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TESIS DOCTORAL

New *in vitro* approaches and technologies to evaluate the biological activity of microbial secondary metabolites in plants, plant pathogens and pests

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Ph.D. program "Biosystems Engineering"

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SUPERVISOR'S REPORT

Dr. **Pedro Antonio Casquero Luelmo**, Dr. **Santiago Gutérrez Martín,** and Dr. **Sara Mayo Prieto,** as supervisors of the Ph. D. Thesis "*New in vitro approaches and technologies to evaluate the biological activity of microbial secondary metabolites in plants, plant pathogens and pests*" carried out by Mr. **Samuel Álvarez García** within the Ph.D. program "Biosystems Engineering" (regulated by the R.D. 99/2011, 28th January), give a favourable report on its submission since it meets the requirements for the dissertation defence.

León, June 2021

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ABSTRACT

This PhD thesis addresses a critical evaluation of some of the most currently used *in vitro* methods to test the biological effects of microbial secondary metabolites, which serve as a starting point for the proposal and assessment of new perspectives and technologies to further improve research on the field.

A comparison of the self-inhibitory and antifungal activities of *Trichoderma* spp. against *F. oxysporum* was performed using membrane assays, demonstrating that these BCAs present *in vitro* similar levels of self-inhibition than the antifungal effects they exert on *F. oxysporum* in the same conditions. This suggests that self-inhibitory traits might be a parameter to consider when searching for putative BCAs *in vitro* using membrane assays.

Additionally, much of the research was centred on the *in vitro* evaluation of interactions mediated by Biogenic Volatile Organic Compounds (BVOCs). Several strains of *Trichoderma harzianum* were used as biological control agent (BCA), and the effects produced by their BVOCs were studied on the common bean (*Phaseolus vulgaris*) phytopathogens *Fusarium oxysporum* and *Rhizoctonia solani*, on the dry bean insect pests *Acanthoscelides obtectus*, and on wheat plantlets (*Triticum aestivum*). In this regard, a new device was developed and tested, named Volatile Organic Compounds Chamber (VOC Chamber). It demonstrated more homogeneity of results, replicability, and versatility than currently used methods. It proved to be also able to reveal significant differences between treatments that were not detected by the traditional methods. Results also gave an insight on the importance of ventilation in these complex volatile interaction between microorganisms, as the growth-inhibitory activity produced by some *Trichoderma* strains on plant phytopathogens switched dramatically between vented and non-vented conditions. Moreover, the referred device has been patented, thus proving its novelty, innovation, and potential industrial interest. This chamber was also used to confront the insect pest *A. obtectus* against *T. harzianum* BVOCs, showing significant differences between fungal strains in both insect mortality and bean damage, as well as between sealed and unsealed conditions. In addition, a further modification of the prototype (intellectually protected as a Utility Model) was developed and tested to evaluate the effects of microbial BVOCs on plants. Experiments using this device

showed that BVOCs from different *T. harzianum* strains exert a diverse activity on the growth and development of wheat plantlets, also proving the usefulness of the technology to perform plant-microbe volatile interaction assays.

Finally, individual BVOCs produced by two *T. harzianum* strains, a wild-type and a transformant that overproduces squalene, were identified and quantified using GC/MS technology. This research showed that the percentage of all eighth major BVOCs emitted by these strains differ significantly between them, which could account for some of their biological activities described in the interaction assays.

INTRODUCTION

1. Sustainable agriculture to tackle present and future challenges

The global population is continually rising, and with it, an ever-increasing demand for food and other agricultural products (Dhankher and Foyer, 2018). The eradication of hunger was, after all, the first of the "Millenium Development Goals" that the United Nations set in 2002 for 2015 (UN, 2016), and is now the second one in the new "Sustainable Development Goals" for 2030 (UN, 2021). Thus, the problem of malnutrition and food access has not yet been solved, and there are already new threats looming in the nearer future, as energetic issues, climate change, and other socio-economic and environmental issues pose a significant challenge to agricultural production, storage, and distribution (Dhankher and Foyer, 2018). Among those factors, abiotic and biotic stresses for crops are of major concern. Phytopathogens and plant pests cause huge damage and losses to both industrial and food crops, threatening food security and access to other agricultural commodities (Pandey *et al.*, 2017). Furthermore, it has been reported that postharvest losses, largely due to storage pests and diseases, may account for the waste of up to 20-30% of the global agricultural production (Dukare *et al.*, 2019). While the use of artificially synthesised chemical products has been the basic strategy in conventional control of plant diseases and pests, the current tendency is moving towards more integrated and ecologically respectful strategies, as many of these compounds have been proven to present risks to the environment and human health alike (Alengebawy *et al.*, 2021; Barascou *et al.*, 2021). In this regard, biological control, including the use of microbial Biological Control Agents (BCAs), stands out as an effective, reliable, and safe strategy in the management of plant diseases and pests for improved resilient agriculture (Kashyap *et al.*, 2017).

2. The common bean (*Phaseolus vulgaris* L.)

2.1. Generalities, importance, and current situation: from global to local

Legumes are essential crops of great importance for human nutrition and domestic animals feeding (de Ron, 2015), they belong to the family Fabaceae or Leguminosae. They are usually consumed as dry seeds that are characterized by high protein content, which together with their worldwide distribution, cultivation and consumption make them vital in human protein intake, especially in developing or low-income countries where animal protein is not generally available or affordable (Sanon *et al.*, 1998). Moreover,

legumes are of the utmost importance in sustainable agriculture, as their general symbiotic interaction with nitrogen-fixing bacteria, broadly known as rhizobia, enables them to fixate inorganic into organic nitrogen. This pivotal trait is the base of rotation or coculturing strategies that allow for the reduction of inorganic nitrogen fertilization (de Ron, 2015).

The common bean (*Phaseolus vulgaris* L.) holds the third position among the most important cultivated legumes for human nutrition, behind soybean (*Glycine max* (L.) Merr.) and peanut (*Arachis hypogea* L.). It presents the greatest range of seed characteristics, growth habits, and maturation time among major food crops, which accounts for its broad distribution in different culture systems and environments (Blair *et al.*, 2010). Dry beans are mostly cultivated in Asia, the Americas, and Africa (Fig. 1), with India, China, and Myanmar being the largest world producers by far (FAO, 2021). Bean varieties cultivated nowadays are the result of a long process of evolution and selection, by which genetic, physiological, and morphological changes have been fixed in the populations as a response to human needs and environmental conditions (Casquero, 1997).

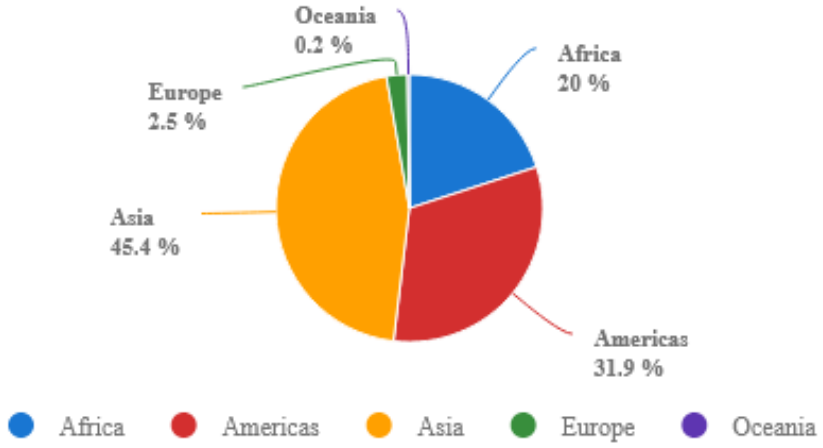

Figure 1. Percentage of the global production of dry bean in each world region according to the Food and Agriculture Organization. Average between 1994 and 2019 (FAO, 2021).

Regarding Europe, the growth of this crop is concentrated in the southern countries of the continent, with Spain in a prominent position (Casquero *et al.*, 2006). Castilla y León is the region with the highest production and within it the province of León (Fig. 2), which accounted for more than 45% of the total cultivated surface and more than 57% of the nationwide annual production in 2019 (data drawn from MAPA, 2019). The socio-

economic characteristics of this province, where small-scale farming is predominant, resulted in the preservation of local varieties until today (Casquero *et al.*, 2006). This conjugation of qualitative and quantitative factors promoted the creation of the Protected Geographic Indication (PGI) "Alubia de La Bañeza-León" (EC Reg. n. 256/2010 published on 26 March 2010, OJEU L880/17).

Figure 2. Map showing the Spanish northwestern Province of León. Highlighted in colors are those shires belonging to the Protected Geographic Indication (PGI) "Alubia de La Bañeza-León", in which these beans are cultivated.

2.2. Fungal pathogens of importance in common bean from the PGI "Alubia de la Bañeza – León

Diverse phytopathogenic fungi can cause diseases to bean crops. In many cases, several pathogens from the genera *Rhizoctonia*, *Pythium*, *Fusarium*, *Thielaviopsis*, *Sclerotinia*, and *Aphanomyces* are found together forming a disease complex (APS, 2005). Infections produced by *Rhizoctonia solani* Kühn, alongside *Fusarium* yellows caused by *Fusarium oxysporum* f. sp. *phaseoli* J.B. Kendr. & W.C. Snyder and root rot caused by *Fusarium solani* f. sp. *phaseoli* (Burkh.) W.C. Snyder & H.N. Hansen are the main fungal diseases in many bean crops (Asensio, 1996). They have an important presence in the production areas located in the province of León, where cause relevant impact on bean yield (Campelo *et al.*, 2007).

Among these fungi, *R. solani* is the most common one in root rots and is found in more than 90% of affected plants (Valenciano *et al.*, 2006). This species is the most

important of the genus and can remain in the field between seasons and persist as a saprophyte in the soil or crop residues, or forming sclerotia (Agrios, 2002). The production of enzymes such as cellulases and pectinases allows its penetration into the plant. *R. solani* usually attacks first the belowground parts of the plant (Fig. 3) and can kill the seed before germination or produce damping-off and death of the plantlets (Hagedorn, 1991). Grown plants and their pods can be also affected by this fungus, causing white or brownish wounds on the external tissues that can sometimes lead to the plant's death (Hall, 1992). These damages are more common in cold wet climates. Generally, fast-growing crops are less susceptible to the fungus, while the infection is more severe when environmental conditions are adverse for the plant (Agrios, 2002).

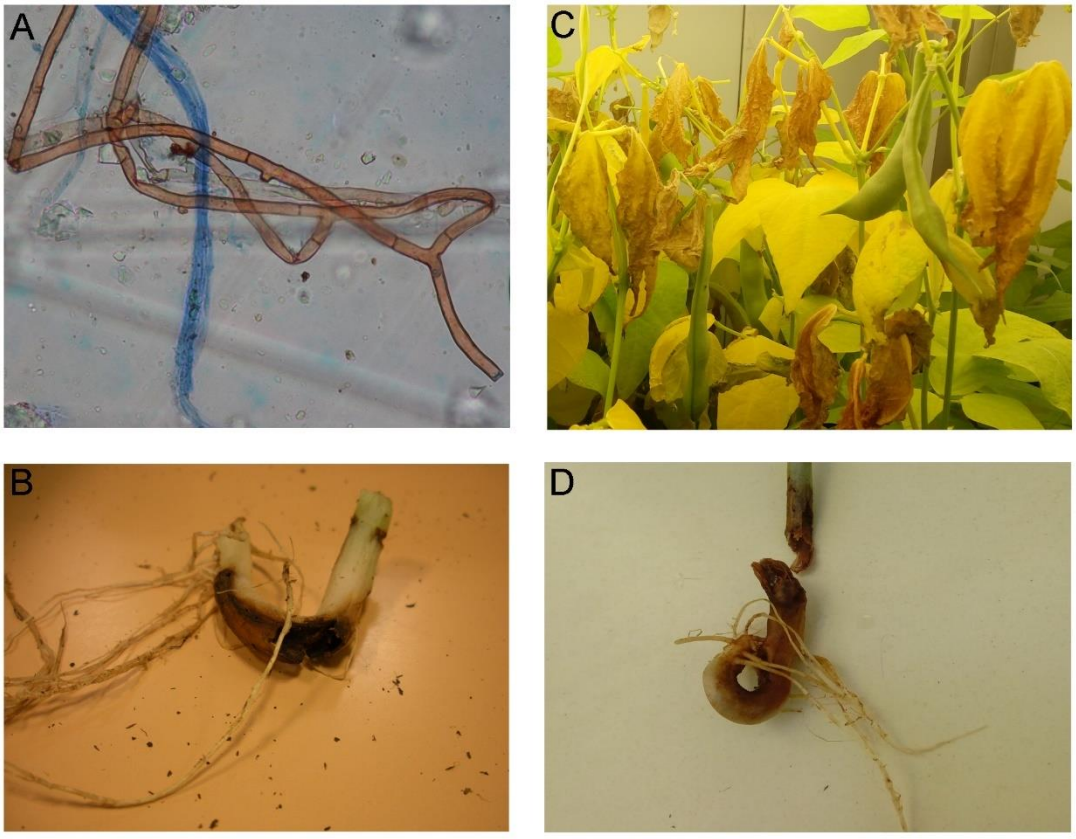

Figure 3. (A) *R. solany* hyphae on bean roots. (B) Wounds caused by *R. solani* on bean roots and hypocotyl. Pictures kindly provided by Dr. Sara Mayo Prieto. (C) Bean plants affected by *F. oxysporum*. (D) Wounds caused by *F. oxysporum* on bean roots and hypocotyl.

F. oxysporum f. sp. *phaseoli*, on the other hand, is an asexual filamentous fungus that causes tracheomycosis in common bean, known as Fusarium wilt and/or yellow wilt. The main symptomatology in the plant is chlorosis followed up by yellowish coloration,

especially on the leaves, and premature senescence of the lower ones. As the infection advances along the vascular system, upper parts of the plant start showing the same symptoms. Affected vascular beams show brown-reddish colour even before the described external symptoms are revealed (Brick *et al.*, 2006). This fungus can also remain in the soil for long periods and infect subsequent crops. The fungal spores germinate, and hyphae enter the plant usually through underground parts. Once the fungus reaches the xylem vessels, it penetrates and moves along them, usually upwards. The vascular flow of the plant can also carry microconidia to other unaffected parts of the organisms. Loses are usually more severe with early infections and wilt than in those cases in which symptoms appear in late phases of the crop (Agrios, 2002).

2.3. *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchidae): An insect pest with important impact in storage dry bean

The bean weevil *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchidae) is a primary insect pest with one of the highest impacts on stored dry seeds, including legumes (Baier and Webster, 1992; Berger *et al.*, 2017), being a huge problem for common dry bean storage (Silva, 2017). This insect evolved in Mesoamerica but it is currently distributed around the globe, affecting modern seed storage facilities (Rodríguez-González *et al.*, 2019) as much as traditional producers in developing countries (Paul *et al.*, 2009). *A. obtectus* may colonize the seeds on the field and later be carried to the silo, or commence its attack directly in storage, where this pest produces the most damage (Baier and Webster, 1992).

Females of the insect lay their eggs on or around the seeds and, after hatching, the larvae carve a hole to penetrate them, where they feed and develop until emerging as new adults. This process can produce exponential growth in populations, able of destroying entire harvests (Berger *et al.*, 2017). The damage to the seeds caused by larvae feeding and the exiting of the adults lowers the overall quality and value of the product, generating important economic losses. It can also affect the embryo, reducing seed germination rates and thus compromising subsequent crop productivity (Silva, 2017). Damaged seeds can be also more easily affected by secondary pests and postharvest diseases (Dukare *et al.*, 2019).

Nowadays, the control of this insect pest is based on the use of synthetic chemical insecticides, especially phosphine and aluminium phosphide. These compounds are generating rising concerns regarding residue persistence, pest resistances, safety, and toxicity to human health and the environment, advising for the convenience of using less harmful control strategies (Vardell *et al.*, 1973; Bogle *et al.*, 2006; Murali *et al.*, 2009; Nayak *et al.*, 2020).

3. Biological control

3.1. Concept, importance, and general mechanisms

Biological control (or Biocontrol) can be defined as the use of biological organisms, their parts, or the substances they produce to control the damage caused by other biological organisms. Organisms employed in biocontrol are named BCAs, and they are being extensively studied for their agricultural applications, in many cases as part of Integrated Pest Management (IPM). Among BCAs, microorganisms hold a pivotal role in agriculture (Pirttilä *et al.*, 2021; Sare *et al.*, 2021). The importance of biological control strategies for crop production has been already mentioned at the beginning of this introduction. As more and more traditional pesticides are being banned due to health and environmental issues, and new resistances to them arise from microbial pathogens and insect pests, the search and use of less harmful novel effective strategies have become of utmost relevance (Kashyap *et al.*, 2017).

Several interesting features and advantages have been highlighted in the use of BCAs and their derived products. When applied correctly, they are more respectful with the environment and, usually, their metabolites present less toxicity and persistence (Tilocca *et al.*, 2020). Many BCAs can be easily produced using affordable processes, which made them ideal also for low-income countries and communities (Paul *et al.*, 2009; Kashyap *et al.*, 2017). BCAs can, in many cases, confront diseases and pests while promoting at the same time plant growth, resistance to biotic and abiotic stresses, and productivity (Hermosa *et al.*, 2013; Maurya, 2020). BCAs are usually less aggressive towards natural beneficial organisms present on the crop than synthetic pesticides (Mayo-Prieto *et al.*, 2020). The diverse and complex mechanisms that each single BCA can commonly unleash against a specific phytopathogen or pest significantly reduce their ability to develop resistances or effective evading strategies (Sood *et al.*, 2020).

Notwithstanding their increasing success, research and study are still needed to further improve the effectiveness and use of these products, as "natural" or "biological" does not automatically mean safer and effective. New biocontrol strategies, BCAs, and novel uses for those already known will be a key factor to overcome many of the present and future challenges in plant yield and to accomplish more efficient and resilient agriculture (Kashyap *et al.*, 2017).

3.2. Mechanisms

As stated above, microbial antagonists used as BCAs in agriculture present a variety of mechanisms of action that, ultimately, suppress or interfere with the normal development of plant pathogens or pests. A large number of microbial species and strains have been identified as BCAs from different taxonomic groups such as bacteria, yeast, and filamentous fungi (Dukare *et al.*, 2019). Each of these organisms presents in many cases more than one antagonistic mechanism that can act synergistically (Sood *et al.*, 2020). In short, these mechanisms are:

Competition. Resources and space are mostly limited in the environment, and crops are not an exception. Competition for these resources shapes the ability of some organisms to control, limit development, or drive phytopathogens out of the niches from where they might infect the plant (Alfiky and Weisskopf, 2021). Competition can even take place on the surface or inside plant tissues (Carro-Huerga *et al.*, 2020; Poveda *et al.*, 2020). Rapid growth and more efficient use of nutrients are the key factors in this process, although they may be coupled with other biocontrol mechanisms (Ruano-Rosa *et al.*, 2016).

Parasitism. It is a mechanism by which an organism lives at the expense of another, the host, that receives harm and may eventually die as a result of this relationship (Dukare *et al.*, 2019). Regarding biological control exerted by microorganisms, it usually involves the release of lytic enzymes, like chitinases and glucanases, to penetrate the host (Sood *et al.*, 2020). In many cases, the BCA grows actively towards its prey in response to chemical stimuli released by it (Alfiky and Weisskopf, 2021). Regarding filamentous fungi, they can present mycoparasitic activity against fungal phytopathogens (John *et al.*, 2010), or entomopathogenic or nematophagous behaviours against pests (Jaber and Ownley, 2018; Poveda *et al.*, 2020).

Plant growth and defence promotion. BCAs can establish relationships with plants and induce their development or enhance their resistance against biotic and abiotic stresses (Kashyap *et al.*, 2017). These interactions may take place in the proximity, the surface, or the interior of the plant, and can be both beneficial for the BCA as well as the plant (Carro-Huerga *et al.*, 2020; Wonglom *et al.*, 2020). The rhizosphere is a hotspot where plant promotion takes place alongside other biocontrol mechanisms (Contreras-Cornejo *et al.*, 2016). BCAs can help to mobilize nutrients that were not readily available for the plant (Vinale *et al.*, 2008; Srivastava *et al.*, 2018), produce secondary metabolites and phytohormones that promote growth and development, or induce plant defensive responses to pathogens and pests (Mayo *et al.*, 2016a,b; Gomes *et al.*, 2020). Systemic acquire resistance (SAR), induced systemic resistance (ISR), or hypersensitive response (HR) have been described among those defensive mechanisms induced by microbial BCAs (Harman, 2006; Sood *et al.*, 2020).

Antibiosis. Secondary metabolites with biotoxic activity are produced and released by microbial BCAs. These compounds are chemically diverse and can be of either volatile or non-volatile nature (Dukare *et al.*, 2019). These compounds mediate complex interactions, affect a wide variety of target organisms, and can serve as biomarkers for strain identification (Poveda, 2021). Steroids, mono and sesquiterpenes, peptaibols, furanes, diketopiperazines, pyrones, benzene derivatives, or polyketides are among those groups with described antibiotic activity (Korpi *et al.*, 2009; Kai *et al.*, 2016; Contreras-Cornejo *et al.*, 2020).

Due to their central role in this Ph.D. thesis, microbial volatiles and their effects will be more extensively treated in the following section.

3.3. Microbial Biogenic Volatile Organic Compounds (BVOCs) for disease control, pest management, and plant stimulation in agriculture

Biogenic Volatile Organic Compounds (BVOCs), also referred to as Volatile Organic Compounds (VOCs), are organic molecules produced and released to the environment by a diverse array of organisms. They have a low molecular mass and boiling point, as well as a high vapour pressure, which allows them to display a gaseous state under environmental conditions (Herrman, 2010). These compounds can act as mediators

in diverse intra and interspecific long-range biological interactions (Effmert *et al.*, 2012; Quintana-Rodríguez *et al.*, 2015), playing significant roles in inter-organismic processes such as competition (Hammerbacher *et al.*, 2019), symbiosis (Kandasamy *et al.*, 2019), recognition (Li *et al.*, 2018), or communication (Markovic *et al.*, 2019). It has been also suggested that BVOCs are a relevant factor for the establishment and sustaining of ecological communities (Li *et al.*, 2020), among which stand out those taking place in the rhizosphere between plants and microbes, including several fungi, such as filamentous (Hung *et al.*, 2015), mycorrhizal ones (Ditengou *et al.*, 2015), as well as bacteria (Wenke *et al.*, 2019) and other microorganisms (Spadaro and Droby, 2016). For example, Li *et al.*, (2020) suggested that soil bacteria developed a collective antagonism against competitors like soilborne fungi. They propose that these traits account for much of soil fungistatic capability, being a general strategy of bacteria to stand their ground and defend their niches against invading fungi.

Therefore, studies regarding microbe-microbe interactions have shown that exposition to BVOCs induces diverse effects in bacteria and fungi. Growth inhibition, growth promotion, as well as morphological or physiological responses have been reported so far (Giorgio *et al.*, 2015; Lo Cantore *et al.*, 2015; Myo *et al.*, 2019; Taylor *et al.*, 2021). *Trichoderma*, *Fusarium*, and *Rhizoctonia* have been reported as some of the most odoriferous filamentous fungi (Loulier *et al.*, 2020). Remarkably, these effects are not only species- or strain-specific but are also highly influenced by the surrounding environmental conditions, which shape the outcome of volatile interactions (Lo Cantore *et al.*, 2015; Kai *et al.*, 2016; Speckbacher *et al.*, 2020). The quantity and nature of the BVOCs produced vary depending on factors as diverse as nutrients and oxygen availability, temperature, pH, age of the population, etc. (Loulier *et al.*, 2020). Chemically, microbial BVOCs are also diverse, with furanes, alkenes, alcohols, mono and sesquiterpenes, aldehydes, esters, ketones, or benzene derivatives among the most common ones (Korpi *et al.*, 2009; Contreras-Cornejo *et al.*, 2020). In addition to their role in the field, BVOCs could be an interesting tool for the control of postharvest microbial diseases (Dukare *et al.*, 2019).

Microbial BVOCs affect plant physiology and can modulate root architecture (Malmierca *et al.*, 2015; Schenkel *et al.*, 2018), flowering (Sánchez-López *et al.*, 2016b), or enhance their capacity to counteract herbivores (Cordovez *et al.*, 2017) and pathogens

by inducing different defence pathways (Riedlmeier *et al.*, 2017; Frank *et al.*, 2021). Additionally, it has been demonstrated that these compounds influence plant metabolism in several ways. They induce the accumulation of substances (Ezquer *et al.*, 2010), modify nutrient uptake (Martínez-Medina *et al.*, 2017), photosynthesis (Ameztoy *et al.*, 2019), or cell wall remodelling (Lee *et al.*, 2019). At molecular level, both transcriptional and post-transcriptional changes in response to BVOCs have been reported (Ameztoy *et al.*, 2019; García-Gómez *et al.*, 2019).

On the other hand, most studies regarding insect response to BVOCs are either focused on plant extracts and essential oils or on attractive-deterrent behavioural phenomena. For instance, some BVOCs have been proven to attract potential insect hosts as well as parasitoids of insect pests (Battaglia *et al.*, 2013; Rodríguez-González *et al.*, 2018; Contreras-Cornejo *et al.*, 2020). Moreover, Kandasamy *et al.*, (2019) reported the attractive capabilities of volatiles produced by microbial symbionts to their insect hosts. The induction of plant resistance against insect attack as a result of microbial BVOCs has been also described (Moisan *et al.*, 2019). By comparison, research on the effects of microbial BVOCs against insects is scarce. Herrera *et al.*, (2015) reported the insecticidal activity of purified fungal BVOCs on *Sitophilus zeamais* Motschulsky, while toxic effects on *Drosophila melanogaster* Meigen have been assessed as a biological model (Inamdar, Zaman, *et al.*, 2014; Zhao *et al.*, 2017). Previous research explored the insecticidal properties of fungal volatile overproducers from the genus *Trichoderma* on *A. obtectus* (Rodríguez-González *et al.*, 2018, 2019, 2020). Nevertheless, as in these experiments fungal strains had physical contact with the insect, results regarding insect mortality cannot be clearly attributed to volatile interactions. Finally, 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.*, 2014).

Different ways to apply BVOCs in agriculture have been proposed, both on the field and in postharvest storage, from the placing of diffusers to the use of genetically modified crops. However, some obvious difficulties have been experienced due to the physical characteristics of these compounds (Maurya, 2020; Thomas *et al.*, 2020; Tilocca *et al.*, 2020).

Thus, BVOCs have been gaining attention in the last years. Nevertheless, the volatile nature of BVOCs makes their *in vitro* study quite challenging and requires

specific laboratory material and procedures. In this regard, there is still a lack of reliable and standardized devices and methods to evaluate some aspects of biological volatile interactions.

Concerning microbe-microbe interactions mediated by BVOCs, three main techniques are being currently used, namely the divided Petri dishes, the plate-within-a-plate or plate-within-a-box systems, and the two sealed-base plates or double dish set (DDS) method. Each of these techniques presents some limitations regarding either growth surface for the microbial strains, easy manipulation and data collection, cross-contamination, homogeneity, and replicability of results, or flexibility in the range of experimental conditions that can be assessed, including gas exchange with the exterior (Dennis and Webster, 1971; Noguchi *et al.*, 2002; Spadaro and Droby, 2016; Alijani *et al.*, 2019). These limitations can strongly influence the outcome of the interaction and thus affect the obtained results (Lo Cantore *et al.*, 2015). In the case of plant-microbe volatile interactions, there are two main technological models, Passive Diffusion Systems where BVOCs move passively from one organism to the other (Park *et al.*, 2015; Ameztoy *et al.*, 2019), or Dynamic Air Stream Systems, in which they are actively channelled controlling flow and directionality (Kai and Piechulla, 2009). Problems regarding cross-contamination, lack of space for plant growth, manipulation, and flexibility are usually found when using the first group, while technical complexity and cost are the main limitations for the second one (Kai *et al.*, 2016).

Interestingly, recent attempts to overcome some of these problems have been proposed using technological innovations. For example, some studies place membranes between the microbial strains to avoid cross-contaminations (El Ariebi *et al.*, 2016). Cernava *et al.*, (2015) published a method for fast-screening of bioactive microbial BVOCs, while, for the study of microbe-insect interactions, Inamdar *et al.*, (2014a) proposed the use of perforated lids to attach separating membranes. These new developments demonstrate that there is a need for adequate material for the performance of BVOC-mediated biological interaction assays.

4. *Trichoderma* spp. Persoon, Fries

Trichoderma is a well-described and ubiquitous fungal genus that thrives in many terrestrial environments, showing high adaptability to a diverse range of ecological

conditions and niches (Contreras-Cornejo *et al.*, 2020; Macías-Rodríguez *et al.*, 2020). *Trichoderma* is present in naturally decaying organic matter, as well as in the soil and the rhizosphere, usually as a saprophytic fungus. However, many species can be found as mycoparasites growing on other fungi, or parasitizing nematodes and insects (Contreras-Cornejo *et al.*, 2020). Some *Trichoderma* strains also grow and develop in symbiosis and endophytic association with plants (Jalali *et al.*, 2017; Macías-Rodríguez *et al.*, 2020).

Some *Trichoderma* strains can pose a significant risk to mushroom cultivation due to their mycoparasitic nature (Kredics *et al.*, 2021). Although uncommon, cases have been also published indicating that some *Trichoderma* can affect human health in very specific conditions, with reported opportunistic infections in immunocompromised patients (Kuhls *et al.*, 1999; Chouaki *et al.*, 2002; Sautour *et al.*, 2018) as well as some allergic reactions (Tang *et al.*, 2003). Nevertheless, most *Trichoderma* spp. strains are considered a harmless and safe microorganism (Sood *et al.*, 2020), and this fungal genus is well known and widely recognized for its capabilities as an eco-friendly and low-cost BCA, as well as a promoter of plant growth and defence (Pereira *et al.*, 2014; Gomes *et al.*, 2020). At least 375 species of this genus have been described to date (2021), with around 50 new ones being reported each year (Cai and Druzhinina, 2021), among which many of them have demonstrated interesting traits in the control of plant diseases and agricultural pests. These characteristics make *Trichoderma* a fundamental element in the interdependent and complex relationships taking place in environments such as the rhizosphere (Contreras-Cornejo *et al.*, 2016; Szczałba *et al.*, 2019), and highlight its potential as a tool for more efficient and resilient agriculture (Kashyap *et al.*, 2017).

4.1. Taxonomy and biology

Trichoderma belongs to the Division Ascomycota and was described for the first time by Persoon in 1794, even though only one of the original four organisms described by this author remains inside the genus (Bissett *et al.*, 2015). The teleomorph of *Trichoderma* has been traditionally labelled as *Hypocrea* Fr (Druzhinina *et al.*, 2011). Nevertheless, the current tendency is to unify both anamorph and teleomorph under a single generic name, abandoning the latter in favour of the former, thus being *Trichoderma* the generally accepted denomination nowadays (Druzhinina *et al.*, 2011; Bissett *et al.*, 2015; Cai and Druzhinina, 2021). *Trichoderma* taxonomy has been defined as (Samuels, 1996):

- Domain: Eukaryota
    - Kingdom: Fungi
    - Subkingdom: Dikarya
        - Phylum (Division): Ascomycota
        - Subphylum (Subdivision): Pezizomycotina
            - Class: Sordariomycetes
            - Subclass: Hypocreomycetidae
                - Order: Hypocreales
                - Family: Hypocreaceae
                - Genus: *Trichoderma* Persoon, Fries

As previously referred, *Trichoderma* is a widespread genus present in a variety of climatic zones (Kredics *et al.*, 2018). Lately, more and more species are being identified in a diversity of specific habitats, from marine (Gal-Hemed *et al.*, 2011; Ruiz *et al.*, 2021) to arid (Redman *et al.*, 2021) or saline ones (Ding *et al.*, 2021) among others.

Being primarily saprophytic filamentous fungi, *Trichoderma* spp. are able of growing using several different compounds and nutritive media as carbon and nitrogen sources, although growth rates and responses can vary greatly between species and strains, as well as according to growth conditions (Singh *et al.*, 2013). *In vitro*, fungi from this genus usually grow fast between 25 and 30ºC, reducing its development rate at lower and higher temperatures. Nevertheless, individual species and strains present a broad range of optimal growing temperatures (Singh *et al.*, 2013), being likely the result of adaptation to specific environments (Shanmugam *et al.*, 2015). Many *Trichoderma* strains sporulate easily in both artificial and natural substrates. This process is favoured by environmental conditions, for example in response to light exposure (Papavizas, 1985). Conidia form on the mycelium, usually turning its characteristic whitish colour into different shades of green and yellow. Conidia are mostly elliptic with a general size of 3-5 x 2-4 µm and appear usually dry (Samuels, 1996). Phialides can be cylindrical or almost round and can appear alone or densely packed depending on the species. Conidiophores are branched and hyphae develop and produce lateral ones (Papavizas, 1985). They can coil or form special structures when parasitizing other fungi as well as insects or nematodes (Atanasova, 2014). The teleomorph is characterized by a discoidal stroma, with perithecia completely submerged, and 16 ascospores (Samuels, 1996).

Trichoderma is also recognized as an industrially important fungal genus due to its ability to produce valuable enzymes and other products. These compounds are used for pharmaceutical, textile, food, or wood industrial processes, among others (Felix *et al.*, 2014; Kredics *et al.*, 2021). Species of this genus produce and liberate an array of hydrolytic enzymes such as proteases, cellulases, or lipases, being employed in biotechnological industrial processes, as in the transformation of biomass into bioethanol, biofuels, and other chemicals (Bischof *et al.*, 2016). In addition, it has been demonstrated that *Trichoderma* can be used in bioremediation, as several strains have shown the ability to detoxify and degrade harmful environmental pollutants, like organochlorides, heavy metals, or pesticides (Sood *et al.*, 2020).

4.2. *Trichoderma* spp. as biocontrol and biostimulant agent in agriculture

Notwithstanding the aforementioned applications, if a defining feature can be applied to the *Trichoderma* genus as a whole, that is its general use in agriculture, plant protection, and biological control in crops and postharvest products (Druzhinina *et al.*, 2011; Sood *et al.*, 2020). As a BCA, *Trichoderma* presents great efficacy in the control of numerous microbial phytopathogens, both bacteria and fungi, as well as agricultural pests such as insects and nematodes (Contreras-Cornejo *et al.*, 2020; Gomes *et al.*, 2020; Poveda *et al.*, 2020). In addition, this fungal genus has been demonstrated to produce other benefits to plant growth, development, and productivity as a biostimulant, enhancing tolerance to biotic and abiotic stresses (Jalali *et al.*, 2017; Ding *et al.*, 2021), activating the plant´s defensive system (Mayo *et al.*, 2016b), or increasing nutrient availability, mobilization, and intake (Vinale *et al.*, 2013). Strains of many *Trichoderma* species and their secondary metabolites are being currently used as BCAs in agriculture, for example, but not restricted to, several strains from species belonging to clades Harzianum, Virens, or Longibrachiatum (Woo *et al.*, 2014; Vinale and Sivasithamparam, 2020).

4.2.1. Competition

Trichoderma can compete for nutrients and space inside specific biological niches with phytopathogenic microorganisms (Carro-Huerga *et al.*, 2020). This trait is especially relevant in the rhizosphere, where *Trichoderma* shows a higher ability to access and absorb nutrients than many soilborne pathogens (Sarrocco *et al.*, 2009; Vargas *et al.*,

2009) thus being able to outgrow them, limit their dissemination, and their ability to reach infection spots in the plant (Alfiky and Weisskopf, 2021). In this regard, *Trichoderma* presents high efficiency in the access and use of common and readily available carbohydrates in the rhizospheres, both complex like chitin or cellulose and simpler like glucose or sucrose (Vargas *et al.*, 2009; Bargaz *et al.*, 2018). The success of *Trichoderma* in this competition against other microorganisms relies also on the solubilization of nutrients by lowering the pH in the soil. For this purpose, the fungus liberates organic acids like fumaric, harzianic, gluconic, and citric. These compounds promote the solubilization of mineral cations and other micronutrients (Vinale *et al.*, 2008). Moreover, this ability to mobilize nutrients is closely related to the plant-growth-promoting capabilities of *Trichoderma* spp.

Additionally, some studies have suggested that plant root exudates may induce *Trichoderma* growth and sporulation, helping it in the competition for resources and niches and playing, therefore, an active role in the complex tritrophic relationships that take place between BCAs, phytopathogens, and plants in the rhizosphere (Macías-Rodríguez *et al.*, 2018). Epiphytic and avirulent endophytic fungi as *Trichoderma* can also compete for space and resources with phytopathogens on the surface or inside plant tissues (Ruano-Rosa *et al.*, 2016; Carro-Huerga *et al.*, 2020; Poveda and Baptista, 2021).

4.2.2. Plant growth and defence promotion

As indicated above, *Trichoderma* can promote plant development by making nutrients more easily available. On top of the vital Fe mobilization exerted by the production of siderophores (Vinale *et al.*, 2013), *T. harzianum* enhanced tomato growth and nutrient uptake via solubilization of phosphate, Cu, Na, and Zn, while *T. asperelloides* also increased the availability of Fe and P (Altomare *et al.*, 1999; Li *et al.*, 2015). Moreover, some *Trichoderma* spp. exhibit additional traits that further enhance plant development and growth, as well as induce resistance to biotic and abiotic stresses. *Trichoderma* strains promote morphological and physiological changes in plants. For instance, the application of different *Trichoderma* species to the rhizosphere increases root or aerial length and biomass, affects lateral root formation, or modifies the number of organs such as leaves and fruits (Malmierca *et al.*, 2015; Mayo-Prieto *et al.*, 2020; Alfiky and Weisskopf, 2021). Several fungi, including *Trichoderma*, modulate plant growth through the emission of BVOCs (Morath *et al.*, 2012; Sood *et al.*, 2020) and a

wide variety of other secondary metabolites (Contreras-Cornejo *et al.*, 2020). Regarding bean development, several *Trichoderma* isolates showed a growth-promoting activity over this plant alongside important antagonism against the pathogen *Rhizoctonia* (Mayo *et al.*, 2015). Interestingly, Mayo-Prieto *et al.*, (2020) demonstrated that not only the *Trichoderma* species and strains but also their specific origin, determine their biocontrol and plant growth promotion activities. In this study fungal isolates from soil enhanced bean growth more effectively than those coming from bean seeds. Endophytic *Trichoderma* also influence growth and development (Bae *et al.*, 2009; Wonglom *et al.*, 2020).

The induction of defensive responses in plants exposed to these fungi or their secondary metabolites is of the utmost importance for the overall success of *Trichoderma* as a BCA. Numerous reports highlight the ability of a great diversity of *Trichoderma* species to enhance plant defence responses against diverse phytopathogens, including bacteria, fungi, and viruses, as well as to induce resistance to pests and herbivore attacks (Poveda *et al.*, 2020). These interactions can trigger systemic acquired resistance (SAR), induced systemic resistance (ISR), or hypersensitive response (HR) (Harman, 2006). In this regard, it has been proposed that the interaction of the fungus with plant receptors can modulate the priming of induced resistance while balancing the cost of both defence and growth processes (Hermosa *et al.*, 2013). Nevertheless, sometimes, the first phase of *Trichoderma*-plant interactions that involve non-pathogenic colonization of plant tissues by the fungus may entail an initial lowering of plant defence responses to allow the establishment of the microorganism (Masunaka *et al.*, 2011; Sood *et al.*, 2020). *Trichoderma* produces a diverse array of enzymes and metabolites that induce plant resistance, including xylanases, chitinases, glucanases, peptaibols, pathogenesis-related proteins, or microbe-associated molecular patterns (MAMPs), such as hydrophobins and BVOCs (Vinale *et al.*, 2008; Hermosa *et al.*, 2013; Schulz-Bohm *et al.*, 2017; Lindo *et al.*, 2020; Poveda *et al.*, 2020; Eslahi *et al.*, 2021). Likewise, members of the *Trichoderma* genus have been described to produce phytohormones such as auxins and gibberellins or to regulate ethylene levels (Jaroszuk-ściseł *et al.*, 2019), cytokinin, salicylic acid, and ABA (Alfiky and Weisskopf, 2021).

Native *Trichoderma* isolates from several species have proven to be able to modulate and enhance *P. vulgaris* L. defensive responses and their metabolome,

including those belonging to the PGI "Alubia de La Bañeza – León" (Pereira *et al.*, 2014; Mayo *et al.*, 2015, 2019; Eslahi *et al.*, 2021). As many of the referred studies prove, these responses are usually intimately related to plant growth and development under biotic stresses. Mayo *et al.*, (2016b) described a group of genes regulated by *T. velutinum* involved in plant defence and demonstrated that this fungal species modifies the overall metabolome of the plant (Mayo-Prieto *et al.*, 2019).

4.2.3. Parasitism

Many *Trichoderma* behave as hyperparasites by parasitizing plant pathogens. *Trichoderma* detects potential hosts without the need for direct interaction, growing in their direction by tropism (Elad *et al.*, 1982; Lu *et al.*, 2004). In mycoparasitism, this early host perception and response plays a key role in the mycoparasitic activity of *Trichoderma* and is driven by host-derived signals (Alfiky and Weisskopf, 2021). These signals are usually constituents of the cell wall or metabolites secreted by the potential host, and trigger antifungal responses in *Trichoderma*. For example, when *T. virens* and *T. atroviride* were confronted with *R. solani*, they responded before physical contact with the activation of genes involved in mycoparasitism (Steyaert *et al.*, 2003). BVOCs produced and emitted by other organisms can act as long-range signals for the recognition of potential hosts. Microbial BVOCs have proven to induce the production of antifungal compounds (Gomes *et al.*, 2020) and mediate recognition and interaction between *Trichoderma* and other organisms (Schulz-Bohm *et al.*, 2017).

Once *Trichoderma* enters in contact with the host, mycoparasitism proper commences (Fig. 4). *Trichoderma* hyphae coil around those of the target, a process followed up by the formation of appressoria (Harman *et al.*, 2004; Harman, 2006), while specific molecules like hydrophobin also help the adhesion to the mycelium (Viterbo and Chet, 2006). Afterwards, *Trichoderma* secretes hydrolytic enzymes (like chitinases, proteases, and glucanases) that degrade the host cell wall, causing its death, and proceeds to the extracellular digestion and absorption of the derived nutrients (Sood *et al.*, 2020). *Trichoderma* also possesses chitin and glucan synthases that are thought to repair the potential damage produced on its own cell wall during these interactions. This cell wall remodeling plays a key role in both *Trichoderma* vegetative growth and biocontrol activity (Kappel *et al.*, 2020). Interestingly, endophytic *Trichoderma* spp. strains also

demonstrated microparasitic activity against plant pathogenic fungi, suggesting that these interactions may well take place inside plant tissues (Carro-Huerga *et al.*, 2020).

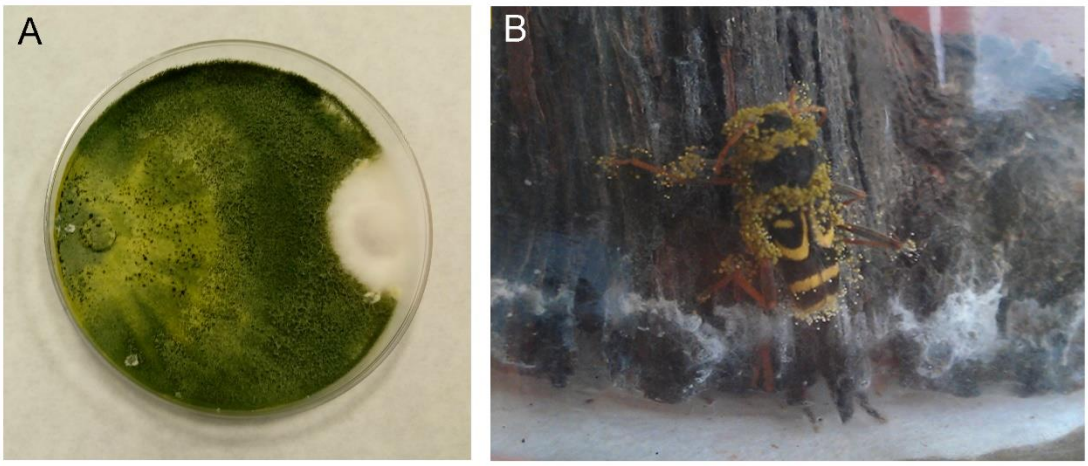

Figure 4. (A) *Trichoderma* sp. on *in vitro* dual confrontation assay against *F. oxysporum*. (B) Sporulating *T. gamsii* on dead adult of the insect pest *Xylotrechus arvicola*. Picture kindly provided by Dr. Álvaro Rodríguez González (Rodríguez-González *et al*., 2018b).

