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Novel culture chamber to evaluate in vitro plant-microbe volatile interactions: Effects of *Trichoderma harzianum* volatiles on wheat plantlets

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ABSTRACT

The field of plant-microbe interactions mediated by Biogenic Volatile Organic Compounds (BVOCs) still faces several limitations due to the lack of reliable equipment. We present a novel device designed to evaluate in vitro plant-microbe volatile interactions, the plant-microbe VOC Chamber. It was tested by evaluating the effects exerted on wheat development by volatiles from three *Trichoderma harzianum* strains, a wild type and two genetically modified strains; one expressing the *tri5* gene, which leads to the synthesis and emission of the volatile trichodiene, and the other by silencing the *erg1* gene, impairing ergosterol production. The wild type and the *erg1*-silenced strain enhanced fresh weight and length of the aerial part, but reduced root dry weight. Interestingly, no differences were found between them. Conversely, the *tri5*-transformant strain reduced root and aerial growth compared to the control and the other strains. No differences were observed regarding chlorophyll fluorescence quantum yield and leaf chlorophyll content, suggesting that the released BVOCs do not interfere with photosynthesis. The plant-microbe VOC Chamber proved to be a simple and reliable method to evaluate the in vitro effects of microbial BVOCs on plant development, perfect for the screening of microorganisms with interesting volatile traits.

1. Introduction

Biogenic Volatile Organic Compounds (BVOCs) are low-weight organic molecules synthetized and emitted by a wide variety of organisms. They present a high vapour pressure, low boiling point, molecular mass, and polarity [1], allowing them to remain in a gaseous state in environmental conditions. BVOCs serve as mediators in several long-range biological interactions, both intra and interspecific ones [2,3]. These volatile dialogues play a significant role in diverse processes such as recognition [4], communication [5], competition [6] or symbiosis [7,8].

BVOCs represent an important factor within the establishment of ecological communities [9], standing out those established between plants and microorganisms. In this regard, rhizosphere microbes can affect plant growth by liberating microbial BVOCs to the environment, as demonstrated for bacteria [10] and several fungi, including *Trichoderma* [11,12], mycorrhizal fungi [13], and others [14,15]. Furthermore, microbial BVOCs can modulate flowering [14], root architecture in plants [16,17], as well as their ability to overcome herbivore attacks [18] and pathogen infections via the induction of different defensive pathways [19,20]. Microbial BVOCs are able to modulate plant metabolism in several ways, for instance by inducing the production or accumulation of specific compounds [21,22], affecting photosynthetic processes [23], nutrient uptake [24], auxin induction, or cell wall remodelling [25]. Besides, microbial BVOCs have been demonstrated to induce changes at both transcriptional and post-transcriptional levels on

Abbreviations: ANOVA, one-way analysis of variance; BVOC, Biogenic Volatile Organic Compound; DAS, Days After Sowing; MGT, Mean Germination Time; PDA, Potato Dextrose Agar; p-mVOC Chamber, plant-microbe Volatile Organic Compounds Chamber.

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plants [23,26]. Moreover, plant BVOCs have been described to propagate defensive signals between infected and healthy tissues and nearby plants, activating defensive systems in preparation for the eventual attack of the pathogen [3,20].

Numerous studies have shown the importance of BVOCs in microbe-microbe interactions. These volatile interactions produce a variety of effects in both bacteria and fungi, from growth promotion [27,28] to growth inhibition [27,29], as well as physiological and morphological changes [30]. Interestingly, some researches have demonstrated that the volatilome of a specific strain can vary widely depending on the culture conditions [27,28,31,32].

During the last years the interest in the study of biological interactions mediated by BVOCs has steadily increased as their importance is being revealed. Nevertheless, researchers still face several limitations and difficulties in the performance of these assays [32], due to the volatile nature of the compounds and the lack of specific, standardized, simple, affordable and reliable equipment. These challenges arise both during the research stages as well as when applying the volatiles on the field as pesticides or fertilizers and in post-harvest disease control [33–35].

Concerning plant-microbe volatile interactions, two main groups of research methodologies can be drawn [32]. One, where the volatiles passively diffuse to the headspace (Passive Diffusion Systems) [3,10,16], and other, where volatiles are actively channelled from the emitter to the plants (Dynamic Air Stream Systems) [36]. These groups can be further subdivided attending to other factors, like the use of purified BVOCs [20,25,37] or the full volatilome produced by growing microbial colonies [20,37]; as well as the difference between open and closed systems, with or without gas exchange with the exterior [32].

In the Dynamic Air Stream Systems, the microbial strains are cultured in either solid or liquid medium, and the BVOCs produced are actively directed to a different container or mini greenhouse, where the plants are grown [32,36]. This system avoids the bidirectional flow of volatiles and allows for good control of the experimental conditions. Nevertheless, its complexity and costly setup seem to lay behind its limited use in comparison to more simple passive systems.

Within the Passive Diffusion Systems, the split or divided Petri dish has been the most favoured methodology [32]. This method allows for an open or closed setup, where differences in ventilation proved to modify the effects produced by the BVOCs [36,38]. Nevertheless, this methodology is prone to cross-contamination between compartments and strongly limits the room available for plant development. In order to overcome this last limitation, a box-in-box, plate-within-a-box or plate-within-plate system is sometimes used [23,26,37]. This method consists of the placement of a lid-free plate with the growing microbial strain into a larger container where the plants are grown. This method relies on non-specific material and therefore is not completely standardized. Additionally, modifications to this general setup have been proposed to target specific plant parts [39].

