted by different microbial strains concerning insect mortality, reproduction, and the damage they produced on bean seeds. Moreover, the flexibility provided by the VOC Chambers regarding the variation of gas exchanged with the environment highlight the importance of ventilation for these volatile-mediated interactions, and the need for methodologies and protocols, like the one here presented, that allow to modify the experimental conditions in which these assays are performed. These characteristics make the VOC Chamber a potential candidate to become a standard methodology to carry out this kind of experiments.

Being a passive diffusion system, VOC Chambers also present some limitations, such us the impossibility of actively control gas flow or the absence of quantitative control of gas exchange with the exterior.

Further research should be conducted to assess whether the results obtained and the microbial strains selected using this promising method could be followed up by the identification of individual or mixed VOCs with putative biocontrol capabilities against insect pests, and their subsequent testing in controlled and natural conditions.

The development of modified protocols based on the present methodology would be of interest to unravel all its potential to provide new insights in the field of microbe-insect volatile interactions.

Declaration of Competing Interest

The authors declare the following financial interests which may be considered as potential competing interests:

Acknowledgements

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