Trichoderma also parasites insects and nematodes (Fig. 4), boosting the study and use of this fungal genus as an effective BCA against agricultural pests. It has been proven that the presence of *T. harzianum* in the rhizosphere and roots halted nematode plant parasitism at different phases of the process. *Trichoderma* can parasite all developmental stages of nematodes either by producing nematode-trapping structures and/or penetrating inside them, thus acting as an endoparasitic fungus (Poveda *et al.*, 2020) and protect the plants (Tiwari *et al.*, 2017; Peiris *et al.*, 2020). On the other hand, *Trichoderma* is a well-known BCA for its entomopathogenic capabilities. The fungal spores can adhere to different parts of the insect and the fungus invades its host through the cuticle or penetrating via intersegmental membranes or the spiracles (Pucheta Díaz *et al.*, 2006). Fungal hyphae develop inside the insect's tissues and spread along the vascular system, liberating hydrolytic enzymes that are a relevant virulence factor. Once the insect dies, *Trichoderma* keeps feeding and eventually grows and sporulate out of it, from where the spores may disseminate and infect new hosts (Rodríguez-González *et al.*, 2020). It has been suggested that important fungal traits related to insect pathogenicity may be shared also in biocontrol against phytopathogens (Ownley *et al.*, 2010). Moreover, evidence indicate that BVOCs produced by *Trichoderma* spp attract numerous insect species

(Rodríguez-González *et al.*, 2018; Contreras-Cornejo *et al.*, 2020), which could make part of the entomopathogenic strategy of these fungi.

4.2.4. Antibiosis

In addition to the referred extracellular enzymes and plant hormones, members of the genus *Trichoderma* spp. synthesize and liberate to their surrounding environment a wide variety of secondary metabolites. These compounds are of diverse chemical nature and carry out numerous and important functions, accounting for complex interactions between the fungus and other more or less neighbouring organisms, many of them being active against a broad spectrum of phytopathogens. Their heterogeneity also makes some of them interesting biomarkers for specific fungal strains (Contreras-Cornejo *et al.*, 2020). While these molecules could be classified in different ways, a basic line is drawn between volatile and non-volatile compounds.

The production of non-volatile soluble or diffusible metabolites with antibiotic activity by *Trichoderma* spp. has been known, studied, and exploited for quite a long time (Harman, 2006; Vinale and Sivasithamparam, 2020). These compounds act at diverse levels in multitrophic interactions against plant pathogens and herbivores in the environment (Macías-Rodríguez *et al.*, 2020). Steroids, sesquiterpenes, peptaibols, diketopiperazines, pyrones, or polyketides are among those groups with described antibiotic activity (Sood *et al.*, 2020). Interestingly, some compounds with antimicrobial activity appear naturally in both soluble and gaseous states (Scarselletti and Faull, 1994; El-Hasan *et al.*, 2018; Phoka *et al.*, 2020).

Related to the topic we are addressing, self-inhibition and autotoxic effects have been described for different organisms (Mazzoleni *et al.*, 2015a, b). They involve toxic and inhibitory effects against members of the clade itself (species, strain, etc.). Most of the proposed mechanisms for these traits rely on the accumulation and release of toxic compounds, while reproduction, quorum sensing, and competition might be important ecological factors behind this behaviour (Hogan, 2006). Some studies explored these processes in filamentous fungi, but most of them focused on concentration-dependent inhibition of spore germination (Chitarra *et al.*, 2004; Gillot *et al.*, 2016). Interestingly, it has been proposed that autotoxicity drives hyphal growth and accounts for the defining radial expansion of fungal colonies (Bottone *et al.*, 1998). In the case of *Trichoderma*

spp., it has been described that *T. harzianum* extracellular self-DNA may be involved in self-inhibitory effects (Mazzoleni *et al.*, 2015b).

Volatiles produced by *Trichoderma* are of diverse nature, being C_8 compounds, aliphatic alcohols, and isoprenoid mono and sesquiterpenes among those commonly reported. Nevertheless, the similarity of their mass spectra makes sometimes difficult their chemical identification (Contreras-Cornejo *et al.*, 2020). BVOCs from *Trichoderma* spp. carry out many different activities in volatile-mediated interactions between this fungal genus and other organisms. As already described, they can induce defensive responses in plants, induce or inhibit plant growth and development, reduce microbial production of toxic compounds, or attract potential hosts (Malmierca *et al.*, 2015a, b; Rodríguez-González *et al.*, 2018; Taylor *et al.*, 2021). In addition, these compounds have been proven to perform antibiotic and inhibitory activity against microbial pathogens and pests for some time (Dennis and Webster, 1971). Although their study is still somehow lacking behind other aspects of *Trichoderma* biology, research in this regard has been growing in number during the last years. For instance, *T. virens* BVOCs inhibited *Botrytis cinerea* Pers. growth (Contreras-Cornejo *et al.*, 2014), *T. virens* and *T. viride* showed antifungal activity against *F. oxysporum* (Li *et al.*, 2018), or *T. harzianum* and other *Trichoderma* sp. against *F. graminearum* in wheat (El-Hasan *et al.*, 2018; Taylor *et al.*, 2021).

Data about the toxic effects of BVOCs on insects are scarce, as researchers have been mostly focusing so far on behavioural aspects (Ponce *et al.*, 2021). Toxic activity from *T. viride* and *T. longibrachiatum* BVOCs has been reported against *Drosophila melanogaster* (Inamdar, Zaman, *et al.*, 2014; Zhao *et al.*, 2017). Rodríguez-González *et al.*, (2018, 2019) reported that volatile-overproducing strains of *T. harzianum* present modify insecticidal activity against *A. obtectus*, nevertheless, as the fungi entered in direct contact with the insects, it is not completely clear if volatile interactions account for the reported increase in mortality.

As indicated above, there is still a need for specific, standardized, and reliable materials, methods, and protocols for the screening of bioactive BVOCs and for the evaluation of microbial volatile effects on other organisms, especially those directly produced by actively growing microbial strains.

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HYPOTHESIS AND OBJECTIVES

Biogenic Volatile Organic Compounds (BVOCs) of microbial origin have been gaining attention in the last years as biocontrol effectors, with a mounting number of publications striving to unveil their biological and ecological roles. Nevertheless, the volatile nature of BVOCs makes their *in vitro* study quite challenging and requires specific laboratory material and protocols. In this regard, there is still a lack of reliable and standardized devices and methods to evaluate microbial volatile effects on other organisms, especially those directly produced by actively growing microbial strains. Current techniques present limitations in the available growing space, cost, manipulation, data collection, cross-contamination, homogeneity and replicability of results, or flexibility in the range of experimental conditions, with special mention to ventilation and gas exchange with the exterior.

It has been described that the presence of enzymes able of repairing and remodelling the fungal cell wall plays a key role in both *Trichoderma* vegetative growth and biocontrol activity. In addition, self-inhibitory or autotoxic traits have been reported in filamentous fungi, which may have an impact on their reproduction and drive hyphal growth, accounting for their defining radial expansion. Nevertheless, little is known about the impact of these self-inhibitory processes in traditional methods used for the selection of fungal strains with putative biocontrol capabilities against phytopathogens through the release of toxic secondary metabolites.

In this context, the hypothesis of the present PhD. thesis is that the *in vitro* growing conditions in which the biological activity of microbial secondary metabolites is tested determine the outcome of the studied interactions, thus research methodologies should take into account these conditions and be able to modulate them.

To address these matters and to solve some of the referred technical problems, the following objectives were pursued in the present PhD. thesis:

I. To evaluate the self-inhibitory activity of *Trichoderma* spp. using *in vitro* membrane assays and to compare it with the antifungal activity exerted in the same conditions against a phytopathogenic *Fusarium oxysporum*.

II. To develop a simple and reliable device to evaluate microbe-microbe and insect-microbe volatile-mediated interactions able to solve some of the existing limitations in the field as well as to better reproduce environmental conditions, and to test its performance using *Trichoderma* strains as BCAs.

III. To develop a simple and reliable device to evaluate plant-microbe volatile-mediated interactions able to solve some of the existing limitations on the field as well as to better reproduce environmental conditions, and to test its performance using *Trichoderma* strains as BCAs.

IV. To identify individual BVOCs emitted by *Trichoderma* spp. that may account for the observed biological activities.

CHAPTER I

Self-inhibitory activity of *Trichoderma* soluble metabolites and their antifungal effects on *Fusarium oxysporum*

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Self-Inhibitory Activity of Trichoderma Soluble Metabolites and Their Antifungal Effects on *Fusarium oxysporum*

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Abstract: Self-inhibitory processes are a common feature shared by different organisms. One of the main mechanisms involved in these interactions regarding microorganisms is the release of toxic diffusible substances into the environment. These metabolites can exert both antimicrobial effects against other organisms as well as self-inhibitory ones. The in vitro evaluation of these effects against other organisms has been widely used to identify potential biocontrol agents against phytopathogenic microorganisms. In the present study, we performed membrane assays to compare the self-inhibitory effects of soluble metabolites produced by several Trichoderma isolates and their antifungal activity against a phytopathogenic strain of *Fusarium oxysporum*. The results demonstrated that *Trichoderma* spp. present a high self-inhibitory activity in vitro, being affected in both their growth rate and the macroscopic structure of their colonies. These effects were highly similar to those exerted against *F. oxysporum* in the same conditions, showing no significant differences in most cases. Consequently, membrane assays may not be very informative by themselves to assess putative biocontrol capabilities. Therefore, different methods, or a combination of antifungal and self-inhibitory experiments, could be a better approach to evaluate the potential biocontrol activity of microbial strains in order to pre-select them for further in vivo trials.

Keywords: self-inhibition; autotoxicity; autoinhibition; membrane assay; secondary metabolites; diffusible metabolites; antifungal; fungi; Trichoderma; Fusarium

1. Introduction

Autotoxicity and self-inhibition have been described as common traits shown by different species, genera, and kingdoms [1]. Some general mechanisms have been proposed to explain these processes, mainly the accumulation and release of different toxic compounds to the environment, and also the self-inhibitory activity of extracellular self-DNA or conspecific DNA [1]. However, competition for resources, quorum sensing, and reproductive issues have been pointed out as the most likely and major ecological factors that motivate this behavior [2].

Regarding filamentous fungi, some studies have been conducted to evaluate self-inhibitory processes, allowing the identification of different autotoxic compounds [3,4]. Nonetheless, most of these studies are focused on the concentration-dependent self-inhibition of fungal spore germination [3–5]. Moreover, autotoxicity has been proposed as a way to drive directional growth, accounting for the

characteristic radial expansion of fungal colonies [6]. As for the genus Trichoderma, there are very few references to self-inhibition. In this regard, most significant studies point to the self-inhibitory effects of *Trichoderma harzianum* mediated by extracellular self-DNA [1]. Besides, to our knowledge, there are no previous studies comparing self-inhibitory and antifungal effects produced in vitro by *Trichoderma* spp. or any other biocontrol agent. Trichoderma is a ubiquitous genus of filamentous fungi, widely used as a biocontrol agent against many phytopathogenic microorganisms [7,8], as well as for crop biostimulation [9,10] and as a general model to sustain crop productivity [11].

On the other hand, *Fusarium oxysporum* is a common endophyte and phytopathogenic filamentous fungus that thrives in numerous important crops [12], *F. oxysporum* f. sp. *phaseoli* being one of the forms that mainly affects bean plants (*Phaseolus vulgaris* L.) [13]. Furthermore, many studies highlight the antifungal activity of soluble metabolites [8,14,15], as well as volatile ones [5,14,16], produced by *Trichoderma* spp. against different Fusarium species.

The hypothesis of this work is that soluble metabolites released in vitro by *Trichoderma* spp. produce both self-inhibitory effects and antifungal effects against *F. oxysporum*. The main objective of the present study is to evaluate the self-inhibitory effects of soluble metabolites produced by several Trichoderma isolates and to compare them to the antifungal activity exerted by the same fungal strains against a phytopathogenic strain of *F. oxysporum*.

2. Materials and Methods

2.1. Microbial Strains and Culture Conditions

Ten Trichoderma strains were used to evaluate the activity of their diffusible metabolites: seven *T. harzianum*, one *T. citrinoviride*, one *T. velutinum*, and one *T. gamsii*. Out of the ten strains, eight of them were isolated from bean (*P. vulgaris* L.) fields belonging to de Protected Geographical Indication (PGI) "Alubia de La Bañeza-León", while the other two were isolated from sugar beet crops growing in the same area [17]. Trichoderma strains were selected in such a way that at least either the fungal species, the place of origin, the bean variety (or crop) they came from, or the source they were isolated from (seed or soil) differed among them (Table 1).

A strain of *F. oxysporum* (F3 from now onwards) was isolated from bean (*P. vulgaris*) fields belonging to the same PGI and was selected for its high virulence against this crop.

All fungal strains were preserved in 50% glycerol spore suspension at −80 °C and stored in the "Pathogens and Antagonists Collection" at the "Pest and Diseases Diagnosis Laboratory" (PALDPD, University of León, León, Spain). All cultures were activated on PDA (Sigma Aldrich, St. Louis, MO, USA) at 25 °C.

Table 1. Code, species, source, and area of origin of the *Trichoderma* isolates used in the assays.

Code	*Trichoderma* Species	Crop	Source	Municipality	Area
T002	*T. harzianum*	Riñón menudo (bean)	seed	Moscas del Páramo	El Páramo
T007	*T. harzianum*	Pinta (bean)	seed	Sueros de Cepeda	Astorga
T008	*T. citrinoviride*	Pinta (bean)	seed	Fresno de la Vega	Esla-Campos
T015	*T. harzianum*	Riñón menudo (bean)	seed	Veguellina de Fondo	El Páramo
T021	*T. harzianum*	Pinta (bean)	seed	Altobar de la Encomienda	El Páramo
T028	*T. velutinum*	Riñón (bean)	soil	Otero de Escarpizo	Astorga
T044	*T. harzianum*	Riñón (bean)	soil	Javares de los Oteros	Esla-Campos
T050	*T. harzianum*	Canela (bean)	soil	Bercianos del Páramo	El Páramo
T055	*T. harzianum*	Sugarbeet	soil	La Milla del Páramo	El Páramo
T057	*T. gamsii*	Sugarbeet	soil	La Milla del Páramo	El Páramo

2.2. In Vitro Evaluation of Antifungal and Self-Inhibitory Activity of Soluble Metabolites Produced by *Trichoderma spp.*

A membrane assay was performed, as described by Mayo et al. (2015) [7], by placing a sterile cellophane membrane to cover the potato-dextrose-agar (PDA, Sigma Aldrich, St. Louis, MO, USA) medium in 90 mm Petri dishes (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). A 6 mm plug from

the edge of a 2 day-old Trichoderma culture was placed in the center of each plate over the cellophane and was grown for 2 days, letting the soluble metabolites diffuse to the medium, yet avoiding its colonization by the fungal mycelium. After that, membrane and fungi were removed, and a 6 mm mycelial plug from the edge of the 2 day-old culture of the same Trichoderma strain was placed in the center of the dish. Controls were performed in the same manner but without growing Trichoderma over the membranes prior to the inoculation. Diameters of the colonies were measured in three different directions with a ruler to the farthest edge of mycelial growth (via visual detection of the most outer hyphal apex) 24 and 48 h post-inoculation (hpi) (Figure 1a). The diameter of each replicate was considered as the mean of its three measurements. The second round of data collection was determined to be 48 hpi, taking into account that the fastest-growing Trichoderma strains reached the edge of the plate at this time.

The inhibitory activity of Trichoderma metabolites on *F. oxysporum* was performed as described above, but inoculating a 6 mm plug from the edge of a 5 day-old *F. oxysporum* culture on the center of the plates after removing the membranes. The diameters of the colonies were measured in the same way, but 3 and 7 days post-inoculation (dpi) (Figure 1b), as the slower mycelial growth of *F. oxysporum* controls reached the same diameter after 3 and 7 days as Trichoderma controls after 24 and 48 h. The second round of data collection was determined to be 7 dpi, taking into account that F3 colonies reached the edge of the plate at this time. From now onwards, measures taken 24 hpi for *Trichoderma* spp. and 3 dpi for F3 will be referred to as the first round of data collection, while 48 hpi for *Trichoderma* spp. and 7 dpi for F3 will be considered to be the second round of data collection, in order to compare self and heterologous inhibition.

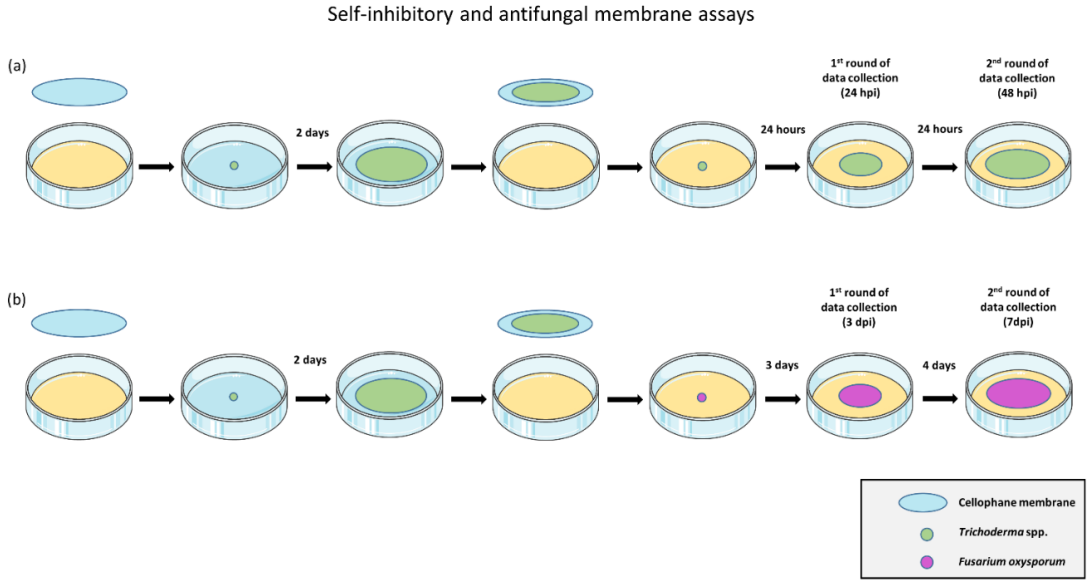

Figure 1. Self-inhibitory and antifungal membrane assays. (**a**) Evaluation of the effects of *Trichoderma* spp. diffusible metabolites on the same Trichoderma strain. (**b**) Evaluation of the effects of *Trichoderma* spp. diffusible metabolites on *F. oxysporum* F3.

Controls were performed in the same manner but without growing *Trichoderma* spp. over the cellophane membrane. The 1st round of data collection was performed when fungal colonies in the controls reached 1/3 of the plate (24 hpi for *Trichoderma* spp. and 3 dpi for *F. oxysporum*). The 2nd round of data collection was performed when fungal colonies in the controls reached the edge of the plate (48 hpi for the fastest *Trichoderma* spp. and 7 dpi for *F. oxysporum*). Diameters were measured with a ruler in three different directions. Four replicates per treatment were performed. All cultures were performed on PDA (Sigma Aldrich, St. Louis, MO, USA) at 25 °C.

2.3. Data Treatment and Statistical Analysis

Microbial growth was considered as the mean diameter of the three measures from each replicate, and 6 mm were subtracted from all measures to avoid distortions produced in the percentage of inhibition (PI) by the diameter of the plugs, as shown by Mutawila et al. (2016) [15]. PIs were estimated in relation to the control using the following equation: PI (%) = [(C − T/C) × 100] [16], where C is the diameter of the control and T is that of each Trichoderma treatment. PIs were analyzed with one-way analysis of variance (ANOVA, $p \leq 0.05$) after confirmation of normality and equality of variances. Subsequently, treatments were contrasted between them and with their controls using Tukey's post hoc test ($p \leq 0.05$). Statistical analyses were performed separately for self-inhibition and antifungal activity of the ten *Trichoderma* spp. (Table 2), as well as to compare self-inhibition and antifungal activity of each Trichoderma isolate (Figure 2). Four replicates were performed for each treatment.

3. Results and Discussion

Under the referred conditions, all Trichoderma isolates showed important antifungal effects on F3 growth (Table 2). *T. harzianum* isolates consistently demonstrated a higher inhibitory activity than the other three Trichoderma species, with PIs ranging from 99.51% to 86.92% at day 3 post-inoculation, and from 95.25% to 89.40% in day 7 post-inoculation. In contrast, *T. citrinoviride* T008 showed lower PI values (70.73% in day 3 and 59.38% in day 7), while *T. velutinum* T028 exerted the lowest inhibition of all strains, with 34.15% in day 3 and a mere 15.89% in day 7. Finally, *T. gamsii* T057 showed similar PI to those of some *T. harzianum* in day 3 (92.90%) with a significant decrease in day 7 (76.27%). All treatments showed significant differences ($p \leq 0.05$) compared to the growth of F3 controls. These results are in accordance with previous studies regarding the antifungal activity of *Trichoderma* spp. on *F. oxysporum* [18,19].

Table 2. PI values (%) of self-inhibition and F3 inhibition produced by Trichoderma isolates. Results are expressed as the mean ± standard deviation (SD) of PI (%) from the four replicates. First round of data collection (24 hpi for *Trichoderma* spp. and 3 dpi for F3), and Second round of data collection (48 hpi for *Trichoderma* spp. and 7 dpi for F3).

		First Round of Data Collection				Second Round of Data Collection			
Code	*Trichoderma*	Self-Inhibition (% ± SD)	Statistics [1]	F3 Inhibition (% ± SD)	Statistics [2]	Self-Inhibition (% ± SD)	Statistics [1]	F3 Inhibition (% ± SD)	Statistics [2]
T002	*T. harzianum*	93.18 ± 1.21	A	99.51 ± 0.56	a	95.04 ± 0.87	A	95.25 ± 1.89	a
T007	*T. harzianum*	91.68 ± 1.89	A,B	98.54 ± 1.26	a	89.87 ± 2.67	A,B	92.93 ± 2.16	a,b
T008	*T. citrinoviride*	74.51 ± 1.66	D	70.73 ± 1.38	e	71.02 ± 1.85	C	59.38 ± 2.93	d
T015	*T. harzianum*	93.86 ± 1.60	A	97.07 ± 1.59	a,b	94.62 ± 1.21	A	89.40 ± 2.28	b
T021	*T. harzianum*	91.43 ± 3.77	A,B	99.02 ± 0.00	a	90.08 ± 4.25	A,B	92.38 ± 0.91	a,b
T028	*T. velutinum*	42.29 ± 1.40	E	34.15 ± 3.03	f	23.57 ± 2.12	D	15.89 ± 1.96	e
T044	*T. harzianum*	90.09 ± 1.79	A,B	91.35 ± 2.55	c,d	89.66 ± 1.71	A,B	92.93 ± 3.22	a,b
T050	*T. harzianum*	91.11 ± 1.48	A,B	88.91 ± 0.51	c,d	89.98 ± 0.94	A,B	93.46 ± 0.42	a,b
T055	*T. harzianum*	87.86 ± 0.97	B	86.92 ± 1.12	d	87.66 ± 1.35	B	90.40 ± 1.11	a,b
T057	*T. gamsii*	82.53 ± 2.91	C	92.90 ± 4.86	b,c	68.74 ± 3.65	C	76.27 ± 2.02	c

[1] Different capital letters indicate significant differences in self-inhibitory activity among the Trichoderma isolates ($p \leq 0.05$). [2] Different lowercase letters indicate significant differences in antifungal activity against F3 among the Trichoderma isolates ($p \leq 0.05$). PIs were estimated in relation to the control using the following equation: PI (%) = [(C − T/C) × 100] [16]. A one-way analysis of variance (ANOVA, $p \leq 0.05$) was performed and differences were estimated using Tukey's post hoc test ($p \leq 0.05$).

Interestingly, the self-inhibitory effects of the tested Trichoderma strains seem to be highly similar to the abovementioned antifungal inhibitory ones on F3. In this regard, five strains (T008, T015, T044, T050, and T055) showed no significant differences between self-inhibitory and antifungal activities during the first round of data collection (Figure 2a). Moreover, this number amounted to seven strains (all *T. harzianum* tested) in the second round of data collection (Figure 2b). The remaining three Trichoderma strains (T008, T028 and T057) showed, however, differences between self and heterologous inhibition, although following a similar trend. Their IPs for both heterologous antifungal and self-inhibitory effects were significantly lower ($p \leq 0.05$) than those of the *T. harzianum* strains (Table 2). Self-inhibitory PIs of *T. harzianum* strains were very high and ranged from 95.04% (T002,

48 hpi) to 87.66% (T055, 48 hpi). However, T008, T028, and T057 showed, respectively, self-inhibitory PIs of 74.51%, 42.29%, and 82.53% after 24 h, and 71.02%, 23.57% and 68.74% after 48 h (Table 2). Additionally, T008 and T028 were the only two strains showing significantly higher self-inhibition than antifungal inhibition against F3. Other strains showed slightly higher self-inhibitory effects than their antifungal ones, but were not statistically significant ($p \leq 0.05$) (Figure 2).

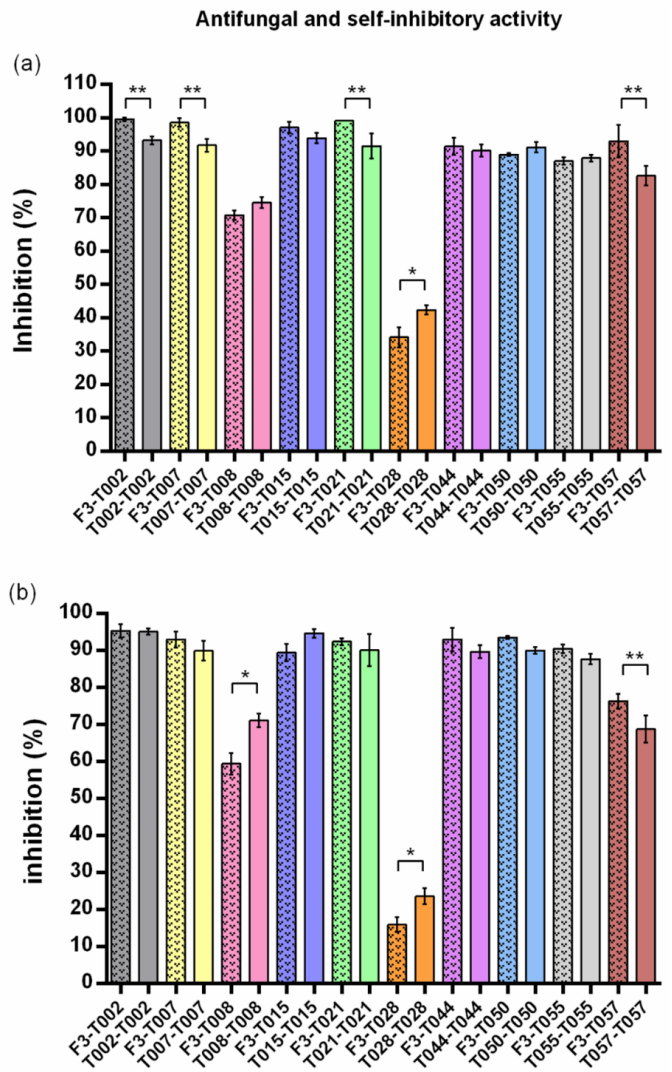

Figure 2. Self-inhibitory activity of the soluble metabolites from the *Trichoderma* spp. evaluated (plain columns), and their antifungal activity against *F. oxysporum* F3 (shadowed columns). (**a**) First round of data collection (24 hpi for Trichoderma and 3 dpi for F3); (**b**) Second round of data collection (48 hpi for Trichoderma and 7 dpi for F3). Columns indicate the mean of PI values (%) and their standard deviation (SD). Each color represents a treatment with a different Trichoderma strain. Shadowed columns represent PI values produced by Trichoderma strains against F3, while plain ones represent self-inhibitory PI values produced by each Trichoderma strain on itself. (*) indicate significantly higher antifungal than self-inhibitory activity ($p \leq 0.05$) within the same Trichoderma isolate. (**) indicate significantly higher self-inhibitory than antifungal activity ($p \leq 0.05$) within the same Trichoderma isolate. No asterisks mean no differences between self-inhibitory and antifungal activity ($p \leq 0.05$). PIs were estimated in relation to the control using the following equation: PI (%) = [(C − T/C) × 100] [16]. A one-way analysis of variance (ANOVA, $p \leq 0.05$) was performed, and differences were estimated using Tukey's post hoc test ($p \leq 0.05$) to compare the self-inhibition and antifungal effects for each Trichoderma strain.

These results seem to indicate that the antifungal and self-inhibitory effects exerted in vitro by *Trichoderma* spp. are closely related and might be general traits shared for both heterologous competition with other fungal species in the environment, as well as ecological adaptations within the same genus, species, and strain. The latter likely being related to directional centrifugal growth of filamentous fungi [6], competition for resources, and/or reproductive processes [3]. The self-inhibitory activity presented here seems to be a non-specific one, and therefore, different from that proposed by Mazzoleni et al. (2015) in *T. harzianum*, mediated by conspecific DNA [1]. These heterologous and autotoxic effects may be related to those observed in fairy-ring-forming basidiomycetes, which have been proposed as one of the mechanisms behind the formation of their characteristic growth rings in the soil and their important influence on the ecosystemic scale [20]. The highly similar effects observed between F3 inhibition and Trichoderma self-inhibition may be partly due to their relative phylogenetic and ecological proximity, being both soilborne filamentous ascomycetes related to the rhizosphere and plants, as epiphytic or endophytic fungi. New studies are needed to elucidate whether these similar effects are also found when comparing other filamentous fungi and less related microorganisms.

The results also point out that self-inhibition varies between Trichoderma strains and species, suggesting that either they produce higher quantity and/or stronger autotoxic metabolites, or they possess different detoxification capabilities to confront these compounds. We propose that, taking into account the similarities presented here between self and heterologous antifungal inhibitory effects within the same strains, the differences seen between strains are mostly due to different antifungal activity and/or the amount rather than different detoxification capabilities. Besides, the high PI values, especially regarding those referred to self-inhibition, are likely due to an extremely high accumulation of soluble metabolites in the PDA medium, derived from the unnatural conditions of the in vitro design. Nevertheless, with the available data, it is not possible to determine whether the observed effects are produced by toxic subproducts of fungal metabolism or secondary metabolites that are purposefully released for antifungal activity. It also is not possible to assure that both the self-inhibitory and the heterologous antifungal activities are produced by the same metabolites, even though the very similar PI values between them seem to point in this direction. New studies should be directed to elucidate these aspects.

In addition, as can be seen in Figure 3, Trichoderma colonies suffered a clearly aberrant growth when exposed to their own diffusible metabolites, showing uncharacteristic arbuscular shapes instead of their common circular colonies with homogeneous edges, clearly observed seven days after inoculation. This aberrant growth in the macroscopic level may indicate microscopic, anatomical abnormalities in the fungal hyphae, as well as physiological alterations [21,22]. Further microscopic and molecular investigations are needed to unveil these anatomical and physiological traits.

It is well known that in vitro assays are only an approximation of more complex natural contexts [23] and that, at least in the field of biological control and microbial interactions, they always need to be completed and validated by subsequent in vivo trials [7,17]. Nevertheless, the overall results here presented pose an additional doubt on membrane assays, and perhaps, on other in vitro related methods used to evaluate fungal soluble metabolites. These experiments are widely used as preliminary studies to help identify new bioactive compounds or microbiological strains with potential biocontrol capabilities [23–26]. However, our findings suggest that membrane assays, at least in the reported conditions, do not seem to be a very informative in vitro indicator of the real biocontrol activity that a fungal strain may exert in natural conditions. Therefore, these assays would still be very useful to identify bioactive compounds, but not so much to select putative biocontrol strains. In this regard, taking into account that most Trichoderma strains tested in the present study showed no significant differences between their self-inhibitory activity and their antifungal activity against *F. oxysporum*, we believe that their high in vitro inhibitory activity alone is not enough to claim a potential biocontrol activity, being likely biased by the unnatural concentrations reached in the plates. Thus, different experimental laboratory designs could be more adequate to evaluate these traits before transitioning to

in vivo and field trials. For example, Kron et al. (2020) [27] demonstrated that ex vivo assays using *Pseudomonas orientalis* mutants against *Erwinia amylovora* were more informative than in vitro assays.

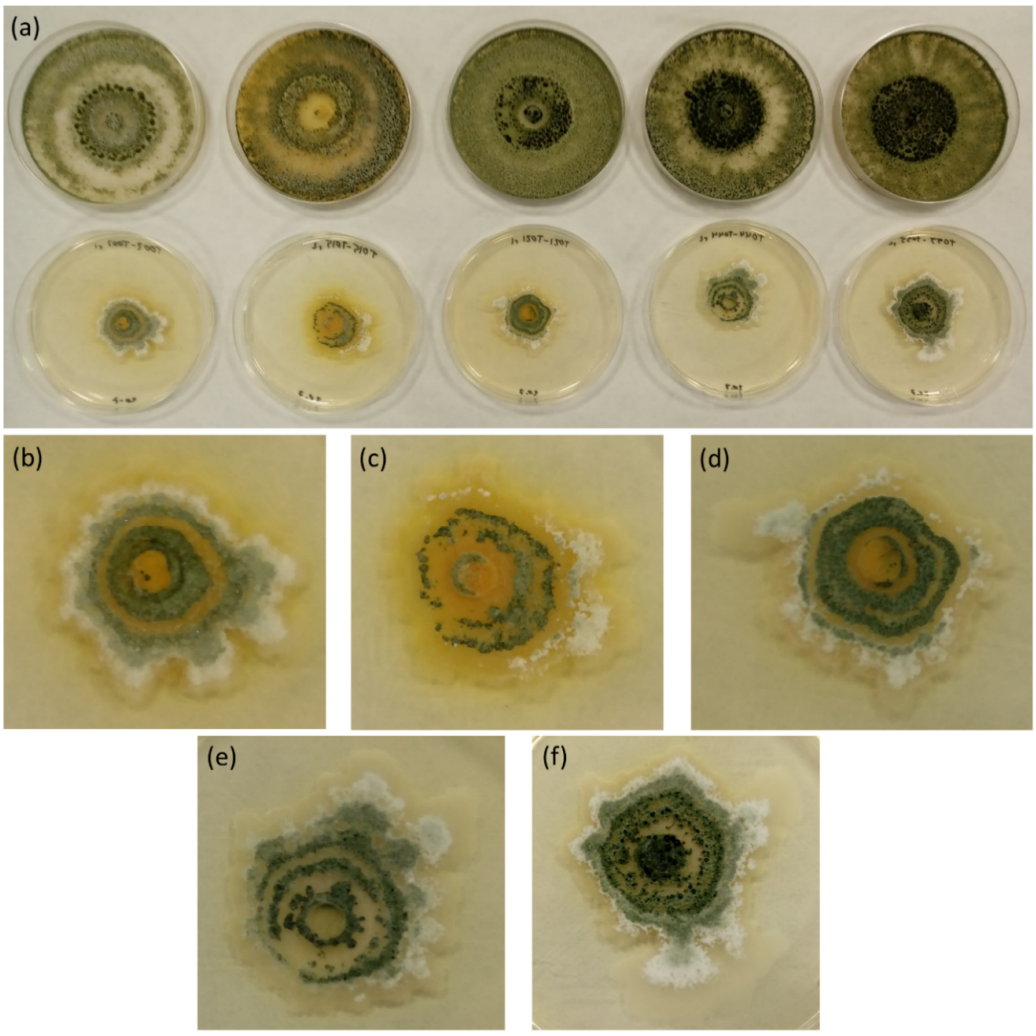

Figure 3. Development of some Trichoderma colonies after 7 days growing on PDA medium with soluble metabolites produced by the strain itself. Aberrant arbuscular shapes can be seen in the treatments. (**a**) Controls without metabolites (upper row; left to right: T002, T015, T021, T044, and T055); Trichoderma treatments (lower row; left to right: T002, T015, T021, T044, and T055), (**b**) T002 detail, (**c**) T015 detail, (**d**) T021 detail, (**e**) T044 detail, and (**f**) T055 detail.

In addition, we suggest that if membrane assays are used to identify potential biocontrol strains, autotoxic studies, as the here presented using the same strains, should be conducted in identical conditions in order to compare and select those microbial agents showing both high antimicrobial activity alongside a lower self-inhibitory one. For example, in our case, while T002, T007, T015, and T021 show very high antifungal effects on the first round of data collection, we may consider discarding T015 as a potential biocontrol strain, as its antifungal activity against F3 is not significantly different from its own self-inhibitory one (Figure 2a). However, during the second round of data collection, T057 was the only strain showing significantly higher heterologous than self-inhibitory effects (Figure 2b). Thus, even though it had a lower antifungal activity than other strains, it might be adequate to select it for further in planta or in vivo experiments. This is further supported by the fact that T057 strain showed the highest difference between antifungal and self-inhibitory activities during the first round (Figure 2a). However, the validity of these hypotheses and proposals need to be

tested by future plant and field trials, as well as by using a wider variety of both biocontrol agents and phytopathogenic strains to assess the presence of the same traits in other microorganisms.

Finally, a deeper understanding of self-inhibitory mechanisms within biocontrol strains, species, and genera might be a key factor to develop commercial pesticides based on a mixture of similar biological control agents. Additionally, this knowledge would be very useful to determine the ideal concentration of the agent when applying it on the field in order to avoid self-inhibitory effects, as well as to select the adequate growth conditions for its industrial production.

4. Conclusions

To summarize, the results presented demonstrate that the majority of the tested *Trichoderma* spp. show, in vitro, a high self-inhibitory activity mediated by secreted soluble metabolites. This activity varied among different strains and species, with *T. harzianum* showing the highest self-inhibitory effects. Trichoderma strains are affected both in their growth rate and the macroscopic structure of their colonies, arguably reflecting microscopic structural and physiological abnormalities in the fungal hyphae. These growth-inhibitory effects are highly similar to those exerted against *Fusarium oxysporum* in the same conditions, especially for *T. harzianum* isolates, indicating a likely comparable mechanism in both cases. In this regard, the great inhibition observed in both cases for most Trichoderma strains is possibly derived from the extremely high concentration of metabolites accumulated in the culture medium. As a result, membrane assays seem to not be very informative by themselves, in regards the direct biocontrol capabilities of the tested strains. Therefore, different methods or a combination of antifungal and self-inhibitory experiments could be a better approach to preliminarily evaluate the potential biocontrol activity of microbial strains in order to pre-select them in vitro for further in vivo trials. This strategy may serve to optimize the process of pre-selection, helping to save time and resources in the subsequent phases of the research. Nevertheless, these conclusions need to be further investigated and confirmed by new assays, involving both in vivo studies and a wider variety of biocontrol and phytopathogenic microbial strains.

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CHAPTER II

Volatile Organic Compound Chamber: a novel technology for microbiological volatile interaction assays

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Volatile Organic Compound Chamber: A Novel Technology for Microbiological Volatile Interaction Assays

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Abstract: The interest in the study of microbiological interactions mediated by volatile organic compounds (VOCs) has steadily increased in the last few years. Nevertheless, most assays still rely on the use of non-specific materials. We present a new tool, the volatile organic compound chamber (VOC chamber), specifically designed to perform these experiments. The novel devices were tested using four *Trichoderma* strains against *Fusarium oxysporum* and *Rhizoctonia solani*. We demonstrate that VOC chambers provide higher sensitivity and selectivity between treatments and higher homogeneity of results than the traditional method. VOC chambers are also able to test both vented and non-vented conditions. We prove that ventilation plays a very important role regarding volatile interactions, up to the point that some growth-inhibitory effects observed in closed environments switch to promoting ones when tested in vented conditions. This promoting activity seems to be related to the accumulation of squalene by *T. harzianum*. The VOC chambers proved to be an easy, homogeneous, flexible, and repeatable method, able to better select microorganisms with high biocontrol activity and to guide the future identification of new bioactive VOCs and their role in microbial interactions.

Keywords: volatile organic compounds; VOCs; trichodiene; squalene; *Trichoderma*; *Fusarium*; *Rhizoctonia*; biocontrol; biological control

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1. Introduction

Volatile organic compounds (VOCs) are small organic molecules produced and emitted by a wide variety of organisms. These compounds often serve as mediators in communication, interaction, and/or competition between organisms [1]. Besides, they play a key role in the establishment of ecological communities and their interrelationships [2]. VOCs have been described as mediating animal, plant, and microbial communication. Regarding animals, VOCs have been widely studied as attractants and repellents [3,4]. Plant VOCs can function as herbivore deterrents [5], while volatile plant–plant interactions have been demonstrated to take part in defence activation against predators [6] and diseases [7]. Concerning microbe–plant interactions, Moisan et al. [8] demonstrated that fungal VOCs from both pathogenic and non-pathogenic strains promoted growth and flowering and modulated the induced systemic response (ISR) of the exposed plants. VOCs from some fungi enhanced plant defences, while others increased their susceptibility to attack by generalist caterpillars. A comprehensive review of the effects produced by bacterial VOCs in plant development can be seen in the work of Kai et al. [9].

Additionally, microbiological volatile interactions have proved to be highly relevant, leading to a wide range of effects, from growth inhibition to growth promotion [1,10]. They

also exert morphological and physiological changes in the fungal and bacterial strains exposed to them [11]. Nevertheless, the importance of microbial volatile organic compounds (mVOCs) was understated for decades, both in terms of little research towards its comprehension and a lack of specific laboratory material for performing VOC assays [12,13]. Fortunately, the study of the role played by these molecules in the biological and ecological interactions between organisms has become increasingly important throughout the last few years [14–18].

Ecologically, rhizosphere microbes seem to regulate plant growth through VOC production, as demonstrated for bacteria [19], *Trichoderma viride* [20], or mycorrhizal fungi [21]. Moreover, fungal VOCs have been proven to affect plant root architecture [22,23] and even the ability of the plant to confront herbivores [24]. Besides, VOCs released by infected plants seem to prepare the defensive systems of nearby plants for the eventual attack of the pathogen [7]. Furthermore, Li et al. [2] revealed a collective volatile-mediated antagonism of soil bacteria against fungi. These authors proposed VOC production as an important bacterial strategy for defending occupied niches against invading fungi, stating that this mechanism may represent a major contribution to soil fungistasis.

In addition, fungal species have been described as producing a wide variety of VOCs: sesquiterpenes, furanes, alkenes, alcohols, phenols, aliphatic hydrocarbons, aldehydes, ketones, benzene derivatives, esters, etc. [9,12,25,26]. In this regard, sesquiterpenes seem to account for an important part of the differences between microorganisms [12]. Moreover, species from the fungal genera *Fusarium*, *Rhizoctonia*, and *Trichoderma* have been identified as among the most odoriferous ones using E-Nose and solid phase microextraction–gas chromatography/mass spectrometry (SPME–GC/MS) [12]. Furthermore, the nature and amount of the VOCs produced are influenced not only by the strain itself but also by external factors such as temperature, oxygen and nutrient availability, pH, incubation period, etc. [12].

Trichoderma is a well-described and widespread fungal genus used as an effective biological control agent (BCA). In this regard, some studies have focused on fungal interactions mediated by *Trichoderma*-produced VOCs [27]. For example, VOCs emitted by *Trichoderma* spp. showed a strong effect against plant pathogenic fungi such as *Botrytis cinerea* and *Alternaria brassicicola* [28]. In the same way, Li et al. [29] tested four *Trichoderma* species, confirming that all of them produced VOCs that inhibited *Fusarium oxysporum* growth, while Speckbacher et al. [30] explored the production of bioactive VOCs from *Trichoderma atroviride* in various conditions and in confrontation with *Rhizoctonia solani* and *F. oxysporum*.

Nowadays, three main methods are currently in use for VOC challenge assays: (i) divided Petri dishes, (ii) the plate-within-a-plate system, and (iii) the double dish set (DDS) method, also known as "two sealed base plates" or "sandwiched Petri plates". The three of them possess certain advantages and limitations. Firstly, the divided Petri dishes are easy to manipulate and technically able to provide vented and non-vented conditions. Nevertheless, their structure poses a serious limitation in the available surface for microbial growth, and they are also highly prone to cross-contamination, as microorganisms, especially filamentous fungi, tend to overcome the dividing walls. Secondly, the plate-within-a-plate system is more difficult to set up, it requires more material and space and, most importantly, it presents important difficulties regarding management and data collection during the experiments. Finally, the two sealed base plates or double dish set (DDS) method [31] seems to be the preferred and most widely used setup for performing the in vitro assessment of volatile-mediated microbial interactions. Nevertheless, this technique presents several problems: risk of cross-contamination, low homogeneity and reproducibility due to incorrect or imperfect fitting, difficult and time-consuming assembling of the replicates, and the impossibility of varying the gas exchange rate with the exterior, producing a strong limitation on flexibility and on the ability to test different experimental conditions concerning ventilation. Thus, as stated by Alijani et al. [32], the results obtained with this technique are only applicable to airtight conditions. Moreover, gas exchange and

oxygen availability play a significant role in microbial development [33] and microbial competition in biocontrol strategies [34]. For this reason, the outcome of the experiments performed with DDS or similar closed techniques may be strongly biased by this lack of ventilation, which could lay behind a significant part of the inhibitory activity detected in these experiments. Therefore, low oxygen concentrations may be distorting the final results, mistakenly assigned to the single bioactivity of VOCs.