For a comprehensive review on methodologies regarding the study of plant-microbe interactions mediated by BVOCs, please see Kai et al. [32]. In addition, systems for the screening of the global BVOCs produced by microorganisms have been also developed [40]. On a larger scale, greenhouses and greenhouse chambers have been used by placing the plants inside them alongside open plates with, either the growing microbial colonies [34] or purified compounds [20].

Notwithstanding the methodologies described so far, the need for new experimental setups in the study of volatile interactions has been highlighted in previous works [28,32]. Therefore, this study presents a novel culture chamber specifically designed to evaluate in vitro plant-microbe volatile interactions, the plant-microbe Volatile Organic Compounds Chamber (p-mVOC Chamber). To test its performance, we used p-mVOC Chamber prototypes to assess the effects produced on wheat (*Triticum aestivum* var. tremie) development by a wild type *Trichoderma harzianum* strain and two of its transformants; a strain that overproduces the volatile trichodiene due to the introduction of the *tri5* gene from *Trichoderma arundinaceum*, and a strain created by silencing the *erg1* gene, leading to the reduction of ergosterol levels and the accumulation of squalene [16,41].

The aforementioned strains were previously developed and described by our group [16,41,42]. These studies demonstrated that, regarding the production of soluble metabolites, the introduction of *tri5* results in increased antifungal activity of *T. harzianum* [16,43], while *erg1* silencing impairs it. Additionally, trichodiene overproduction was demonstrated to increase the antifungal activity of *Trichoderma* BVOCs against *Fusarium oxysporum* and *Rhizoctonia solani* [28], and to reduce the biosynthesis of the mycotoxin deoxynivalenol by *Fusarium graminearum* [37]. For its part, *erg1* silencing led to lower inhibition activity on *F. graminearum and R. solani*, and BVOCs from this strain exerted a growth-promoting effect on *F. oxysporum* when tested in vented conditions [28]. Moreover, a recently published study reports that *erg1* silencing in *T. harzianum* leads to an increased insecticidal activity of this strain regarding the production of volatile compounds against the insect pest *Acanthoscelides obtectus* [44]. These results strongly suggest that the silencing of the *er1* gene, with the subsequent accumulation of squalene and reduction in ergosterol levels, affect the synthesis and emission of BVOCs by *T. harzianum*.

Previous reports indicate that trichodiene functions as a signalling volatile between *Trichoderma* and plants, strongly inducing the expression of systemic defence genes related to the salicylic acid-dependent pathway in tomato plants [16], and exerting a modest effect on wheat disease response [37]. Trichodiene also reduced the differentiation of tomato lateral roots and the growth of its aerial part [43]. Besides, same authors demonstrated that squalene acts as a concentration-dependent elicitor of defence-related genes in tomato, and its overproduction enhanced the ability of *T. harzianum* to colonize tomato roots. Contrary, trichodiene overproduction seemed to produce the opposing effect, reducing plant root colonization by *T. harzianum* [43]. Finally, it has been proposed that the ratio of ergosterol/squalene production affected the ability of *T. harzianum* to colonize plants [41].

The primary objective of this study was to evaluate the reliability and efficacy of the novel p-mVOC Chamber to carry out plant-microbe volatile interactions assays. For this purpose, the effects produced by BVOCs from three *T. harzianum* strains on early stages of wheat growth and development were tested.

2. Materials and methods

2.1. plant-microbe VOC Chamber (p-mVOC Chamber): technical description

The device presented in this study is a culture chamber specifically designed to evaluate in vitro plant-microbe volatile interactions. This technology has been intellectually protected via a Utility Model in the Spanish Office of Patents and Trademarks (Oficina Española de Patentes y Marcas; Application Number: U202131032; N/Ref.: 2020/50419; publication date: 14/07/2021) and derives from a previous invention developed to perform microbial volatile interactions assays [28], which has been patented by the University of León (Spain) with the number ES 2708899 B2 (Spanish Office of Patents and Trademarks) and the international PCT/ES2019/070475.

The p-mVOC Chamber is comprised of three parts; a minor plate (Fig. 1, number 1) with the dimensions of a 90 mm Petri dish; a major vessel (Fig. 1, number 2) that can present different sizes; and a perforated central piece (Fig. 1, number 3) that acts as a double lid with a hole in the middle (Fig. 1, number 11), holding together and connecting the headspaces of both the plate and the vessel. The plate and the vessel's mouth have the same diameter, while the central piece presents a slightly larger one, with two lateral walls projecting upwards and downwards (Fig. 1, number 7) hence being able to hold and house the opening of both receptacles. The central hole (Fig. 1, number 11) connects the two receptacles, thus allowing volatile compounds to freely