Notwithstanding the aforementioned limitations, several authors have proposed the potential usefulness of VOCs for biotechnological and field applications, for example in biocontrol [14], as bio-fumigants [15], and other uses [35]. As these studies have been carried out in airtight environments, claims made regarding the usefulness of these microbial VOCs can only be accepted for the tested conditions until new trials are carried out. Surprisingly, it appears that almost no studies have been conducted so far to elucidate the effect that different ventilation produces on the microbiological interactions mediated by VOCs. Lo Cantore et al. [36] demonstrated that these interactions need to be approached in vitro by using non-airtight conditions. Their findings showed significant differences in the effect of bacterial volatiles using sealed and non-sealed divided Petri dishes. This need for the general testing of new conditions is further supported by the findings of Speckbacher et al. [30], who demonstrated the influence of light variation in the VOC production of *T. atroviride*.

In this regard, a few interesting modifications and alternative methods have already been proposed in the study of volatile interactions. For instance, Gershow et al. [3] presented a system used to deliver gaseous stimuli to study small animal navigation. Closer to the present study, some authors placed dialysis membranes between the plates to avoid cross-contamination when using the DDS method [37,38], or manually perforated plate lids to place these membranes on them [39]. Finally, Cernava et al. [40] developed a novel system for the fast screening and detection of bioactive microbial VOCs. Albeit definitely useful for their intended purposes, these last proposals still rely on non-specific materials, and they lack the ability to provide some important features to the experiments.

The VOC chambers presented here provide a standardized method for performing volatile assays, allowing for easier and faster use, better experimental homogeneity and replicability, and more flexibility regarding ventilation conditions. To prove the previous statements, we have compared the DDS method and two structurally different VOC chambers, namely non-vented and vented VOC chambers. The design of these new devices is derived from previous experiences in our research group. In this regard, we selected a wild type (WT) *T. harzianum* strain and three of its transformants that were previously developed, described, and their antifungal activity assessed in our group [41–43]. Furthermore, our group has also reported the attractive and repellent activity of these fungal strains against the insect pest *Acanthoscelides obtectus* [4]. In the present work, we confronted these BCAs against two phytopathogenic fungal strains isolated in our laboratory: *F. oxysporum* F3 and *R. solani* R43 [44].

Finally, we hypothesize that variations in ventilation produce significant effects on interactions between filamentous fungi mediated by VOCs, and that the herein presented VOC chambers are a more effective way to perform these kinds of experiments than the traditional methods. Hence, the main goals of this study are: (i) to present a novel technology specifically designed to perform microbiological VOC assays, (ii) to demonstrate its advantages (easy, homogeneous, flexible, and repeatable) in comparison to the traditional DDS method, and (iii) to further prove the importance of non-tightly closed environments in microbe–microbe interactions mediated by VOCs.

2. Materials and Methods

2.1. VOC Chamber: General Technical Description

The novel device presented in this article is a culture chamber (Figure 1) specifically designed to perform microbiological competition and interaction assays mediated by VOCs. It has been patented with the number ES 2708899 B2 in the Spanish Office of

Patents and Trademarks and has been submitted internationally with the PCT number PCT/ES2019/070475. The system is derived from the double dish set (DDS), also known as a "sandwiched Petri plates assay" or "two sealed base plate system" [31] and is comprised of three parts; two base plates equal to 90 mm standard Petri dishes (Figure 1, numbers 1 and 2), and a perforated central piece (Figure 1, number 3) that acts as a double lid, holding together and connecting the aforementioned plates facing each other. This central piece has a slightly larger diameter than the plates, and two lateral walls projecting upwards and downwards (Figure 1, number 7), thus being able to hold and house both plates. It has a central hole (Figure 1, number 11) that connects the headspaces of both plates, thus allowing VOCs to freely move from one culture to the other.

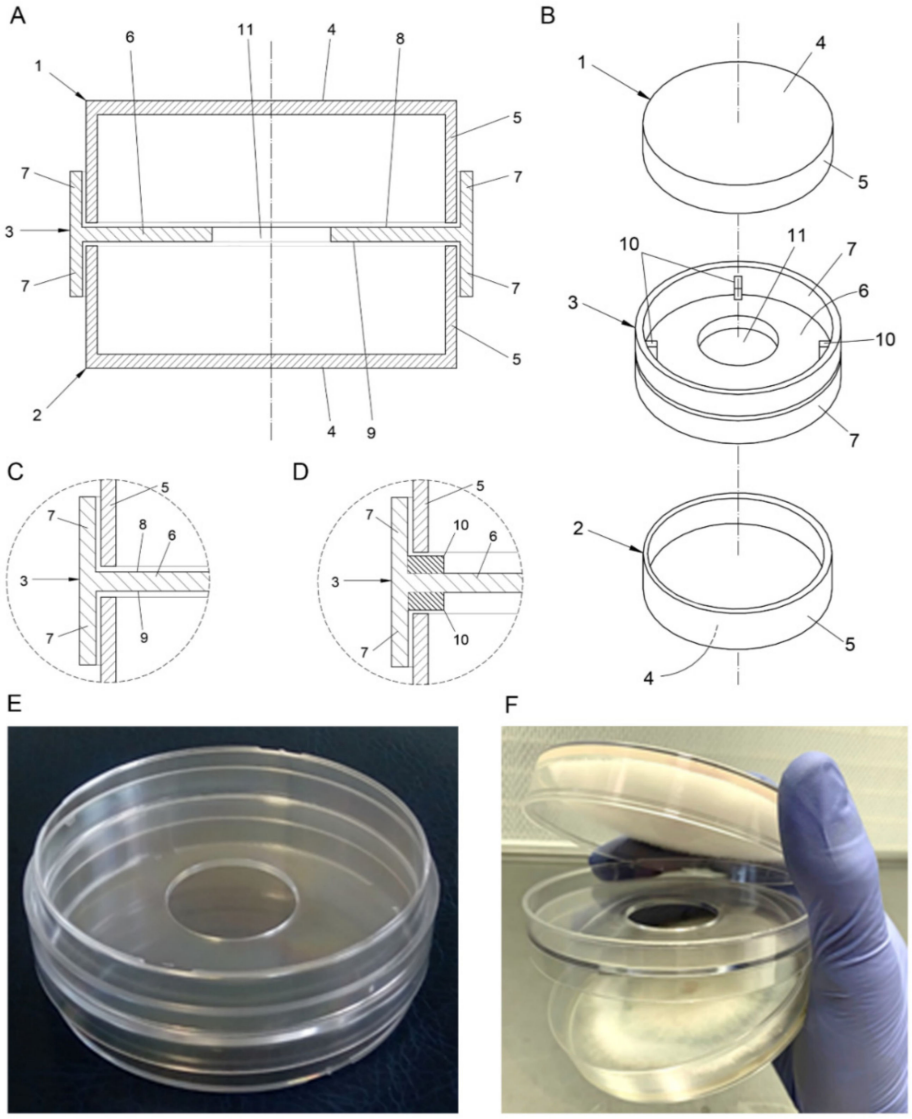

Figure 1. (**A**) Frontal cross-section, non-vented VOC chamber. (**B**) Explosive view, vented VOC chamber. (**C**) Detail of the union between the plates and the central piece in the non-vented VOC chambers. (**D**) Detail of the union between the plates and the central piece with flanges in the vented 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. (**E**) Closed empty VOC chamber. (**F**) Opened VOC chamber with *F. oxysporum* (upper plate) and *T. harzianum* (lower plate).

In order to perform mVOC interaction assays, the two base plates are used as regular Petri dish plates to pour culture media and grow microorganisms on. The lower plate is placed facing upwards, while the perforated central piece rests on the top of it as a lid would do. Finally, the upper plate rests on the upper horizontal face (Figure 1, number 8) of the central piece facing downwards. Therefore, both plates are firmly set facing each other and held by the central piece, which allows gas exchange between them through its hole.

The intermediate wall (Figure 1, number 6) of the central piece can present a flat surface on both sides like in a non-vented Petri dish (Figure 1D), thus limiting the gas exchange with the exterior and therefore allowing for a higher concentration of VOCs inside the chamber or, otherwise, can present a vented configuration (Figure 1C) with small flanges (Figure 1C, number 10) on both sides of the edge of the central piece's intermediate wall, making both plates rest a little up from the bottom and thus allowing for a higher rate of gas exchange between the chamber and the exterior. This vented structure helps to reduce VOC concentration inside the chamber and ensures oxygen availability for the growing microorganisms, whereupon it can provide closer conditions to those present in some natural environments. This vented/non-vented configuration is one of the main new features provided by the VOC chamber, widening the range of experimental conditions previously available using the traditional DDS methodology.

Additionally, a membrane or filter (Figure S2, number 14) can be placed covering the hole of the central piece to avoid cross-contamination between cultures. Membranes or filters could potentially limit the crossing of certain active biological molecules, therefore acting as a way to sort the VOCs that can reach the opposing microorganisms. Neither filters nor membranes were used in the present study to cover the central hole.

Divided Petri dishes (Figure S1, number 15), while not tested in the present work, could be used to evaluate multilateral interactions between more than two microbiological strains. Further modifications of the prototype regarding the central piece or the plates and the dimensioning of the prototype are available in the supplementary materials.

2.2. VOC Chamber: Prototypes

The base plates used for the present research were obtained from 90 mm Petri dishes, while the central pieces were produced from polystyrene crystal by J.D. Catalán S.L. (Arganda del Rey, Madrid, Spain) using a plastic injection steel mold made specifically for this purpose. These central pieces (Figure 1, number 3) were 92 mm in diameter with a 30 mm central hole (Figure 1, number 11). Its peripheral walls (Figure 1, number 7) had a height of 15 mm and the plastic thickness was 1 mm in all parts of the piece.

Non-vented VOC chambers presented a central piece (Figure 1, number 3) with flat surfaces on both sides, while vented VOC chambers had 3 flanges (Figure 1, number 10) on both sides of the edge of the central piece's horizontal wall. These flanges were 0.7 mm in height, leaving a space between the plates and the central piece, thus allowing for gas exchange between the chamber and the exterior, as explained in the previous section. Pictures of VOC chambers are presented in Figure 1E,F.

2.3. Microbial Strains and Culture Conditions

Two *Trichoderma* strains were used as parental strains: *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) (T34 from now onwards), a well-established biocontrol agent, and *T. harzianum* E20, a transformant derived from T34 by silencing squalene epoxidase encoding gene *erg1*, which lead to the accumulation of squalene and produced low levels of ergosterol [41]. Expression of the *T. arundinaceum* tri5 gene in these strains resulted in the selection of two transformants: T34-5.27 and E20-5.7, respectively, which showed high levels of trichodiene production [42,43]. This last compound is the first and the only volatile intermediate in trichothecene biosynthesis and is considered a non-phytotoxic VOC. Furthermore, as a result of the *erg1* gene silencing, with

the subsequent accumulation of farnesyl diphosphate (FDP) in the E20 strain, production of trichodiene was significantly higher in the strain E20-5.7 than in T34-5.27 [42,43].

Regarding phytopathogenic strains, *F. oxysporum* F3 and *R. solani* R43 were isolated from bean (*Phaseolus vulgaris* L.) fields belonging to the Protected Geographical Indication (PGI) "Alubia de La Bañeza-León" (Spain), and selected for their high virulence against this crop [44]. These strains are stored in the Pathogens and Antagonists Collection at the Pest and Diseases Diagnosis Laboratory (PALDPD, University of León, León, Spain). *Trichoderma* and *F. oxysporum* strains were preserved in 50% glycerol spore suspension at −80 °C, while *R. solani* was kept at 4 °C in slanted assay tubes with PDA (potato dextrose agar, Difco Becton Dickinson, Sparks, MD, USA) and sealed with Parafilm®. All cultures were activated by culturing on PDA at 25 °C.

2.4. In Vitro Evaluation of VOC-Mediated Interactions between R. solani R43 and Trichoderma Using DDS Method, Vented, and Non-Vented VOC Chambers

A "double dish set" (DDS) method was performed as described by Dennis and Webster (1971) with some modifications. 6 mm plugs from the fresh edge of 2-day-old *Trichoderma* colonies were placed on the centre of 90 mm Petri dishes containing 18 mL of PDA, and were left to grow for 48 h at 25 °C. After that, *R. solani* R43 6 mm plugs from the edge of 3-day-old colonies were inoculated on the centre of 18 mL PDA Petri dishes. The respective lids of both *Trichoderma* and *R. solani* R43 were immediately removed, setting the plates facing each other and sealing both with two layers of Parafilm® (Merck KGaA, Darmstadt, Germany). *Trichoderma* strains were always placed on the lower plate, facing upwards, while the pathogen was grown on the upper plate, and, therefore, facing downwards. This technique creates a tightly closed chamber where VOCs concentrate and move freely to and from both strains.

Assays with non-vented and vented VOC chambers were carried out in the same manner, except for the use of non-vented and vented central pieces, respectively, to hold and connect both the upper and lower plates. Besides this, the non-vented VOC chambers were sealed with two layers of Parafilm®, while the vented ones were not.

Four repetitions were performed for each treatment and technique, and all of them were incubated at 25 °C. Control treatments were performed in the same way using PDA without *Trichoderma* in the lower plates. Colony diameters of *R. solani* R43 were measured in three different directions (Figure 2) with a ruler after 1, 2, 3, and 4 days after inoculation, considering the growth for each replicate as the mean of the aforementioned three measures (expressed in mm). Two sets of experiments were performed separately, both using T34, T34-5.27, E20, and E20-5.7 as biocontrol agents.

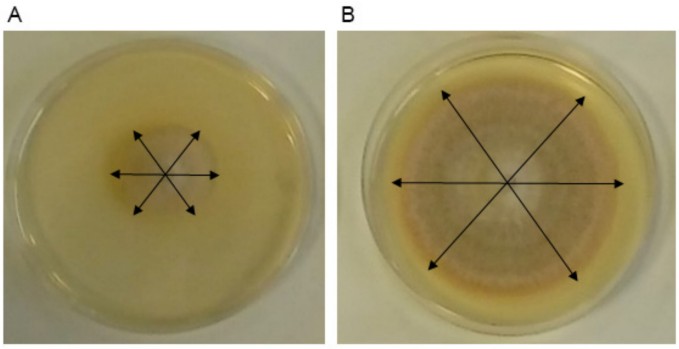

Figure 2. (**A**,**B**) *R. solani* R43 growth previously exposed to *T. harzianum* VOCs. The arrows represent the three diameters measured (in mm) per replicate. Their mean −6 (mm) represents the diameters used for growth comparisons and to calculate the percentage of inhibition (PI) for each treatment: PI = [(C − T) / C] × 100. Where C is the diameter of the control and T the diameter of the treatment.

2.5. In Vitro Evaluation of VOC-Mediated Interactions between F. oxysporum F3 and Trichoderma Using the DDS Method, Vented, and Non-Vented VOC Chambers

The same protocol, as described in the previous section, was performed to evaluate the effect of *Trichoderma* spp. VOCs on *F. oxysporum* F3. Due to the slower rate of growth showed by this pathogen, plugs from 5-day-old *F. Oxysporum* F3 colonies were used in these assays, and data collection was carried out after 3, 5, and 7 days post-inoculation.

2.6. Data Treatment and Statistical Analysis

Microbial growth was considered as the mean diameter from each replicate, and 6 mm were subtracted from all measures to avoid distortions produced in the percentage of inhibition (PI) by the diameter of the plugs, as shown by Mutawila et al. [45]. PIs were calculated in relation to the control using the following equation proposed by Gotor-Vila et al. [46]: PI = [(C − T) / C] × 100. Where C is the diameter of the control and T that of the treatment (Figure 2).

Microbial growth data (Diameter −6 mm) obtained from the VOC-mediated interaction assays were analysed with one-way analysis of variance (ANOVA, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$) after confirmation of the normality and equality of variances. Subsequently, *Trichoderma* treatments were contrasted between them and with the control within each technique/method (DDS, non-vented, and vented) using Tukey's post hoc test ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$).

Moreover, the coefficient of variation (CV) or relative standard deviation (RSD) was calculated separately for each treatment and method to evaluate their precision and reproducibility. The equation being CV = σ/μ, in which σ represents the standard deviation and μ the mean for each treatment. The CV obtained were analysed with one-way analysis of variance (ANOVA, $p \leq 0.05$) after confirmation of the normality and equality of variances and were contrasted between the three methods within *R. solani* R43 and *F. oxysporum* F3 confrontation assays using Tukey's post hoc test ($p \leq 0.05$). All statistical analyses were performed using SPSS v24.0 (IBM).

3. Results

3.1. Homogeneity and Replicability of the Results

In this work, we have evaluated the effects of VOCs produced by several Trichoderma spp. isolates on the development of two phytopathogenic fungi, and we have compared the results obtained by using the routine DDS method and the novel VOC chambers, both non-vented and vented versions, in order to elucidate their influence over the results.

The higher homogeneity provided by the new methodology is clearly shown by the lower coefficient of variation (CV) obtained in both *R. solani* R43 and *F. oxysporum* F3 assays (Table 1). In this regard, non-vented and vented VOC chambers presented a CV significantly lower than the results obtained with the DDS method, both using *R. solani* R43 as well as *F. oxysporum* F3. Additionally, there were no significant differences in CV between vented and non-vented VOC chambers. These results indicate that the use of the VOC chamber provides a higher degree of homogeneity and replicability than the traditional DDS method. The comparison of the results from experiment 1 and experiment 2 revealed significant differences between them, thus both were analysed and are presented separately (Table 1).

Table 1. Coefficient of variation (CV) from *R. solani* R43 and *F. oxysporum* F3 growth using the DDS method, non-vented, and vented VOC chambers. In the table are represented the CV mean, its standard deviation (SD), and its standard error (SE). The results were analysed with one-way analysis of variance (ANOVA, $p \leq 0.05$) and Tukey's post hoc test ($p \leq 0.05$). Different capital letters represent significant differences between treatments. The results from experiment 1 and experiment 2 are represented separately.

		Coefficient of Variation R43 Experiment 1		
Method	Mean	SD	SE	Statistics
DDS	0.156	0.083	0.019	A
Non-vented	0.048	0.024	0.005	B
Vented	0.057	0.040	0.009	B
		Coefficient of Variation R43 Experiment 2		
Method	Mean	SD	SE	Statistics
DDS	0.10	0.056	0.012	A
Non-vented	0.040	0.016	0.004	B
Vented	0.069	0.039	0.009	B
		Coefficient of Variation F3 Experiment 1		
Method	Mean	SD	SE	Statistics
DDS	0.062	0.037	0.010	A
Non-vented	0.035	0.027	0.007	B
Vented	0.026	0.014	0.004	B
		Coefficient of Variation F3 Experiment 2		
Method	Mean	SD	SE	Statistics
DDS	0.050	0.015	0.004	A
Non-vented	0.021	0.008	0.002	B
Vented	0.031	0.015	0.004	B

3.2. Volatile Activity of Trichoderma Against R. solani R43 and F. oxysporum F3. Statistical Differences in Growth Results Using the DDS Method, Non-Vented, and Vented VOC Chambers

When R43 growth was evaluated in confrontation with the different *T. harzianum* strains, the DDS method showed clear differences between the treatments and the control ($p \leq 0.001$) and was also able to expose some statistical differences between trichodiene-overproducing strains (T34-5.27 and E20-5.7) and their parental strains (T34 and E20). Nevertheless, these differences were less significant than those observed using non-vented and vented VOC chambers and disappeared towards the last few days of the experiments (Figure 3A,D, Tables S1 and S2). For the same pathogenic strain, the non-vented VOC chambers rendered equally clearly significant differences between the treatments and the control ($p \leq 0.001$) and, additionally, showed these differences between trichodiene-overproducing strains and their parental strains throughout the whole length of the experiment, mostly with $p \leq 0.001$ (Figure 3B,E, Tables S1 and S2). Finally, the results obtained using the vented VOC chambers produced quite a different statistical output, indicating that, in these open conditions, the parental strains (T34 and E20) do not present significant inhibitory activity against *R. solani* R43 for the most part, while the trichodiene-overproducing strains retain their significant inhibitory activity ($p \leq 0.001$) (Figure 3C,F, Tables S1 and S2). The vented VOC chambers showed statistical differences between trichodiene-producing strains and their parental strains up to day 4 in both experiments ($p \leq 0.001$), except for the comparison between E20 and E20-5.7 during days 2 and 3 in experiment 2, which showed differences with $p \leq 0.01$. Moreover, *F. oxysporum* F3 growth exposed to the different *T. harzianum* strains using the DDS method showed statistical differences between the *Trichoderma* treatments and the control ($p \leq 0.01$ and $p \leq 0.001$). Nevertheless, it failed to show, in most cases, statistical differences between trichodiene-overproducing strains (T34-5.27

and E20-5.7) and their parental strains (T34 and E20). In experiment 1 these differences were only present between E20 and E20-5.7 on day 3 ($p \leq 0.05$), while in experiment 2, they were also presented on day 3 comparing T34 and T34-5.27, but in both cases disappeared towards the last few days of the experiment (Figure 4A,D, Tables S3 and S4). For the same pathogenic strain, the non-vented VOC chambers rendered significant differences between the trichodiene-overproducing strains and the control ($p \leq 0.01$ and $p \leq 0.001$), but not so much between the parental strains and the control treatment, especially in the case of E20, which, in most cases, grouped with the control. Additionally, the results using the non-vented VOC chambers showed clear differences between trichodiene-overproducing strains and their parental strains throughout the whole length of the experiment, mostly with $p \leq 0.001$ and $p \leq 0.01$ (Figure 4B,E, Tables S3 and S4). Finally, the results obtained using the vented VOC chambers produced quite a different statistical output, indicating that in these open conditions the parental strains (T34 and E20) do not only not present significant inhibitory activity against *F. oxysporum* F3, but exert a significant promotion of its growth compared to the control ($p \leq 0.01$ and $p \leq 0.001$). On the contrary, the trichodiene-overproducing strains retain their significant inhibitory activity ($p \leq 0.05$, and $p \leq 0.01$ in experiment 1, and $p \leq 0.001$ in experiment 2) (Figure 4C,F, Tables S3 and S4). The vented VOC chambers showed statistical differences between trichodiene-producing strains and their parental strains up to day 7 in all cases for both experiments ($p \leq 0.001$).

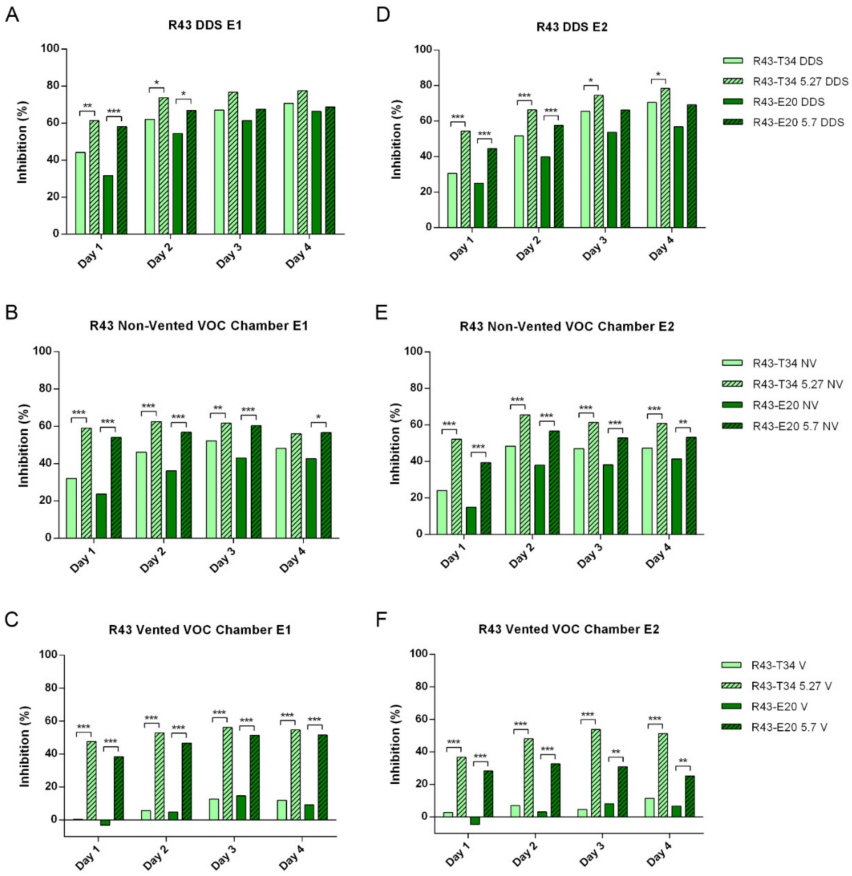

Figure 3. Inhibition percentages (PI) of *R. solani* R43 exposed to *T. harzianum* T34, T34-5.27, E20, and E20-5.7 VOCs after 1, 2, 3, and 4 days using (**A,D**) the DDS method (DDS, upper row), (**B,E**) the non-vented VOC chambers (NV, middle row), and (**C,F**) the vented VOC chambers (V, lower row). (**A–C**) Experiment 1, left column; (**D–F**) Experiment 2, right column. Statistical differences between each parental strain (T34 and E20) and their respective trichodiene-overproducing transformant (T34-5.27 and E20-5.7) were obtained from mycelial growth using ANOVA and Tukey's post hoc test, and are represented between treatments within the same method and day by *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$. No asterisks mean no significant differences. $n = 4$. Please note that the scales in the Y-axis of the figures representing vented VOC chambers (**C,F**) are compressed compared to the DDS (**A,D**) and non-vented ones (**B,E**), due to the presence of negative results.

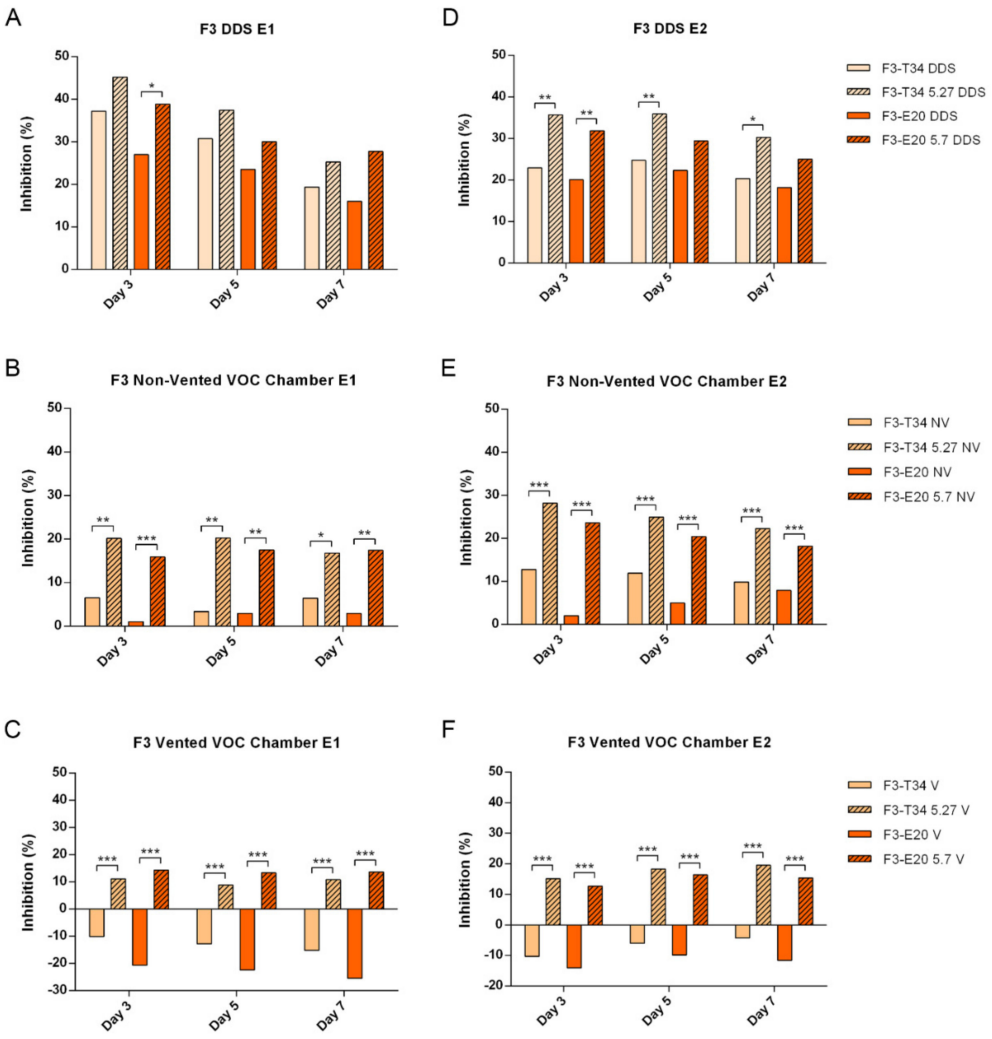

Figure 4. Inhibition percentages (PI) of *F. oxysporum* F3 exposed to *T. harzianum* T34, T34-5.27, E20, and E20-5.7 VOCs after 3, 5, and 7 days using (**A**,**D**) the DDS method (DDS, upper row), (**B**,**E**) the non-vented VOC chambers (NV, middle row), and (**C**,**F**) the vented VOC chambers (V, lower row). (**A**–**C**) Experiment 1, left column; (**D**–**F**) Experiment 2, right column. Statistical differences between each parental strain (T34 and E20) and their respective trichodiene-overproducing transformant (T34-5.27 and E20-5.7) were obtained from mycelial growth using ANOVA and Tukey's post hoc test, and are represented between treatments within the same method and day by *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$. No asterisks mean no significant differences. $n = 4$. Please note that the scales in the Y-axis of the figures representing vented VOC chambers (**C**,**F**) are compressed compared to the DDS (**A**,**D**) and non-vented ones (**B**,**E**), due to the presence of negative results.

3.3. Volatile Activity of Trichoderma Against R. solani R43 and F. oxysporum F3. Quantitative and Qualitative Differences in Percentage of Inhibition (PI) Using the DDS Method, Non-Vented, and Vented VOC Chambers

Firstly, it can be seen from Figure 3 that PI are consistently lower in vented conditions in comparison to the non-vented VOC chamber, and especially compared to the results obtained from the DDS treatments. In this regard, *R. solani* R43 exposed to the *Trichoderma* volatiles showed values of PI mostly between 50% and up to more than 78% using the DDS method (Figure 3A,D). These PI values were generally lower when using the non-vented VOC chambers, being between 32% and 65% (Figure 3B,E). As stated in the previous section, *T. harzianum* strains that overproduce trichodiene (T34-5.27 and E20-5.7) showed a significantly higher PI than their parental strains. Finally, the results obtained using the

vented VOC chambers with *R. solani* R43 showed much lower PI values with maximums of around 56% exerted by T34-5.27 and 53% by E20-5.7, to mere 12% and 14% for T34 and E20, respectively (Figure 3C,F). On the other hand, *F. oxysporum* F3 exposed to *Trichoderma* volatiles showed a generally lower PI than that demonstrated against R43. When using the DDS method, all treatments significantly inhibited *F. oxysporum* F3 growth, with PI values between 16% and 25% for E20, to between 25% and 45% for T34 5.27 (Figure 4A,D). The results rendered by the use of non-vented VOC chambers demonstrated a lower inhibitory activity of the *Trichoderma* volatiles against *F. oxysporum* F3 than the DDS method. In this regard, the parental strains (T34 and E20) showed very low PI, albeit statistically significant in some cases compared to the control. Using the same method, the trichodiene-overproducing strains presented significantly higher PI than their parental ones, while still lower than the one they presented in DDS conditions by themselves (Figure 4B,E). Finally, using the vented VOC chambers, T34-5.27 and E20-5.7 presented lower, but still statistically significant, PI against *F. oxysporum* F3 compared to the control. By contrast, T34 and, especially, E20 showed important negative PI, indicating a completely opposite growth-promoting activity on *F. oxysporum* F3, being between −4% and −15% for T34 and from around −10% to −25% for E20 (Figure 4C,F). This increase in growth rate was statistically significant in all days and both experiments for E20 and for the most part when confronting *F. oxysporum* F3 to T34. All growths, PIs, and P values are represented in the Supplementary Tables S1, S2, S3, and S4. These results obtained comparing the three setups revealed that VOC's effects, and the fungal responses to them, are strongly affected, both quantitatively and qualitatively, by the modification of the experimental conditions. Ventilation and gas exchange with the exterior seem to play a very important role in microbe–microbe volatile interactions.

3.4. Effects of erg1 Silencing and T. arundinaceum tri5 Expression on the Volatile Activity of T. harzianum T34 against R. solani R43 and F. oxysporum F3

Regarding the activity of the VOCs produced by the wild type T34, results show a high inhibitory effect against *R. solani* R43 in both DDS and non-vented VOC chamber conditions (Figure 5). Nonetheless, as previously stated, using vented VOC chambers, the PI dropped dramatically, and its growth was not statistically different from the control treatment in most cases (Supplementary Tables ST1 and ST2). Additionally, this wild type strain showed a consistent growth inhibition activity against *F. oxysporum* F3 with DDS, and some inhibitory activity with non-vented VOC chambers, while demonstrating an opposite effect of growth promotion in vented conditions (Supplementary Tables S3 and S4). From this starting point, the results reveal a decrease in the inhibitory activity shown by the E20 strain compared to the WT. This decrease is consistent for *R. solani* R43 in all days and using any of the three techniques, but only statistically significant in some cases using DDS and especially non-vented VOC chambers (Tables S1 and S2). Regarding *F. oxysporum* F3, the DDS method failed to show statistical differences between T34 and E20, while non-vented VOC chambers were able to reveal them in some cases. However, using vented VOC chambers, the transformant E20 showed an increase in its growth-promoting activity on *F. oxysporum* F3 compared to WT T34 (Tables S3 and S4).

The results obtained from the T34-5.27 and E20-5.7 transformants revealed that the introduction of the *tri5* gene significantly increased the inhibitory effects of the overall VOCs produced by both strains. As stated, these effects were less significant when using the DDS method, which failed to find consistent statistical differences between the transformed strains and their parental ones. On the contrary, assays using non-vented and vented VOC chambers showed the relevant effect of this genetic modification, being not only able to increase the inhibitory capacity of the *Trichoderma* strains in non-vented VOC chambers but also producing an inversion of effects in vented ones, from significant growth promotion by T34 and E20 to significant growth inhibition by T34-5.27 and E20-5.7 (Figure 4, Tables S3 and S4).

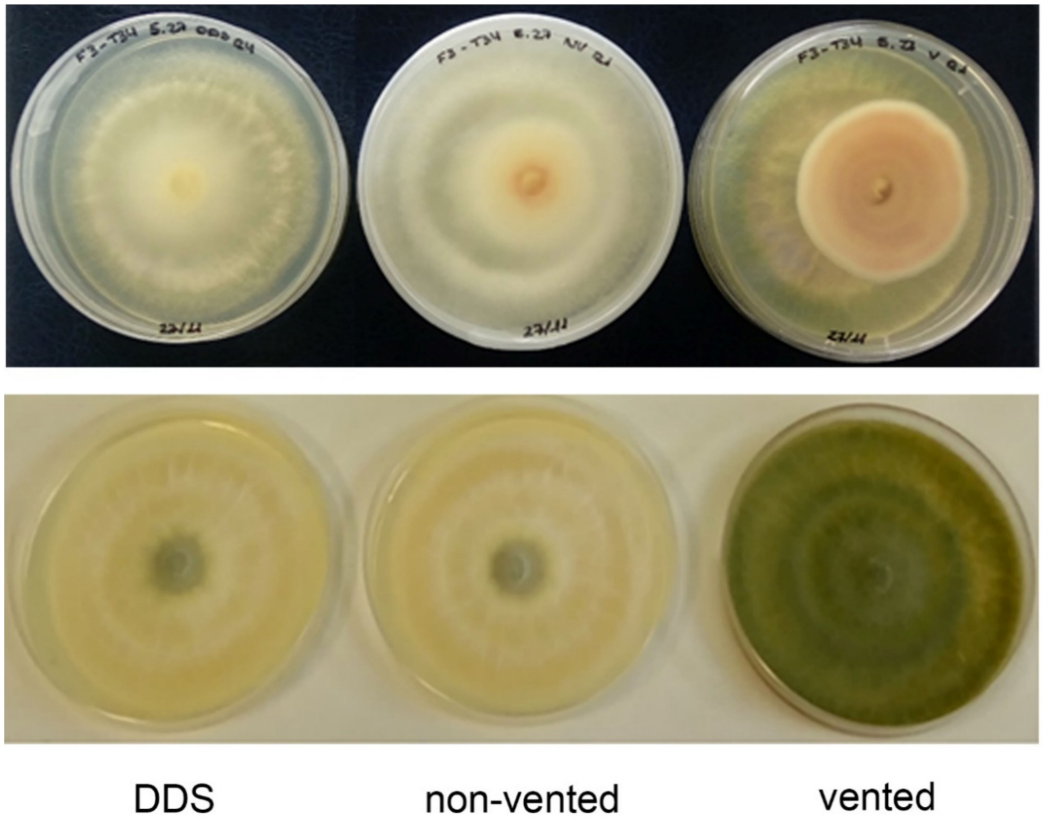

Figure 5. Fungal growth and mycelial aspect after 7 days in volatile assay. *F. oxysporum* F3 in confrontation with T34-5.27 (upper row). *T. harzianum* T34-5.27 after confrontation with *F. oxysporum* F3 (lower row). Both images show results from the DDS method (left column), non-vented VOC chamber (central column), and vented VOC chamber (right column). *F. oxysporum* F3 mycelium shows a faint growth, difficult to perceive, and a loss of coloration in DDS and non-vented VOC chambers, while *T. harzianum* shows thicker growth and more sporulation in vented conditions.

3.5. Additional Effects Produced by the Used of DDS, Non-Vented and Vented VOC Chambers

Pathogens cultivated in DDS and, to a lesser extent, in non-vented VOC chambers presented a fainter growth, especially on the edges (Figure 5). This effect hindered data collection and was not observed when vented VOC chambers were used. Additionally, in the vented VOC chambers, the pathogens presented very clear compact edges that were easier to measure. This faint growth phenomenon was present in both pathogens but was stronger in *F. oxysporum* F3 than in *R. solani* R43 and was also observed in the DDS and non-vented controls. Moreover, not only the pathogens but also *Trichoderma* strains showed a fainter growth and less or no sporulation in DDS and non-vented conditions (Figure 5), indicating as well that their development is affected by the lack of gas exchange with the exterior. Moreover, *Trichoderma* volatiles produced a loss of pigmentation in *F. oxysporum* F3 mycelia. This effect was altered by the different ventilation setups, being non-existent in vented conditions and higher in non-vented and, especially, in DDS ones (Figure 5).

4. Discussion

As previously stated, regarding microbe–microbe VOC interactions, the DDS method [31] seems to be the preferred system in many recent studies [47]. A few interesting modifications and alternative methods have been proposed to date [37,39,40]. On the other hand, oxygen availability and its concentration play a key role in microbe development [33], and thus it must be of the utmost importance regarding microbial interactions. Besides, many of these ecological interactions take place in non-tightly closed environments. Hence, new experiments taking into account different ventilation conditions are needed. For that purpose, the novel VOC chambers described in this work were compared with the routine

DDS method, evaluating the effects of VOCs produced by four *Trichoderma* strains against two phytopathogenic filamentous fungi: *R. solani* R43 and *F. oxysporum* F3.

Many previous studies using tightly closed routine methods, including the modifications stated before, reported important antimicrobial inhibitory activities exerted by VOCs, using bacteria [11,46,47], fungi [29,48,49], or yeast [50] as biocontrol agents. Our findings demonstrated an overall significantly higher inhibitory activity of VOCs from *Trichoderma* on *R. solani* than on *F. oxysporum*. These differences are consistent within all tested *Trichoderma* treatments and the three methodologies employed. Our findings are also in accordance with those described by Kashyap et al. [51], who reported inhibition percentages of around 60% when confronting *R. solani* to different *Trichoderma* strains, while Kai et al. [52] described *R. solani* inhibition between 80% and 99% when confronted to bacterial VOCs. In addition, Giorgio et al. (2015) tested different fungal pathogens against bacterial VOCs and reported *R. solani* to be one of the most inhibited fungi, while the two *F. oxysporum* strains evaluated appeared to be among the less inhibited ones. Moreover, as we report in this study, these authors also described a loss of pigmentation in *F. oxysporum* mycelia in the presence of microbial VOCs. However, our results indicate that *F. oxysporum* F3 does not suffer this loss in vented conditions. Interestingly, this alteration could be of significance to both metabolic aspects of the fungi, and also for some features related to the pathogen's virulence and its antimicrobial capacity, as the pinkish-purple naphthoquinones produced by *F. oxysporum* were demonstrated to possess antimicrobial activity [53,54]. Moreover, a recent study evaluated the effects of the overproduction of trichodiene by *T. harzianum* on *Fusarium graminearum*, finding that it decreases its production of the mycotoxin deoxynivalenol [55].

4.1. Differences in Selectivity and Homogeneity Between Methods

The present findings highlight the capacity of the VOC chambers to reveal significant differences between treatments that were not shown up by the traditional DDS method. Therefore, VOC chambers demonstrated higher selectivity between treatments in almost all of the circumstances tested thus far, revealing new relevant information that could not be detected using the DDS method. This is a very important feature that will allow for better selecting those microorganisms with higher biocontrol potential in terms of VOC production, as well as to guide the future identification of new bioactive volatile compounds, and the investigation of their effects at molecular, genetic, and physiological levels. Therefore, the VOC chambers could become a very useful tool both for applied and basic research regarding volatile interactions. Furthermore, our study indicates that the coefficient of variation (CV) obtained from the data using non-vented and vented VOC chambers provides a higher degree of homogeneity than the DDS method.

4.2. Quantitative and Qualitative Effects of Different Ventilation Conditions

The results presented in this study comparing the three setups (DDS, non-vented, and vented VOC chambers) revealed that VOC's effects and the fungal responses to them are strongly affected, both quantitatively and qualitatively, by the modification of the experimental conditions. Ventilation and gas exchange with the exterior indeed seem to play a very important role in fungus–fungus volatile interactions. Besides, the higher PI showed in DDS and non-vented assays appeared to come not only from a higher accumulation of toxic volatiles, but also from the significant reduction in oxygen in the headspace. This being supported by the fainter growth and physiological alterations presented by the fungi in airtight conditions. This effect should not be surprising, as oxygen concentration is of the utmost importance regarding microbial development [33], and could be exacerbated by the use of fast-growing biocontrol strains that would consume the available oxygen at a faster pace, like *Trichoderma* spp. This lack of oxygen could lie behind a significant part of the inhibitory activity detected in DDS and non-vented conditions in the present study, as well as of that reported previously in other studies using similar techniques. This could therefore jeopardize and distort the final results,

mistakenly assigned to the single bioactivity of VOCs. Nevertheless, it should be taken into account that oxygen limitation can also induce microbial secondary metabolite formation, as reported by Clark et al. [56], and that effective competition for oxygen can be vital to controlling pathogens in certain conditions [34].

Furthermore, the present paper reports a surprising switch from inhibitory effect to growth-promoting effect produced by T34 and E20 VOCs on *F. oxysporum* F3 when using vented VOC chambers. This change could represent adaptive traits developed by the pathogenic and/or the biocontrol fungal strains in non-tightly closed natural environments, which, up until now, were concealed in vitro by the quasi-isolating characteristics of the DDS method. Promoting effects exerted by VOCs had already been reported in *Fusarium* spp. [57], but to our knowledge, this is the first time that ventilation conditions have been proven to produce qualitative changes from inhibitory to promoting effects using filamentous fungi as biocontrol agents against fungal pathogens. Interestingly, our findings are in contradiction with those obtained by Lo Cantore et al. [36], who used divided Petri dishes and reported an increase in the mycelia growth of *Pleurotus ostreatus* and *Pleurotus eryngii* when exposed to VOCs produced by *Pseudomonas tolaasii* in non-vented conditions, as opposed to an inhibitory effect in vented ones. This disparity of results may partially be due to the fast-growing rate of *Trichoderma* spp. in comparison to bacterial cultures, and therefore, faster oxygen consumption in the headspace. Albeit contradictory, both results pose a strong case towards the convenience of completing past and future VOC-mediated competition studies to assess the effect of different ventilation conditions on the microbial interactions mediated by VOCs. To summarize, three major findings indicate different effects of *Trichoderma* VOCs over the two pathogenic strains tested. Firstly, the overall significantly higher inhibitory activity of VOCs from *T. harzianum* on *R. solani* R43 than on *F. oxysporum* F3. Secondly, the general reduction of inhibitory activity in vented conditions compared to less open ones. Additionally, and thirdly, the most striking result obtained, the switch from inhibitory to growth-promoting effect of T34 and, especially, of E20 VOCs on *F. oxysporum* F3 in vented conditions.

All these findings may be rooted in ecological adaptations developed by both the pathogens and the *Trichoderma* strains, leading to complex microbiological interactions and the coevolution of the different fungal strains in their diverse natural environments. In this regard, DDSs and non-vented VOC chambers could be considered to represent environments where airflow and gas exchange face important limitations [32]. For instance, the conditions faced by microorganisms in some storing facilities [15,58] or the microbial interactions taking place in flooded areas or in soils with a clay texture. On the other hand, we believe that vented VOC chambers give very valuable information about the behaviour of microorganisms when exposed to volatile interactions in less tightly closed environments, as in soils with a sandy texture, aerial parts of the plant's surface, and other open-air conditions. New studies should be conducted to elucidate how and why some microorganisms, as *F. oxysporum* F3 in our case, react in such different ways when facing VOCs from other microbial strains in vented and non-vented conditions, as well as to explain its link with the ecological interactions that might be explained by this behaviour.

4.3. Effects of the erg1 Silencing and tri5 Expression

Comparing to T34, the results using the E20 strain revealed a decrease in its inhibitory activity, being statistically significant in some cases. In fact, a slight promoting effect was observed for E20 in vented conditions, but without significant differences to the control. These findings suggest that the silencing of *erg1* and the subsequent reduction in ergosterol levels and the increase in squalene production may lead to lower production of toxic VOCs. It may indicate that the ergosterol route is implicated in the production of volatile compounds with antifungal activity, most likely of triterpene nature, but still unidentified. This is in agreement with the findings described previously where a reduction of bioactive activity was reported for the same transformants but in relation to soluble metabolites [41]. The high antifungal activity exerted by the wild type strain T34 on *R.*

solani R43, and to a lesser extent on *F. oxysporum* F3, could derive from the production of some bioactive VOCs already described for *T. harzianum* and other species. In this regard, dimethyl disulfide and dimethyl trisulfide were identified by Elkahoui et al. [59] as important VOCs against *R. solani*. *T. harzianum* can produce also 3-octanone and 1-octen-3-ol, 2-phenylethyl alcohol and isopentyl acetate [29], compounds with fungistatic and fungicidal activity. Additionally, previous studies showed the production of 6-pentyl-2H-pyran-2-one (6PP) by several *Trichoderma* spp., including *T. harzianum*. 6PP is a secondary metabolite with inhibitory activity against several plant pathogenic fungi such as *R. solani* and *F. oxysporum* [60]. Nevertheless, it is impossible to pinpoint any specific compound without proper identification and individual testing, as hundreds of VOCs produced by *T. harzianum* and related species have been described so far [61,62].

A similar outcome was observed with *F. oxysporum* F3 using DDS and non-vented VOC chambers, with important PI when confronted to the T34 strain, and a reduction of this activity when using E20, albeit not statistically significant in some cases when using the DDS method. Nevertheless, both T34 and E20 strains demonstrated a completely opposite effect when they were tested in vented conditions. These vented treatments produced a significant growth promotion on *F. oxysporum* F3. Moreover, E20 showed an increase in this growth-promoting activity in comparison to the original T34 strain. In addition to the previous explanation, this could mean that the silencing of the *erg1* gene not only reduces the production of antifungal volatiles, but may also increase the production of growth-promoting bioactive compounds, at least in vented conditions. This could mean that squalene or some derived compound exerts this promoting activity in *F. oxysporum*. Alternatively, the explanation could be that the same compounds showing inhibitory activity in closed circumstances exert promoting effects when they act in vented ones. Notwithstanding, a combination of various complex and synergistic interactions could lay behind the reported effects, as stated by Strobel et al. [63]. VOCs may not be the only factor to explain the effects observed using these strains, as inorganic volatiles like CO_2, NH_3, or HCN could also be responsible for part of this basal activity in the wild type strain and, therefore, in its transformants. Possible changes in the pH of the growth medium due to the presence of inorganic volatiles could also account for some of the activity.

On the other hand, the results obtained from the T34-5.27 and E20-5.7 transformants revealed that the heterologous expression of *T. arundinaceum tri5* significantly increased the inhibitory effects of the overall VOCs produced by both strains. Interestingly, when using the DDS method, this effect was barely noticeable and not statistically significant in many cases. Indicating, probably, that the maximum inhibitory activity in tightly closed environments is reached without the need for this modification. On the contrary, assays using non-vented and vented VOC chambers showed the relevant effect of this modification, being not only able to increase the inhibitory capacity of the *Trichoderma* strains, but also reverting the effects observed using a vented VOC chamber, from the aforementioned significant growth promotion of T34 and E20 over *F. oxysporum* F3, to its significant inhibition. These findings support the results described by Malmierca et al. [42,43], where an increase in the antifungal activity of *Trichoderma* was also found using the same transformant strains against several fungal phytopathogens using confrontation and membrane assays but non-volatile confrontation assays. Besides, a recent study conducted by Taylor et al. [55] demonstrated that the overproduction of trichodiene by *T. harzianum* reduced the production of the mycotoxin deoxynivalenol by *F. graminearum*.

4.4. Additional Observations and Final Overview

Finally, VOC chambers were demonstrated to be easier and faster to use than the traditional DDS method. The time required to assemble the VOC chambers was much lower than the time needed to correctly place both Petri plates using the DDS method and seal them with parafilm. This is especially true for the vented chambers, while the non-vented prototypes used in this study still needed to be sealed with parafilm to ensure a fairly closed environment. Nevertheless, the time needed for the assembling of non-

vented VOC chambers could be significantly reduced by its industrial production using an ad hoc locking system (e. g., Figure S2, numbers 10 and 11). Future assays could be performed with VOC chambers using membranes of filters to cover the central orifice, thus avoiding cross-contamination or even allowing for eventual compound sorting. These new features could be attached commercially or added in the laboratory. Moreover, the VOC chambers are not limited to use with filamentous fungi, nor even to microorganisms, but could be useful for evaluating VOC interactions involving other types of organisms. The VOC chamber could present new configurations and features with an outlet or adaptor to allow the direct extraction of the VOCs retained in the headspace, trapping them for ulterior analysis by GC/MS. Additionally, new future uses could arise from the scientific community for this novel technology.

In the end, the overall evidence from this study indicates that ventilation and gas exchange play a very important role in the microbial interactions mediated by VOCs. Furthermore, our findings highlight the limitations of the traditional DDS method on the performance of these experiments, demonstrating that VOC chambers have the ability to reveal differences that were not detected with the traditional method, showing higher selectivity between treatments in almost all of the tested circumstances, in addition to higher homogeneity of results. Tightly closed methods, such as the DDS, seem to be unable to reproduce the interactions developed between organisms in open environments. Therefore, we strongly believe that past and future VOC studies should be completed using the new techniques presented here. Thus, allowing for the assessment of the effect of different ventilation conditions on the microbial interactions mediated by volatile compounds. Nevertheless, we do not imply that previous studies using DDS or related methods are wrong, but rather that their findings and derived conclusions can be only applied to the airtight conditions tested, as already stated by some authors [32]. Therefore, the present work provides useful novel tools to solve these limitations, and opens up the opportunity for new trials to complete that past research, and to obtain a deeper understanding of microbial interactions. Consequentially, further and more specific investigations are strongly recommended for unveiling the very complex VOC-mediated microbial interactions that take place in different ecosystems and conditions, alongside their ecological, molecular, physiological, and genetic implications.

5. Patents

The device herein presented has been patented with the number ES 2708899 B2 in the Spanish Office of Patents and Trademarks and submitted with the international PCT number PCT/ES2019/070475. Thus far, additional patent applications have been already submitted to the following countries: United States of America, Canada, Japan, and the European Union. European Patent Office; Application Number 19846227.7 – 1132.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7040248/s1, Supplementary Figure S1, Supplementary Figure S2, Supplementary Figure S3, Plan 1A, Plan 2A, Plan 3A, Plan 1B, Plan 2B, Supplementary Table S1, Supplementary Table S2, Supplementary Table S3, and Supplementary Table S4.

Author Contributions: Conceptualization, S.Á.-G., P.A.C., and S.G.; methodology, S.Á.-G.; validation, S.Á.-G., S.M.-P., and Ó.G.-L.; formal analysis, P.A.C.; investigation, S.Á.-G., G.C.-H., and Á.R.-G.; resources, P.A.C. and S.Á.-G.; data curation, S.M.-P. and Ó.G.-L.; writing—original draft preparation, S.Á.-G.; writing—review and editing, P.A.C. and S.G.; visualization, Ó.G.-L.; supervision, P.A.C.; project administration, P.A.C. and S.Á.-G.; funding acquisition, P.A.C. and S.Á.-G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study are available in Supplementary materials and additional data can be obtained from the corresponding author upon reasonable request. All microbiological strains used in this study will be made available to researchers upon reasonable request. After publication, 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). Correspondence and requests for materials should be addressed to S.A.-G.

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Conflicts of Interest: The authors declare the existence of financial competing interests derived from the patent held by the institution (University of León) as the patent applicant. The authors of the present manuscript share part of the patent as inventors: 55% Samuel Álvarez-García, 7% Pedro A. Casquero, 7% Santiago Gutiérrez, 7% Sara Mayo-Prieto, 7% Guzmán Carro-Huerga, 7% Óscar González-López, 5% Álvaro Rodríguez-González. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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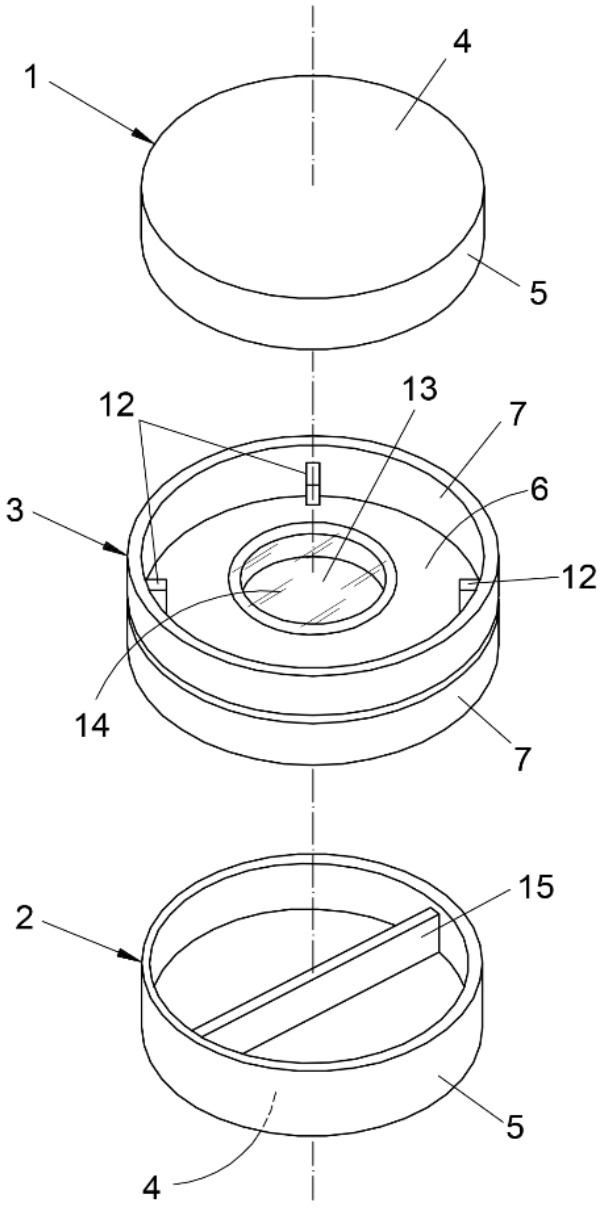
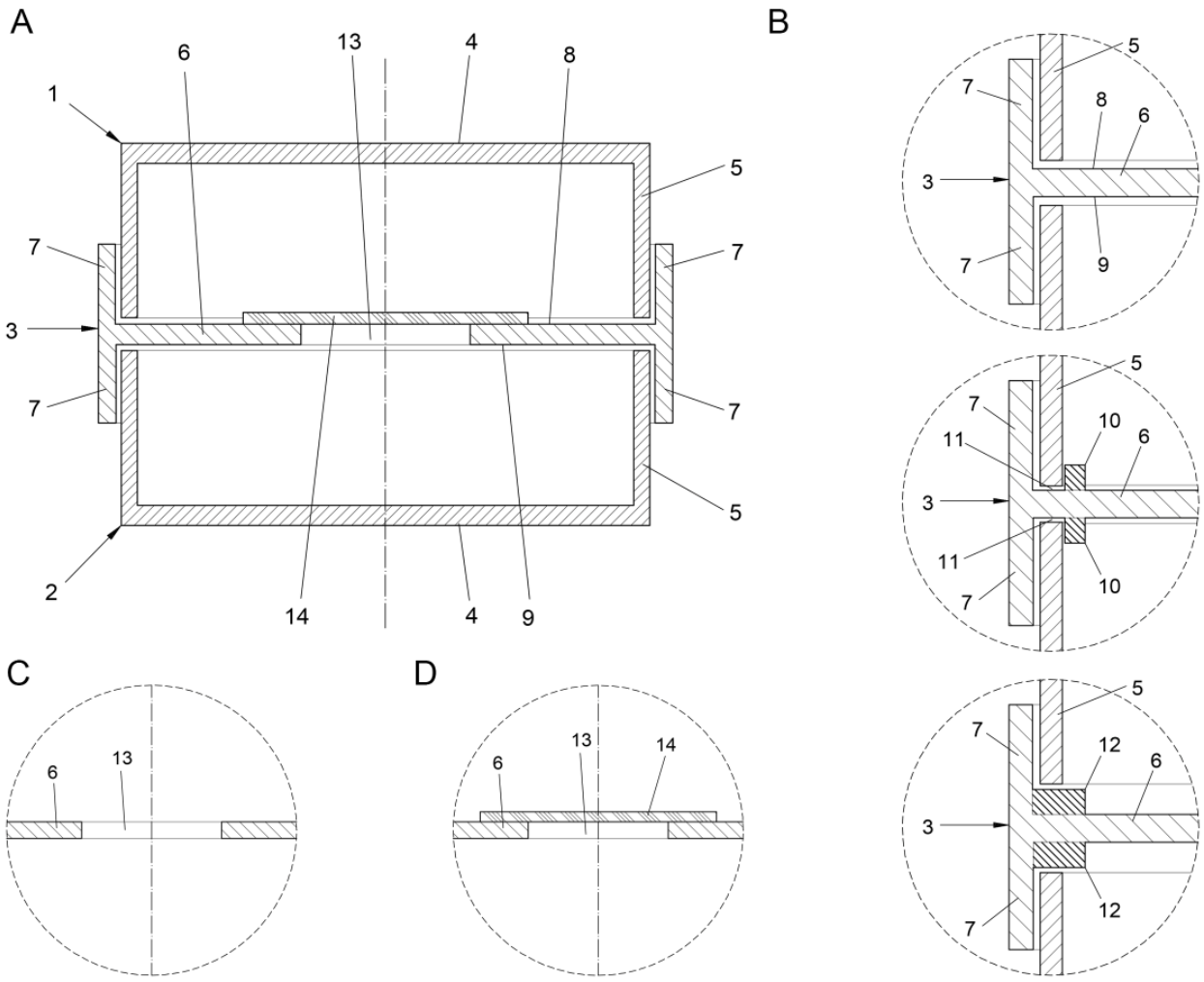
Annex to CHAPTER II

Supplementary material

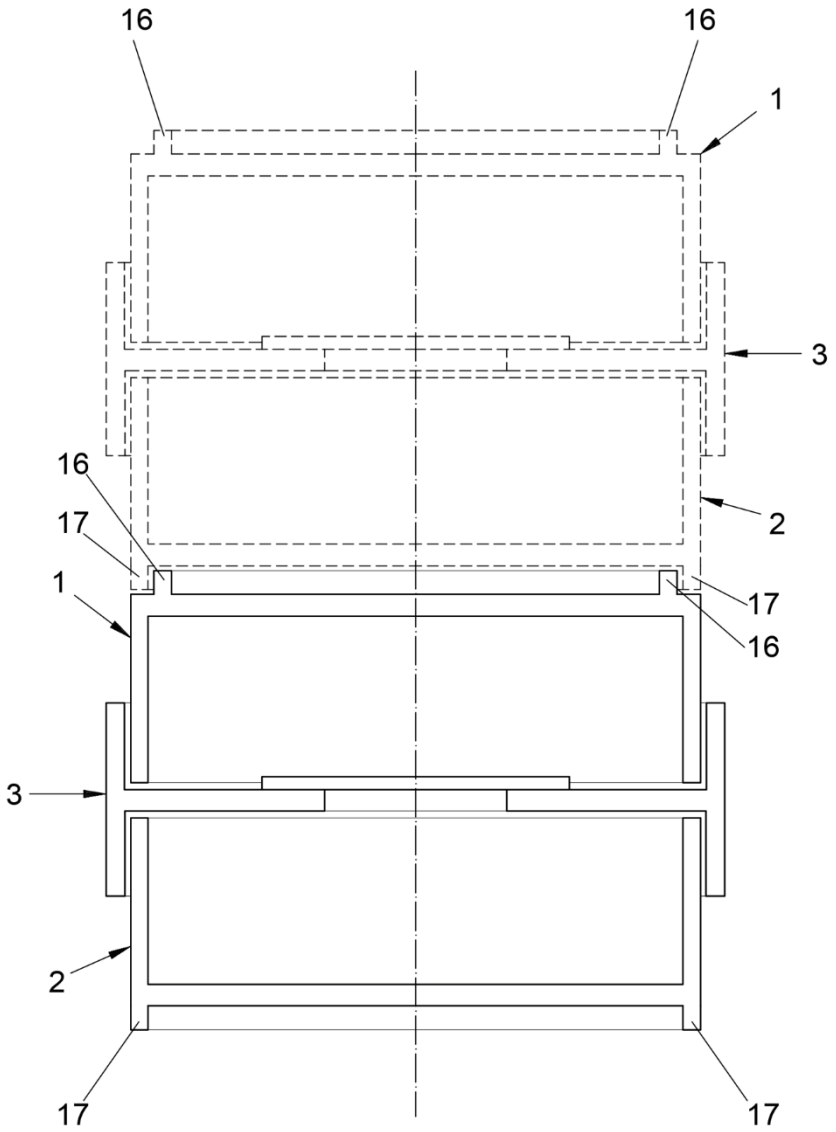
SUPPLEMENTARY MATERIALS



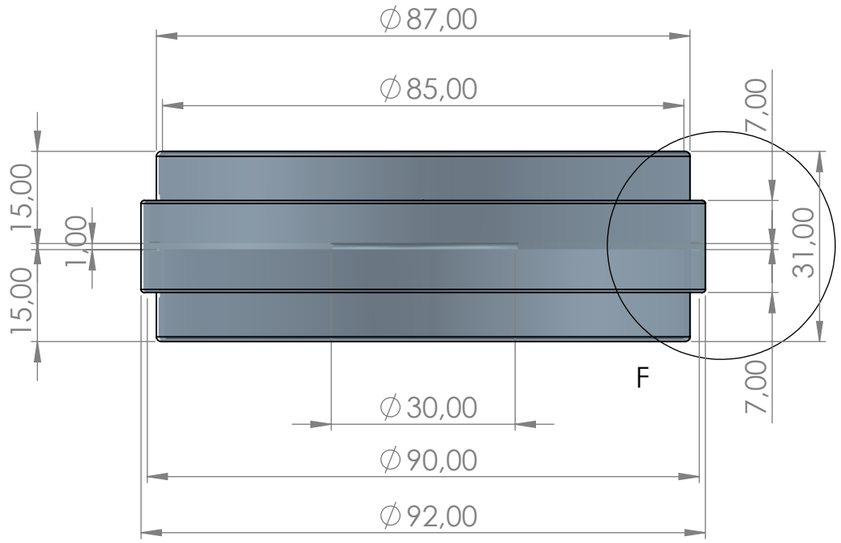
Supplementary Figure SF1. Explosive View. Vented VOC Chamber. 1: upper plate; 2: lower plate; 3: central piece; 4: upper and lower walls (plates); 5: perimeter wall (plates); 6: intermediate wall (central piece); 7: lateral walls (central piece); 8: upper face (not shown. See SF2); 9: lower face (not shown. See SF2); 10: internal edge (not shown. See SF2); 11: shelter (not shown. See SF2); 12: ventilation flanges; 13: central hole; 14: membrane or filter (optional. Not used in this study); 15: septum for divided Petri dishes (optional. Not used in this study).



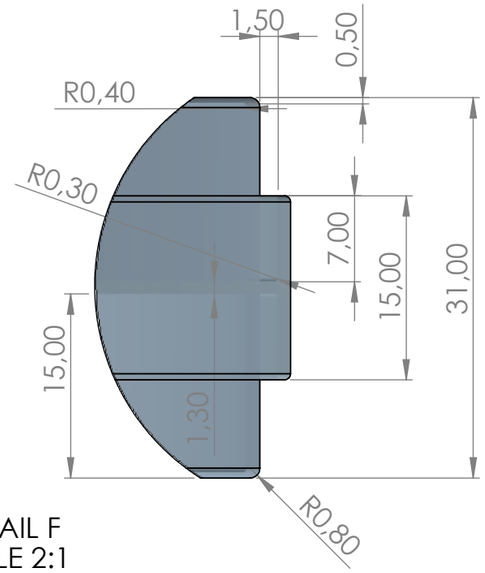
Supplementary Figure SF2. (A) Frontal Cross-section. Non-Vented VOC Chamber. **(B)** Detail of union between plates and central piece in Non-Vented VOC Chambers (upper circle), Non-Vented VOC Chamber with locking system (central circle), Vented VOC Chamber with flanges (lower circle). **(C)** Detail of central hole without membrane or filter. **(D)** Detail of central hole with membrane or filter attached. 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 (central piece); 9: lower face (central piece); 10: internal edge (locking system. Optional. Not used in this study); 11: shelter (locking system. Optional. Not used in this study); 12: ventilation flanges; 13: central hole; 14: membrane or filter (optional. Not used in this study).



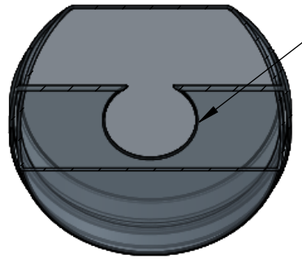
Supplementary Figure SF3. Frontal Cross-section of two Non-Vented VOC Chambers stacked. 1: upper plate; 2: lower plate; 3: central piece; 16: upper inner ring for stacking chambers (optional. Not used in this study); 17: lower outer ring for stacking chambers (optional. Not used in this study).



FRONT VIEW

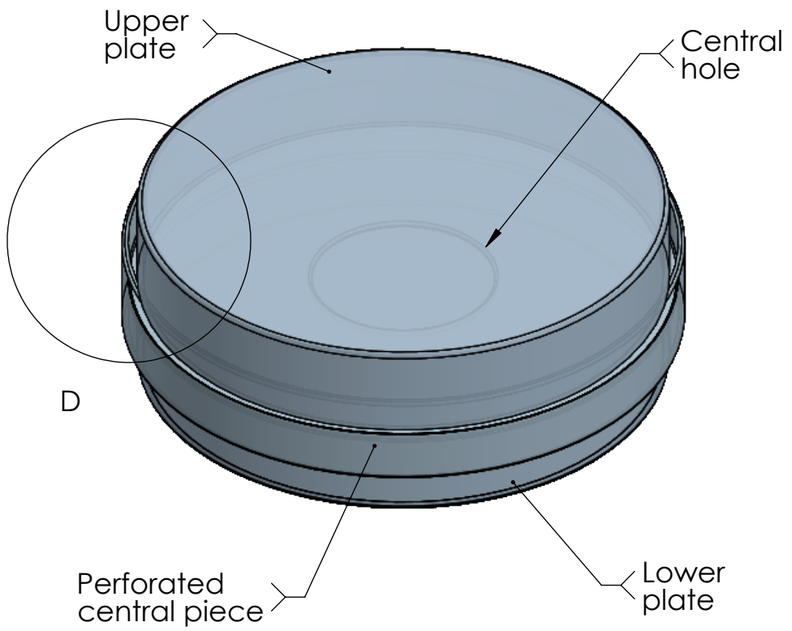


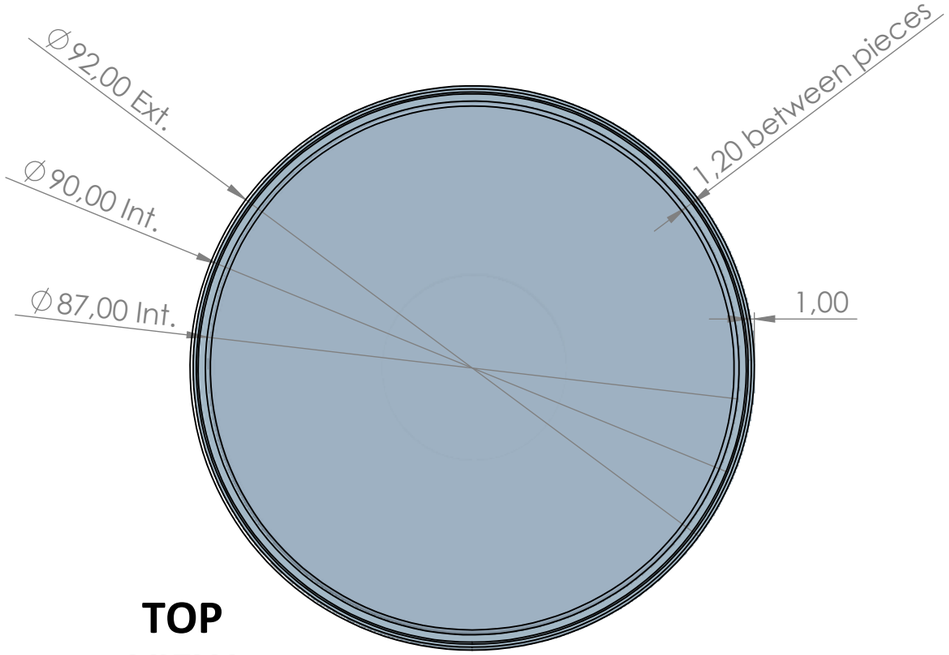
DETAIL F
SCALE 2:1



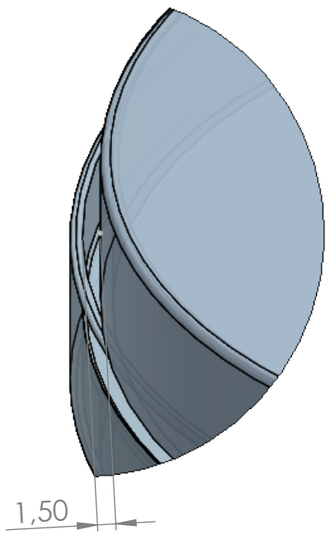
SECTION B-B
SCALE 1 : 2

DETAIL UNION BETWEEN CENTRAL PIECE AND PLATE





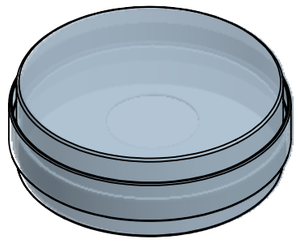
TOP VIEW



DETAIL D
SCALE 2:1

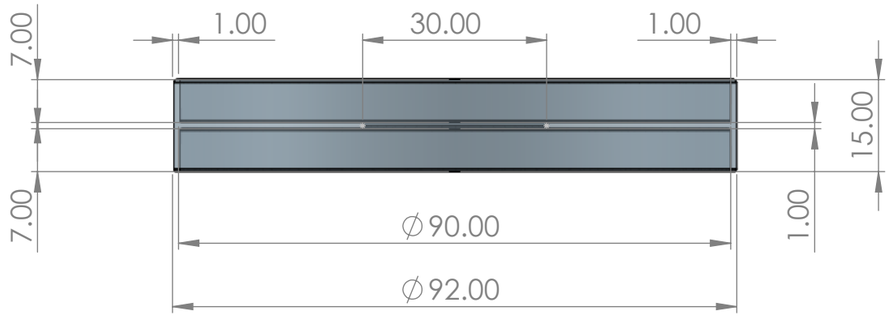
AUXILIARY VIEW

UNIVERSITY OF LEÓN			
VOLATILE ORGANIC COMPOUNDS CHAMBER - VOC CHAMBER			
PLAN:	Full Non - Vented VOC Chamber		
SCALA:	1 : 1	Plan drawn by: Mario del Val Martínez	Plan: **1A**
DATE:	April 2020		



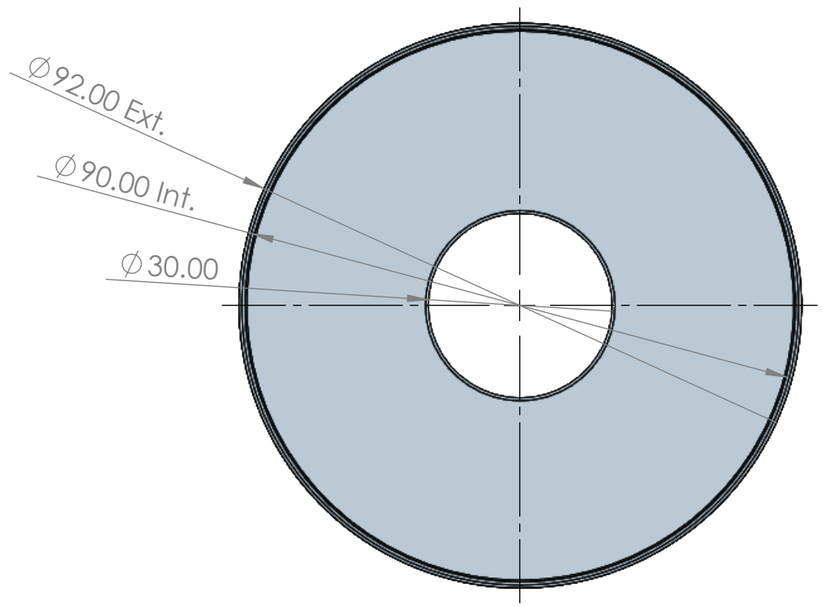
Scale:
1/2

FULL CHAMBER

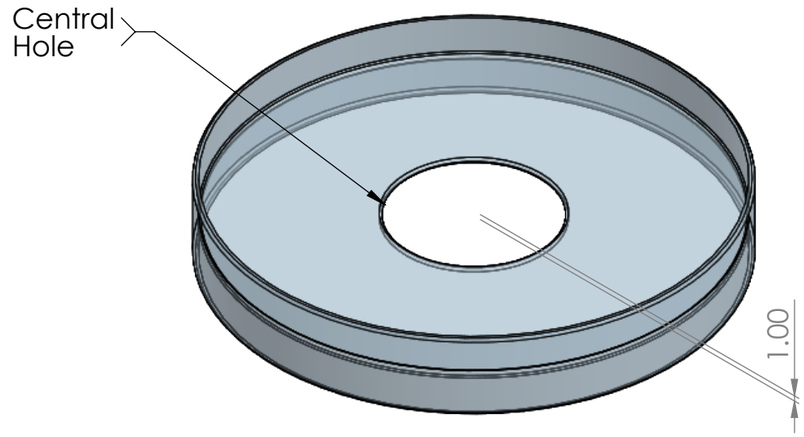


FRONT VIEW

1 x Central piece per full chamber

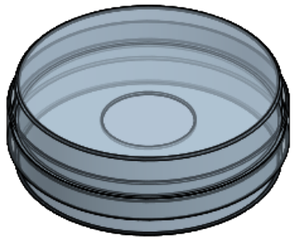


TOP VIEW



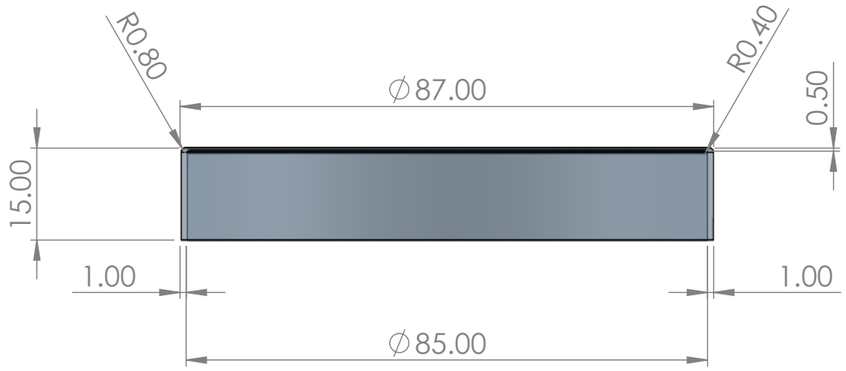
AUXILIARY VIEW

UNIVERSITY OF LEÓN			
VOLATILE ORGANIC COMPOUNDS CHAMBER VOC CHAMBER			
PLAN:	Perforated central piece		
SCALE:	1 : 1	Plan drawn by: Mario del Val Martínez	Plan: **2A**
DATE:	April 2020		



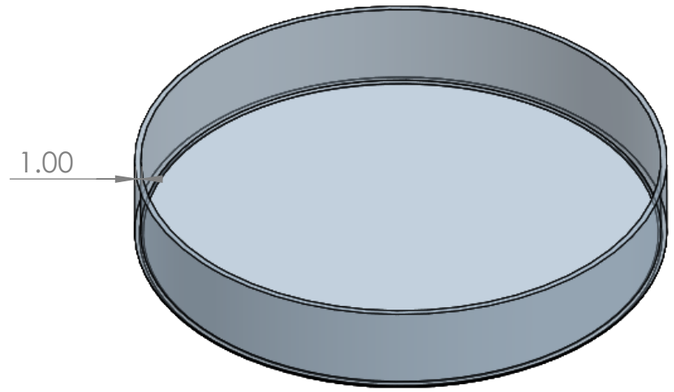
Scale:
1/2

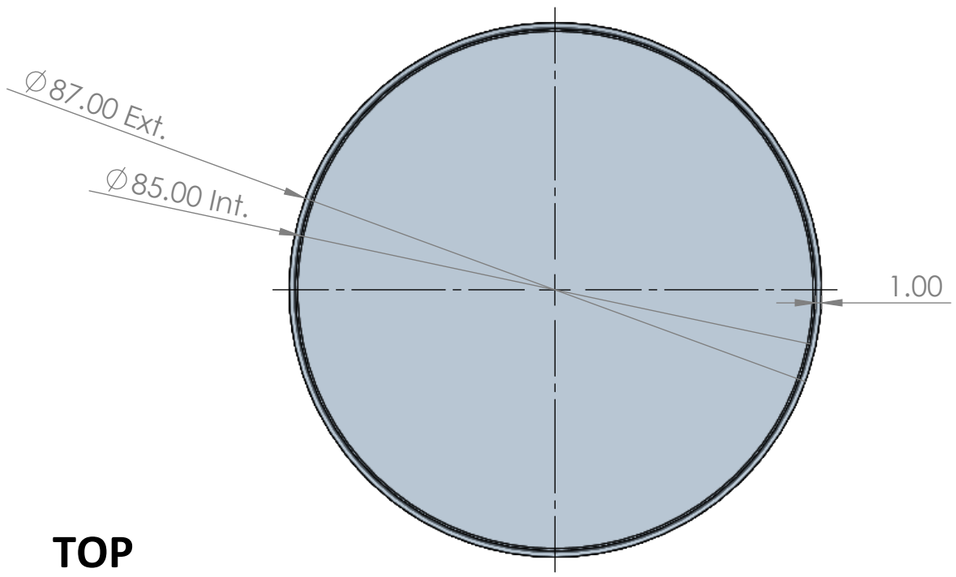
FULL CHAMBER



FRONT VIEW

2 x Petri plates per full chamber

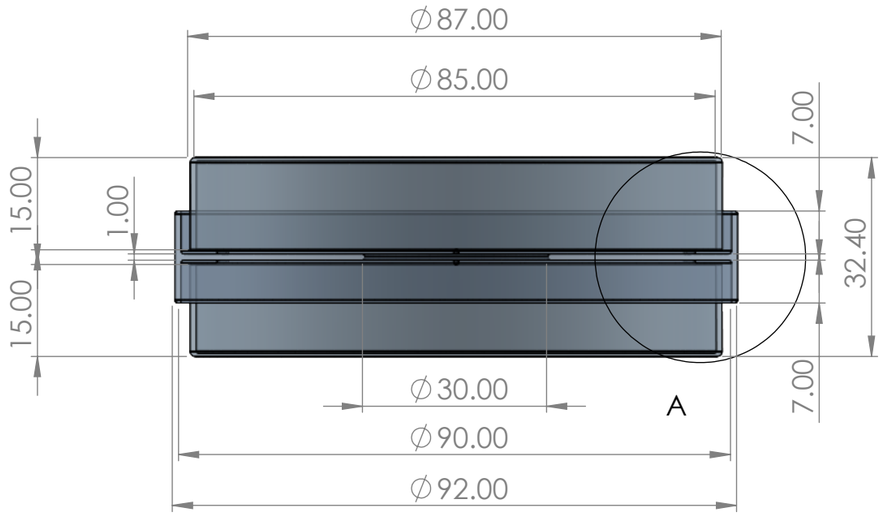




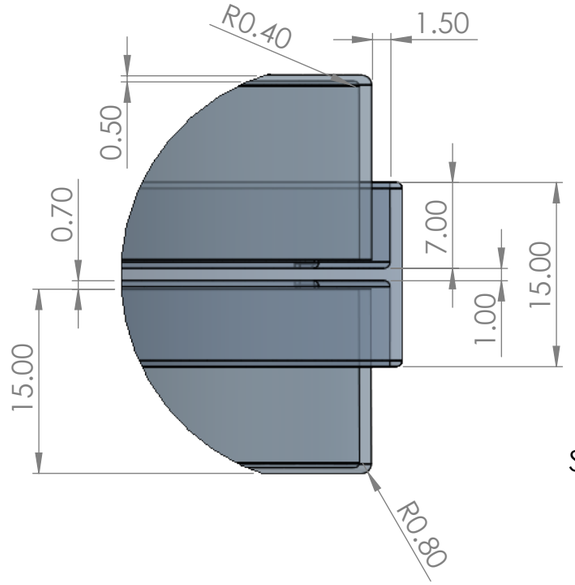
TOP VIEW

AUXILIARY VIEW

UNIVERSITY OF LEÓN			
VOLATILE ORGANIC COMPOUNDS CHAMBER VOC CHAMBER			
PLAN:	Plates		
SCALE:	1 : 1	Plan drawn by: Mario del Val Martínez	Plan: **3A**
DATE:	April 2020		

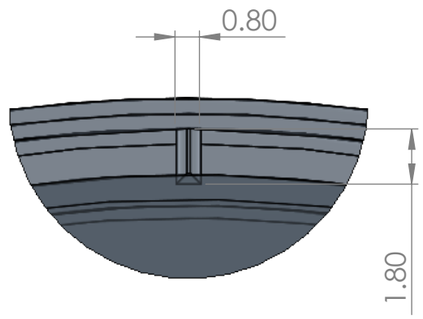


FRONT VIEW



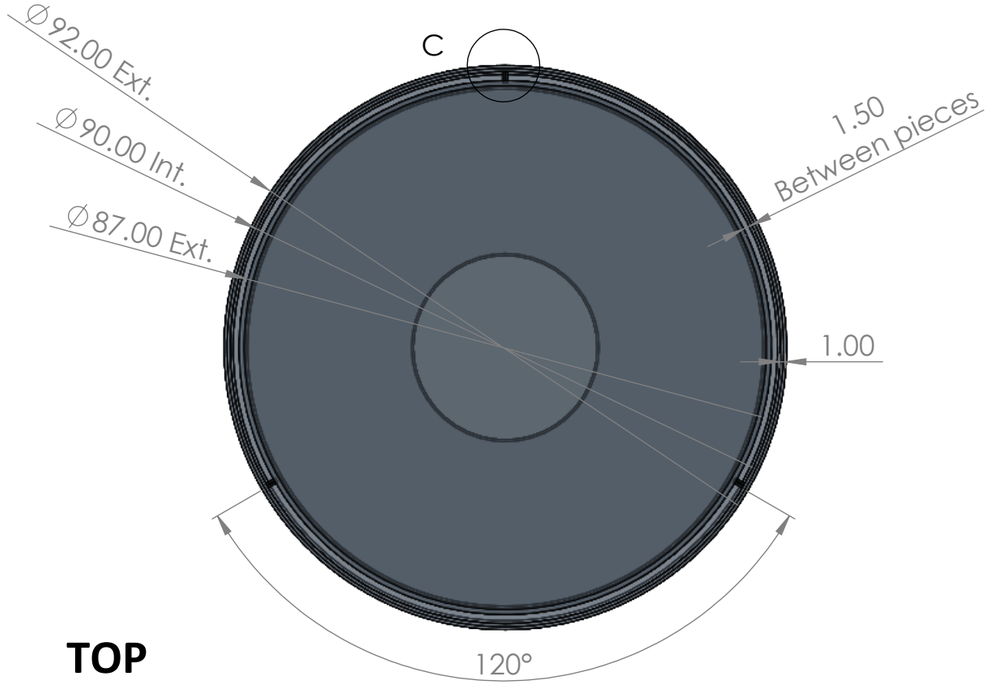
DETAIL A
SCALE 2:1

AUXILIARY VIEW

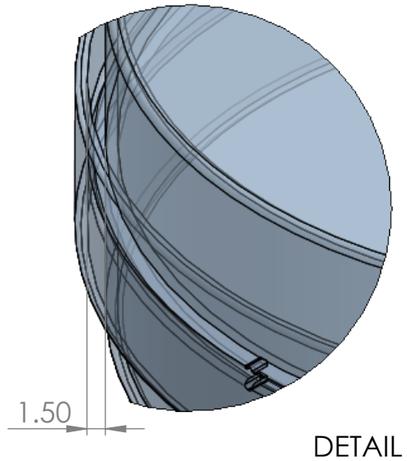


DETAIL C
SCALE 5:1

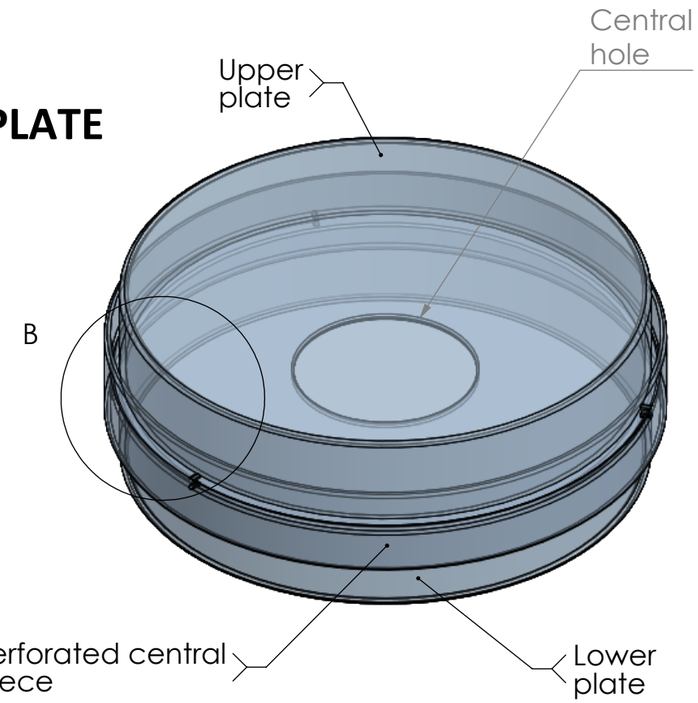
DETAIL UNION BETWEEN CENTRAL PIECE AND PLATE



TOP VIEW

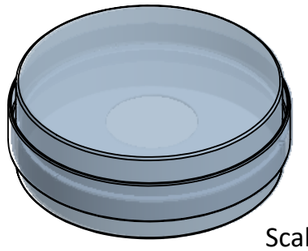


DETAIL B
SCALE 2:1



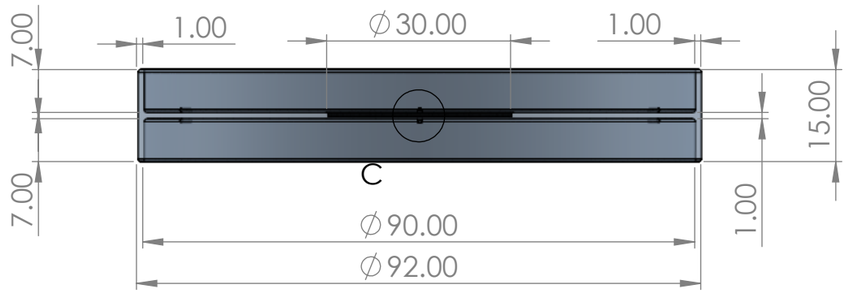
UNIVERSITY OF LEÓN			
VOLATILE ORGANIC COMPOUNDS CHAMBER VOC CHAMBER			
PLAN:	Full Vented VOC Chamber		
SCALA: 1 : 1		Plan drawn by: Mario del Val Martínez	Plan: **1B**
DATE: April 2020			

1 x Central piece per full chamber

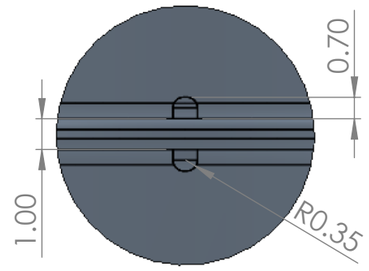


Scale: 1/2

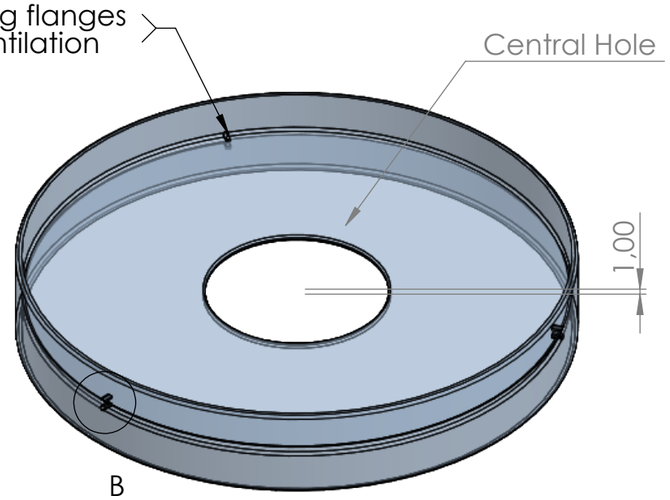
FULL CHAMBER



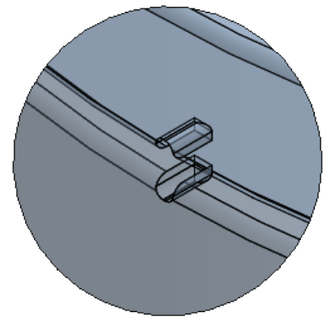
FRONT VIEW



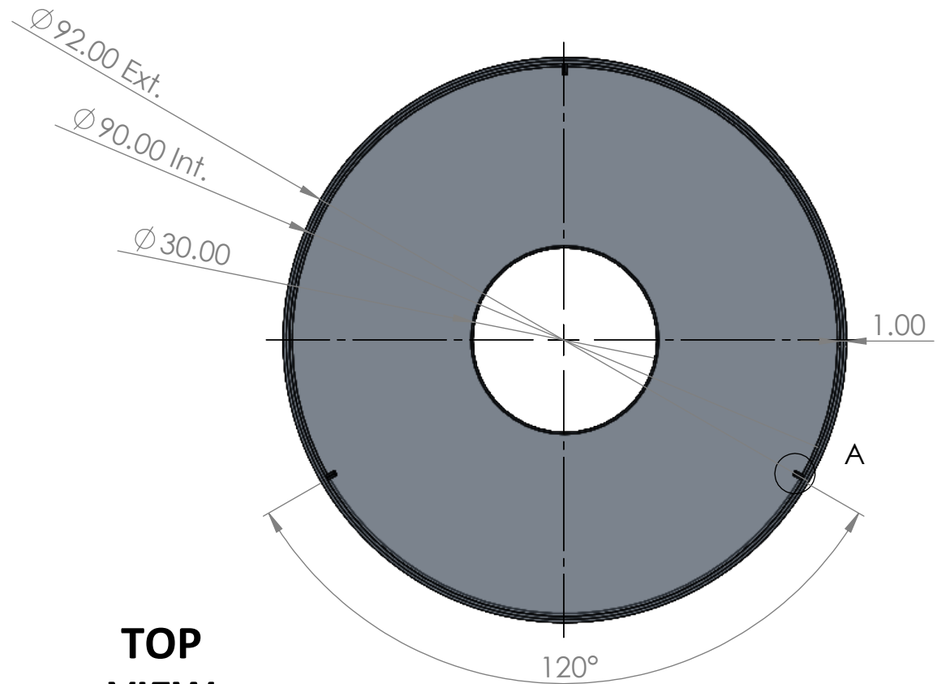
DETAIL C
SCALE 5:1



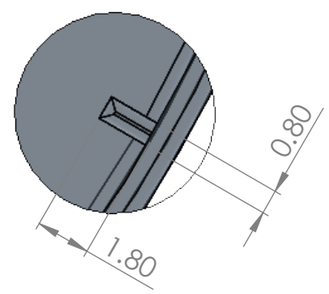
AUXILIARY VIEW



DETAIL B
SCALE 5:1



TOP VIEW



DETAIL A
SCALE 5 :1

UNIVERSITY OF LEÓN			
VOLATILE ORGANIC COMPOUNDS CHAMBER VOC CHAMBER			
PLAN:	Perforated central piece		
SCALA:	1 : 1	Plan drawn by: Mario del Val Martínez	Plan: **2B**
DATE:	April 2020		

Supplementary table ST1. Experiment 1. Growth (mm) and Percentage of inhibition (PI) of *R. solani* R43 confronted by *T. harzianum* T34, T34-5.27, E20 and E20-5.7 using DDS method (first row), Non-Vented VOC Chambers (second row), and Vented VOC Chambers (third row); after 1, 2, 3, and 4 days post-inoculation. PI was calculated in relation to the control using the following equation proposed by Gotor-Vila et al. (2017): [(C-T) / C] x 100. Where C is the diameter of the controls and T that of the treatments. PI is expressed as the mean of the four replicates. P values were obtained from growth results using one-way analysis of variance (ANOVA), after confirmation of normality and equality of variances, and were contrasted using Tukey's *post hoc* test. Capital letters represent the differences between treatments with P ≤ 0.05; P ≤ 0.01; and P ≤ 0.001, respectively. All statistical analyses were performed using SPSS 24.0 (IBM).