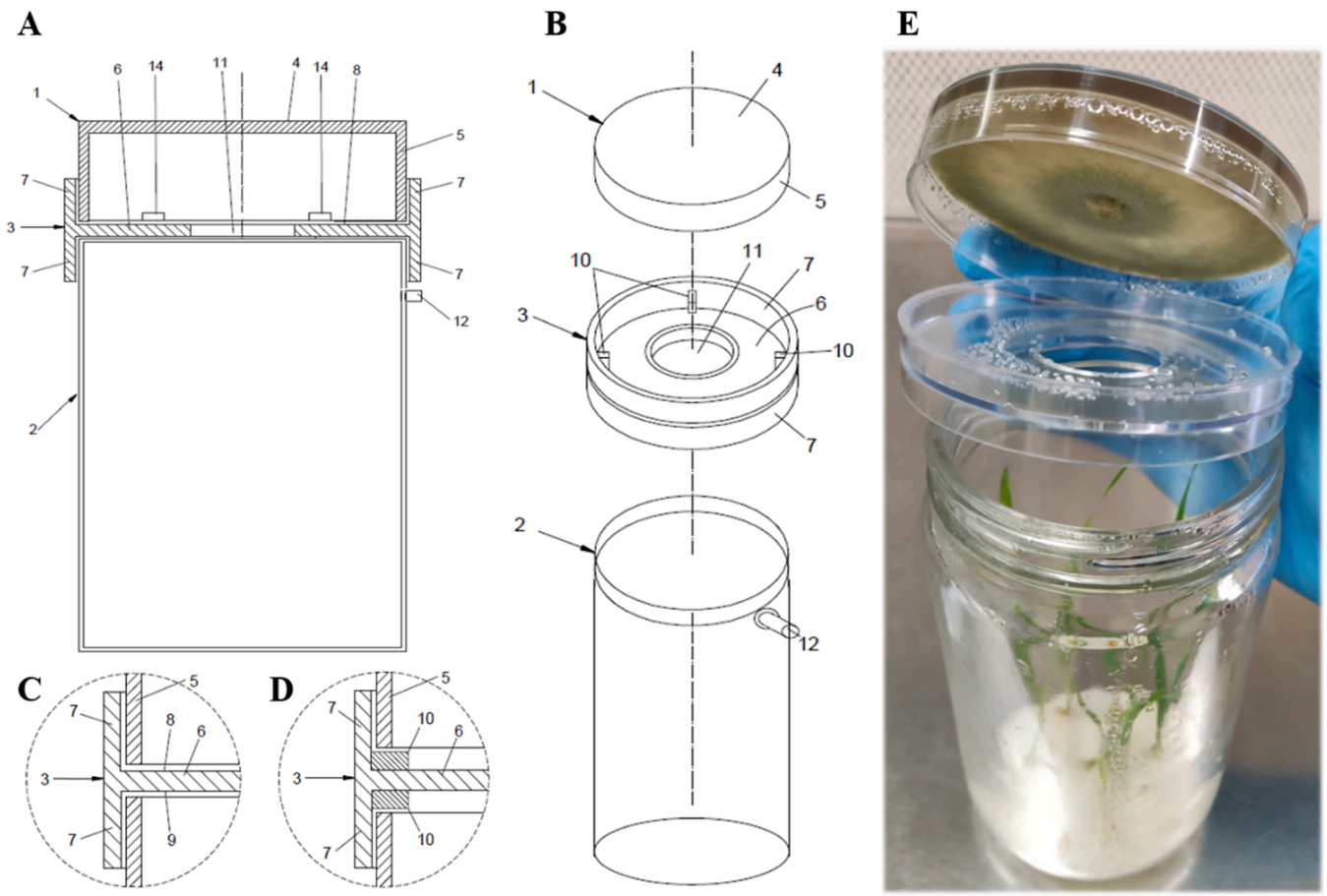


Fig. 1. (A) Frontal Cross-section. Non-Vented p-mVOC Chamber. (B) Explosive View. Vented p-mVOC Chamber (not used in this study). (C) Detail of union between plates and central piece in Non-Vented p-mVOC Chambers. (D) Detail of union between plates and central piece with flanges in Vented p-mVOC Chambers (not used in this study). 1. minor plate; 2. major vessel; 3. central piece; 4. base wall (plate); 5. perimeter wall (plate); 6. intermediate wall (central piece); 7. lateral walls (central piece); 8: upper face (intermediate wall); 9: lower face (intermediate wall); 10. ventilation flanges (Vented p-mVOC Chambers, not used in this study); 11. central hole; 12. Gas-extraction outlet (not used in this study). (E) Opened p-mVOC Chamber with *T. harzianum* (minor plate) and wheat (*Triticum aestivum* var. tremie) plantlets (major vessel), note that for this study the central hole was covered with GF/A glass fibre Whatman filters.

move from one space to the other.

To perform volatile interaction assays, the plate is usually employed to culture the microbial strains on solid growth medium, while the vessel harbours the growing plants (Fig. 2). The vessel is placed facing upwards, with the central piece resting on top of it as a lid would do. The upper plate is placed in turn facing downwards on top of the central piece. In this way, both receptacles are firmly set facing each other, hold in place by the central piece that allows gas exchange through its hole (Fig. 1).

The p-mVOC Chamber can offer vented and non-vented configurations. In its non-vented conformation (Fig. 1C), the central piece presents an intermediate wall with flat surfaces (Fig. 1C, numbers 8 and 9), limiting gas exchange with the exterior and thus allowing for the build-up of higher BVOCs concentration inside the chamber. On the vented conformation (Fig. 1D) the central piece presents small flanges on the edges of the intermediate wall (Fig. 1, number 10), like a Petri dish with vents does. This configuration allows an increased gas exchange rate with the environment, reducing the overall concentration of BVOCs inside the chamber and ensuring oxygen availability for the organisms growing inside. Additionally, a filter or membrane can be placed on top of the central piece (Supplementary Fig. S6a and b, number 13), covering its hole with the aim of avoiding cross-contamination as well as for eventual compound sorting. Further alternative modifications of the basic prototype are available in supplementary materials (Supplementary Figs. S1–S6).