R43 EXPERIMENT 1

		day 1						day 2						day 3						day 4					
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Double Dish Set	CC	17.92	1.23	-	A	A	A	34.9167	4.11	-	A	A	A	45.50	3.19	-	A	A	A	54.58	2.63	-	A	A	A
	R43/T34	10.00	1.05	44.19	C	BC	BC	13.25	1.91	62.05	BC	B	B	15.00	2.94	67.03	B	B	B	16.00	3.47	70.69	B	B	B
	R43/T34-5.27	6.92	0.17	61.40	D	D	C	9.16667	1.35	73.75	D	B	B	10.58	2.04	76.74	B	B	B	12.25	3.41	77.56	B	B	B
	R43/E20	12.25	1.29	31.63	B	B	B	15.9167	3.98	54.42	B	B	B	17.58	5.03	61.36	B	B	B	18.42	5.82	66.26	B	B	B
	R43/E20-5.7	7.50	0.58	58.14	D	CD	C	11.5833	1.69	66.83	BC	B	B	14.75	2.32	67.58	B	B	B	17.08	2.91	68.70	B	B	B
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Non-Vented VOC Chaber	CC	19.75	0.74	-	A	A	A	36.67	1.12	-	A	A	A	51.25	1.50	-	A	A	A	63.58	1.71	-	A	A	A
	R43/T34	13.42	0.32	32.07	C	B	B	19.75	0.32	46.14	C	C	BC	24.50	1.40	52.20	C	C	BC	33.00	3.32	48.10	BC	BC	BC
	R43/T34-5.27	8.08	0.32	59.07	D	C	C	13.75	1.03	62.50	D	D	D	19.67	1.31	61.63	D	D	C	28.00	2.19	55.96	CD	C	BC
	R43/E20	15.08	0.83	23.63	B	B	B	23.42	0.63	36.14	B	B	B	29.25	0.63	42.93	B	B	B	36.50	1.11	42.60	B	B	B
	R43/E20-5.7	9.08	0.63	54.01	D	C	C	15.83	0.58	56.82	D	D	CD	20.33	1.45	60.33	D	D	C	27.56	1.68	56.66	D	C	C
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Vented VOC Chaber	CC	19.08	1.00	-	A	A	A	40.83	0.79	-	A	A	A	60.83	1.69	-	A	A	A	71.75	1.07	-	A	A	A
	R43/T34	19.00	0.98	0.44	A	A	A	38.50	3.49	5.71	A	A	A	53.08	7.05	12.74	A	A	A	63.08	2.57	12.08	B	B	B
	R43/T34-5.27	10.00	0.27	47.60	B	B	B	19.25	0.32	52.86	B	B	B	26.67	0.47	56.16	B	B	B	32.50	0.58	54.70	C	C	C
	R43/E20	19.67	1.31	-3.06	A	A	A	41.11	4.74	4.90	A	A	A	51.89	7.82	14.70	A	A	A	65.11	2.59	9.25	B	B	AB
	R43/E20-5.7	11.75	0.50	38.43	B	B	B	21.75	1.40	46.73	B	B	B	29.58	2.47	51.37	B	B	B	34.75	2.13	51.57	C	C	C

Supplementary table ST2. Experiment 2. Growth (mm) and Percentage of inhibition (PI) of *R. solani* R43 confronted by *T. harzianum* T34, T34-5.27, E20, and E20-5.7 using DDS method (first row), Non-Vented VOC Chambers (second row), and Vented VOC Chambers (third row); after 1, 2, 3, and 4 days post-inoculation. PI was calculated in relation to the control using the following equation proposed by Gotor-Vila et al. (2017): [(C-T) / C] x 100. Where C is the diameter of the controls and T that of the treatments. PI is expressed as the mean of the four replicates. P values were obtained from growth results using one-way analysis of variance (ANOVA), after confirmation of normality and equality of variances, and were contrasted using Tukey's *post hoc* test. Capital letters represent the differences between treatments with P ≤ 0.05; P ≤ 0.01; and P ≤ 0.001, respectively. All statistical analyses were performed using SPSS 24.0 (IBM).

R43 EXPERIMENT 2

		day 1						day 2						day 3						day 4					
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Double Dish Set	CC	15.33	0.47	-	A	A	A	31.42	1.03	-	A	A	A	49.25	2.63	-	A	A	A	62.92	3.30	-	A	A	A
	R43/T34	10.67	0.72	30.43	B	B	BC	15.17	1.11	51.72	C	C	BC	17.00	2.09	65.48	C	BC	BC	18.58	2.46	70.46	C	BC	BC
	R43/T34-5.27	7.00	0.47	54.35	C	C	D	10.58	1.29	66.31	D	D	D	12.58	2.51	74.45	C	C	C	13.58	3.38	78.41	C	C	C
	R43/E20	11.50	1.11	25.00	B	B	B	18.92	1.26	39.79	B	B	B	22.83	2.76	53.64	B	B	B	27.17	3.25	56.82	B	B	B
	R43/E20-5.7	8.50	0.43	44.57	C	C	CD	13.33	0.98	57.56	C	CD	CD	16.67	1.76	66.16	C	BC	BC	19.42	3.01	69.14	C	BC	BC
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Non-Vented VOC Chaber	CC	15.67	0.72	-	A	A	A	38.42	1.13	-	A	A	A	50.25	2.41	-	A	A	A	65.00	3.80	-	A	A	A
	R43/T34	11.92	0.32	23.94	C	C	B	19.83	0.64	48.37	C	C	C	26.67	1.36	46.93	C	C	BC	34.25	3.01	47.31	BC	BC	B
	R43/T34-5.27	7.50	0.33	52.13	E	E	D	13.25	0.32	65.51	E	E	E	19.42	0.57	61.36	D	D	D	25.50	0.88	60.77	D	D	C
	R43/E20	13.33	0.47	14.89	B	B	B	23.83	0.58	37.96	B	B	B	31.08	0.74	38.14	B	B	B	38.08	0.96	41.41	B	B	B
	R43/E20-5.7	9.50	0.33	39.36	D	D	C	16.67	0.98	56.62	D	D	D	23.67	1.25	52.90	C	C	CD	30.42	1.29	53.21	CD	CD	BC
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Vented VOC Chaber	CC	14.75	1.40	-	A	A	A	31.92	1.42	-	A	A	AB	51.26	2.33	-	A	A	A	60.67	0.98	-	A	A	A
	R43/T34	14.33	1.28	2.82	A	A	A	29.67	1.61	7.05	A	A	AB	48.67	4.21	4.58	A	A	A	53.67	4.15	11.54	AB	AB	AB
	R43/T34-5.27	9.33	0.27	36.72	B	B	B	16.58	1.87	48.04	C	C	C	23.58	3.66	53.76	C	C	B	29.58	3.07	51.24	C	C	C
	R43/E20	15.42	0.42	-4.52	A	A	A	30.92	2.47	3.13	A	A	A	46.83	5.27	8.17	A	A	A	56.58	6.29	6.73	A	A	AB
	R43/E20-5.7	10.58	0.50	28.25	B	B	B	23.22	0.51	32.64	B	B	BC	35.22	1.02	30.94	B	B	AB	45.33	1.45	25.27	B	B	B

Supplementary table ST3. Experiment 1. Growth (mm) and Percentage of inhibition (PI) of *F. oxysporum* F3 confronted by *T. harzianum* T34, T34-5.27, E20, and E20-5.7 using DDS method (first row), Non-Vented VOC Chambers (second row), and Vented VOC Chambers (third row); after 3, 5, and 7 days post-inoculation. PI was calculated in relation to the control using the following equation proposed by Gotor-Vila et al. (2017): [(C-T) / C] x 100. Where C is the diameter of the controls and T that of the treatments. PI is expressed as the mean of the four replicates. P values were obtained from growth results using one-way analysis of variance (ANOVA), after confirmation of normality and equality of variances, and were contrasted using Tukey's *post hoc* test. Capital letters represent the differences between treatments with P ≤ 0.05; P ≤ 0.01; and P ≤ 0.001, respectively. All statistical analyses were performed using SPSS 24.0 (IBM).

F3 EXPERIMENT 1		day 3						day 5						day 7					
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Double Dish Set	CC	30.25	0.96	-	A	A	A	46.08	1.69	-	A	A	A	57.67	4.25	-	A	A	A
	F3/T34	19.00	2.19	37.19	BC	BC	BC	31.92	4.38	30.74	BC	BC	B	46.50	2.67	19.36	B	B	AB
	F3/T34-5.27	16.58	1.29	45.18	C	C	C	28.83	0.43	37.43	C	C	B	43.08	0.92	25.29	B	B	B
	F3/E20	22.08	1.17	27.00	B	B	B	35.25	1.10	23.51	B	B	B	48.42	1.66	16.04	B	B	AB
	F3/E20-5.7	18.50	1.60	38.84	C	BC	BC	32.25	1.52	30.02	BC	BC	B	41.67	4.51	27.75	B	B	B
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Non-Vented VOC Chaber	CC	25.67	1.72	-	A	A	A	39.58	4.03	-	A	A	A	53.67	3.99	-	A	A	A
	F3/T34	24.00	0.98	6.49	A	AB	AB	38.25	0.88	3.37	A	A	AB	50.22	0.38	6.42	A	AB	A
	F3/T34-5.27	20.50	0.33	20.13	B	C	B	31.58	0.79	20.21	B	B	B	44.67	0.58	16.77	B	B	A
	F3/E20	25.42	0.63	0.97	A	A	A	38.42	0.96	2.95	A	A	AB	52.11	0.84	2.90	A	A	A
	F3/E20-5.7	21.58	0.83	15.91	B	BC	B	32.67	1.12	17.47	B	B	AB	44.33	0.58	17.39	B	B	A
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Vented VOC Chaber	CC	26.25	0.57	-	C	C	C	37.67	0.88	-	C	B	BC	49.78	2.52	-	C	B	B
	F3/T34	28.92	0.79	-10.16	B	B	B	42.50	1.69	-12.83	B	A	AB	57.33	0.33	-15.18	B	A	A
	F3/T34-5.27	23.33	0.47	11.11	D	D	D	34.33	1.39	8.85	D	BC	C	44.42	2.11	10.77	D	C	B
	F3/E20	31.67	0.61	-20.63	A	A	A	46.08	1.26	-22.35	A	A	A	62.44	0.69	-25.45	A	A	A
	F3/E20-5.7	22.50	0.64	14.29	D	D	D	32.67	0.67	13.27	D	C	C	43.00	0.33	13.62	D	C	B

Supplementary table ST4. Experiment 2. Growth (mm) and Percentage of inhibition (PI) of *F. oxysporum* F3 confronted by *T. harzianum* T34, T34-5.27, E20, and E20-5.7 using DDS method (first row), Non-Vented VOC Chambers (second row), and Vented VOC Chambers (third row); after 3, 5, and 7 days post-inoculation. PI was calculated in relation to the control using the following equation proposed by Gotor-Vila et al. (2017): [(C-T) / C] x 100. Where C is the diameter of the controls and T that of the treatments. PI is expressed as the mean of the four replicates. P values were obtained from growth results using one-way analysis of variance (ANOVA), after confirmation of normality and equality of variances, and were contrasted using Tukey's *post hoc* test. Capital letters represent the differences between treatments with P ≤ 0.05; P ≤ 0.01; and P ≤ 0.001, respectively. All statistical analyses were performed using SPSS 24.0 (IBM).

F3 EXPERIMENT 2		day 3						day 5						day 7					
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Double Dish Set	CC	26.17	1.29	-	A	A	A	41.08	0.88	-	A	A	A	53.67	2.16	-	A	A	A
	F3/T34	20.17	1.40	22.93	BC	BC	BC	30.92	1.87	24.75	B	B	B	42.75	3.14	20.34	B	BC	B
	F3/T34-5.27	16.83	0.64	35.67	D	D	C	26.33	1.44	35.90	C	C	B	37.42	1.13	30.28	C	C	B
	F3/E20	20.92	1.00	20.06	B	B	B	31.92	2.15	22.31	B	B	B	43.92	1.85	18.17	B	B	B
	F3/E20-5.7	17.83	0.96	31.85	CD	CD	BC	29.00	1.52	29.41	BC	BC	B	40.25	1.73	25.00	BC	BC	B
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Non-Vented VOC Chaber	CC	25.42	0.57	-	A	A	A	38.42	1.20	-	A	A	A	52.75	1.26	-	A	A	A
	F3/T34	22.17	0.79	12.79	B	B	B	33.83	0.43	11.93	C	B	B	47.54	1.23	9.87	B	B	B
	F3/T34-5.27	18.25	0.17	28.20	C	C	C	28.83	0.43	24.95	E	C	C	41.00	0.33	22.27	C	C	C
	F3/E20	24.92	0.57	1.97	A	A	A	36.50	0.79	4.99	B	A	A	48.58	1.00	7.90	B	B	B
	F3/E20-5.7	19.42	0.57	23.61	C	C	C	30.58	0.50	20.39	D	C	C	43.17	0.58	18.17	C	C	C
		GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS			GROWTH (mm)		INHIBITION (%)	STATISTICS		
	TREATMENT	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001	Mean	SD	Mean	P ≤ 0.05	P ≤ 0.01	P ≤ 0.001
Vented VOC Chaber	CC	24.25	0.50	-	B	B	B	38.75	0.74	-	B	B	A	52.58	2.28	-	B	B	A
	F3/T34	26.75	0.50	-10.31	A	A	AB	41.08	1.20	-6.02	AB	AB	A	54.83	1.55	-4.28	B	AB	A
	F3/T34-5.27	20.78	0.57	15.12	C	C	C	31.89	1.12	18.28	C	C	B	42.56	0.61	19.49	C	C	B
	F3/E20	27.67	0.27	-14.09	A	A	A	42.58	1.62	-9.89	A	A	A	58.67	1.00	-11.57	A	A	A
	F3/E20-5.7	21.17	1.29	12.71	C	C	C	32.42	1.83	16.34	C	C	B	44.50	2.01	15.37	C	C	B

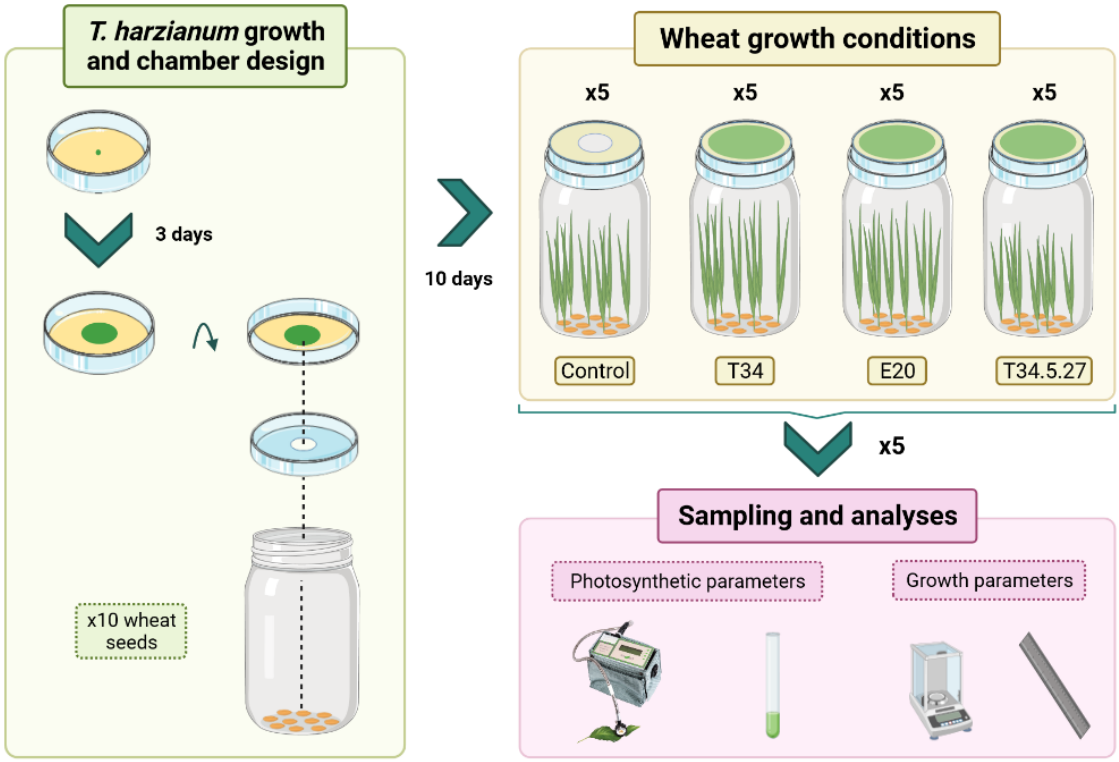
CHAPTER III

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

Highlight

Novel chamber provides a simple, homogeneous, and replicable method to evaluate plant-microbe volatile interactions. *Trichorderma harzianum* volatiles, including squalene- and trichodiene-overproducing strains, affected wheat development without interfering photosynthetic parameters.

Graphical abstract



Abstract

The interest in plant-microbe interactions mediated by Biogenic Volatile Organic Compounds (BVOCs) is increasing. Nevertheless, researchers still face several limitations in the performance of these assays 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. The technology was tested by evaluating the effects on wheat development of BVOCs produced by three *Trichoderma harzianum* strains, a wild type and two transformants that overproduce trichodiene and squalene respectively. Both the wild type and the squalene-overproducer enhanced fresh weight and length of the aerial part, but reduced root dry weight. Interestingly, no differences were found between them, suggesting that squalene overproduction does not modify the activity of *T.*

harzianum BVOCs on wheat plantlets. Trichodiene overproduction 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 these 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.

Keywords: BVOCs, plant-microbe, plant-microbe VOC Chamber, squalene, technology, *Trichoderma,* trichodiene, volatile interactions, Volatile Organic Compounds.

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

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 (Herrman, 2010), 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 (Effmert *et al.,* 2012; Quintana-Rodriguez *et al.,* 2015). These volatile dialogues play a significant role in diverse processes such as recognition (Li *et al.,* 2018), communication (Markovic *et al.,* 2019), competition (Hammerbacher *et al.,* 2019) or symbiosis (Werner *et al.,* 2016; Kandasamy *et al.,* 2019).

BVOCs represent an important factor within the establishment of ecological communities (Li *et al.,* 2020), 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 (Wenke *et al.,* 2019) and several fungi, including *Trichoderma* (Hung *et al.,* 2013; Sood *et al.,* 2020), mycorrhizal fungi (Ditengou *et al.,* 2015), and others (Sánchez-López *et al.,* 2016*a*; Moisan *et al.,* 2019). Furthermore, microbial BVOCs can modulate flowering (Sánchez-

López *et al.*, 2016*a*), root architecture in plants (Malmierca *et al.*, 2015*a*; Schenkel *et al.*, 2018), as well as their ability to overcome herbivore attacks (Cordovez *et al.*, 2017) and pathogen infections via the induction of different defensive pathways (Riedlmeier *et al.*, 2017; Frank *et al.*, 2021). Microbial BVOCs are able to modulate plant metabolism in several ways, for instance by inducing the production or accumulation of specific compounds (Ezquer *et al.*, 2010; Li *et al.*, 2011), affecting photosynthetic processes (Ameztoy *et al.*, 2019), nutrient uptake (Martínez-Medina *et al.*, 2017), auxin induction, or cell wall remodelling (Lee *et al.*, 2019). Besides, microbial BVOCs have been demonstrated to induce changes at both transcriptional and post-transcriptional levels on plants (Ameztoy *et al.*, 2019; García-Gómez *et al.*, 2019). 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 (Quintana-Rodriguez *et al.*, 2015; Frank *et al.*, 2021).

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 (Lo Cantore *et al.*, 2015; Álvarez-García *et al.*, 2021) to growth inhibition (Lo Cantore *et al.*, 2015; Myo *et al.*, 2019), as well as physiological and morphological changes (Giorgio *et al.*, 2015). Interestingly, some researches have demonstrated that the effects exerted by the volatilome of a specific strain can vary widely depending on the culture conditions (Lo Cantore *et al.*, 2015; Speckbacher *et al.*, 2020; Álvarez-García *et al.*, 2021), indicating that the outcome of volatile interactions seem to differ regarding the environmental conditions where they take place (Kai *et al.*, 2016; Álvarez-García *et al.*, 2021). Moreover, Li *et al.*, (2020) demonstrated that soil bacteria present a collective antagonism against fungal communities, and proposed that volatile emissions play a key role in the strategy of bacterial niche-defence against invading fungi, accounting for a significant part of soil fungistasis.

As it can be seen from the above references, during the last years the interest in the study of microbe-microbe and plant-microbe 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 (Kai *et al.*, 2016), 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 (Dukare *et al.*, 2019; Song *et al.*, 2019; Thomas *et al.*, 2020). Therefore, some methodologies have been developed to this day, a few relying on specific materials and others adapting lab material intended originally for other purposes.

Concerning plant-microbe interactions mediated by BVOCs, two main groups of methodologies can be drawn (Kai *et al.*, 2016). One, where the volatiles passively diffuse to the headspace (Passive Diffusion Systems) (Malmierca *et al.*, 2015*a*; Quintana-Rodriguez *et al.*, 2015; Wenke *et al.*, 2019), and other, where volatiles are actively channelled from the emitter to the plants (Dynamic Air Stream Systems) (Kai and Piechulla, 2009). These groups can be further subdivided attending to other factors, like the use of purified BVOCs (Lee *et al.*, 2019; Frank *et al.*, 2021; Taylor *et al.*, 2021) or the full volatilome produced by growing microbial colonies (Frank *et al.*, 2021; Taylor *et al.*, 2021); as well as the difference between open and closed systems, with or without gas exchange with the exterior (Kai *et al.*, 2016).

With regards to Dynamic Air Stream Systems, these are more complex and expensive technologies, that require pumps, pipes and a power source. The microbial strain is 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 (Kai and Piechulla, 2009; Kai *et al.*, 2016). 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 rare 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 (Kai *et al.*, 2016). This method allows for an open or closed setup, usually by using or not parafilm to seal the plates, and the difference in ventilation conditions proved to modify the effects produced by the BVOCs (Kai and Piechulla, 2009, 2010). 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 (Ameztoy *et al.*, 2019; García-Gómez *et al.*, 2019; Taylor *et al.*, 2021). This method consists of the placement of a lid-free plate with the growing microbial strain into a larger container (whether a jar, a box or a larger Petri plate) where

the plants are grown. This method relies on non-specific material for BVOC evaluation and therefore is not completely standardized. Additionally, modifications to this general setup have been proposed to target specific plant parts. For example, Park *et al.*, (2015) placed the microbial culture in a plastic container under an above-head compartment where the plants were grown. In this way, the microbial BVOCs diffused passively into the soil, affecting specifically the root system.

For a comprehensive review on methodologies regarding the study of plant-microbe interactions mediated by BVOCs, with a special focus on the rhizosphere, please see Kai *et al.*, (2016). In addition, systems for the screening of the global BVOCs produced by microorganisms have been also developed (Guo *et al.*, 2020).

On a larger scale, standard and miniature greenhouses and greenhouse chambers have been used by placing the plants inside them alongside open plates with, either the growing microbial colonies to evaluate the effects of their BVOCs (Song *et al.*, 2019) or the purified compounds (Frank *et al.*, 2021). Similar approaches were selected to evaluate the volatile-mediated induction of resistance between plants (Quintana-Rodriguez *et al.*, 2015).

Notwithstanding the methodologies described so far, the need for new experimental setups in the study of volatile interactions has been highlighted in previous works (Kai *et al.*, 2016; Álvarez-García *et al.*, 2021). 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 evaluate 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 (Malmierca *et al.*, 2015*a*; Lindo *et al.*, 2020).

The aforementioned strains were previously developed and described by our group (Cardoza *et al.*, 2006; Malmierca *et al.*, 2015*a*,*b*). These studies demonstrated that trichodiene overproduction accounts for an increase in antifungal activity via soluble metabolites (Malmierca *et al.*, 2015*a*,*b*), while lower ergosterol levels and the subsequent

accumulation of squalene reduced the bioactivity of the *Trichoderma* soluble metabolites (Cardoza *et al.*, 2006). Additionally, trichodiene overproduction was demonstrated to increase the antifungal activity of *Trichoderma* BVOCs against *Fusarium oxysporum* and *Rhizoctonia solani* (Álvarez-García *et al.*, 2021). This same trichodiene-overproducing strain reduced the biosynthesis of the mycotoxin deoxynivalenol by *Fusarium graminearum* and showed enhanced biocontrol activity against this phytopathogenic fungus (Taylor *et al.*, 2021). For its part, *erg1* silencing led to lower inhibition activity on *F. graminearum and R. solani*. Furthermore, this last modification was responsible for switching the inhibitory activity produced on *F. oxysporum* by the wild type strain into a significant growth-promoting one when the experiments were carried out in vented conditions (Álvarez-García *et al.*, 2021).

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 (Malmierca *et al.*, 2015*a*), and exerting a modest effect on wheat disease response (Taylor *et al.*, 2021). Trichodiene also reduced the differentiation of tomato lateral roots and the growth of its aerial part (Malmierca *et al.*, 2015*b*). 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* (Malmierca *et al.*, 2015*b*). Finally, it has been proposed that the ratio of ergosterol/squalene production affected the ability of *T. harzianum* to colonize plants (Lindo *et al.*, 2020).

The primary objective of this study is to prove that the novel p-mVOC Chamber is a reliable and efficient method to assess plant-microbe volatile interactions. For this purpose, the putative effects of squalene and trichodiene-overproducing strains of *T. harzianum* on early stages of wheat growth and development were tested using p-mVOC Chamber prototypes.

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 is currently in the process of intellectual protection (Submission Number: U202131032; Spanish Office of Patents and Trademarks) and derives from a previous invention developed to perform microbial volatile interactions assays (Álvarez-García *et al.*, 2021), which has been already 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 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).

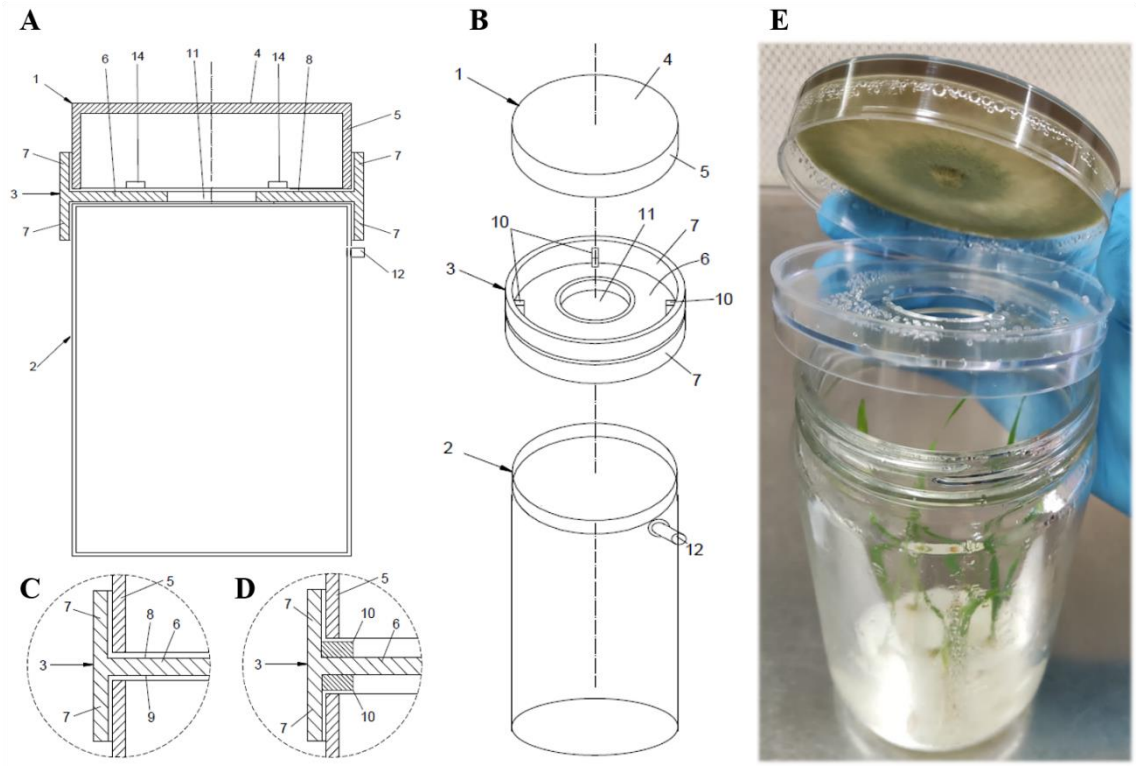


Figure 1. (A) Frontal Cross-section. Non-Vented p-mVOC Chamber. (B) Explosive View. Vented p-mVOC Chamber (not used in this study). (C) Detail of union between plates and central piece in Non-Vented p-mVOC Chambers. (D) Detail of union between plates and central piece with flanges in Vented p-mVOC Chambers (not used in this study). 1. minor plate; 2. major vessel; 3. central piece; 4. upper walls (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 central hole was covered with GF/A glass fibre Whatman filters.

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 Figures S6a and S6b, 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 Fig. S1 to 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 Fig. 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 from now). It is a studied and well-known fungal strain with extensive biocontrol activity (Malmierca *et al.*, 2015*a*; Taylor *et al.*, 2021; Álvarez-García *et al.*, 2021). Additionally, two transformants previously obtained from the aforementioned wild type strain were also tested. On one hand, *T. harzianum* E20 is a transformant strain derived from T34 by silencing the *erg1* gene, which is responsible for encoding squalene epoxidase. This modification led to the accumulation of squalene and subsequent lower ergosterol levels (Cardoza *et al.*, 2006). 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 (Malmierca *et al.*, 2015*a*,*b*).

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 sec (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.

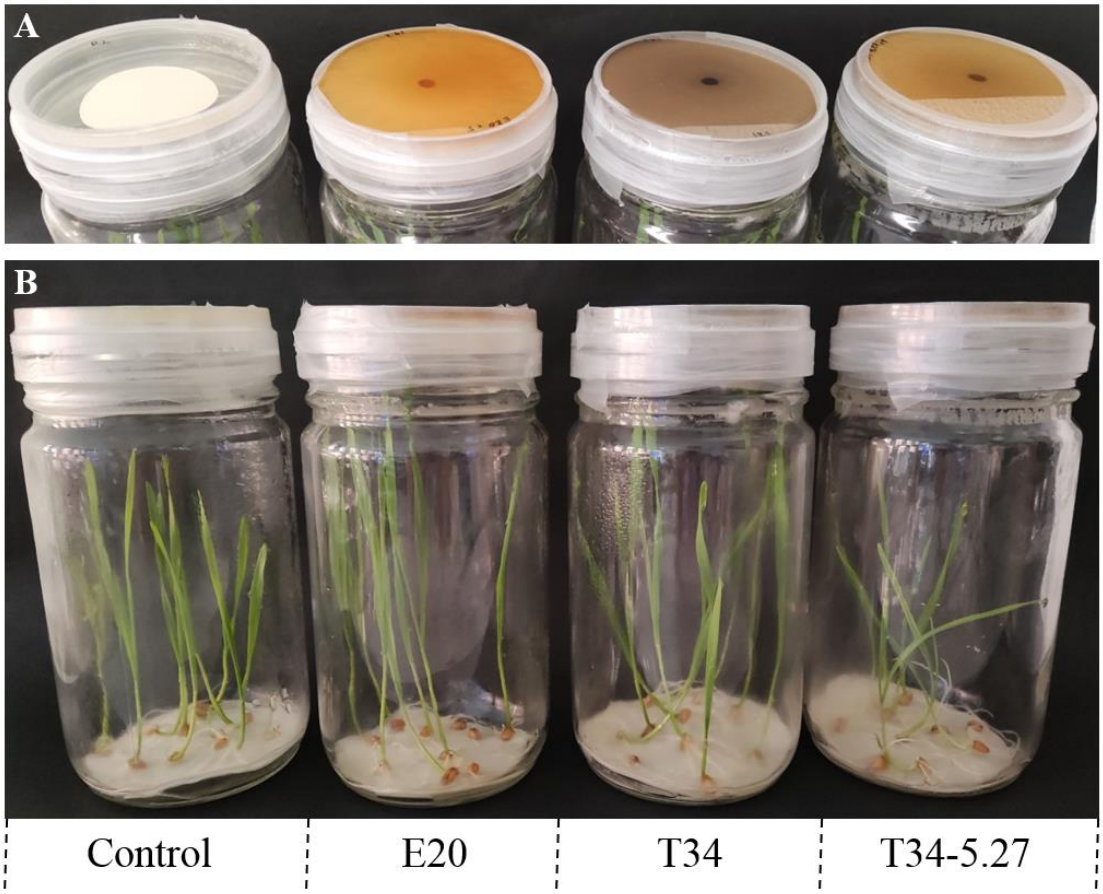


Figure 2. Assembled p-mVOC Chambers as used in this study. (A) Upper-frontal view of the Petri plates with PDA and the growing *T. harzianum* strains ten days after sowing. (B) Full frontal view of the mounted chambers with the growing wheat plantlets ten days after sowing. From left to right: control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

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).

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 (Arnon, 1949).

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

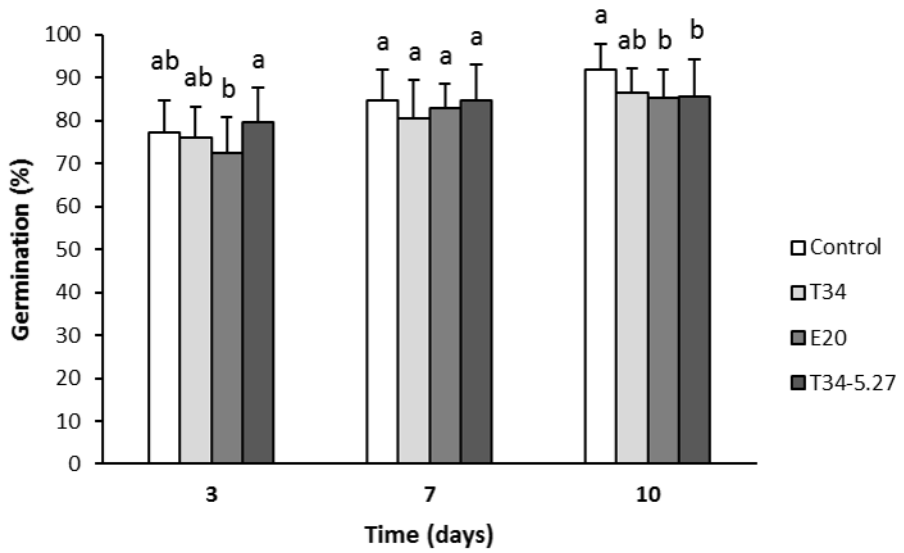
2.5. Data treatment and statistical analysis

After testing the normality of the data series by Kolmogorov-Smirnov´s test and the equality of variances by Levene´s 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 that overproduced the volatiles trichodiene and squalene, T34-5.27 and E20 respectively.

A



B

	Control	T34	E20	T34-5.27
Mean Germination Rate (seeds/day)	3.85 ± 0.54 a	3.69 ± 0.52 a	3.66 ± 0.14 a	3.46 ± 0.26 a

Figure 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 Rate. 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.

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. Contrary, the overproduction of trichodiene and squalene by the transformants reduced the germination rate of wheat seeds: ten DAS with T34-5.27, and three and ten DAS with E20, while no differences were seen seven DAS (Fig. 3). 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 (Hung *et al.*, 2014; Lee *et al.*, 2019). In addition, Ogura *et al.*, (2000) proved that important soil-related microbial BVOCs, like geosmin and 2-methylisoborneol, inhibit germination in Brassicaceae. The referred findings highlight the complex volatile interaction taking place between microorganisms and plants, resulting from a balance between the positive, negative and neutral effects that individual BVOCs exert on plant development (Hung *et al.*, 2014).

Regarding plantlet growth and development, our results indicate that BVOCs produced by *T. harzianum* affect the fresh weight gain and length of the wheat aerial part (Fig. 4A,B). Both parameters significantly increased in plantlets exposed to T34 and E20 volatiles when compared to non-exposed controls. Differences in biomass accumulation of the aerial part were not observed, as T34 and E20 exposed plantlets did not differ in dry weight with the control treatment. Conversely, T34-5.27 volatiles produced the opposing effect, significantly reducing the biomass accumulation measured as dry weight, as well as reducing the fresh weight gain and the total length of the aerial part in comparison to both the control treatment and the other two fungal strains (Fig. 4A,B,C).

Root fresh weight was barely affected by the treatments (Fig. 4E), showing only statistical differences between T34 and T34-5.27 exposed plantlets. Root dry weight was higher in the control and significantly lower in plantlets exposed to T34, E20 and T34-5.27, being the last one in turn also significantly lower than the former two fungal strains (Fig. 4F). Finally, a statistical reduction in root length was found in plantlets exposed to T34-5.27 BVOCs when compared to the control, T34 and E20 treatments, which in turn did not show significant differences among them (Fig. 4D).

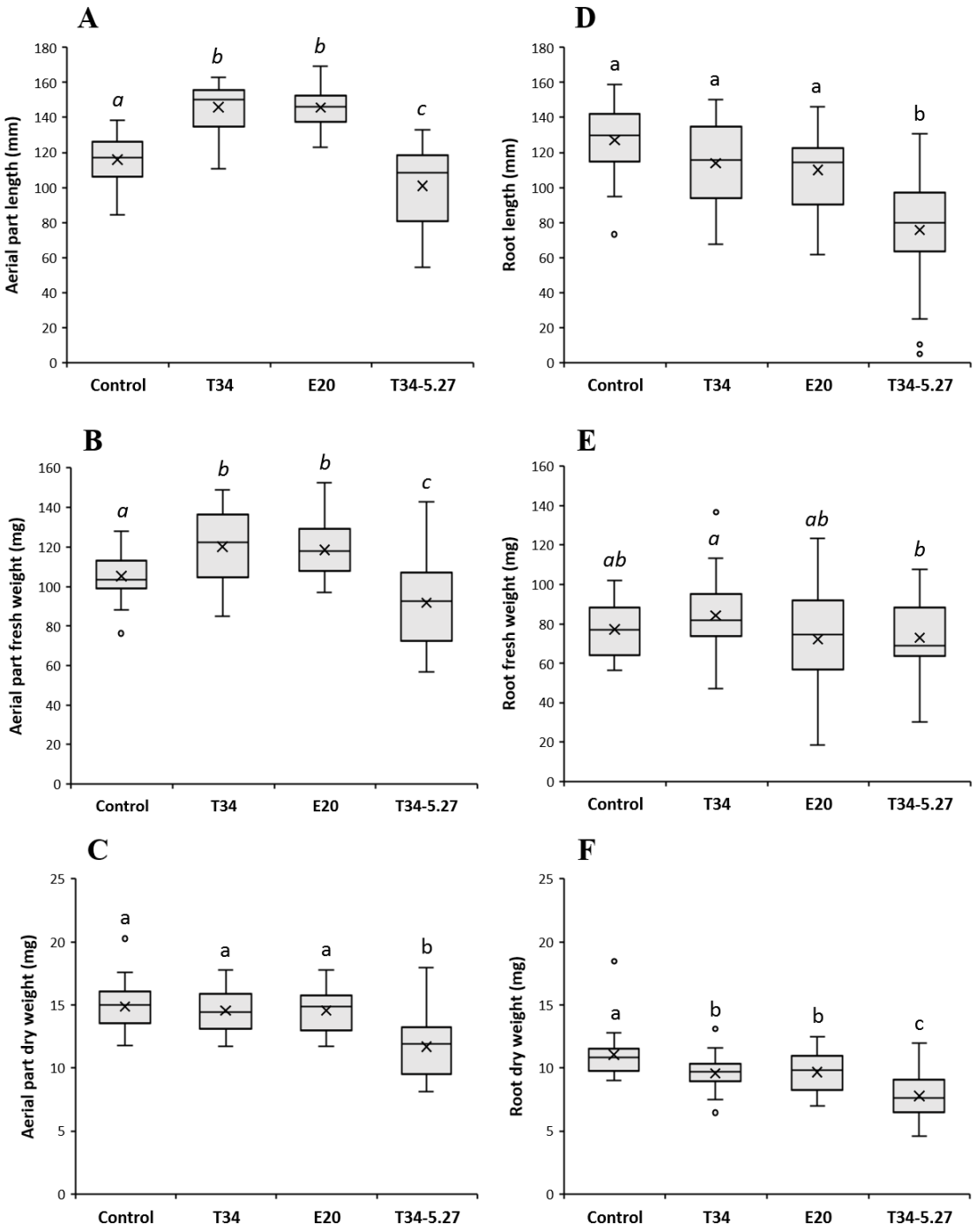


Figure 4. Effect of BVOCs produced by T34 (wild-type), E20, and T34-5.27 *T. harzianum* strains on growth parameters of wheat plantlets ten days after sowing. Control treatment indicates wheat seeds exposed to an uncultured plate. Aerial part: left column; Root: right column. The effect on (A, D) total 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.

To summarize, BVOCs from the *T. harzianum* wild type T34, and its squalene-overproducer transformant E20, significantly enhanced the growth of the aerial part of wheat plantlets compared to the control, both in terms of fresh weight and length. In contrast, the same treatments reduced root dry weight gain. 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. On the contrary, BVOCs from T34-5.27 significantly reduced the biomass accumulation (dry weight) and total length of aerial part and roots. These results suggest a general inhibitory effect in plantlet development derived from the expression of the orthologous gene *Tri5* and the subsequent overproduction of trichodiene. Surprisingly, these results contradict the findings of Taylor *et al.,* (2021), who reported that wheat seeds soaked with T34 and T34-5.27 conidia showed no visual effects on plant growth and development. On the contrary, 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 (Malmierca *et al.*, 2015*b*). 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 (Desjardins *et al.*, 2007; Cardoza *et al.*, 2015). Nevertheless, it must be taken into account that the levels of trichodiene-derived phytotoxic trichothecenes (Desjardins *et al.*, 2007; Barúa *et al.*, 2019; Flynn *et al.*, 2019) may increase in the trichodiene-overproducing strains and thus account for part of the effects observed in the present and previous studies. Therefore, further research should be conducted in this direction.

Our results seem to be also partially in contradiction with those reported by Lindo *et al.*, (2020). In this work, the treatment of tomato plants with squalene/ergosterol concentrations comparable to those produced by the T34 wild type strain, did not affect the aerial growth. However, squalene/ergosterol levels corresponding to those of squalene-overproducer E20 significantly reduced this parameter. The reported discrepancies between these studies may derive from the use of different plant species and/or different methodologies, as in our work the potential effects of squalene overproduction might be affected by other BVOCs or inorganic volatiles emitted by the growing strains. Besides, squalene overproduction has been related to fungal growth

promotion (Álvarez-García *et al.*, 2021), while trichodiene overproduction was described to enhance the antifungal activity of *T. harzianum* against phytopathogenic fungi, and to reduce their mycotoxin production (Taylor *et al.*, 2021; Álvarez-García *et al.*, 2021).

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, including trichodiene overproduction, 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. This is further supported by the observed corresponding increase in shoot length. On the contrary, reduction in dry weight of both aerial part and root caused by T34-5.27, indicate that this trichodiene-overproducing strain affects biomass accumulation on wheat. Previous investigations reported that BVOCs emitted by phylogenetically diverse microorganisms promote photosynthesis via complex processes involving abscisic acid and cytokinin (Sánchez-López *et al.*, 2016*b*; Ameztoy *et al.*, 2019). Besides, these authors demonstrated that fungal BVOCs produced global changes in the expression of photosynthesis-related proteins in *A. thaliana*, as well as an increase in chlorophyll accumulation (Ameztoy *et al.*, 2019). Therefore, changes in the photosynthetic rate not related to chlorophyll fluorescence quantum yield and leaf chlorophyll content, cannot be completely ruled out as responsible for part of the observed results without further research.

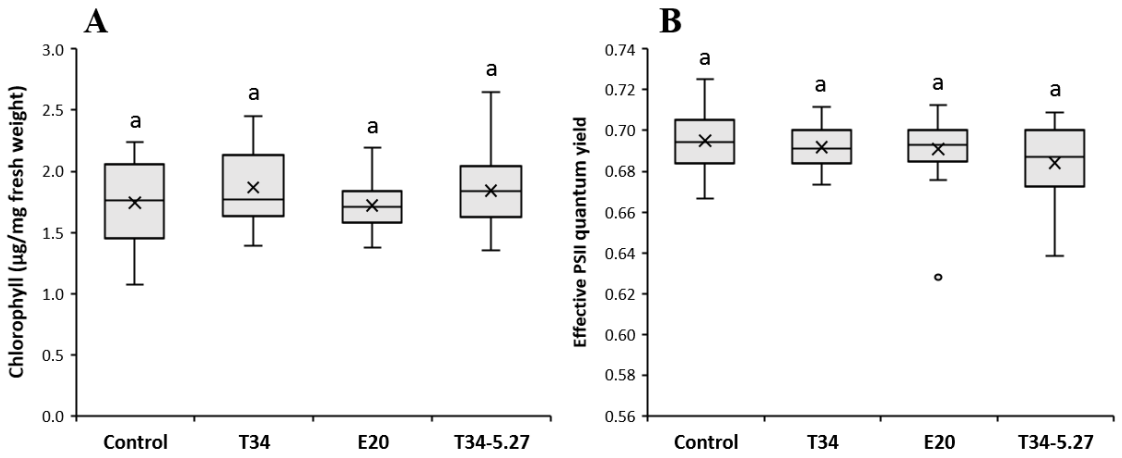


Figure 5. Effect of BVOCs produced by T34 (wild-type), E20, and T34-5.27 *T. harzianum* strains on (A) total leaf chlorophyll content and (B) effective quantum yield of chlorophyll of wheat plantlets ten days after sowing. Control treatment indicates wheat seeds exposed to an uncultured plate. Boxplots are as described in Fig 4. Different letters indicate significant differences (Tukey´s test for one-way ANOVA, $p < 0.05$) (n = 25).

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, ergosterol and squalene have been demonstrated to trigger the transcription of plant defence-related genes in a concentration-dependent manner (Malmierca *et al.*, 2016; Lindo *et al.*, 2020). The ergosterol to squalene ratio can also influence *T. harzianum* colonization of plants (Lindo *et al.*, 2020), and squalene overproduction enhanced the ability of *T. harzianum* to colonize tomato roots (Malmierca *et al.*, 2015*b*). 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 (Malmierca *et al.*, 2015*a*) and wheat (Taylor *et al.*, 2021), and reducing root colonization (Malmierca *et al.*, 2015*b*).

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. 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 (Kai and Piechulla, 2009, 2010; Giorgio *et al.*, 2015; Álvarez-García *et al.*, 2021).

Moreover, these chambers could 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.

In summary, the p-mVOC Chamber is 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, and to guide the future identification of new bioactive volatile compounds and their role in plant-microbe ecological relationships. In our opinion, the p-mVOC has the potential to become a standard method for the evaluation of *in vitro* plant-microbe volatile interactions. Nevertheless, the technology presents also 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. Besides, even though vessels with different volumes can be used, plant growth is still limited, especially for fast-growing species or long-time experiments.

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 (Hammerbacher *et al.*, 2019). 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. Finally, new uses and applications could arise from the scientific community for the p-mVOC Chambers.

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Conflicts of Interest

The authors declare the existence of financial competing interests derived from the utility model submitted by the University of León regarding the described technology. 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 and materials 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. After publication, 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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CHAPTER IV

Effects of trichodiene and squalene overproduction on volatile-mediated interactions between *Trichoderma harzianum* and *Acanthoscelides obtectus*: an *in vitro* model to evaluate the impact of microbial volatile compounds on dry grain storage pests

Abstract

Biological interactions mediated by Biogenic Volatile Organic Compounds (BVOCs) have been gaining attention during the last years. Although extensive focus is currently given to the control of insect pests using natural molecules, the field of volatile interactions between microorganisms and insects has been largely neglected thus far. We present a novel protocol to assess the effects of microbial BVOCs directly produced by growing strains on dry grain insect pests and the seed damage they produce. VOC Chambers were used to evaluate both sealed and unsealed conditions. Four *Trichoderma harzianum* strains, a wild type and three of its transformants that overproduce the volatiles trichodiene and squalene were tested against *Acanthoscelides obtectus*. Results demonstrated that ventilation plays an important role in these interactions. All fungal strains significantly increased insect 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. Squalene overproducing strains caused significantly higher adult mortality than the other fungal strains in sealed conditions, and lower insect emergence in both sealed and unsealed ones. Bean damage produced by insects was also lower when exposed to BVOCs from these strains in sealed conditions. On the contrary, trichodiene overproduction did not show enhanced toxicity or significant reduction of insect emergence or bean damage in the tested conditions. Therefore, *T. harzianum* BVOCs, especially those from squalene overproducing strains, may be of use, either individually or blended, for the biological control of *A. obtectus* infestation in dry grain storing facilities. VOC Chambers demonstrated to be an effective method for the screening of volatile mediated interactions between growing microbial strains and insect pests, useful in the search for new putative bioactive microbial BVOCs for integrated pest management.

Highlights

- *Trichoderma harzianum* volatiles increase *Acanthoscelides obtectus* mortality
- *T. harzianum* volatiles reduce *A. obtectus* emergence and bean damage
- Squalene overproduction rises insect mortality, reduces emergence and bean damage
- Ventilation plays a key role in microbe-insect volatile interactions
- VOC Chambers are a reliable technology to test microbe-insect volatile interactions

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 (Sanon *et al.*, 1998; Casquero *et al.*, 2006). This species originated in Mesoamerica and is nowadays widely distributed around the world (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.*, 2009a). The weevil attack can start on the fields or directly in storage, where the pest produces most of the damage (Baier and Webster, 1992). Female adults lay their eggs between the seeds and the larvae bore a hole to penetrate them, where they feed and develop until a new adult emerges (Berger *et al.*, 2017). The damage caused to the seeds lowers the quality of the product, generates important economic losses, and can even affect their ability to germinate, therefore jeopardizing further crop productivity (Paul *et al.*, 2009a).

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 resistances (Herrera *et al.*, 2015), have led towards Integrated Pest Management perspectives, including biological control as an important trait to address these challenges. In this regard, the role of Biogenic Volatile Organic Compounds (BVOCs) and their biocontrol capabilities have been gaining attention during the last years (Morath *et al.*, 2012; Cordovez *et al.*, 2015; El Ariebi *et al.*, 2016; Schulz-Bohm *et al.*, 2017; Dukare *et al.*, 2019; Ponce *et al.*, 2021). 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-Rodríguez *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.*, 2009b; 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 behavioural responses exerted on insects by individual or mixed BVOCs (Gershow *et al.*, 2012; Bueno *et al.*, 2020; 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). Kandasamy et al., (2019) reported the attraction exerted on the spruce bark beetle, *Ips typographus*, by BVOCs emitted from growing colonies of their symbiotic fungi, while Moisan *et al.*, (2019) demonstrated that fungal BVOCs induce plant resistance against insect pests, highlighting the role played by these metabolites in complex interspecific recognition and communication processes. In addition, microbial BVOCs produce also toxicity on insects, although studies in this regard are substantially less abundant. Herrera *et al.*, (2015) demonstrated that isolated fungal BVOCs have insecticidal properties against *Sitophilus zeamais*. Inamdar *et al.*, (2014) and Zhao *et al.*, (2017) reported toxic effects of fungal volatiles on *Drosophila melanogaster*. Regarding *A. obtectus*, Rodríguez-González *et al.*, (2018; 2019) reported that fungal strains overproducing BVOCs as trichodiene or squalene altered mortality rates and adult emergence from treated bean seeds. Nevertheless, in this case, fungal spores were in contact with the insects, making it difficult to determine whether the observed outcomes were the result of volatile

interactions or not. 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.*, 2014).

Among those biological control agents (BCAs) producing active BVOCs, *Trichoderma* stands out as one of the most studied and effective fungal genres (Guo *et al.*, 2020; Macías-Rodríguez *et al.*, 2020). Several investigations have demonstrated different effects produced by *Trichoderma* volatiles on plants and their defensive response (Hung *et al.*, 2013; Cardoza *et al.*, 2015; Lee *et al.*, 2016, 2019). *Trichoderma* BVOCs proved to exert as well a strong influence on microbial metabolism, growth, and development (Mutawila *et al.*, 2016; Wang *et al.*, 2018; Dukare *et al.*, 2019; Álvarez-García *et al.*, 2021; Taylor *et al.*, 2021). Regarding their activity on insects, *Trichoderma* BVOCs have been assayed on *Drosophila* as a model organism (Inamdar *et al.*, 2014; 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. Behavioural 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 other traits like 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; Rojht *et al.*, 2012b; Ponce *et al.*, 2021). Most of these techniques could be adequate to evaluate purified volatiles but are ill-suited when evaluating the effects of directly produced BVOCs by growing microbes. In this regard, Inamdar *et al.*, (2014) 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 compromise homogeneity and replicability. 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 (Kai *et al.*, 2016; Piechulla and Schnitzler, 2016; Álvarez-García *et al.*, 2021). In this regard, specific VOC Chambers have been developed to perform standardized volatile-mediated interaction assays and have been tested in microbe-microbe (Álvarez-García *et al.*, 2021). Moreover, these authors already proposed the possibility of employing this VOC Chambers to evaluate volatile interactions involving other organisms, including insects.

Therefore, in this study, we used VOC Chambers (Álvarez-García *et al.*, 2021) to expose *A. obtectus* adults to the VOCs 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.*, 2015; Lindo *et al.*, 2020). These strains have been also 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.*, 2015; 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 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 if the rise in mortality and the subsequent reduction in insect emergence and bean damage were directly caused by the microbial BVOCs or rather involved other interactions between the insects and the fungal strains, 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 hypothesize also that trichodiene and squalene overproduction by *T. harzianum* modifies the outcome of these volatile interactions. Our final hypothesis was that *T. harzianum* BVOCs could be used as potential biofumigants to reduce the damage produced by *A. obtectus* in stored dry grains. Therefore, our goals were: i) to determine the effects of *T. harzianum* BVOCs, and trichodiene and squalene overproduction by *T. harzianum*, 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

In this study, four *T. harzianum* strains were used to evaluate the effects of their VOCs 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.*, 2015; Taylor *et al.*, 2021). *T. harzianum* E20 derives from T34 by silencing the gene *erg1*,

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 channelling 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 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 reproduce in 4 l glass jars containing the aforementioned bean seeds and were kept in a controlled chamber with 25±1ºC, 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* VOCs 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 plates separated by a perforated intermediate piece that holds them in place facing one another, and thus forming a chamber that allows the flow of VOCs 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. Plugs (6 mm in diameter) from the fresh edge of 3 days-old colonies of the four *T. harzianum* strains were placed in the centre of Petri dishes containing 18 ml of PDA. After two days of growth at 25ºC, 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 autoclaved cellophane membrane and filter paper on top to allow the pass of fungal volatiles through the hole, while avoiding at the same time the potential contamination by fungal spores and the fall of insects or bean seeds to the lower plate. Non-cultivated Petri plates with 18ml of PDA were used as control treatment. 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.

Two different sets of experiments were performed. A first one in which both Petri plates were 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 BVOCs concentration inside the camber (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 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 were kept inside the VOC Chambers with the *Trichoderma* or control plates for 16 days in a controlled chamber with 25±1ºC and relative humidity of 60±5%, and the mortality was daily recorded. After this period, the VOC Chambers were flipped over and the intermediate pieces plus the plates with the fungal cultures were removed and discarded. The other plates, now containing the bean seeds, were covered with a new lid after removing the *A. obtectus* cadavers. These insects were stored at -80ºC for later microbial isolation assays. The Petri plates containing the bean seeds were kept in the same conditions (25±1ºC, relative humidity of 60±5%) to allow a new generation of insects to fully 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 25ºC.

2.4 Effects produced by *Trichoderma* VOCs 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. The count was stopped at this point to ensure that third-generation insects were not included in the results and taking into account 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 x 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, and five replicates were performed per treatment. Experiments were performed separately in sealed and unsealed conditions following the same general protocol. The number of dead insects per replicate was recorded each day and the cumulative percentage of mortality calculated as Mortality (%) = number of dead insects / 20 x 100. Afterwards, daily emergence was recorded, and 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. At the end of the experiment, the number of affected beans (with at least one exit hole), the total number of holes per replicate (40 beans), and the number of holes per individual affected bean were recorded. The percentage of weight loss was calculated by comparing the initial and final weights of the beans in each replicate.

Data normality and equality of variances were assessed using Kolmogorov-Smirnov´s and Levene´s 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 performed using IBM SPSS Statistics 26.

3. Results and discussion

3.1 Effects produced by *Trichoderma* VOCs on the mortality of *A. obtectus* adults

The results presented in Fig. 1A show that the VOCs 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 (Fig. 1A). In this regard, T34-5.27 was the only one not showing statistically significant differences with the control. Additionally, this trichodiene-overproducing strain neither presented differences with its parental strain T34. In turn, the T34 wildtype strain showed significant differences with 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 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 just on day 2, and around 80% on day 4 (Fig. 1A). 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 (Lanzón *et al.*, 1994; Dutton *et al.*, 2002; Jiang *et al.*, 2015), 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; Spanova and Daum, 2011; Jiang *et al.*, 2015). Squalene is also sometimes accumulated as a final product inside the organism (Jahaniaval *et al.*, 2000; Spanova and Daum, 2011). Therefore, without further research it, cannot be determined if squalene directly accounts for the observed insecticidal effects, or rather they are exerted by derived metabolites synthetised as by-products of the referred modification.

Concerning trichodiene overproduction, E20-5.7 produced slightly lower mortality than E20, although not statistically significant for the most part. This absence of statistically significant differences in mortality between trichodiene overproducing strains and their parental ones seems to indicate that this compound does not possess a significant insecticidal activity in the tested conditions. Moreover, the general lower mortality produced by these strains compared to their parental ones, while small and not significant for the most part, may even indicate a reduction of the overall insecticidal properties of *T. harzianum* BVOCs for unknown reasons when expressing the *Tri5* gene. Besides, oxygen availability likely influences the results in these sealed conditions (Kai *et al.*, 2016; Álvarez-García *et al.*, 2021), 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 oxygen availability must be involved in the observed insecticidal activity.

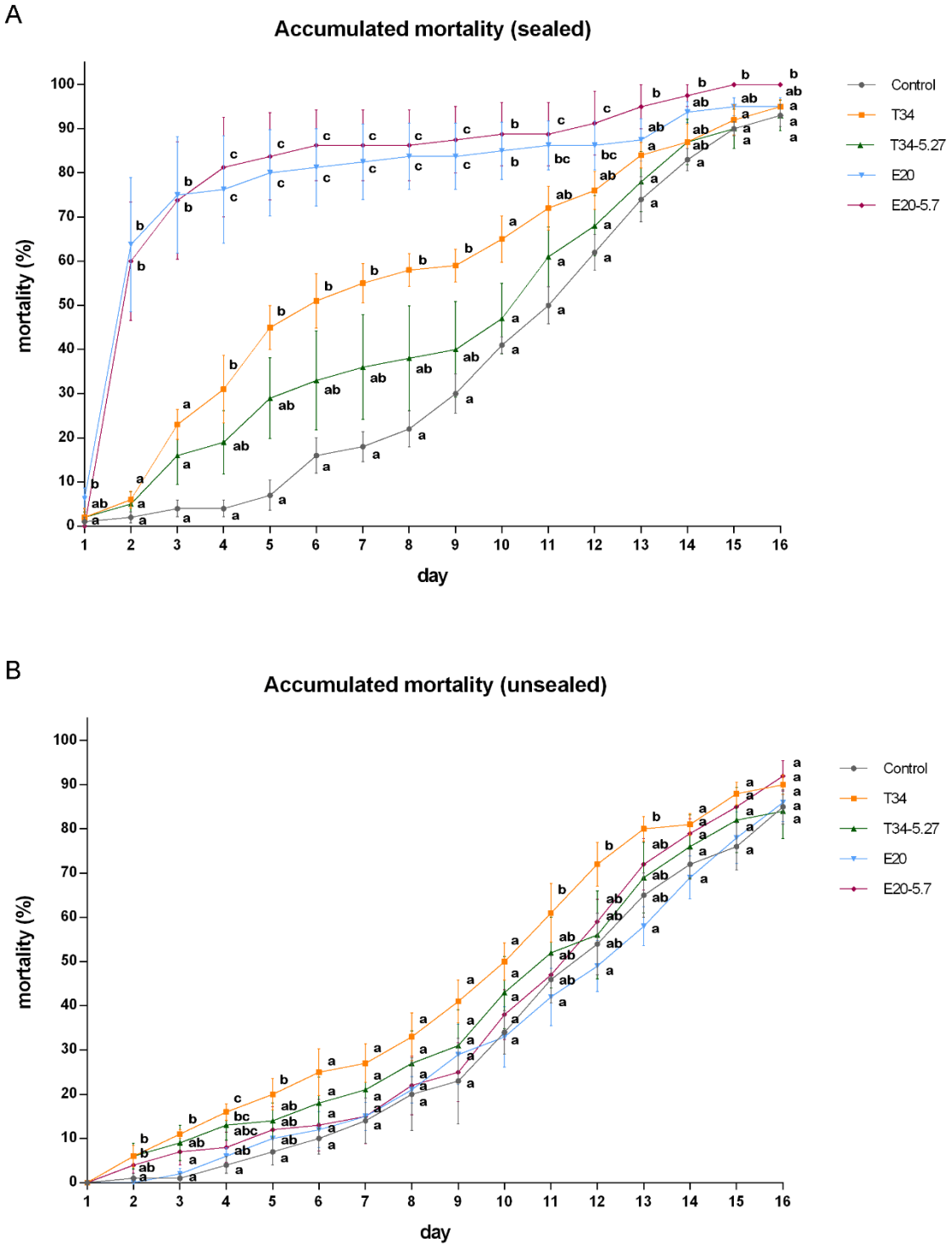

Figure 1. Accumulated mortality (%) of *A. obtectus* adults in (A) sealed and (B) unsealed conditions exposed for 16 days to BVOCs produced by *T. harzianum* strains T34 (wild type, squares, orange); T34-

5.27 (trichodiene overproducer, face-up triangles, green); E20 (squalene overproducer, face-down triangles, blue); E20-5.7 (squalene and trichodiene overproducer, diamonds, red); and untreated control (uncultured PDA medium, circles, grey). Data are represented as the accumulated percentage of death 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 ($p \leq 0.05$).

Regarding unsealed conditions (Fig. 1B), mortality was generally lower than in sealed ones for all treatments but the control and T34-5.7, which showed similar trends in both cases. Differences between treatments and the control were minimal in these unsealed conditions. Only the WT T34 stood out with a slightly higher mortality rate than the control and E20, mostly during the first days. Therefore, between sealed and unsealed conditions, a huge reduction in the insecticidal properties of E20 and E20-5.7 VOCs was observed (Fig. 1), suggesting that the previously described effects of the *Erg1* silencing and squalene accumulation on insect mortality are only applicable in tightly 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 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) compared to their non-trichodiene producing parental strains. Besides, the production of the toxin deoxynivalenol by *Fusarium graminearum* was reduced in plants inoculated with trichodiene-overproducing *Trichoderma* strains (Taylor *et al.*, 2021). These reported effects somehow contrast with the lower insect mortality obtained in the present study. Moreover, previous research showed that VOCs emitted by the squalene overproducing strain E20 presented lower antimicrobial activity than the WT and the trichodiene overproducers, especially in vented conditions, where this strain significantly promoted *Fusarium oxysporum* growth (Álvarez-García *et al.*, 2021). Results reported in chapter IV of the present PhD. Thesis show a growth-promoting activity produced by E20 BVOCs on wheat plantlets. Therefore, the herein presented results seem to indicate an opposed trend of trichodiene and squalene effects on insects compared to those produced on microorganisms and plants. (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 squalene overproducers, that significantly repelled them. This, combined with the enhanced insecticidal effects we described for E20 and E20-5.70 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 might be further supported by previous works that show the importance of olfaction in granivorous pests and the ability of the weevil to respond to BVOCs (Khelfane-Goucem *et al.*, 2014; Giunti *et al.*, 2018).

Rodríguez-González *et al.*, (2018) reported that trichodiene-overproducing strains increased insect mortality compared to their parental strains (also the squalene overproducer 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 demonstrated important insecticidal properties, these strains may present reduced infectious capability due to membrane changes derived from the blocking of ergosterol production, as well as to other metabolic alterations related to squalene and FDP accumulation, for example, protein prenylation or quorum sensing (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

As described, after the 16-day exposition to fungal BVOCs, insects were removed, and bean seeds were placed in Petri dishes to follow up the emergence of second-generation adults. Day 1 was set for this assay when the first insect emerged, and thereof, emergence was daily recorded until day 23.

Accumulated emergence was lower in sealed than in unsealed conditions for all treatments, including the control (Fig. 2), indicating that oxygen limitation likely reduces the reproductive rate of the insects. Nevertheless, this reduction was sharper when insects were exposed to *T. harzianum* BVOCs. In sealed conditions (Fig. 2A), 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 these two groups in several days, especially 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 well with those regarding mortality, as the higher mortality produced by the squalene-overproducing 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.

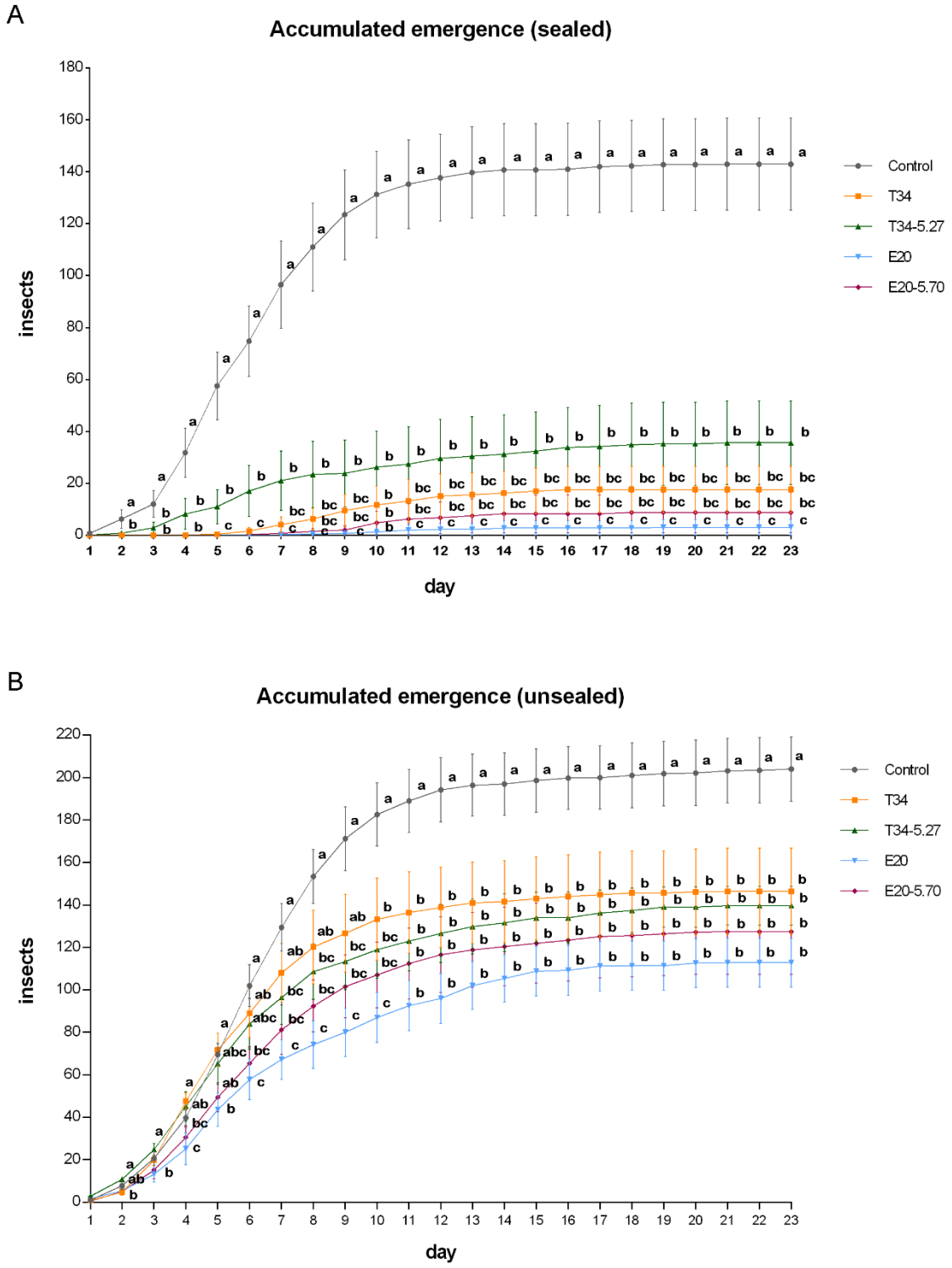

Figure 2. Accumulated emergence of *A. obtectus* adults in (A) sealed and (B) unsealed conditions during 23 days after exposition to BVOCs produced by *T. harzianum* strains T34 (wild type, squares, orange);

T34-5.27 (trichodiene overproducer, face-up triangles, green); E20 (squalene overproducer, face-down triangles, blue); E20-5.7 (squalene 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 ($p \leq 0.05$).

Insect emergence in unsealed conditions was also significantly reduced by all fungal treatments compared to the control (Fig. 2B). Nevertheless, this reduction was not as sharp as in sealed ones, suggesting that fungal VOCs affect *A. obtectus* reproduction also in more open environments, though not as much as in tightly 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 that well with the observed mortality. As described, very small differences were observed between treatments regarding mortality in unsealed conditions. Moreover, albeit not statistically significant in many cases, T34 and T34-5.27 were the treatments that presented higher insect mortality. 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 the squalene-overproducers BVOCs in early stages of insect development (eggs, larvae) or derive from behavioural changes in exposed *A. obtectus* adults that may affect their reproductive rate. In this regard, several studies have demonstrated that BVOCs exert toxic effects on pre-adult 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). Importantly, Rodríguez-González *et al.*, (2018) described a deterrent effect of E20 towards *A. obtectus* females when spores were sprayed over bean seeds. These authors

also reported an increase in *A. obtectus* emergence from beans sprayed with T34-5.27 and E20-5.7 trichodiene-overproducing strains in comparison to their parental ones, with T34 rendering the lowest insect emergence and bean weight loss (Rodríguez-González *et al.*, 2019), even though the trichodiene-overproducers caused a higher adult mortality. However, some results in the referred study may be influenced by the attraction and repellency induced by the different fungal strains. Additional research should be conducted to further clarify these aspects.

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

After 23 days of insect emergence, the following parameters were recorded: the number of affected seeds (with at least 1 exit hole) and the subsequent percentage of affected beans; the total number of holes per replicate; the number of holes per affected bean; and the final weigh, from which the percentage of bean weigh loss was calculated comparing the initial and final ones on each replicate.

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 VOCs, 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 one (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, being impossible to calculate as most of the replicates did not show affected beans at all (Table 1A). Finally, weight loss was significantly reduced by all treatments compared to the control, with a reduction of 14.56% for 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 caused 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, as well, lower bean damage.

Aluminum phosphide and phosphine have been used for decades as the main active compounds in the control of pests inside storage facilities, while their insecticidal activity and dynamics have been extensively tested against many insects, including *A. obtectus* (Hole *et al.*, 1976; Hasan and Reichmuth, 2004; Arora and Srivastava, 2021; Arora *et al.*, 2021). Nevertheless, rising concerns regarding pest resistances, residue persistence, safety, and toxicity that poses a serious hazard to human health, advice for the search of more natural and less harmful compounds (Vardell *et al.*, 1973; Bogle *et al.*, 2006; Murali *et al.*, 2009; Pérez Navero *et al.*, 2009; Nayak *et al.*, 2020). In this regard, BVOCs could be an adequate potential substitute, as phosphine acts in gaseous form (although mostly applied using tablets or granules) inside airtight storing facilities. The application of these compounds could be also combined with controlled atmospheres. Therefore, our results suggest that volatiles produced by these *T. harzianum* strains, especially those by E20, either individually or blended, could be potentially used as biofumigants against *A. obtectus* inside storing facilities with tightly closed environments.

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 (squalene overproducer); E20-5.7 (squalene 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**	5.86	a	**143.5**	18.21	a	**5.46**	0.25	a	**14.56**	1.89	a
T34	**16**	4.65	bc	**17**	8.57	b	**2.27**	0.43	b	**3.57**	0.84	bc
T34-5.27	**24.5**	7.22	b	**36.2**	16.12	b	**3**	0.64	b	**5.65**	1.29	b
E20	**4.38**	4.38	c	**2.5**	2.50	b	**NA**	-	-	**1.93**	0.30	c
E20-5.7	**10**	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	**67.5**	3.26	a	**200**	15.49	a	**7.39**	0.35	ab	**14.17**	1.86	a
T34	**58**	5.33	b	**153**	23.55	ab	**6.47**	0.62	ab	**10.75**	2.10	a
T34-5.27	**43.5**	3.32	c	**135.6**	11.91	b	**7.84**	0.57	a	**9.94**	1.09	a
E20	**48**	3.10	bc	**116.2**	12.10	b	**6.12**	0.71	b	**10.63**	1.16	a
E20-5.7	**47.5**	7.37	bc	**131.6**	23.35	b	**6.91**	0.42	ab	**9.83**	1.37	a

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 treatments (58%) and T34-5.27 the lowest (43.5%). In this case, no differences were shown between the squalene-overproducers and the other strains. 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 but 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 number of holes per affected bean, no differences were observed between any treatment 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 redounded in 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 mortality and the lowest insect emergence in sealed conditions. Nevertheless, likewise the observed pattern regarding 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 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 bean in the case of E20. This evidence indicates that fungal VOCs are also able to reduce bean damage produced by *A. obtectus* in open environments, as well as reinforces the expressed hypothesis that processes other than toxicity on adults play a key role in the described results. Therefore, volatiles emitted by the assessed *T. harzianum* strains, especially those of E20, might be also useful to reduce the damage produced by *A. obtectus* inside storing facilities with more open atmospheres.

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, in order to design effective strategies to produce and apply them 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.

4. Conclusions

To summarize, the VOC Chambers proved to be an effective and reliable method to evaluate the direct volatile-mediated interactions between growing microorganisms and insects, representing a useful model for the screening of putative bioactive microbial BVOCs against dry grain storage pests. These devices provided flexibility regarding ventilation, allowing to test both sealed and unsealed conditions. *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, suggesting that ventilation and gas exchange play an important role in microbe-insect volatile interactions. The squalene-overproducer E20 and its derived strain E20-5.7 significantly increased mortality in comparison to the wild-type T34 and its transformant T34-5.7 in sealed conditions, indicating that the silencing of *erg1* gene and the subsequent accumulation of squalene enhance the insecticidal activity of *T. harzianum* BVOCs. These strains also lowered insect emergence and bean damage in both 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. Considering the aforementioned results, *T. harzianum* BVOCs, especially those from E20, might be useful as alternative natural biofumigants to mitigate the damage produced on dry grain by *A. obtectus* infestation inside storage facilities.

Annex to CHAPTER IV

Preliminary identification and quantification of Biogenic Volatile Organic Compounds produced by *T. harzianum* T34 and E20

1. Hypothesis and Objectives

After evaluating the volatile effects produced by the wild-type *Trichoderma harzianum* strain T34 and three of its transformants, namely T34-5.27, E20, and E20-5.7, on the phytopathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum*, on wheat plantlets, and on the insect pest *Acanthoscelides obtectus*, it was determined that the bioactivity of their BVOCs varies among them. The BVOCs emitted by the trichodiene-overproducing strains T34-5.27 and E20-5.7, both expressing the *T. arundinaceum tri5* gene, have been already studied in previous research (Malmierca *et al.*, 2015; Taylor *et al.*, 2021). However, to our knowledge, no studies have been carried out so far regarding the production of specific BVOCs derived from the *erg1* gene-silenced strain *T. harzanum* E20.

As the *T. harzanum* strains T34 and E20 showed significant differences of bioactivity in volatile-mediated interactions against fungi and insects, with E20 exerting lower antifungal activity and higher insecticidal one, we hypothesize that the emission of BVOCs by these strains varies either in the nature of the compounds, their quantity or both. Therefore, our goal was to identify and compare using GC/MS technology the production of specific BVOCs by these two *T. harzianum* strains, which may account for their differential volatile-mediated bioactivity.

2. Material and Methods

As stated, two *T. harzianum* strains were evaluated in the present study to preliminary identify, quantify, and compare their production of BVOCs using gas chromatography and mass spectrometry (GC/MS) technology. *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) was included as the parental wild-type strain (T34 from now on) for this study, while *T. harzianum* E20 is a transformant strain derived from T34 by silencing the *erg1* gene, which is responsible for encoding squalene epoxidase. This modification leads to the accumulation of squalene and a reduction in ergosterol levels (Cardoza *et al.*, 2006). For the present study, the microorganisms were kept in 50% glycerol spore suspension at -80ºC and were grown by culturing on Potato Dextrose Agar (PDA) medium at 25ºC.

50 ml flasks containing 10 ml of PDA and left horizontally until the medium solidified. Thereafter, a 6 mm plug taken from a three days-old fresh fungal colony was placed in the center of the medium inside the flasks, which were subsequently closed and left inside a growing chamber for three days in darkness at 25ºC. For the control, non-cultured flasks with 10 ml of PDA were used. After three days of growth, extraction was performed through headspace solid-phase microextraction (HS-SPME), and GC/MS analyses were carried out. Five replicates were performed per treatment.

Fungal BVOCs were adsorbed to a divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS 50/30 µm) SPME fiber (Supelco, Bellefonte, USA), and the sampling procedure was carried out as described by Malheiro *et al.*, (2018) with some modifications. Thus, samples were conditioned for 5 min at 25ºC prior to the exposition of the SPME fiber to their headspace, which lasted for 30 minutes in the same conditions. Afterwards, BVOCs were eluted in the GC/MS injection port for 1 min by thermal desorption (220ºC) and the fiber was left in there for a further 10 min to clean up and prepare it for the following injection. Analyses were conducted using a Shimadzu GC-2010 Plus gas chromatographer coupled with a Shimadzu GC/MS-QP2010 SE mass spectrometer detector with a TRB-5MS (30 m×0.25 mm×0.25 µm) column (Teknokroma, Spain). The injection was performed manually in spitless mode, using helium (Praxair, Portugal) as mobile phase at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min. A temperature ramp was set up as follows: 40ºC (1 min); plus 2ºC/min until reaching 220ºC. The temperature at the ionization source was kept at 250ºC with an energy of 70 eV, and an ionizing current of 0.1 kV. The range of electron ionization for mass spectra acquisition was *m/z* 35-500. For identification, mass spectra were compared with those of the NIST 11 chemical database (National Institute of Standards and Technology; U.S. Department of Commerce). Peak areas were obtained integrating the reconstructed chromatogram using the specific ion base for each peak (100% m/z intensity) to determine the percentage of compounds in the sample.

Analyses of the statistical difference between means were performed comparing the percentage of each selected compound in the headspace of both fungal strains by *Student's t-test*, after confirming normality and that equal variances could be assumed. IBM SPSS Statistics 26 was used for the statistical analyses.

3. Results and Discussion

For this preliminary analysis, seven majoritarian compounds were selected after integrating all peak areas from the chromatograms. The selected BVOCs were those amounting to more than 1% of the total integrated area of the chromatograms. Ordered by retention time, these compounds were identified as: 1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Isobutyl acetate; 1-Butanol, 3-methyl-, acetate; Phenylethyl Alcohol; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- (Table 1). Additionally, a further compound identified as -Benzeneacetic acid, ethyl ester- has been included because, although it did not reach the 1% threshold, it was deemed potentially relevant as its amount was almost 5 times higher in E20 than in T34 (Table 1). Out of the 65 detected peaks, these eight compounds accounted for more than 90% of the total volatile emission in both strains.

Table 1. Majoritarian compounds from the GC/MS analysis of the volatilome produced by T34 and E20 strains. Compounds are ordered by retention time, and the peak area (%) represents the qualitative proportional percentage of each one compared to the total integrated area (mean ± SE). P values indicate the statistical significance regarding compound richness between the two fungal strains, calculated using *Student's t-test*. Asterisks (*) mark the fungal strain that produces the higher percentage of each compound.

		Peak area (%)		
Compound	**Ret. Time**	***T. harzianum* T34**	***T. harzianum* E20**	**p value**
1-Propanol, 2-methyl-	3.40	16.32 ± 0.45	23.85 ± 2.73 *	0.0180
Acetoin	4.90	5.55 ± 0.19	12.09 ± 1.27 *	0.0007
1-Butanol, 3-methyl-	5.65	7.96 ± 0.25	15.19 ± 1.36 *	0.0006
Isobutyl acetate	6.85	18.66 ± 1.6 *	9.73 ± 1.74	0.0070
1-Butanol, 3-methyl-, acetate	11.60	41.11 ± 2.72 *	21.86 ± 3.91	0.0042
Phenylethyl Alcohol	27.15	2.93 ± 0.29	5.07 ± 0.81 *	0.0292
Benzeneacetic acid, ethyl ester	36.50	0.06 ± 0.006	0.29 ± 0.04 *	0.0003
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	48.15 & 48.50	1.23 ± 0.05	2.59 ± 0.5 *	0.0173

As it can be seen from Table 1, production of all seven majoritarian BVOCs, as well as Benzeneacetic acid, ethyl ester, showed statistically significant differences between both fungal strains regarding their percentage in the samples. The wild-type strain T34 showed a significantly higher production of Isobutyl acetate (18.66 ± 1.6%) and 1-Butanol, 3-methyl-, acetate (41.11 ± 2.72%). Conversely, E20 showed significantly higher production of the other six compounds, namely 1-Propanol, 2-methyl- (23.85 ± 2.73%); Acetoin (12.09 ± 1.27%); 1-Butanol, 3-methyl- (15.19 ± 1.36%); Phenylethyl Alcohol (5.07 ± 0.81%); Benzeneacetic acid, ethyl ester (0.29 ± 0.04%); and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- (2.59 ± 0.5%).

The decrease in the production of Isobutyl acetate; 1-Butanol, 3-methyl-, acetate; or both may account for the reduced antifungal activity of E20 against *R. solani* and *F. oxysporum*. Interestingly, the lowering of these compounds or the overproduction of any of the other six (1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-) could be one of the reasons of the enhanced growth-promoting effects showed by this strain on *F. oxysporum* when tested in vented conditions. Nevertheless, as the present quantification was carried out in airtight conditions due to technical limitations, it cannot be assured if the observed differences in the proportion of these BVOCs remain the same in vented ones. In addition, complex interactions between the effects exerted by more than one of these compounds, as well as other non-described minority BVOCs, could account for the overall described effects. Therefore, further research is needed in this regard.

BVOCs produced in sealed conditions by E20 showed significantly higher insecticidal properties against *A. obtectus* adults than those produced by T34. Insects exposed to E20 volatile compounds caused lower damage on dry bean (*Phaseolus vulgaris*) seeds than those exposed to BVOCs from T34 in both sealed and unsealed conditions. This could indicate that one or more of the six identified BVOCs overproduced by E20 (1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-) may account for the increase of insecticidal properties. However, as already stated for the putative antifungal activity, other compounds produced by these fungal strains in lower quantities could not be entirely ruled out as responsible for a relevant part of their volatile-mediated bioactivity. Therefore, further research and analysis are needed to complete the partial picture herein presented.

Considering these results and the previously reported volatile-mediated bioactive effects of the tested *T. harzianum* strains, it would be of great interest to test the potential antifungal activity of purified Isobutyl acetate and 1-Butanol, 3-methyl-, and, especially, the putative insecticidal activity of 1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- against *A. obtectus* and other insect pests.

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DISCUSSION

As an ever-increasing amount of traditional pesticides are being banned due to health and environmental issues, resistances to them arise from microbial pathogens and insect pest, and new environmental issues threaten agricultural yield. Biological Control strategies, including the use of microbial Biological Control Agents (BCAs), stand out as an effective, reliable, and safe strategy in the management of plant diseases and pests for improved resilient agriculture (Kashyap *et al.*, 2017). Among microorganisms, members of the fungal genus *Trichoderma* are widely recognized as effective BCAs in the control of plant diseases and pests, both in the field and in postharvest (Sood *et al.*, 2020; Kredics *et al.*, 2021), as well as for the promotion of plant defence and growth (Pereira *et al.*, 2014; Gomes *et al.*, 2020). In this regard, the production and emission of volatile and non-volatile microbial metabolites to the environment play a key role in the biocontrol capabilities of *Trichoderma* spp. and other BCAs.