This technology can also be used to evaluate the effects of microbial BVOCs on plant parts, organs and tissues, as well as, conversely, to assess the effects of plant volatiles on microbial strains. Additionally, the device can be turned round (Supplementary Figs. S2 and S4), placing the plate at the bottom and the vessel at the top when the experimental design requires so.

2.2. p-mVOC Chamber prototypes used in the study

In the present study, only non-vented prototypes (Fig. 1C) were used. The central pieces were specifically manufactured in polystyrene crystal by J.D. Catalán S.L. (Arganda del Rey, Madrid, Spain) with a steel mould for plastic injection. These pieces had a diameter of 92 mm, a 30 mm central hole (Fig. 1, number 11), no flanges for ventilation, and 15 mm-high peripheral walls (Fig. 1, number 7). The plastic thickness in all parts of the piece was 1 mm.

Plastic Petri dishes without vents (90 mm in diameter) were used as minor plates for microbial growth, while plants were cultured in 946 ml glass vessels with a mouth diameter of 89 mm (Phytotech labs, Lenexa, KS, USA).

2.3. Fungal material and culture conditions

Trichoderma harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) was employed as the parental wild type strain (T34

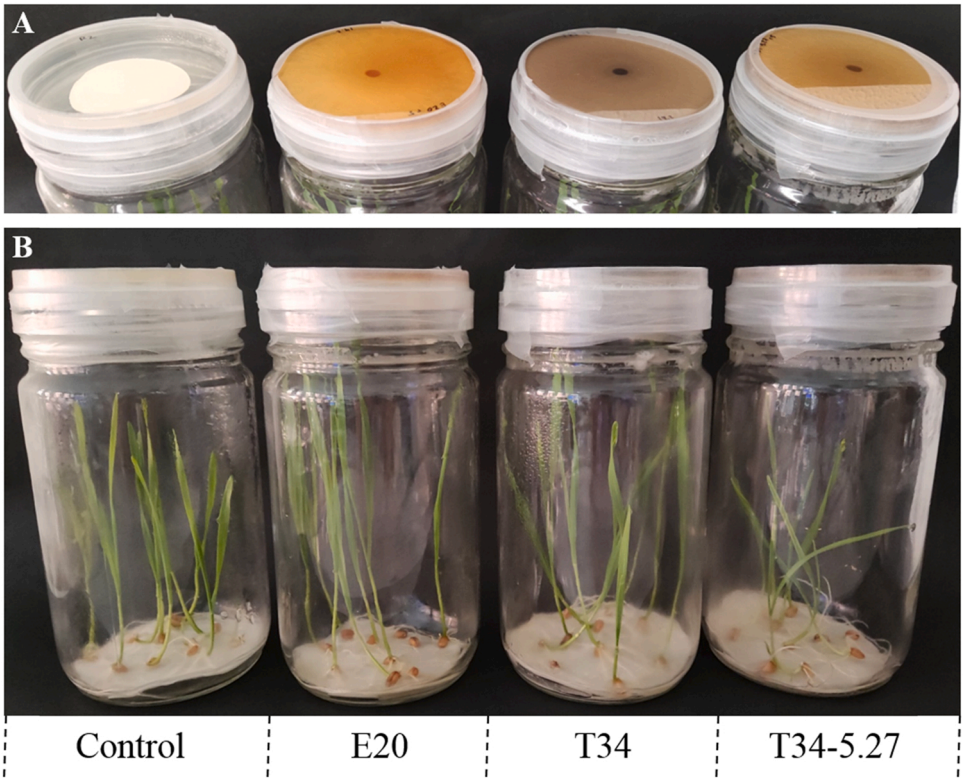


Fig. 2. Assembled p-mVOC Chambers as used in this study. (A) Upper-frontal view of the Petri plates with PDA and the growing *T. harzianum* strains ten days after sowing. (B) Full frontal view of the mounted chambers with the growing wheat plantlets ten days after sowing. From left to right: control treatment (uncultured plate), E20, T34 and T34–5.27.

from now). It is a studied and well-known fungal strain with extensive biocontrol activity [16,28,37]. Additionally, two genetically modified strains previously obtained from the aforementioned wild type strain were also tested. On one hand, *T. harzianum* E20 is a mutant strain derived from T34 by silencing the *erg1* gene, which is responsible for encoding a squalene epoxidase. This modification led to the accumulation of squalene and subsequent lower ergosterol levels [42]. On the other hand, *T. harzianum* T34–5.27 is a T34 transformant that expresses the *tri5* gene from *T. arundinaceum*, this gene encodes a terpene cyclase that is responsible for the cyclization of farnesyl diphosphate into trichodiene, resulting in the overproduction of this metabolite, which is the only intermediate volatile in the trichothecene biosynthetic pathway [16,43].

Fungal strains were stored in spore suspension (−80 °C, 50% glycerol) 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 Potato Dextrose Agar medium (PDA) at 25 °C.