The effects of these metabolites, mainly those belonging to the secondary metabolism, on plant pathogens and pests have been extensively studied, especially those of non-volatile soluble nature. However, the research of BVOCs has been gaining momentum lately. In many cases, the *in vitro* study of these chemical interactions presents important limitations, including the control of diverse environmental factors that may affect the results, among which the unnatural extremely high accumulation of toxic compounds inside the experimental unit could be highlighted. Therefore, the initial hypothesis of this PhD. thesis was that the *in vitro* growing conditions in which the biological activity of microbial secondary metabolites is tested determine the outcome of the studied interactions, thus research methodologies should take into account these conditions and be able to modulate them. In this context, the *in vitro* self-inhibitory activity of soluble metabolites produced by *Trichoderma* spp. was assessed and compared to the toxic effects exerted against a phytopathogenic *F. oxysporum* strain. In addition, the main focus of the present study was the development and evaluation of new devices able to solve some of the existing technological problems in the research of microbe-microbe, microbe-plant, and microbe-insect interactions mediated by BVOCs.

Concerning self-inhibition, although the release of lytic enzymes and toxic metabolites is of the utmost importance for microbial biocontrol (Sood *et al.*, 2020; Vinale and Sivasithamparam, 2020), another key factor in the effectiveness of *Trichoderma* regarding antibiosis and mycoparasitic mechanisms lies in its ability to overcome its own toxic effects by producing cell wall remodelling enzymes (Kappel *et al.*, 2020).

Nevertheless, little is known about the impact of these self-inhibitory processes in traditional methods used for the selection of fungal strains with putative biocontrol capabilities against phytopathogens through the release of toxic secondary metabolites. The here presented results seem to indicate that, in membrane assays, fungal strains are exposed to growing conditions so stressful that, in many cases, even the BCA proper produces self-inhibitory effects as high as those inhibitory ones exerted on the phytopathogen. It is reasonable to think that this would not be the case in most natural conditions. The relationship between self-inhibitory and antimicrobial effects might be also conditioned by the phylogenetic closeness of the specific strains used in the experiments, with more related ones likely, although not necessarily, rendering more similar outcomes.

The results obtained using this methodology to confront a pathogen to a BCA, surely provide useful information regarding the presence of toxic compounds, which could be further researched and potentially used. However, concerning the selection of fungal strains as candidates to be applied directly in biocontrol on the field, results seem to indicate that, at least in the reported conditions and with the tested strains, hardly any relevant conclusion could be drawn from a traditional membrane assay alone. We propose that, if used at all for this purpose, membrane assays should be coupled with self-inhibitory ones, in order to select not only those strains that produce a higher inhibition to the pathogen but also those that present a better ability to overcome their own toxic effects, which again, has been defined as a central factor in *Trichoderma* biocontrol activity (Kappel *et al.,* 2020). Thus, we suggest that a significantly lower self-inhibitory effect than the antimicrobial one showed *in vitro* by a microbial strain could potentially be a useful criterion for further selecting putative BCAs during the first stages of a research. Alternative methods to *in vitro* membrane assays or a combination of antifungal and self-inhibitory experiments could be a better approach to preliminarily evaluate the potential biocontrol activity of microbial strains to pre-select them for further in vivo trials. Nevertheless, these hypotheses should be put to test by proving if the use of this criterion improves the selection of bioactive BCAs. Therefore, further research on these self-inhibitory processes, their potential use, and their mechanisms in *Trichoderma*-pathogen interactions would be of great interest.

Regarding volatile interactions, the research of microbial BVOCs and the biological effects they exert on other organisms has been gaining attention in the last years. Many

BVOCs have been reported as important mediators in several different biological interactions. Nevertheless, this research still presents some difficulties and limitations derived from the volatile nature of these compounds and the still reduced range of available methodologies. These problems include lack of specific and reliable material, difficult manipulation, reduced room for the development of the organisms, lack of flexibility, homogeneity, and replicability, or risk of cross-contamination. Moreover, a major drawback that was deemed necessary to solve is that most methodologies do not allow for the control or modulation of gas exchange with the exterior, as they are performed in sealed conditions and do not present vented configurations. These airtight environments may widely distort the final results of these interactions, jeopardizing in some cases the accuracy of the drawn conclusions regarding the actual potential of these volatile traits for biocontrol purposes (Alijani *et al.,* 2019). This point connects with the expressed main hypothesis regarding the importance of the *in vitro* growing conditions in which biocontrol interactions take place and the necessity of research methodologies able to modulate them.

In order to solve some of the referred limitations, two new devices were developed, intellectually protected, and their performance was tested. The first one is the Volatile Organic Compounds Chamber (VOC Chamber), designed originally for microbial interactions mediated by BVOCs but that proved to be also useful to test insect-microbe volatile interactions. The second one, the plant-microbe VOC Chamber, is a derived design from the previous one that allows for the growing of plantlets and the evaluation of the effects exerted by microbial BVOCs on plants, seeds, and plant parts.

VOC Chambers provided a higher homogeneity and replicability of results compared to the traditional DDS method when confronting microorganisms, as they showed a consistently lower coefficient of variation for all tested conditions and fungal strains. This characteristic alone would be by itself an important improvement. In addition, the use of non-vented and vented VOC Chambers revealed significant differences in growth inhibitory activity among *T. harzianum* strains that were not present in the tightly closed environment of the traditional method, where higher inhibition was exerted by all strains, likely due to the unnatural high accumulation of toxic BVOCs. Moreover, completely new biological responses to *T. harzianum* BVOCs were shown by *F. oxysporum* in vented conditions, where the wild type T34 and the squalene-overproducer E20 not only did not inhibited *F. oxysporum* growth but significantly

promoted it compared to the control treatment. These results back the original hypothesis that experimental conditions in *in vitro* volatile assays, especially those regarding ventilation and gas exchange, highly affect the outcome of the research. This is further supported by additional changes showed by the fungal strains between closed and more vented environments, such as those in colouration and sporulation. Therefore, fungal strains show different growth and physiological responses when exposed to BVOCs in diverse ventilation scenarios, proving that methodologies able to modulate these conditions like the described VOC Chambers are of great use.

All these findings may be rooted in ecological adaptations developed by both partners, the pathogens and the *T. harzianum* strains, leading to complex microbiological interactions and to the coevolution of the different fungal strains in their diverse natural environments. In this regard, DDSs and non-vented VOC chambers could be considered to represent environments where airflow and gas exchange face important limitations (Alijani *et al.*, 2019). For instance, the conditions faced by microorganisms in some storing facilities (Dukare *et al.*, 2019) or the microbial interactions taking place in flooded areas or soils with a clay texture. On the other hand, we believe that vented VOC chambers give very valuable information about the behaviour of microorganisms when exposed to volatile interactions in less tightly closed environments, as in soils with a sandy texture, aerial parts of the plant's surface, and other open-air conditions. Additionally, the ability of *Trichoderma* spp. to detect volatile and non-volatile cues from potential hosts and to respond growing by tropism towards them has been extensively described (Elad and Chet, 1982; Lu *et al.*, 2004; Alfiky and Weisskopf, 2021), as well as the attractive effects of some microbial BVOCs on insects (Rodríguez-González *et al.*, 2018; Ponce *et al.*, 2021). In this regard, we wonder if the growth promotion exerted by T34 and E20 on *F. oxysporum* could be a similar trait, that *T. harzianum* uses to attract a potential fungal host. Hence, new studies should be conducted to answer these questions and to elucidate how and why some microorganisms react in such different ways when facing BVOCs from other microbial strains in vented and non-vented conditions, as well as to explain their link to the ecological interactions that might lay behind this behaviour.

VOC Chambers proved to be also a useful tool to evaluate the effects of BVOCs produced by growing microbial strains on insect pests. While many studies have been conducted regarding the effects of BVOCs on insect behaviour (Ponce *et al.*, 2021), or the insecticidal activity of purified volatile compounds, little has been done to evaluate

the insecticidal properties of BVOCs directly produced by growing microbial strains. In this regard, the assays we performed using VOC Chambers to expose *A. obtectus* adults to *T. harzianum* BVOCs directly emitted by the growing fungal colonies in both sealed and unsealed conditions. As observed with the phytopathogens, the toxic or insecticidal activity of the *T. harzianum* BVOCs decreased when the experiments were carried out in more open conditions, in contrast to the high insect mortality observed in sealed ones. In the light of these results, ventilation and gas exchange also seem to clearly play a pivotal role in volatile interactions between microbial BCAs and insects, thus methodologies that allow modulating these conditions, like the here presented VOC Chambers, can improve the study of these biological interactions. As stated, very few studies have been reported regarding the effects exerted on insects by BVOCs directly produced by growing microbial strains, and therefore, no standardized materials and procedures have been reported so far, with only a few interesting proposals using non-specific lab gear manually modified (Inamdar *et al.*, 2014; Zhao *et al.*, 2017). Therefore, there was a gap in both materials and methodologies to perform these assays in a homogeneous and standardized way, which can be solved using the here presented VOC Chambers, as proved by the reported results.

VOC Chambers demonstrated to be able to reveal statistically significant differences regarding insecticidal activity between different fungal strains, between fungal strains and the control treatment, and also among the same fungal strain when studied in sealed and unsealed conditions. This further support the usefulness and versatility of the VOC Chambers to perform this kind of research. Moreover, the effect of the exposition to fungal BVOCs on the damage produced by *A. obtectus* on bean seeds was also studied. Differences were revealed in both sealed and unsealed conditions, being higher in the former. Regarding unsealed conditions, the absence of significant toxicity to the adults, alongside a reduction in the bean damage produced by the insects, seem to indicate that either behavioural factors or toxicity to other insect developmental stages are also involved in these interactions. Thus, VOC Chambers can be employed as well to evaluate the effect of microbial BVOCs on the damage produced by insect pests on storage products. Moreover, we believe that p-mVOC Chambers could be used also to evaluate the combined effects of microbial BVOCs on plants and insect pests at the same time.

To summarize, the use of VOC Chambers to perform direct microbe-insect volatile interaction assays could be of great interest for the screening of microbial strains with putative volatile biocontrol activity, designed as the first stage for further identification of specific BVOCs with the potential to be employed against insect pests.

Concerning the use of the p-mVOC Chamber, this device demonstrated its reliability for the evaluation of plant-microbe interactions mediated by microbial BVOCs. It proved to be a technology easy to set up and convenient for its employment inside phytotrons or growth chambers used for plant research. Only non-vented conditions were evaluated, so further research using vented chambers is highly recommended to understand the implications of gas exchange on these interactions, especially considering CO_2 flows that may affect plant growth and development. Wheat plantlets responded differently to BVOCs produced by different *T. harzianum* strains regarding germination, root and aerial part length, and weight (fresh and dry), while did not show statistical differences regarding chlorophyll content or quantum yield. p-mVOC Chambers could be also used to evaluate tripartite interactions mediated by BVOCs in which BCAs, plants, and phytopathogenic microorganisms or plant pests participate at the same time. Thus, 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.

As it has been mentioned, for the evaluation of the new technologies and the study of biological interactions mediated by BVOCs, four *T. harzianum* strains were employed. A wild type (T34), a squalene overproducer (E20), and two trichodiene-overproducing strains derived from the former two (T34-5.27 and E20-5.7). These strains had been already developed and described in previous works (Cardoza *et al.*, 2006; Malmierca *et al.*, 2015a; Malmierca *et al.*, 2015b). *F. oxysporum*, *R. solani*, *A. obtectus*, and *T. aestivum* var. tremie were exposed to BVOCs from growing colonies of these strains using VOC Chambers or p-mVOC Chambers, although E20-5.7 was not employed with plants.

The BVOCs emitted by these strains seem to differ widely in their biological effects exerted on different organisms, suggesting that their genetic modifications and subsequent metabolic implications modify either the nature or quantity of the produced volatiles. Interestingly, these results indicate that filamentous fungi, plants, and insects respond very differently to the same BVOCs.

On one hand, the BVOCs produced by the trichodiene overproducers T34-5.27 and E20-5.7 showed a significant increase in antifungal activity against *R. solani* and *F. oxisporum* compared to the control and their parental strains. Although growth inhibition generally decreased in all treatments as ventilation increased, differences between trichodiene-producing and non-producing strains increased in more open conditions using VOC Chambers. In this regard, while E20 and T34 promoted *F. oxysporum* growth in vented VOC Chambers, E20-5.7 and T34-5.27 significantly inhibited it. Additionally, wheat plantlets responded similarly to the BVOCs emitted by T34-5.27, which significantly reduced root and aerial part length, as well as fresh and dry weight, compared both to the control treatment and to T34 and E20 BVOCs. If these similar responses showed by fungal phytopathogens and plants are produced by the same BVOCs and/or are the result of comparable mechanisms is a question that should be addressed by conducting new experiments. Conversely, the effects exerted by T34-5.27 and E20-5.7 BVOCs on *A. obtectus* adults did not significantly differ from those produced by their parental strains. Therefore, changes in BVOC production induced in these trichodiene-overproducing strains seem not to produce a significant increase or reduction of their insecticidal properties against *A. obtectus*. The effects on insect emergence followed a similar pattern, with minor differences between trichodiene producers and non-producers, in which T34-5.27 and E20-5.7 showed slightly higher insect emergence than their parental strains. These results suggest that insects respond very differently to fungal BVOCs than other fungal strains and plants.

Even though previous reports showed the extremely high production of trichodiene by T34-5.27 and E20-5.7 *T. harzianum* strains and tested the effects of this compound on fungal strains and tomato plants (Malmierca *et al.*, 2015a; Malmierca *et al.*, 2015b), it cannot be assured with certainty that, in the present case, this compound is the responsible for the observed effects.

On the other hand, E20 BVOCs showed a general reduction in antifungal activity against *R. solani* and *F. oxysporum*. This strain and T34 even promoted *F. oxysporum* growth in vented conditions, although E20 produced statistically higher promotion than the wild type. This suggests that the blocking of *erg1* in *T. harzianum* may increase the production of BVOCs with growth-promoting activity on *F. oxysporum* when interacting in open environments. Similarly, E20 and T34 BVOCs showed significant growth-promoting activity on the aerial part of wheat plantlets compared to the control, but

without differences between them. These results could point out a similar mechanism in growth-promoting activity exerted by *T. harzianum* BVOCs both on plants and filamentous fungi, in which the same individual compounds may play the same role. Nevertheless, this hypothesis needs to be proven by evaluating the individual effects of purified BVOCs on these organisms. Plants could be also affected by CO_2 production.

Concerning the assays performed against insects, the results obtained using the squalene-overproducing strains were completely the opposite. E20 and E20-5.7 BVOCs showed a huge increase in insecticidal activity against *A. obtectus* adults compared to T34 and T34-5.27 when tested in sealed conditions, although this effect disappeared in unsealed ones. These strains also reduced the emergence of new insects from bean seeds and reduced the damage produced by *A. obtectus* on the beans. The presented results back up the expressed idea that insect responses to fungal BVOCs are quite different from those shown by other fungal strains and plants. Additionally, the insecticidal properties exerted by E20 and E20-5.7 suggest that these strains or their purified BVOCs could be used to develop biological control strategies against *A. obtectus*, with the aim of reducing the important damage produced on dry seeds by this insect pest, especially in storage facilities.

In this regard, BVOCs produced by T34 and E20 were identified and compared using GC/MS. Although still preliminary, the obtained results indicate that at least the proportion of the majoritarian individual BVOCs in the volatilome of the two strains significantly vary between them. T34 produces a higher percentage of Isobutyl acetate and 1-Butanol, 3-methyl-, acetate than E20; while E20 emits a higher percentage of 1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- than the wild type T34. This differential production of specific BVOCs may account for some of the volatile-mediated bioactive traits described for these *T. harzianum* strains, as well as for the differences reported between them. For example, one or more of the BVOCs overproduced by E20 could be responsible for the growth-promoting activity exerted on *F. oxysporum* by this fungal strain, or the responsible for the enhanced insecticidal properties showed against *A. obtectus*. However, other non-described BVOCs produced by these fungal strains in lower quantities could not be entirely ruled out as responsible for a relevant part of their volatile-mediated bioactivity.

Considering these results and the previously reported volatile-mediated bioactive effects of these *T. harzianum* strains, it would be of great interest to revise and to test the potential antifungal activity of purified Isobutyl acetate and 1-Butanol, 3-methyl-, and, especially, the putative insecticidal activity of 1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- against *A. obtectus* and other insect pests.

All the discussed evidence proves that the *in vitro* growing conditions in which the biological activity of microbial secondary metabolites is tested determine the outcome of the studied interactions. Therefore, research methodologies should take into account these conditions and be able to modulate them, as demonstrated by the use of vented and non-vented VOC Chambers. Finally, these new devices proved to be an effective, flexible, simple, replicable, and reliable methodology to evaluate microbe-microbe, plant-microbe, and insect-microbe interactions mediated by BVOCs, pointing out their potential to become a standard method for the performance of these assays. Moreover, new uses and applications for these devices could arise from the scientific community in the future.

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CONCLUSIONS

FIRST: Non-volatile soluble metabolites produced and released by *Trichoderma* spp. strains isolated from bean (*P. vulgaris*) fields belonging to the PGI "Alubia de La Bañeza-León" significantly inhibit the growth of a *F. oxysporum* bean phytopathogenic strain on *in vitro* membrane assays.

SECOND: All tested *Trichoderma* spp. strains show self-inhibitory activity mediated by non-volatile soluble metabolites, significantly inhibiting their own hyphal growth and altering the morphology of the fungal colony on *in vitro* membrane assays. These growth-inhibitory effects are mostly comparable to those exerted against *F. oxysporum* in the same conditions.

THIRD: A new Volatile Organic Compound Chamber (VOC Chamber) has been designed (Patent number: ES 2708899 B2), which proved to be an efficient and reliable device for the evaluation of microbe-microbe and insect-microbe volatile interactions.

FOURTH: This technology is less time consuming than the traditional double dish set (DDS) method, significantly increases the homogeneity and replicability of results, reveals differences not detected by the traditional methods, and allows for a more flexible set of experimental conditions, especially regarding ventilation and gas exchange with the environment.

FIFTH: Inhibitory effects produced by Biogenic Volatile Organic Compounds (BVOCs) emitted by *T. harzianum* strains on *R. solani* and *F. oxysporum* differ widely between vented, non-vented, and airtight conditions. Growth inhibitory effects generally decrease as ventilation increases, to the point that inhibition exerted by BVOCs from some *T. harzianum* strains on *F. oxysporum* in airtight conditions switch to growth promotion in vented ones.

SIXTH: *T. harzianum* BVOCs present insecticidal properties against *A. obtectus* and reduce the damage produced by the insect on bean seeds, being these effects generally lower with increased ventilation.

SEVENTH: A new plant-microbe Volatile Organic Compound Chamber (p-mVOC Chamber) has been designed (Utility Model application number: U202131032), which proved to be a reliable method for the evaluation of plant-microbe volatile interactions

and for the screening of microbial strains that produce BVOCs with putative bioactive effects on plants.

EIGHTH: The silencing of the *erg1* gene in *T. harzianum*, with the subsequent reduction of ergosterol and increase of squalene levels, reduces the antifungal activity of the BVOCs produced by this strain against *R. solani* and *F. oxysporum*, enhances their insecticidal properties against *A. obtectus*, reducing bean damage caused by this insect, and does not modify their growth-promoting activity on wheat plantlets, all compared to those effects produced by the wild type.

NINTH: The introduction of the *tri5* gene from *T. arundinaceum* in *T. harzianun*, with the subsequent overproduction of the volatile trichodiene, increases the antifungal activity of the BVOCs produced by these strains against *R. solani* and *F. oxysporum*, does not modify their insecticidal properties against *A. obtectus*, and enhances growth inhibition of wheat plantlets, all compared to those effects produced by the wild type.

TENTH: The silencing of the *erg1* gene in *T. harzianum* induces significant differences in the amount of specific BVOCs produced by this strain compared to those of the wild type, with lower emission of Isobutyl acetate and 1-Butanol, 3-methyl-, acetate; and higher emission of 1-Propanol, 2-methyl-; Acetoin; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Benzeneacetic acid, ethyl ester; and Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-. These differences may account for some of the specific volatile-mediated bioactive traits described for these *T. harzianum* strains.

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APPENDICES

Nuevos enfoques y tecnologías para evaluar la actividad biológica de los metabolitos secundarios microbianos en plantas, patógenos de plantas y plagas

RESUMEN

INTRODUCCIÓN

1. Agricultura sostenible frente a los retos presentes y futuros

La población mundial no deja de crecer y con ella la demanda de alimentos y otros productos agrícolas (Dhankher y Foyer, 2018). La erradicación del hambre es uno de los ejes centrales de los "Objetivos de Desarrollo Sostenible" que plantea la ONU para 2030. A pesar de ello, este problema aún no ha sido solucionado y existen una serie de riesgos para su consecución derivados del cambio climático, problemas energéticos y otros factores socioeconómicos y ambientales para la producción agrícola. Entre dichos factores, los estreses bióticos y abióticos a los que los cultivos se ven sometidos representan un elemento importante. Las enfermedades y plagas agrícolas causan grandes daños y pérdidas tanto a cultivos industriales como alimentarios (Pandey *et al.*, 2017). Además, estos agentes biológicos también afectan a los productos tras la recolección, pudiendo suponer la pérdida de hasta el 30% de los mismos (Dukare *et al.*, 2019). En este sentido, tradicionalmente se han venido utilizando productos fitosanitarios de síntesis química. Sin embargo, debido a los efectos perniciosos que estos productos presentan en muchos casos hacia el medio ambiente y la salud humana, está comenzando a prohibirse la utilización de muchos de ellos (Alengebawy *et al.*, 2021). De este modo, es necesario la búsqueda de alternativas para la protección de cultivos y productos agrícolas que sean más respetuosas con el medio ambiente y supongan un menor riesgo para la salud humana. Entre las estrategias más importantes a este respecto destacan los Agentes de Control Biológico como una estrategia fiable, efectiva y más segura (Kashyap *et al.*, 2017).

2. La judía común (*Phaseolus vulgaris* L.)

Las leguminosas son cultivos de extraordinaria importancia para la nutrición humana y la alimentación de animales (De Ron, 2015). Pertenecen a la familia vegetal Fabaceae. Se trata de productos con alto contenido proteínico y una amplia distribución, lo que hace de ellos un cultivo clave, sobre todo en aquellos países en vías de desarrollo donde el acceso a la proteína animal no está generalizado (Sanon *et al.*, 1998). Además, las leguminosas son capaces de fijar nitrógeno atmosférico inorgánico, de modo que su cultivo enriquece el suelo en este elemento esencial para el desarrollo de las plantas. Es

por esto que las leguminosas son clave en las estrategias de rotación de cultivos y en una agricultura sostenible (Casquero *et al.*, 2006).

Por otro lado, la judía común (*Phaseolus vulgaris* L.) ocupa el tercer puesto entre las leguminosas más importantes de las dedicadas al consumo humano, ya que su cultivo se extiende por todos los continentes y es fuente de proteínas, vitaminas y minerales. La Comunidad Autónoma de Castilla y León es la más importante productora de judía en España y en ella destaca la provincia de León, donde se encuentra la Indicación Geográfica Protegida (IGP) "Alubia de La Bañeza-León". Esta provincia supuso más del 45% de la superficie cultivada de toda España y más del 57% de la producción nacional en 2019 (MAPA, 2021).

La alubia se ve afectada por diversas enfermedades y plagas. Entre ellas, los hongos filamentosos fitopatógenos representan un grupo con especial relevancia. En muchos casos, microorganismos de los géneros *Rhizoctonia*, *Pythium*, *Fusarium, Thielaviopsis*, *Sclerotinia*, y *Aphanomyces* atacan juntos formando un complejo patogénico. En muchos cultivos *Rhizoctonia solani* Kühn, junto con el amarilleamiento producido por *Fusarium oxysporum* f. sp. *phaseoli* J.B Kendr. & W.C. Snyder y la podredumbre radicular causada por *Fusarium solani* f. sp. *phaseoli* (Burkh) W.C. Snyder & H.N. Hansen son las principales enfermedades fúngicas. En los últimos años la alubia en la IGP "Alubia de la Bañeza-León" se ha visto afectado por estas micosis, asociadas prácticamente a la totalidad de las plantas afectadas por podredumbre radicular en estas comarcas (Valenciano *et al.*, 2006).

En lo referente a *R. solani*, la infección de la planta se produce a través de heridas o por un recubrimiento de un órgano por el micelio, el cual penetra hasta la epidermis. La producción de enzimas líticas es muy importante en este proceso. Una vez infecta el hongo se ramifica rápidamente alcanzando las células de los tejidos próximos. Es un patógeno más agresivo en condiciones adversas para la planta, con temperaturas entre 15 y 18 ºC y con el suelo húmedo (Agrios, 2002). La enfermedad produce lesiones en los cotiledones, en el hipocotilo y en las raíces. El efecto más característico de este hongo es el conocido como caída o "damping-off" de las plántulas jóvenes. El hongo puede permanecer entre campañas agrícolas en el campo en forma de esclerocios o micelio, colonizando restos vegetales, plantas perennes o el suelo (Hall, 1992).

En el caso de *F. oxysporum* f. sp. *phaseoli*, se trata de un hongo filamentosos asexual que causa traqueomicosis en la judía común, conocido como amarilleamiento o marchitez de la judía. La sintomatología más características es la clorosis seguida de un amarilleamiento, que comienza en las hojas más bajas y va subiendo por la planta a medida que avanza la infección. Los haces vasculares muestran tonos marrones y rojizos, incluso antes de presentarse los síntomas externos (Singh and Schwartz, 2011). La infección comienza generalmente bajo el suelo y cuando llega a los vasos del xilema se desplaza por ellos para invadir el resto de la planta. Las pérdidas que produce son generalmente más importantes cuando la infección se da en las etapas tempranas de desarrollo de las plantas (Agrios, 2002).

Por otro lado, *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae: Bruchidae) en un insecto coleóptero que actúa como plaga primaria en granos almacenados, entre ellos de forma importante en la alubia (Berger *et al.*, 2017; Rodríguez-González *et al.*, 2019). Este insecto evolucionó en Mesoamérica y se ha distribuido por todo el mundo, produciendo grandes pérdidas en leguminosas. El insecto puede colonizar las semillas en campo o directamente hacerlo en el almacén, donde produce la mayor parte del daño (Baier y Webster, 1992). Las hembras pones los huevos y las larvas perforan un orificio por el que penetran en la semilla. Se desarrollan en el interior alimentándose de las mismas hasta que emergen como nuevos adultos. Este proceso reduce la calidad y el valor comercial del producto, e incluso puede condicionar su futura germinación (Paul *et al.*, 2009). Además, el daño generado favorece la aparición de plagas secundarias y enfermedades postcosecha (Dukare *et al.*, 2019).

3. Control Biológico

El control biológico, o biocontrol, puede ser definido como el uso de organismos biológicos, sus partes, o sustancias que producen para controlar el daño causado por otros organismos biológicos. Como ya se ha indicado, se trata de una estrategia que se viene desarrollando mucho en las últimas décadas con motivo de la búsqueda de alternativas ecológicas y saludables al uso de productos fitosanitarios de síntesis química. Entre los agentes de control biológico empleados los microorganismos presentan un papel central en agricultura (Pirttilä *et al.*, 2021; Sare *et al.*, 2021).

Estos organismos presentan un conjunto de características ventajosas. Cuando se

aplican de forma correcta suelen ser más respetuosos con el medio ambiente, y sus metabolitos son menos tóxicos y persistentes que numerosos productos de síntesis química (Tilocca *et al.*, 2020). Muchos de ellos pueden ser producidos de forma sencilla y barata (Paul *et al.*, 2009). En muchas ocasiones presentan efectos beneficiosos aditivos, controlando enfermedades y a la vez mejorando el desarrollo y crecimiento de las plantas (Maurya, 2020). Son menos agresivos contra otros organismos beneficiosos para el cultivo y su diversidad de mecanismos dificulta el desarrollo de resistencias por parte de os fitopatógenos.

Existen cuatro mecanismos generales por los cuales los agentes de control biológico pueden mejorar la sanidad y rendimiento de los cultivos:

Competición: dado que el espacio y os recursos son limitados en la naturaleza, la competencia por los mismo puede servir como limitante para el desarrollo de plagas y enfermedades. Esta competición permite controlar e incluso expulsar a los organismos dañinos de las zonas desde donde podrían afectar a las plantas (Alfiky y Weisskopf, 2021). Además, este proceso se puede dar incluso en la superficie o el interior de los propios órganos vegetales (Carro-Huerga *et al.*, 2020; Poveda *et al.*, 2020).

Parasitismo: mediante este mecanismo un agente de control biológico parasita al organismo fitopatógeno, alimentándose de él y pudiendo llegar a causarle la muerte. La producción de enzimas líticas y la capacidad de remodelar la propia pared celular son claves en este proceso en lo que a los hongos filamentosos se refiere (Sood *et al.*, 2020). Los microorganismos pueden presentar actividad parasítica frente a hongos, insectos y nemátodos (John *et al.*, 2010; Jaber and Ownley, 2018; Poveda *et al.*, 2020).

Inducción del crecimiento y las defensas de la planta: los agentes de control biológico pueden presentar diversos mecanismos que fomentan el desarrollo más vigoroso y la productividad de los cultivos, tanto desde el exterior como desde el interior de las plantas. En este proceso tiene relevancia la movilización de nutrientes en el suelo, así como la producción de metabolitos secundarios microbianos y de hormonas vegetales (Vinale *et al.*, 2008).

Antibiosis: consiste en la producción de enzimas y metabolitos secundarios con actividad tóxica contra otros organismos. Estos compuestos pueden ser de naturaleza

volátil o no volátil, e conllevan complejas interacciones, afectando a un amplio rango de seres vivos (Dukare *et al.*, 2019).

Debido al papel central que juegan en esta tesis, los compuestos orgánicos volátiles biogénicos (COVBs), así como las técnicas empleadas en su estudio, se tratarán a continuación en mayor profundidad:

Los COVBs son pequeñas moléculas de bajo peso molecular que en condiciones ambientales se presentan en forma gaseosa. Estos compuestos actúan como mediadores en diversas interacciones biológicas como competición (Hammerbacher *et al.*, 2019), simbiosis (Werner *et al.*, 2016; Kandasamy *et al.*, 2019), reconocimiento (Li *et al.*, 2018) o comunicación (Markovic *et al.*, 2019). También se ha propuesto que son importantes en el establecimiento de comunidades ecológicas (Li *et al.*, 2020). Entre los productores de COVBs destacan los microorganismos, tanto bacterias, como hongos filamentosos y levaduras. Los estudios sobre interacciones entre microorganismos mediadas por COBVs han demostrado que ejercen diversos efectos, que no dependen solo de las cepas intervinientes sino también de las condiciones ambientales (Lo Cantore *et al.*, 2015; Speckbacher *et al.*, 2020). Químicamente pueden ser muy diversos, por ejemplo, alcoholes, cetonas, alquenos, furanos, sesquiterpenos, ésteres, derivados bencénicos, etc.

Los volátiles microbianos también pueden afectar a las plantas de diversos modos. Así, se ha demostrado que pueden modular su fisiología (Sánchez-López *et al.*, 2016), incrementar su resistencia a herbívoros (Cordovez *et al.*, 2017) y patógenos vegetales induciendo respuestas defensivas (Frank *et al.*, 2021). También pueden favorecer la acumulación de compuestos o la nutrición de la planta, la fotosíntesis o la remodelación de la pared celular (Ameztoy *et al.*, 2019; García-Gómez *et al.*, 2019; Lee *et al.*, 2019). Finalmente, los COVBs microbianos también afectan a insectos. Sin embargo, la mayoría de los estudios a este respecto se han centrado en su capacidad atractiva o repelente, atendiendo a cuestiones etológicas (Contreras-Cornejo *et al.*, 2020). Comparativamente, pocos estudios se han realizad para analizar los efectos insecticidas de estos compuestos.

En lo referente a la metodología, para el cultivo de microorganismos es de vital importancia la gestión de los flujos gaseosos y sus aplicaciones (Spadaro y Droby, 2016; Alijani *et al.*, 2019). Los ensayos de competencia entre microorganismos se han convertido en herramientas imprescindibles para la caracterización *in vitro* de cepas con

capacidad de control biológico y para la detección de compuestos bioactivos producidos por las mismas. Sin embargo, las metodologías existentes para la investigación de interacciones mediadas por COVBs presentan una serie de limitaciones y problemas relacionadas con falta de espacio, poca flexibilidad, imposibilidad de permitir intercambio de gases, contaminaciones cruzadas, dificultad de manejo, etc.

4. *Trichoderma* spp. Persoon, Fries

Trichoderma un género fúngico bien conocido de amplia distribución que se medra principalmente en ambientes terrestres, mostrando una gran adaptabilidad a diversas condiciones ecológica (Contreras-Cornejo *et al.*, 2020). Se encuentra presente en material orgánica en descomposición, normalmente como un hongo saprófito. Sin embargo, también puede crecer parasitando otros hongos, nematodos e insectos. Algunos *Trichoderma* también se desarrollan en simbiosis con plantas (Jalali *et al.*, 2017; Macías-Rodríguez *et al.*, 2020).

Algunos miembros de este género han sido descritos como un riesgo para el cultivo de hongos (Kredics *et al.*, 2021), o como patógenos oportunistas en humanos inmunodeprimidos (Sautour *et al.*, 2018). Sin embargo, esto son casos aislados y mayoritariamente *Trichoderma* es considerado un hongo no peligroso y con importantes capacidades de control biológico de plagas y enfermedades agrícolas, así como promotor del crecimiento y los sistemas defensivos de las plantas (Sood *et al.*, 2020).

En la actualidad se han descrito unas 375 especies de este género, con aproximadamente 50 nuevas identificadas cada año (Cai y Druzhinina, 2021). *Trichoderma* pertenece a la División Ascomycota, y su teleomorfo ha sido nombrado tradicionalmente como *Hypocrea*. No obstante, la tendencia actual es referir ambos teleomorfo y anamorfo con un único nombre genérico, siendo *Trichoderma* el empleado de forma general (Bissett *et al.*, 2015).

Como se ha dicho, este género fúngico presenta interés industrial como productor de enzimas y otros compuestos empleados en diversos procesos de base biotecnológica. Por otro lado, destaca su capacidad para combatir enfermedades y plagas vegetales relacionadas con grandes pérdidas en agricultura, así como su capacidad para inducir el crecimiento y desarrollo de las plantas y mejorar su sanidad general, produciendo

importantes incrementos en el rendimiento de los cultivos (Kredics *et al.*, 2021).

Trichoderma tiene capacidad de inducir estos efectos mediante diferentes modos de acción. Miembros de este género han demostrado importante capacidad de competencia por recursos y espacio con organismos patógenos de plantas, incluso en el interior de las mismas (Carro-Huerga *et al.*, 2020). Para ello, *Trichoderma* ha demostrado mayor capacidad de acceder a nutrientes que muchos de los organismos fitopatógenos. Además, la gran velocidad a la que se desarrolla el micelio del hongo permite a este ocupar nichos de modo eficiente (Sarrocco *et al.*, 2009). Adicionalmente, se ha demostrado que esta movilización de nutrientes fomenta el crecimiento de las plantas a la vez que priva de ellos a otros microorganismos, y que los exudados de las plantas a su vez pueden favorecer el establecimiento de *Trichoderma* en su entorno (Vinale *et al.*, 2013).

Como se ha dicho, estos hongos son también capaces de promover el crecimiento de las plantas y sus sistemas defensivos. Para ello, además de la movilización de nutrientes, *Trichoderma* produce otra serie de compuestos, tanto volátiles como no volátiles, que inducen el desarrollo de las plantas. Del mismo modo, también se ha descrito la producción y emisión de hormonas vegetales con efectos similares (Alfiky and Weisskopf, 2021). La interacción de este hongo con diversas plantas ha demostrado que es capaz de inducir resistencia sistémica adquirida (RSA), resistencia sistémica inducida (RSI) y respuesta de hipersensibilidad (RH) (Harman, 2006).

Finalmente, *Trichoderma* puede enfrentarse a microorganismos patógenos o plagas mediante mecanismos de parasitismo y/o antibiosis. Para ello es capaz de reconocer posibles huéspedes y desarrollarse hacia ellos, infectándoles cuando entra en contacto con ellos (Alfiky and Weisskopf, 2021). Durante este proceso emite enzimas hidrolíticas que degradas las paredes celulares de los organismos atacados (Gomes *et al.*, 2020). Por otro lado, un elemento importante de estas interacciones es la capacidad de *Trichoderma* de sobreponerse a la toxicidad de sus propios compuestos mediante una intensa actividad de remodelación de su pared celular (Kappel *et al.*, 2020). A este mecanismo se suma la liberación al medio de compuestos volátiles y no volátiles con propiedades tóxicas frente a una gran variedad de organismos (Vinale and Sivasithamparam, 2020).

HIPÓTESIS Y OBJETIVOS

La hipótesis general de esta tesis doctoral es que las condiciones de crecimiento in vitro en las cuales se evalúa la actividad biológica de los metabolitos secundarios microbianos determina el resultado final de las interacciones estudiadas, por lo que las metodologías de investigación empleadas para ello deben tener en cuenta estas condiciones y ser capaces de modularlas.

Para evaluar dicha hipótesis se plantearon los siguientes objetivos generales:

I. Evaluar la actividad auto-inhibitoria de *Trichoderma* spp. empleando ensayos de membrana *in vitro* y compararla con la actividad antifúngica producida por estos hongos frente a *Fusarium oxysporum* en las mismas condiciones.

II. Desarrollar un dispositivo sencillo y eficiente para evaluar las interacciones entre diferentes microorganismos, y entre microorganismos e insectos, mediadas por Compuestos Orgánicos Volátiles Biogénicos (COVBs), y probar su correcto funcionamiento empleando para ello aislados de *Trichoderma* como agentes de control biológico (ACBs).

III. Desarrollar un dispositivo sencillo y eficiente para evaluar las interacciones entre plantas y microorganismos mediadas por Compuestos Orgánicos Volátiles Biogénicos (COVBs), y probar su correcto funcionamiento empleando para ello aislados de *Trichoderma* como agentes de control biológico (ACBs).

IV. Identificar COVBs individuales emitidos por *Trichoderma* spp. que puedan ser los responsables de las actividades biológicas observadas.

METODOLOGÍA

1. Organismos empleados

En la presente tesis se ha empleado un aislado del hongo *F. oxysporum* que demostró actividad patogénica en plantas de judía común (*P. vulgaris*), al que se denominó F3, y un aislado de *R. solani* patógeno de judía. Ambos procedentes de campos correspondientes a la IGP "Alubia de La Bañeza-León". Para los ensayos de autotoxicidad se emplearon 10 aislados del *Trichoderma* spp., siete de ellos pertenecientes a la especie *T. harzianum*, y siendo los tres restantes *T. citrinoviride*, *T. velutinum* y *T. gamsii*. Todos ellos procedían de campos de la IGP "Alubia de La Bañeza-León".

En lo referente a los ensayos de interacción mediada por compuestos volátiles empleando los nuevos dispositivos VOC Chamber y p-mVOC Chamber, se emplearon cuatro cepas de *T. harzianum*. La cepa silvestre *T. harzianum* CECT 2413 (Colección Española de Cultivos Tipo, Valencia, España), a la que se llamó T34; y tres transformantes derivados de ella. Éstas cepas fueron desarrolladas en trabajos previos dentro de este grupo de investigación (Cardoza *et al.*, 2006; Malmierca *et al.*, 2015a, b). E20 consiste en la cepa silvestre a la que se le ha bloqueado el gen *erg1* que codifica la escualen epoxidasa, lo que lleva a la sobreacumulación de escualeno y la reducción de ergosterol. A su vez, T34 y E20 son los parentales a partir de los que se obtuvieron T34-5.27 y E20-5.7 mediante la introducción del gen *tri5* de *T. arundinaceum*, que codifica una terpeno ciclasa. Esta modificación induce la sobreproducción del compuesto volátil trichodieno, que forma parte de la ruta biosintética de los trichotecenos.

En los experimentos con plantas se emplearon semillas de trigo (*Triticum aestivum* var. *tremie*). Por último, como insecto plaga para evaluar el efecto de los COVBs fúngicos se empleó el gorgojo de la alubia (*Acanthoscelides obtectus* (Say) Coleoptera: Chrysomelidae: Bruchidae). Estos insectos fueron capturados en almacenes de semilla pertenecientes a la IGP "Alubia de La Bañeza-León".

2. Evaluación de la actividad antifúngica de autoinhibitoria de los metabolitos secundarios no volátiles producidos por *Trichoderma* spp. mediante ensayos de membrana

Los ensayos de membrana se desarrollaron con la metodología descrita por Mayo *et al.*, (2015). Para ello se colocó una membrana de celofán estéril sobre pacas Petri con medio Patata Dextrosa Agar (PDA). Sobre cada una de ellas se dispuso un disco de 6 mm de diámetro extraído del borde en crecimiento de colonias de los diferentes aislados de *Trichoderma* spp. Para los controles se realizó el mismo procedimiento en placas sin cultivar. Los hongos se dejaron crecer por 2 días a 25 ºC para que los metabolitos producidos difundieran hacia el medio. Tras esto, el celofán junto con el micelio fúngico se retiró e inmediatamente después se sembraron discos de 6 mm del borde activo del aislado de *F. oxysporum*. El diámetro de las colonias se midió a los 3 y 7 días de crecimiento. El procedimiento para la evaluación de la autotoxicidad o autoinhibición fue el mismo, pero colocando de nuevo un disco de la misma cepa de *Trichoderma* en lugar de *F. oxysporum*. Las medidas en este caso se realizaron a las 24 y 48 horas debido a la mayor velocidad de crecimiento de este hongo. Se realizaron 5 réplicas por tratamiento.

3. Evaluación del prototipo "VOC Chaber" y de los efectos producidos por COVBs de *T. harzianum* sobre *F. oxysporum* y *R. solani*.

Se compararon la técnica rutinaria de placas Petri enfrentadas (DDS) con VOC Chamber ventiladas y no ventiladas. Para ello se llenaron las placas Petri con 18 ml de medio PDA. Discos de 6 mm de las distintas cepas de *T. harzianum* (T34, E20, T34-5.27 y E20-5.70) fueron sembrados en el centro de las placas y se dejaron crecer durante 48 horas. Tras esto se sembraron en nuevas placas discos de 6 mm de *F. oxysporum* y *R. solani* y se procedió al montaje de los diferentes dispositivos. Las cámaras formadas mediante el método DDS y las "VOC Chamber" no ventiladas fueron selladas con parafilm. Los controles se realizaron sustituyendo la placa con *T. harzianum* por placas no cultivadas con 18 ml de PDA. Tras esto, las cámaras con los hongos se colocaron a 25 ºC. En el caso de *R. solani* se midió el crecimiento radial de las colonias tras 1, 2, 3 y 4 días; mientras que en el caso de *F. oxysporum* estas se realizaron tras 3, 5 y 7 días. Se llevaron a cabo 5 réplicas por tratamiento.

4. Evaluación del prototipo "p-mVOC Chaber" y de los efectos producidos por COVBs de *T. harzianum* sobre plántulas de trigo (*Triticum aestivum* var. *tremie*)

En primer lugar, las cepas de *T. harzianum* (T34, E20, T34-5.27 y E20-5.70) se sembraron mediante la técnica ya descrita en el apartado anterior en placas Petri con 18 ml de PDA. Estos hongos se dejaron crecer durante 72 horas a 25 ºC. Pasado este tiempo, 10 semillas de trigo previamente desinfectadas fueron colocadas sobre 4 capas de papel de filtro (73 g m^{-2}) en el interior de recipientes de vidrio de 946 ml. Inmediatamente después se montaron las cámaras colocando en la parte inferior los recipientes con las semillas, sobre cada uno de ellos una pieza central de la "p-mVOC Chamber" con el orificio central cubierto por un Filtro Whatman (47 mm), y sobre cada una de estas, boca abajo, se colocó una placa con hongo tras serle retirada la tapa. Las cámaras montadas se sellaron con parafilm y se colocaron en una cámara de crecimiento (23-23 ºC, fotoperiodo de 16:8 y 3000 lux (41 µmol m^{-2} s^{-1}) durante 10 días. Se realizaron 5 réplicas por tratamiento y el experimento se repitió 5 veces.