2.4. Use of p-mVOC Chambers to assess the effects of T. harzianum BVOCs on wheat seed germination, plantlet growth and photosynthetic parameters

To evaluate the effects of the BVOCs produced by the three *T. harzianum* strains on wheat, experiments were conducted using the described p-mVOC Chamber prototypes as follows. Petri dishes were filled with 18 ml of PDA and a 6 mm plug from the fresh edge of a 3 days-old active fungal colony was placed in the centre of each plate and left to grow for three days inside a growth chamber at 25ºC in darkness.

For wheat growing, 946 ml glass vessels were used. Four layers of filter paper (73 g/m^{-2}) were placed in the bottom of glass vessels, soaked with sterile water (10 ml) and autoclaved (121ºC; 1 atm; 20 min). Surface-disinfected seeds of wheat (*Triticum aestivum* var. tremie) were disposed inside each glass vessel (ten seeds per vessel) on top of the filter paper. Surface disinfection was carried out by treating seeds with 70% ethanol for 30 s (100 ml/300 seeds). After ethanol treatment, seeds were washed with 50 ml of sterile distilled water (x1), further treated with 0.8% NaClO for 20 min and then washed again with 50 ml of sterile distilled water (x5) before sowing.

Immediately after sowing, a non-vented central piece was placed on top of each vessel's mouth, and the plates with the 3 days-old growing fungi were placed, in turn, upside down on top of them after covering the central hole with a GF/A glass fibre Whatman filter (47 mm diameter) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) to avoid spore cross-contamination. Uncultured Petri dishes with 18 ml of PDA were used as control treatment. Finally, the central piece was sealed to the plate and the vessel using three layers of Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid) (Fig. 2). All procedures were carried out in sterile chamber. Therefore, a gas chamber was formed comprising the headspaces of both the plate and the vessel, connected through the filter-covered central hole, allowing the diffusion of BVOCs from one culture to the other.

The assembled p-mVOC Chambers were randomly distributed inside a phytotron and grown at 23–25ºC, under photoperiodic conditions (16:8) and 3000 lux (≈ 41 μmol m^{-2} s^{-1}) for 10 days. Seed germination was recorded 3, 7 and 10 days after sowing (DAS). Mean germination time (MGT) was calculated for each treatment following the formula described by Ellis and Roberts, (1980) [45].

The chambers were removed thereafter, and plant growth and photosynthetic parameters were measured and recorded. Five seedlings per treatment were used to evaluate growth parameters. Root and aerial part length were measured with a ruler. Fresh weight and dry weight from the root system and aerial part were measured using a precision balance (Mettler Toledo; 220 g x 10^{-4} g). After fresh weight recording, samples were dried at 60ºC (Selecta Drying Oven; J. P. Selecta; Abrera, Barcelona) until weight remained unchanged and then weighted again

to record dry weight.

Besides, three seedlings per treatment were set aside for chlorophyll fluorescence quantum yield quantification using a PAM-fluorometer (Teaching-PAM; Walz; Effeltrich, Germany), and they were then stored at − 80ºC for future evaluation of leaf chlorophyll content. The total chlorophyll content of leaf tissue was assayed on 80% acetone extracts by using the Arnon method [46].

Five replicates were performed per treatment and the experiment was carried out five times in the same conditions (n = 25).

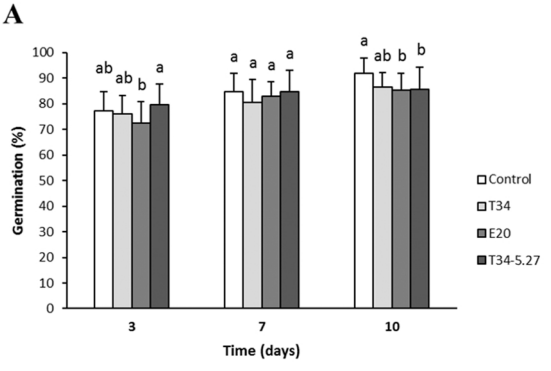
2.5. Data treatment and statistical analysis

After testing the normality of the data series by Kolmogorov-Smirnovś test and the equality of variances by Levenés test, they were analysed by using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($p \leq 0.05$). A non-parametric Kruskal–Wallis H-test was used followed by a Mann-Whitney U-test ($p \leq 0.05$) when data were not normally distributed or homoscedastic. All statistical analyses were performed using IBM SPSS Statistics 26.

3. Results and discussion

In the present study, the novel p-mVOC Chamber was tested by evaluating the effects produced on wheat development by BVOCs from three *T. harzianum* strains, a wild type (T34) and two transformants, T34–5.27, which overproduces the volatile trichodiene, and E20, which shows higher levels of squalene and reduced ergosterol ones.

Results concerning wheat seed germination show that none of the treatments affects germination speed (expressed as MGT) in the tested conditions. Although a slight decrease in MGT was recorded for BVOCs exposed seeds, no significant differences were found (Fig. 3B). Contrary, when wheat seeds were exposed to T34–5.27 and E20 strains, a reduction in the germination rate was recorded at ten DAS (Fig. 3A). Previous studies demonstrated that some individual fungal BVOCs inhibit seed germination in *Arabidopsis thaliana*, while others did not produce any significant effects on this parameter, and just one promoted seedling formation [25,47]. In addition, Ogura et al. [48] proved that important soil-related microbial BVOCs, like geosmin and 2-methylisoborneol, inhibit germination in Brassicaceae.



B

	Control	T34	E20	T34-5.27
Mean Germination Time (days)	3.85 ± 0.54 a	3.69 ± 0.52 a	3.66 ± 0.14 a	3.46 ± 0.26 a

Fig. 3. Effect of BVOCs produced by T34 (wild-type), E20, and T34–5.27 *T. harzianum* strains on wheat germination expressed as (A) germination percentage along time and (B) Mean Germination Time. Control treatment indicates wheat seeds exposed to an uncultured plate. Data represent average ± sd (n = 25 in Fig. 3.A and n = 5 in Fig. 3.B). Different letters indicate significant differences (Mann–Whitney U test for Kruskal–Wallis, $p < 0.05$). In Fig. 3.A, comparisons were carried out among data collected at three, seven and ten days after sowing.

Regarding growth parameters (Fig. 4) our results indicate that BVOCs produced by the *T. harzianum* wild type T34 and its E20 mutant significantly enhanced length (Fig. 4A) and fresh weight gain (Fig. 4B) of wheat aerial part compared to the control. The length and fresh weight of roots (Fig. 4D, E) was not significantly altered by these treatments. Dry weight gain, a growth parameter directly related to biomass accumulation and therefore net photosynthesis [52] was only significantly affected in the roots of T34 and E20 exposed plantlets (Fig. 4F).

Interestingly, no differences were found between T34 and E20 for any of the studied parameters, suggesting that squalene overproduction and the subsequent reduction in ergosterol levels do not modify the overall activity of *T. harzianum* BVOCs on wheat plantlets.

Conversely, T34–5.27 volatiles significantly reduce the length and dry weight gain of the aerial part (Fig. 4A, C) and roots (Fig. 4D, F), and the fresh weight gain of the aerial part (Fig. 4B) when compared to non-exposed controls, and to T34 and E20 treatments. These results suggest a general inhibitory effect in plantlet development derived from the expression of *tri5* and the subsequent overproduction of trichodiene. Our results regarding trichodiene overproduction tie well with studies wherein this compound reduced root development, lateral roots differentiation, and the growth of aerial part in tomato [43]. Altogether, these evidences may point out to a putative phytotoxicity of trichodiene against both mono and dicotyledonous plants, even though it had been previously labelled as the only non-phytotoxic metabolite in the biosynthetic route of trichothecenes [49,50], and previous studies reported that wheat seeds soaked with T34 and T34–5.27 conidia showed no visual effects on plant growth and development [37].

Besides, *erg1* silencing has been related to fungal growth promotion [28] and increased insecticidal activity [44] via BVOC production, while trichodiene overproduction via *tri5* expression was described to enhance the antifungal activity of *T. harzianum* against phytopathogenic fungi, and to reduce their mycotoxin production [28,37].

In this study, no differences were observed in chlorophyll fluorescence quantum yield and leaf chlorophyll content (Fig. 5), likely suggesting that the changes induced by the *T. harzianum* BVOCs, affect plant growth without interfering with photosynthetic processes. Therefore, alternative targets to photosynthesis involving cell division and elongation should be considered for these compounds. In this regard, the increase of wheat aerial fresh weight produced by T34 and E20 and the negligible effect on its dry weight suggest that turgor-driven cell elongation may account for these effects.

Changes in the photosynthetic rate not related to chlorophyll fluorescence quantum yield and leaf chlorophyll content cannot be completely ruled out. It should be bear in mind, that sealed conditions (Fig. 2) in a non-vented configuration (Fig. 1A, C) for the device have been used. For long-term experiments of fungal-plant coculture in the above conditions, variations in the atmospheric composition of the culture chamber are expected [26]. Particularly, a CO_2-fertilization effect due to fungal respiration it is likely to occur [36]. By this mean an increase in net photosynthetic rate [51] and therefore in dry mass accumulation would be expected for wheat plantlets cocultured with fungal strains when compared to non-exposed controls. In the present study, no data about variations on CO_2 levels are available, what certainly may limit the interpretation of these results. However, results provided here do not seem to fit in a CO_2 fertilization scenario as no stimulatory effects on dry weight gain were observed for fungal-cocultured plantlets. On the contrary, when significant changes were observed, an inhibition on this parameter was recorded (Fig. 4C for T34–5.27 exposed plantlets; Fig. 4F).

New studies involving, among others, molecular and genetic analyses are needed to unveil the complex volatile interaction taking place inside these chambers and their correlation with results reported in previous studies. In this respect, previous reports of trichodiene overproducing-strains and the purified compound indicate that trichodiene also functions as a signalling volatile between *Trichoderma* and plants, inducing the expression of systemic defence genes in tomato [16]

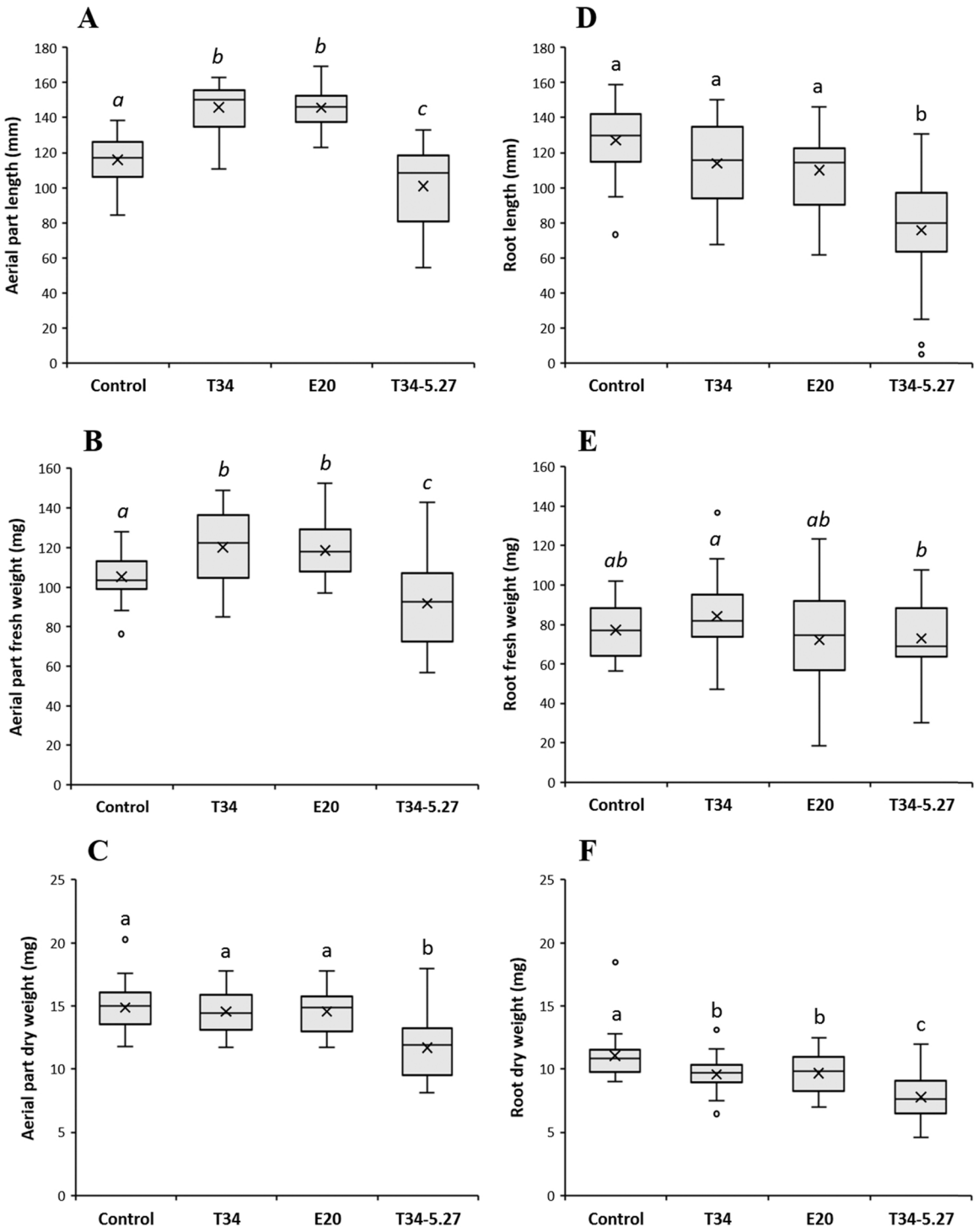


Fig. 4. Effect of BVOCs produced by T34 (wild-type), E20, and T34–5.27 *T. harzianum* strains on growth parameters of wheat plantlets ten days after sowing. Control treatment indicates wheat seeds exposed to an uncultured plate. Aerial part: left column; Root: right column. The effect on (A, D) total length, (B, E) fresh weight and (C, F) dry weight gain of (A, B and C) aerial part and (D, E and F) roots of wheat plantlets is plotted. Boxplots show median and confidence intervals. Means are represented by crosses. Different letters indicate significant differences (in italic: Mann–Whitney U test for Kruskal–Wallis, $p < 0.05$; non-italic: Tukey's test for one-way ANOVA, $p < 0.05$) (n = 25).

and wheat [37], and reducing root colonization [43].

As for the methodology itself, the p-mVOC Chamber demonstrated to be a reliable and efficient method to evaluate in vitro the effects of volatiles directly produced by growing microbial strains on plant development. They provide homogenous and replicable results and allow easy and fast manipulation and arrangement, thus making for a perfect screening method. The p-mVOC Chamber is also easy to place on racks inside growth chambers due to its upright structure. Moreover, the central piece's design enables the adequate placement of membranes or filters covering its central hole to avoid cross-contamination. Filters could be also used for eventual compound sorting. In addition, the chamber provides flexibility to the experimental design, as culture vessels of different shape, material and volume can be coupled according to the specific needs of each assay. Moreover, these chambers could

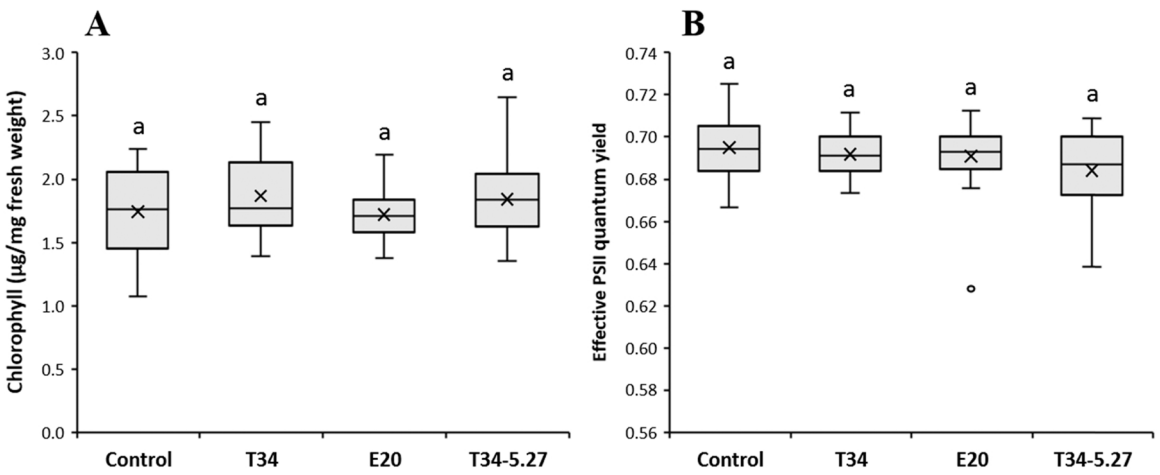


Fig. 5. Effect of BVOCs produced by T34 (wild-type), E20, and T34–5.27 *T. harzianum* strains on (A) total leaf chlorophyll content and (B) effective quantum yield of chlorophyll of wheat plantlets ten days after sowing. Control treatment indicates wheat seeds exposed to an uncultured plate. Boxplots are as described in Fig. 4. Different letters indicate significant differences (Tukey's test for one-way ANOVA, $p < 0.05$) (n = 25).

easily become an affordable device, as only the central piece is of new design, while traditional 90 mm Petri dishes and plant culture vessels could be used for the upper and lower receptacles.

Gas exchange and ventilation with the exterior can be modified as well using vented (Fig. 1D) or non-vented (Fig. 1C) configurations (central pieces with or without vents (Fig. 1, number 10)), further increasing the range of potential experimental conditions. The importance of ventilation control in volatile assays, while not tested in the present work, has been previously reported for both microbe-microbe and plant-microbe interactions [28,30,36,38]. Additionally, as mentioned before, in the case of long-term plant-fungal coculture experiments, the monitoring of gas composition inside the culture chamber or the use of VOCs-absorbing charcoal would aid to solve problems with the misinterpretation of results as pointed out by García-Gómez et al. [26]. The technology also presents some limitations. BVOC diffusion occurs only passively without the possibility of controlling the gas flow, which is always bidirectional and cannot be directed to specific plant organs. In addition, the growing of the BVOC-producing microbial strain in the upper part of the chamber may result in the induction of stronger effects on the aerial part of the plant compared to the rest of the organism. Besides, even though vessels with different volumes can be used, plant growth is still limited, especially for fast-growing species or long-time experiments. New protocols and designs would be of much interest to help circumvent these problems.

As referred, up until now, we have only evaluated physiological parameters in the plants. Further research is needed to unveil the genetic and molecular traits that lay behind the interactions taking place inside the chambers in order to fully understand their ecological and biological implications. Furthermore, volatile dialogues are bidirectional, therefore, the effects produced by plant BVOCs on the microbial strains should also be evaluated in the future [6]. Additional studies should be conducted using different plant species, isolated plant organs and tissues, microbial strains, culture conditions and technical setups, to better assess all the potential and limitations of this novel technology.

4. Conclusions

The *T.harzianum* wild type and the *erg1* (squalene epoxidase) silenced strains enhanced fresh weight and length of the wheat aerial part and reduced root dry weight compared to the control, while not showing any differences between them. Conversely, the expression of the *tri5* gene (involved in trichodiene production) significantly impaired root and aerial growth compared to the control and the other two strains. No differences were observed in any case regarding chlorophyll fluorescence quantum yield and leaf chlorophyll content, suggesting that the released BVOCs do not interfere with photosynthesis.

The p-mVOC Chamber proved to be a simple, homogeneous, flexible, and replicable method to evaluate the in vitro effects of microbial BVOCs on plant development, as well as to select microorganisms with interesting volatile traits. It might also be useful to guide the future identification of new bioactive volatile compounds and their role in plant-microbe ecological relationships. Thus, the p-mVOC Chamber could become a standard methodology for the evaluation of in vitro plant-microbe volatile interactions. Moreover, as this field is gaining interest, new uses and applications for these devices may arise from the scientific community.

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Authors' contributions

S.A-G., P.A.C. and A.E. developed the technology; S.G developed the microbial strains and designed the culture conditions; S.A-G. and A.E. planed the experimental design; S.A-G. and A.E. performed the experiments; S.A-G. and A.E. wrote the manuscript; A.M-R. performed data curation, data analysis and produced the figures; S.A-G., A.M-R., S.G., P. A.C. and A.E. 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 utility model submitted by the University of León regarding the described technology (Oficina Española de Patentes y Marcas; N/Ref.: 2020/50419; publication date: 14/07/2021). Some of the authors of the present manuscript share part of the intellectual

protection as inventors: 52% Samuel Álvarez García, 10% Pedro A. Casquero, and 30% Antonio Encina.

Data Availability

The data that support the findings of this study are available from the corresponding authors (Antonio Encina and Samuel Álvarez-García) 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).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111286.

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