La germinación de las semillas se contabilizó a los 3, 7 y 10 días. Tras esto, se procedió a medir el peso fresco y longitud de la raíz y la parte aérea de cinco plantas de cada réplica. Se secaron a 60 ºC hasta que el peso se mantuvo constante, momento en el que se procedió a evaluar el peso seco de las muestras. Otras 3 plantas fueron empleada para analizar el rendimiento cuántico de fluorescencia de la clorofila usando un fluorímetro PAM. Tras esto se almacenaron a -80 ºC para la futura evaluación del contenido en clorofila, que se llevó a cabo mediante el método de Arnon (Arnon, 1949).

5. Empleo de "VOC Chambers" para la valuación de los efectos producidos por COVBs de *T. harzianum* sobre *A. obtectus* y los daños que este produce sobre alubias

Para este experimento se emplearon cámaras no ventiladas. En primer lugar, se sembraron discos de 6 mm de las cuatro cepas de *T. harzianum* en placas Petri con 18 ml de PDA. Los hongos se dejaron crecer durante 3 días antes de exponer a los insectos a los COVBs producidos por estos. Los controles se realizaron usando placas Petri con 18 ml de PDA no cultivadas. Por otro lado, los recipientes donde se mantienen las poblaciones de *A. obtectus* fueron limpiados de adultos 3 días antes del experimento, de modo que los insectos añadidos tuvieran una edad homogénea controlada de entre 0 y 3 días.

Adicionalmente, se contaron y pesaron grupos de 40 alubias de la variedad "Riñón Menudo" pertenecientes a la IGP "Alubia de La Bañeza-León" para su introducción junto con los insectos en cada una de las réplicas experimental.

Las cámaras se montaron del siguiente modo: la placa con el hongo se emplazó boca arriba retirándosele la tapa, sobre ella se colocó la pieza intermedia de la VOC Chamber, cuya superficie superior, incluyendo el orificio central se cubrió con una membrana de celofán y un papel de filtro estériles, de modo que permitiesen el paso de los COVBs pero impidieran a los insectos acceder a la parte inferior, evitando también contaminaciones cruzadas por esporas fúngicas. Sobre el papel se colocaron 40 alubias y 20 adultos de *A. obtectus* sin sexar. Finalmente, estos se cubrieron con una placa Petri vacía boca abajo. En la disposición sellada del experimento el contacto entre ambas placas y la pieza central fue sellada empleando parafilm. Para la disposición no sellada solamente la placa inferior con el hongo fue sellada con parafilm, mientras que la superior conteniendo las alubias y los insectos se mantuvo sin sellar, para permitir un mayor intercambio de gases con el exterior. Las cámaras se colocaron a 25ºC en oscuridad. La mortalidad de los insectos fue evaluada diariamente hasta pasar 16 días. Se realizaron 5 réplicas por tratamiento y condición (sellada y no sellada).

Tras esto, las placas inferiores y las piezas centrales se descartaron y los cadáveres de los insectos fueron retirados de la placa conteniendo las alubias, que se cerraron con nuevas tapas. Estos cadáveres se cultivaron en medio Rosa de Bengala-Cloranfenicol, para comprobar que no había entrado en contacto físico con los *T. harzianum*. Por otro lado, las placas con las 40 semillas de cada réplica se colocaron de nuevo a 25ºC en oscuridad hasta que el primer adulto de la F1 emergió de las mismas. Este momento fue determinado como día 1 del experimento de emergencia. A partir de aquí, la emergencia de nuevos adultos se contabilizó diariamente hasta el día 23, habiendo cesado la misma de forma general en torno al día 20. Finalmente, las alubias de cada réplica se pesaron para calcular la pérdida de peso sufrida y se contó el número de alubias afectadas, el número de orificios por alubia individualmente y el número total de orificios por réplica. De este modo se dispuso de datos relevantes sobre los daños producidos por *A. obtectus* sobre alubias estando expuesto a COVBs de diferentes cepas fúngicas.

6. Identificación y cuantificación de los COVBs producidos por *T. harzianum* T34 y E20 mediante (CG/EM)

Las cepas fúngicas se sembraron y dejaron crecer a 25ºC durante 3 días en frascos de 50 ml con 10 ml de agar en posición horizontal. Tras esto, la extracción se llevó a cabo microextracción en fase sólida (HS-SPME) y se analizó empleando Cromatografía de gases y Espectrometría de Masas (CG/EM). La fibra de adsorción empleada fue divinylbenzeno/carbonex/polydimethylsiloxano (DVB/CAR/PDMS 50/30 µm) SPME, empleando la metodología de muestreo descrita por Malheiro *et al.*, (2018). Las muestras se mantuvieron a 25ºC durante 5 minutos y posteriormente se expuso la fibra durante 30 minutos. Tras extraerse, ésta se eluyó en el puerto de inyección del CG/EM mediante desorción térmica a 220ºC. El análisis se realizó en un cromatógrafo Shimadzu GC-2010 unido a un detector espectrómetro Shimadzu GC/MS-QP2010 SE. Se empleó helio como fase móvil a 30 cm/s. Se programó una rampa de temperatura partiendo de 40ºC más 2ºC/min hasta alcanzar 220ºC. La temperatura de la fuente de ionización fue de 250ºC, con una energía de 70 eV y corriente de ionización de 0.1 KV. El rango de ionización para los espectros de masa fue de 35-500 m/z. Para su identificación, los espectros fueron comparados a la base de datos del NIST 11, y el porcentaje de los picos determinado integrando el área empleando el ion base específico para cada uno de ellos.

7. Análisis estadísticos

De forma general, para todos los conjuntos de datos se evaluó la normalidad y homocedasticidad de las muestras empleando para ello el método de Kolgomorov-Smirnov y la prueba de Levene. Los datos que cumplieron estas condiciones se analizaron análisis de varianza de una vía (ANOVA) seguidos de la prueba *post hoc* de Tukey, mayoritariamente aplicando $p \leq 0.05$, aunque para la comparativa de metodologías entre DDS y VOC Chambers se evaluaron para $p \leq 0.05$; $p \leq 0.01$ y $p \leq 0.001$.

En los datos de los experimentos con insectos la prueba de contraste empleada fue la de Diferencia Mínima Significativa ($p \leq 0.05$). En el caso de tratarse de únicamente dos muestras de datos se utilizó la prueba de *t* de Student ($p \leq 0.05$). Por último, en el caso de no cumplirse las condiciones de normalidad o de igualdad de varianzas, se empleó la prueba no paramétrica H de Kruskal-Wallis, seguida de la prueba U de Mann-Whitney ($p \leq 0.05$). Para todos los análisis se empleó el programa IBM SPSS Statistics 26.

8. Dispositivos desarrollados para la realización de ensayos *in vitro* de interacción mediada por COVBs

Se describen a continuación las invenciones que han sido desarrolladas y patentadas para llevar a cabo de forma fiable y eficiente los experimentos de interacción mediada por compuestos volátiles. Dado que son el elemento metodológico central de la tesis, su descripción se realizará en este resumen de forma más pormenorizada.

8.1. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles (VOC Chamber)

Es objeto de la invención una cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles que comprende: un primer receptáculo, un segundo receptáculo, y una pieza central entre el primer receptáculo y el segundo receptáculo. Los receptáculos comprenden una pared externa y una pared perimetral perpendicular a la pared externa, y la pieza central comprende una pared intermedia con al menos un orificio, y dos paredes laterales que se proyectan desde el perímetro de la pared intermedia en direcciones opuestas. En la placa objeto de la invención las paredes laterales de la pieza central rodean las paredes perimetrales de los receptáculos y las paredes perimetrales de los receptáculos se apoyan en la pared intermedia.

En otra realización, la pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde al menos una de las caras comprende salientes. En otra realización, la pieza central comprende un reborde interno en al menos una de las caras de la pared intermedia, tal que el reborde interno y la pared lateral configuran un alojamiento para el extremo de la pared externa de un receptáculo. En otra realización, la pared intermedia comprende una pluralidad de pestañas en la primera cara y/o en la segunda cara donde se apoya la pared externa de un receptáculo. En otra realización, comprende una membrana o filtro cubriendo el orificio de la pared intermedia de la pieza central, donde la membrana o filtro son porosos permitiendo el paso del aire y compuestos volátiles, y bloqueando el paso de elementos de mayor tamaño.

La cámara comprende un primer reborde situado en la pared externa del primer receptáculo, y un segundo reborde de diámetro ligeramente diferente situado en la pared

externa del segundo receptáculo, tal que las paredes externas de un primer receptáculo y un segundo receptáculo, el primer reborde encaja en el segundo reborde impidiendo el movimiento relativo entre dos placas de cultivo apiladas. En otra realización la cámara comprende al menos un tabique en al menos un receptáculo, tal que el tabique divide en partes independientes el interior de dicho receptáculo.

Descripción de los dibujos

Las distintas referencias numéricas que se encuentran reflejadas en las figuras y el texto corresponden a los siguientes elementos:

1. primer receptáculo,
2. segundo receptáculo,
3. pieza central,
4. pared externa,
5. pared perimetral,
6. pared intermedia,
7. pared lateral,
8. primera cara,
9. segunda cara,
10. reborde interno,
11. alojamiento,
12. pestañas,
13. orificio,
14. membrana o filtro,
15. tabique,
16. primer reborde, y
17. segundo reborde.

Reivindicaciones

1. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles, **caracterizada por** que comprende:

- un primer receptáculo (1),
- un segundo receptáculo (2), y
- una pieza central (3) entre el primer receptáculo (1) y el segundo

receptáculo (2),

donde los receptáculos (1, 2) comprenden una pared externa (4) y una pared perimetral (5) perpendicular a la pared externa (4), y donde la pieza central (3) comprende una pared intermedia (6) con al menos un orificio (13), y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en direcciones opuestas, tal que las paredes laterales (7) de la pieza central (3) rodean las paredes perimetrales (5) de los receptáculos (1, 2) y las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan en la pared intermedia (6) de la pieza central (3).

2. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde la primera cara (8) y la segunda cara (9) son lisas.

3. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde al menos una de las caras (8, 9) comprende una pluralidad de pestañas (12) donde se apoya la pared perimetral (5) de un receptáculo (1, 2), facilitando así el intercambio gaseoso con el exterior. .

4. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 3, **caracterizada por** que la pieza central (3) comprende un reborde interno (10) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde interno (10) y la pared lateral (7) configuran un alojamiento (11) para el extremo de la pared perimetral (5) de uno o ambos receptáculos (1, 2) quedando así enclavados.

5. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 4, **caracterizada por** que comprende una membrana o filtro (14) cubriendo el orificio (13) de la pared intermedia (6) de la pieza central (3), donde la membrana o filtro (14) es porosa permitiendo el paso

del aire y compuestos volátiles, y bloqueando el paso de elementos de mayor tamaño.

6. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 5, **caracterizada por** que comprende: un primer reborde (16) situado en la pared externa (4) del primer receptáculo (1), y un segundo reborde (17) situado en la pared externa (4) del segundo receptáculo (2), donde enfrentado las paredes externas (4) de un primer receptáculo (1) y un segundo receptáculo (2), el primer reborde (16) encaja en el segundo reborde (17) impidiendo el movimiento relativo entre dos placas de cultivo apiladas.

7. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 6, **caracterizada por** que comprende al menos un tabique (15) en al menos un receptáculo (1, 2), tal que el tabique (15) divide en partes independientes el interior de dicho receptáculo (1, 2).

8.2. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo (p-mVOC Chamber)

La presente invención se refiere a una cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo. La cámara de cultivo objeto de la invención es de aplicación en el campo de la investigación *in vitro* en botánica, fisiología vegetal y agricultura, y más concretamente en la categoría de recipientes y cámaras para el cultivo *in vitro* de plantas o partes de plantas.

La cámara de cultivo permite evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo; así como el efecto de estos mismos compuestos volátiles sobre plagas o enfermedades de plantas cuando se encuentran interaccionando con estas; o, adicionalmente, la evaluación de los efectos producidos por compuestos volátiles emitidos por plantas o alguna de sus partes sobre otros organismos.

La presente cámara comprende: un primer receptáculo, un segundo receptáculo, y una pieza central entre el primer receptáculo y el segundo receptáculo. Los receptáculos comprenden una pared externa y una pared perimetral perpendicular a la pared externa, y donde la pieza central comprende una pared intermedia con al menos un primer orificio, y dos paredes laterales que se proyectan desde el perímetro de la pared intermedia en

sentidos opuestos, tal que las paredes laterales de la pieza central rodean las paredes perimetrales de los receptáculos y las paredes perimetrales de los receptáculos se apoyan en la pared intermedia de la pieza central. La pared perimetral del primer receptáculo tiene una altura diferente a la pared perimetral del segundo receptáculo.

En la cámara de cultivo la pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde la primera cara y la segunda cara son lisas. La pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde al menos una de las caras puede comprender una pluralidad de pestañas donde se apoya la pared perimetral de un receptáculo, facilitando así el intercambio gaseoso con el exterior.

La pieza central puede comprender un reborde interno en al menos una de las caras de la pared intermedia, tal que el reborde interno y la pared lateral configuran un alojamiento para el extremo de la pared perimetral de uno o ambos receptáculos quedando así enclavados. Además, la pieza central puede comprender un reborde en al menos una de las caras de la pared intermedia, tal que el reborde y la pared intermedia están configurados para alojar una membrana o filtro y/o para evitar el goteo de líquidos desde la primera cara. También puede comprender una membrana o filtro cubriendo el primer orificio de la pared intermedia de la pieza central, donde la membrana o filtro es porosa y permite el paso del aire y compuestos volátiles, y bloquea el paso de elementos de mayor tamaño.

La pared perimetral de al menos un receptáculo puede comprender un segundo orificio cubierto por un septo, diafragma o válvula, tal que el segundo orificio impide la libre salida de gases al exterior a través de dicho segundo orificio y permite la extracción de los mismos del interior de la cámara mediante punción con elementos de tipo jeringuilla o unión mediante un tubo. Finalmente, puede presentar al menos un tabique en al menos un receptáculo, tal que el tabique divida en partes independientes el interior de dicho receptáculo.

Descripción de los dibujos

Las distintas referencias numéricas que se encuentran reflejadas en las figuras y el texto corresponden a los siguientes elementos:

1. primer receptáculo,
2. segundo receptáculo,
3. pieza central,
4. pared externa,
5. pared perimetral,
6. pared intermedia,
7. pared lateral,
8. primera cara,
9. segunda cara,
10. pestañas,
11. primer orificio,
12. segundo orificio,
13. membrana o filtro,
14. reborde,
15. reborde interno,
16. alojamiento

Reivindicaciones

1. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo, que comprende un primer receptáculo (1), un segundo receptáculo (2), y una pieza central (3) entre el primer receptáculo (1) y el segundo receptáculo (2), donde los receptáculos (1, 2) comprenden una pared externa (4) y una pared perimetral (5) perpendicular a la pared externa (4), y donde la pieza central (3) comprende una pared intermedia (6) con al menos un primer orificio (11), y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos, tal que las paredes laterales (7) de la pieza central (3) rodean las paredes perimetrales (5) de los receptáculos (1, 2) y las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan en la pared intermedia (6) de la pieza central (3), caracterizado por que la pared perimetral (5) del primer receptáculo (1) tiene una altura diferente a la pared perimetral (5) del segundo receptáculo (2).

2. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según la reivindicación 1, caracterizada por que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde la primera cara (8) y la segunda cara (9) son lisas.

3. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según la reivindicación 1, caracterizada por que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde al menos una de las caras (8, 9) comprende una pluralidad de pestañas (10) para apoyo de la pared perimetral (5) de un receptáculo (1, 2) para intercambio de gases con el exterior.

4. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 3, caracterizada por que la pieza central (3) comprende un reborde interno (15) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde interno (15) y la pared lateral (7) configuran un alojamiento (16) para el extremo de la pared perimetral (5) de uno o ambos receptáculos (1, 2) quedando así enclavados.

5. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 4, caracterizada por que la pieza central (3) comprende un reborde (14) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde (14) y la pared intermedia (6) están configurados para alojar una membrana o filtro (13).

6. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 5, caracterizada por que comprende una membrana o filtro (13) cubriendo el primer orificio (11) de la pared intermedia (6) de la pieza central (3).

7. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 6, caracterizada por que la pared perimetral (5) de al menos un receptáculo (1, 2) comprende un segundo orificio (12) cubierto por un septo, diafragma o válvula, configurado para extraer volátiles.

8. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 7, caracterizada por que comprende al menos un tabique en al menos un receptáculo (1, 2), tal que el tabique divide en partes independientes el interior de dicho receptáculo (1, 2).

PRINCIPALES RESULTADOS Y DISCUSIÓN

1. Evaluación de la actividad antifúngica de autoinhibitoria de los metabolitos secundarios no volátiles producidos por *Trichoderma* spp. mediante ensayos de membrana

En ensayos de membrana, *Trichoderma spp*. demostró una intensa actividad antifúngica frente a *F. oxisporum*. Esta actividad fue significativamente mayor para los aislados de *T. harzianum* que para el resto; *T. velutinum*, *T. gamsii* y *T. citrinoviride*. Por otro lado, los resultados obtenidos en el ensayo de autoinhibición fueron muy similares. En el primer día de la toma de datos 5 de los 10 *Trichoderma* no mostraron diferencias entre autoinhibición y la inhibición sobre *F. oxysporum*. Esta proporción subió a 7 de 10 en el segundo día de toma de medidas, mostrando este efecto todos los *T. harzianum* estudiados. Además, de entre los aislados que mostraron diferencias, dos de ellos

presentaron mayor autoinhibición que actividad antifúngica frente a *F. oxysporum*.

Estos resultados sugieren que en las condiciones *in vitro* que se producen en los ensayos de membrana, la extremadamente alta concentración de sustancias tóxicas que se acumulan no solo afectan de forma significativa a otros microorganismos, sino también al propio ACB. DE este modo, no parece que los resultados obtenidos puedan ser razonablemente extrapolados a condiciones naturales, poniendo además en tela de juicio la utilidad real de estos ensayos por si mismos para seleccionar preliminarmente aislados fúngicos con potencial actividad como ACB. En este sentido, ensayos de autotoxicidad podrían ser de utilidad para afinar de forma más precisa esta selección de ACBs, eligiéndose aquellas que no sólo producen la mayor inhibición sobre el patógeno, sino que también tengan mayor capacidad de resistir sus propios metabolitos tóxicos.

2. Evaluación del prototipo "VOC Chaber" y de los efectos producidos por COVBs de *T. harzianum* sobre *F. oxysporum* y *R. solani*.

En primer lugar, las nuevas cámaras de cultivo "VOC Chambers" mostraron una reducción significativa del coeficiente de variación respecto al método tradicional de placas enfrentadas (DDS), indicando que aportan una mayor homogeneidad de resultados a estos experimentos, lo cual de por sí ya es una característica relevante. Adicionalmente, tanto el modelo ventilado como el no ventilado pusieron de manifiesto diferencias estadísticamente significativas entre cepas de *T. harzianum* que no fueron reveladas por el método DDS, indicando que la nueva tecnología tiene una mayor capacidad de discernir diferencias en la actividad antifúngica de diferentes COVBs.

A nivel general, la actividad inhibitoria sobre los patógenos se redujo con el incremento de intercambio de gases entre el sistema y el exterior. Además, el estudio de la actividad de los COVBs de *T. harzianum* sobre *F. oxysporum* empleando cámaras ventiladas mostró que la inhibición de crecimiento que producen estos COVBs sobre el patógeno en condiciones selladas se convierte en una actividad promotora del crecimiento en condiciones ventiladas para las cepas T34 y E20. Por otro lado, las cepas productoras de trichodieno (T34-5.27 y E20-5.7) produjeron un incremento significativo de la inhibición en comparación con sus parentales. Sin embargo, no se puede asignar con seguridad este efecto al trichodieno sin realizar nuevos experimentos empleando el compuesto puro. Estas cepas mostraron actividad inhibitoria incluso en condiciones

ventiladas.

Estos resultados muestran que las cámaras de cultivo “VOC Chambers” son una tecnología fiable y eficiente para la realización de ensayos de competencia microbiana mediados por COVBs, y que estos dispositivos podrían convertirse en un estándar metodológico en investigación. Además, nuevos usos podrían ser desarrollados por la comunidad científica para estos dispositivos.

3. Evaluación del prototipo “p-mVOC Chaber” y de los efectos producidos por COVBs de *T. harzianum* sobre plántulas de trigo (*Triticum aestivum* var. *tremie*)

Las cámaras de cultivo “p-mVOC Chamber” mostraron ser un método funcional para la evaluación de los efectos producidos por COVBs microbianos sobre semillas y plántulas. En este sentido se observaron diferencias moderadas en la tasa de germinación de semillas de trigo en los tratamientos control y en aquellos expuestos a COVBs de *T. harzianum*.

T34 y E20 indujeron significativamente el crecimiento en longitud de la parte aérea en comparación con el control, así como su peso fresco. Estas cepas no modificaron la longitud y el peso fresco de la raíz, pero si redujeron el peso seco de estas últimas. Por otro lado, la cepa productora de trichodieno T34-5.27 redujo significativamente la longitud, el peso fresco y el peso seco de la parte aérea, y redujo igualmente de forma significativa la longitud y el peso seco de la raíz, todo en referencia a los datos obtenidos para el tratamiento control sin hongo. Además, se observaron importantes diferencias entre esta cepa de *T. harzianum* y las otras dos, con T34-5.27 produciendo una reducción generalizada de los parámetros de crecimiento medidos en las plántulas de trigo. En lo referente a indicadores de fotosíntesis no se observaron diferencias significativas entre ninguno de los tratamientos ni en contenido total de clorofila ni en rendimiento cuántico. Esto parece indicar que las diferencias observadas a nivel de desarrollo no estarían relacionadas con efectos sobre los procesos fotosintéticos de la planta.

4. Empleo de "VOC Chambers" para la valuación de los efectos producidos por COVBs de *T. harzianum* sobre *A. obtectus* y los daños que este produce sobre alubias

Los COVBs producidos por las diferentes cepas de *T. harzianum* produjeron un significativo incremento respecto al control en la mortalidad de adultos de *A. obtectus* expuestos a los mismos en condiciones selladas, mientras que, en condiciones no selladas, donde se produce una mayor ventilación e intercambio de gases con el exterior, apenas se observaron diferencias en la mortalidad. Las cepas superproductoras de escualeno E20 y E20-5.7 produjeron un incremento muy significativo de actividad insecticida respecto a T34 y T34-5.27 en condiciones selladas. El incremento en la mortalidad de adultos se tradujo en una menor emergencia de insectos de las alubias en la siguiente generación. De nuevo, esta reducción fue mayor para E20 y E20-5.7. Cabe destacar que, a pesar de no observarse diferencias en la actividad insecticida sobre los adultos en condiciones no selladas, sí se redujo significativamente el número de nuevos insectos emergidos, siendo de nuevo E20 y E20-5.7 los que produjeron una mayor reducción. Esto parece indicar que los COVBs de *T. harzianum* producen toxicidad sobre otras fases de desarrollo del insecto (huevos, larvas) o que inducen cambios en el comportamiento reproductivo de los mismos.

En lo referente a los daños producidos por *A. obtectus* en las alubias, estos se redujeron cuando los insectos se encontraban expuestos a los COVBs microbianos. En condiciones selladas la reducción fue significativamente mayor en aquellos expuestos a E20 y E20-5.7, en paralelo al incremento de mortalidad y reducción en emergencia. En condiciones no selladas los daños en las alubias también se redujeron significativamente con respecto al control, pero no de forma tan marcada ni para todos los parámetros. Se redujeron significativamente el porcentaje de alubias afectadas y el número total de orificios, pero esta reducción no fue estadísticamente significativa en el caso del número de orificios por alubia afectada ni el porcentaje total de pérdida de peso.

Los resultados referidos sugieren que uno o varios COVBs producidos por las cepas de *T. harzianum* estudiadas podrían ser útiles para el control de *A. obtectus* como plaga de alubias y otros granos almacenados, con el objetivo de reducir las pérdidas causadas por este insecto. Estos compuestos podrían ser una alternativa natural y menos tóxica que los compuestos de síntesis química empleados como insecticidas en la actualidad. Por

tanto, nuevos estudios empleando COVBs aislados y purificados de estas cepas frente a *A. obtectus* y otros insectos plaga de almacén serían de gran interés.

5. Identificación y cuantificación preliminar de los COVBs producidos por *T. harzianum* T34 y E20 mediante (CG/EM)

Se identificaron y cuantificaron mediante CG/EM los COVBs producidos por las cepas T34 y E20 en condiciones selladas. Posteriormente se compararon los niveles de producción para cada uno de ellos. Preliminarmente, se analizaron estas diferencias entre los 8 compuestos mayoritarios. T34 mostró una producción significativamente mayor de Isobutyl acetate (18.66 ± 1.6%) y 1-Butanol, 3-methyl-, acetate (41.11 ± 2.72%), mientras que E20 produjo significativamente mayor cantidad de 1-Propanol, 2-methyl- (23.85 ± 2.73%); Acetoin (12.09 ± 1.27%); 1-Butanol, 3-methyl- (15.19 ± 1.36%); Phenylethyl Alcohol (5.07 ± 0.81%); Benzeneacetic acid, ethyl ester (0.29 ± 0.04%); y Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- (2.59 ± 0.5%). La suma de estos 8 compuestos supuso para ambas cepas más del 90% de COVBs producidos.

Como se ha referido en el apartado anterior, cabe esperar que COVBs producidos en diferente proporción por estas cepas de *T. hazianum* sean los responsables de las diferentes actividades biológicas que han sido descritas en los capítulos anteriores. Sin embargo, otros compuestos minoritarios también podrían ser responsables total o parcialmente. Por tanto, es necesario completar estos resultados preliminares con nuevos análisis y evaluaciones de los efectos de los compuestos aislados.

CONCLUSIONES

PRIMERA: Los metabolitos solubles no volátiles producidos y liberados por cepas de *Trichoderma* spp. aisladas de campos de judía común (*P. vulgaris*) pertenecientes a la IGP "Alubia de la Bañeza-León inhiben significativamente el crecimiento de un aislado fitopatogénico de *Fusarium oxysporum* en ensayos *in vitro* de membrana.

SEGUNDA: Todos los aislados de *Trichoderma* spp. evaluados muestran actividad auto-inhibitoria mediada por metabolitos solubles no volátiles en ensayos *in vitro* de membrana, viéndose significativamente inhibido su crecimiento micelial y alterada la morfología de las colonias fúngicas. Estos efectos inhibitorios del crecimiento son mayoritariamente comparables a aquellos ejercidos sobre *Fusarium oxysporum* en las mismas condiciones.

TERCERA: Una nueva Cámara de Compuestos Orgánicos Volátiles (VOC Chamber) ha sido diseñada (Número de patente: ES 2708899 B2), la cual demuestra ser un dispositivo fiable y eficiente para la evaluación de interacciones volátiles entre diferentes microorganismos y entre microorganismos e insectos.

CUARTA: Esta nueva tecnología consume menos tiempo en su empleo que el método tradicional de dos placas Petri enfrentadas (DDS), incrementa significativamente la homogeneidad y replicabilidad de los resultados, permite detectar diferencias no detectadas por métodos tradicionales y aporta flexibilidad a las condiciones experimentales, especialmente en lo referente a ventilación e intercambio de gases con el entorno.

QUINTA: Los efectos inhibitorios producidos por Compuestos Orgánicos Volátiles Biogénicos (COVBs) emitidos por aislados de *T. harzianum* sobre *R. solani* y *F. oxysporum* difieren considerablemente entre condiciones ventiladas, no ventiladas y selladas. Los efectos inhibitorios sobre el crecimiento decrecen de forma general a medida que la ventilación se incrementa, hasta el punto de que la inhibición producida por COVBs de *T. harzianum* sobre *F. oxysporum* en condiciones selladas pasa a ser promoción del crecimiento en condiciones ventiladas.

SEXTA: Los COVBs emitidos por *T. harzianum* presentan propiedades insecticidas frente a *Acanthoscelides obtectus* y reducen los daños producidos por este insecto sobre

alubias, siendo estos efectos generalmente menores en condiciones de mayor ventilación.

SÉPTIMA: Una nueva Cámara de Compuestos Orgánicos Volátiles planta-microorganismo (p-mVOC Chamber) ha sido diseñada (Número de solicitud del Modelo de Utilidad: U202131032), la cual demuestra ser un método eficiente para la evaluación de las interacciones entre plantas y microorganismos mediadas por COVBs y para la búsqueda de aislados microbianos productores de COVBs con posible actividad biológica sobre plantas.

OCTAVA: El silenciamiento del gen *erg1* en *T. harzianum*, con la subsecuente reducción en los niveles de ergosterol e incremento de los de escualeno, reduce la actividad antifúngica de los COVBs producidos por esta cepa frente a *R. solani* y *F. oxysporum*, incrementa sus propiedades insecticidas contra *A. obtectus*, reduciendo el daño producido por este insecto en alubias, y no modifica su actividad promotora del crecimiento en plántulas de trigo, todo ello en comparación con los efectos producidos por la cepa silvestre.

NOVENA: La introducción del gen *tri5* de *T. arundinaceum* en *T. harzianum*, con la subsecuente sobreproducción del volátil trichodieno, incrementa la actividad antifúngica de los COVBs producidos por esta cepa sobre *R. solani* y *F. oxysporum*, no modifica sus propiedades insecticidas frente a *A. obtectus* e incrementa su inhibición del crecimiento de plántulas de trigo, todo ello en comparación con los efectos producidos por la cepa silvestre.

DÉCIMA: El silenciamiento del gen *erg1* en *T. harzianum* induce diferencias significativas en la cantidad de COVBs específicos producidos por esta cepa en comparación con los de la silvestre, con menor emisión de Isobutyl acetato y 1-Butanol, 3-methyl-, acetato; y mayor emisión de 1-Propanol, 2-methyl-; Acetoina; 1-Butanol, 3-methyl-; Phenylethyl Alcohol; Ácido benzeneacético, ethyl ester; y Naphthaleno, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-. Estas diferencias pueden ser responsables de algunos de los efectos volátiles descritos para estas cepas de *T. harzianum*.

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Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles

PATENTE

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TÍTULO
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Cumplidos los requisitos previstos en la vigente Ley 24/2015, de 24 de julio, de Patentes, se expide el presente TÍTULO, acreditativo de la concesión de la Patente de Invención. La solicitud ha sido tramitada y concedida con realización del Informe sobre el Estado de la Técnica y **con Examen Sustantivo** de los requisitos de patentabilidad establecidos en la Ley.

Se otorga al titular un derecho de exclusiva en todo el territorio nacional, bajo las condiciones y con las limitaciones previstas en la Ley de Patentes. La duración de la patente será de **veinte años** contados a partir de la fecha de presentación de la solicitud (10/08/2018).

La patente se concede sin perjuicio de tercero y sin garantía del Estado en cuanto a la validez y a la utilidad del objeto sobre el que recae.

Para mantener en vigor la patente concedida, deberán abonarse las tasas anuales establecidas, que se pagarán por años adelantados. Asimismo, deberá explotarse el objeto de la invención, bien por su titular o por medio de persona autorizada de acuerdo con el sistema de licencias previsto legalmente, dentro del plazo de cuatro años a partir de la fecha de presentación de la solicitud de patente, o de tres años desde la publicación de la concesión en el Boletín Oficial de la Propiedad Industrial, aplicándose el plazo que expire más tarde.

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(54) Título: **CÁMARA DE CULTIVO PARA ENSAYOS MICROBIOLÓGICOS DE COMPETENCIA MEDIANTE COMPUESTOS VOLÁTILES**

(57) Resumen:

Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles, que comprende un primer receptáculo (1), un segundo receptáculo (2), y una pieza central (3) entre el primer receptáculo (1) y el segundo receptáculo (2), donde los receptáculos (1, 2) comprenden una pared externa (4) y una pared perimetral (5) perpendicular a la pared externa (4), y donde la pieza central (3) comprende una pared intermedia (6) con al menos un orificio (13), y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos, tal que las paredes laterales (7) de la pieza central (3) rodean las paredes perimetrales (5) de los receptáculos (1, 2) y las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan en la pared intermedia (6).

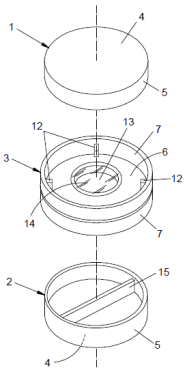

FIG. 1

DESCRIPCIÓN

CÁMARA DE CULTIVO PARA ENSAYOS MICROBIOLÓGICOS DE COMPETENCIA MEDIANTE COMPUESTOS VOLÁTILES

Campo de la invención

La presente invención se refiere a una cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles. La cámara de cultivo objeto de la invención es de aplicación en el campo de las técnicas de análisis microbiológicos, y más concretamente al de las placas de cultivo o placas Petri.

Antecedentes de la invención

Las placas de cultivo, también conocidas como placas Petri, son recipientes de diversos tamaños (con diámetros que varían entre los 50 mm y los 150 mm), generalmente poco profundos y circulares, pudiendo presentar sin embargo otras formas y/o tamaños.

Las placas Petri son el elemento fundamental empleado en el cultivo de microorganismos en medio sólido.

En la actualidad los materiales más habituales en la fabricación de estas placas son materiales plásticos, existiendo no obstante placas Petri de vidrio u otros materiales.

Una placa Petri se compone de dos elementos básicos: una base donde se sitúa el medio de cultivo correspondiente, y una tapa que cubre a la anterior.

Las formas en las que se ajustan o encajan estos dos elementos son variadas, pudiendo mantenerse sueltas una respecto a la otra o fijarse entre ellas mediante diversos elementos de anclaje (apriete mecánico o enclavamiento mecánico mediante rotación o enganche), evitando en este último caso la contaminación de los cultivos por apertura o caídas accidentales. Además, el cierre puede ser completo, generando así un espacio estanco en el interior y evitando o limitando el intercambio de gases con el exterior de la placa (placas no ventiladas), o pueden existir diversos salientes o protuberancias en alguno de los dos elementos que generan un espacio suficiente entre los mismos para que exista un determinado intercambio o flujo de gases entre el interior y el exterior de la placa (placas ventiladas).

Para el cultivo de microorganismos es de vital importancia la gestión de los flujos gaseosos y sus aplicaciones, entre las que se encuentran:

- aportar la necesaria concentración de oxígeno para aquellos microorganismos aerobios o limitar la misma para aquellos anaerobios;
- regular el secado del medio una vez este es depositado en la placa Petri, evitando la condensación de humedad en el caso de las placas ventiladas o evitando el excesivo secado en aquellos cultivos de larga duración en las placas no ventiladas; y
- regular la salida al exterior de compuestos volátiles producidos por los microorganismos en cultivo.

Los ensayos de competencia entre microorganismos se han convertido en herramientas imprescindibles para la caracterización *in vitro* de cepas con capacidad de control biológico y para la detección de compuestos bioactivos producidos por las mismas. Estos experimentos se pueden desarrollar en medio sólido (rutinariamente en placas Petri) o líquido (empleando otras técnicas y materiales). De forma general, existen tres tipos de ensayos *in vitro* de competencia entre microorganismos en medio sólido:

- experimentos de competencia directa, en los que las cepas de microorganismos entran en contacto directo;
- experimentos de competencia a través de compuestos solubles, en los que cepas de microorganismos se enfrenta únicamente a compuestos solubles producidos por otras cepas sin existir contacto físico directo entre las mismas; y
- experimentos de competencia a través de volátiles, en los que cepas de microorganismos se enfrentan únicamente a compuestos volátiles producidos por otras cepas sin existir contacto físico directo entre las mismas.

En la bibliografía científica se han descrito los siguientes medios para realizar este tipo de experimentos de competencia a través de compuestos volátiles:

- placas Petri subdivididas, son placas con uno o más tabiques que dividen la superficie de la misma en varias partes donde pueden cultivarse microorganismos sin que entren en contacto. Estos sistemas limitan el espacio de crecimiento para cada cepa, impidiendo que puedan cultivarse individualmente en el centro de la placa y no son viables para medir el crecimiento radial de las colonias de microorganismos. También existe riesgo

de contaminación cruzada, ya sea a través de esporas o bien porque el micelio es capaz de sobrepasar el tabique de separación.

- introducción de las placas Petri abiertas dentro de recipientes más grandes. Este sistema es complejo, supone un gasto extra y dificulta mucho la manipulación y la toma de medidas durante el periodo en que se desarrolla el experimento.
- disposición de dos placas Petri enfrentadas, donde cada una de las cepas se cultiva en el centro de una placa Petri (de rutina) y posteriormente, tras retirar las tapas de las mismas, se unen los límites superiores de ambas placas enfrentadas mediante algún tipo de adhesivo plástico. De este modo ambos cultivos quedan enfrentados en un espacio cerrado (una especie de cámara formada por el volumen de las dos placas), creciendo uno en la parte interna inferior (generalmente el microorganismo productor de volátiles) y otro en la parte interna superior de la cámara (generalmente el microorganismo sobre el que se evalúa el efecto de los compuestos). Este es uno de los sistemas más empleados para la evaluación de la actividad de los volátiles producidos por hongos de control biológico. Permite una cierta manipulación del material una vez se ha iniciado el experimento, realizar medidas del crecimiento de las colonias y tomar fotografías de las mismas.

Sin embargo, la opción de disponer dos placas Petri enfrentadas plantea una serie de problemas, derivados todos ellos de la no existencia de un dispositivo comercial específico diseñado para llevar a cabo este tipo de experimentos:

- existencia de contaminación cruzada: por esporas, crecimiento de micelio sobre las paredes de las placas (llegando a contaminar el cultivo enfrentado) o por contacto entre las partes aéreas de los micelios. El experto medio en análisis microbiológicos comprende que estas contaminaciones distorsionan los resultados del experimento. Se puede colocar una membrana intermedia para evitar la contaminación, sin embargo la disposición de la misma es compleja y difícil de sistematizar dado que no hay estructuras específicas de anclaje o sujeción.
- dificultad en la disposición enfrentada de las placas ya que no existen placas Petri diseñadas para esta función, por lo que no encajan de forma adecuada y esto dificulta el manejo y la propia reproducibilidad del experimento, además no existe forma de asegurar que el encaje haya sido perfecto o, por el contrario,

hayan quedado pequeños espacios sin contacto entre los bordes de ambas placas.

- imposibilidad de regular la entrada de aire o intercambio de gases con el exterior, en estos experimentos con placas enfrentadas no es posible hacer uso de estas diferentes disposiciones debido a que han de descartarse las tapas, y los bordes de las bases se deben unir con algún tipo de adhesivo.
- gasto innecesario de material al descartarse las tapas de las placas empleadas por carecer de uso en este tipo de experimentos.

La presente patente/modelo de utilidad se dirige a solucionar los citados problemas generados en la puesta a punto y desarrollo de los experimentos de competencia entre microorganismos a través de compuestos volátiles. Se describe una novedosa cámara de cultivo especialmente diseñada para la realización de estos experimentos

Descripción de la invención

Es objeto de la invención una cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles que comprende: un primer receptáculo, un segundo receptáculo, y una pieza central entre el primer receptáculo y el segundo receptáculo.

Los receptáculos comprenden una pared externa y una pared perimetral perpendicular a la pared externa, y la pieza central comprende una pared intermedia con al menos un orificio, y dos paredes laterales que se proyectan desde el perímetro de la pared intermedia en sentidos opuestos. En la placa objeto de la invención las paredes laterales de la pieza central rodean las paredes perimetrales de los receptáculos y las paredes perimetrales de los receptáculos se apoyan en la pared intermedia.

En la cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles objeto de la invención la pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde la primera cara y la segunda cara son lisas.

En otra realización de la cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles objeto de la invención la pieza central comprende un reborde interno en al menos una de las caras de la pared intermedia, tal

que el reborde interno y la pared lateral configuran un alojamiento para el extremo de la pared externa de un receptáculo.

En otra realización de la cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles objeto de la invención la pared intermedia comprende una pluralidad de pestañas en la primera cara y/o en la segunda cara donde se apoya la pared externa de un receptáculo.

En otra realización la cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles comprende una membrana o filtro cubriendo el orificio de la pared intermedia de la pieza central, donde la membrana o filtro son porosos permitiendo el paso del aire y compuestos volátiles, y bloqueando el paso de elementos de mayor tamaño.

La cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles objeto de la invención comprende un primer reborde situado en la pared externa del primer receptáculo, y un segundo reborde de diámetro ligeramente diferente situado en la pared externa del segundo receptáculo, tal que las paredes externas de un primer receptáculo y un segundo receptáculo, el primer reborde encaja en el segundo reborde impidiendo el movimiento relativo entre dos placas de cultivo apiladas.

En otra realización la cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles objeto de la invención comprende al menos un tabique en al menos un receptáculo, tal que el tabique divide en partes independientes el interior de dicho receptáculo.

Breve descripción de los dibujos

Para complementar la descripción que seguidamente se va a realizar y con objeto de ayudar a una mejor comprensión de las características de la invención, se acompaña a la presente memoria descriptiva de un juego de dibujos en base a los que se comprenderán más fácilmente las innovaciones y ventajas del dispositivo objeto de la invención.

La figura 1 muestra una vista en explosión de la cámara de cultivo objeto de la invención.

La figura 2 muestra una vista en sección de la cámara de cultivo objeto de la invención montada.

La figura 3 muestra secciones de dos formas de realización de los medios de unión entre los dos receptáculos y la pieza central y una tercera sección mostrando las pestañas para proporcionar una cámara de cultivo con aireación.

La figura 4 muestra en detalle el orificio central de la pared intermedia horizontal del elemento intermedio, con una variante en la que dicho orificio está abierto, y otra variante con dicho orificio cubierto por una membrana o filtro.

La figura 5 muestra dos placas de cultivos apiladas.

Las distintas referencias numéricas que se encuentran reflejadas en las figuras corresponden a los siguientes elementos:

1. primer receptáculo,
2. segundo receptáculo,
3. pieza central,
4. pared externa,
5. pared perimetral,
6. pared intermedia,
7. pared lateral,
8. primera cara,
9. segunda cara,
10. reborde interno,
11. alojamiento,
12. pestañas,
13. orificio,
14. membrana o filtro,
15. tabique,
16. primer reborde, y
17. segundo reborde.

Descripción detallada de la invención

La cámara de cultivo objeto de la invención comprende un primer receptáculo (1) y un segundo receptáculo (2) y una pieza central (3) que se sitúa entre el primer receptáculo (1) y el segundo receptáculo (2).

Los receptáculos (1, 2), en la realización preferente de la invención, tienen forma de revolución, y es el elemento en el que se coloca el medio nutritivo para el cultivo. La pieza central (3), en la realización preferente de la invención, también tiene forma de revolución y un diámetro ligeramente superior al diámetro de los receptáculos (1, 2).

Los receptáculos (1, 2) comprenden una pared externa (4) que, en la realización preferente de la invención, es de forma circular y lisa en su cara interna, y también comprenden una pared perimetral (5) que surge perpendicular a la pared externa (4).

La pieza central (3) comprende una pared intermedia (6) y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos. La pared intermedia (6) comprende una primera cara (8) hacia el primer receptáculo (1) y una segunda cara (9) hacia el segundo receptáculo (2).

Para fijar la pieza central (3) y los receptáculos (1, 2) la pieza central (3) puede comprender un reborde interno (10) en al menos una de las caras (8, 9) de la pared intermedia (6) de modo que el reborde interno (10) y la pared lateral (7) configuran un alojamiento (11) para el extremo de la pared perimetral (5) de un receptáculo (1, 2) quedando así enclavados ambos elementos.

Además, la pared intermedia (6) aloja un orificio (13), que es circular en la realización preferente de la invención. El diámetro del orificio (13) en la realización preferente de la invención, es aproximadamente de un tercio del diámetro de la pared intermedia (6). Sin embargo, es posible que en lugar de alojar un único orificio (13) la pared intermedia (6) aloje más de un orificio (13) que tenga otras formas y tamaños diferentes al propuesto en la realización preferente, mientras que permita el libre intercambio gaseoso entre ambos receptáculos (1, 2) de la cámara de cultivo y limite las posibilidades de contaminación cruzada de los microorganismos en cultivo.

El orificio (13) de la cámara de cultivo objeto de la invención, puede estar cubierto por una membrana o un filtro (14), que son porosos de modo que permite el paso del aire y compuestos volátiles, pero bloquea el paso de elementos de mayor tamaño, como las

esporas fúngicas. Estos elementos, membrana o filtro (14), pueden presentarse adheridos de forma industrial al contorno del orificio (13), a una parte o la totalidad de alguna de las caras (8, 9) de la pared intermedia (6) de la pieza central o también puede fijarse por medios mecánicos a cualquiera de las caras (8, 9) de la pared intermedia (6).

En la realización preferente de la invención, las dimensiones de la pared perimetral (5) de ambos receptáculos (1, 2) son idénticas, aunque existen realizaciones alternativas en las que las dimensiones de la pared perimetral (5) de cada receptáculo (1, 2) son distintas entre sí.

En la realización preferente de la invención, las dimensiones de la pared externa (4) de los receptáculos (1, 2) son idénticas, aunque existen realizaciones alternativas en las que las dimensiones de las dos paredes externas (4) son distintas.

En la realización preferente de la invención, las dimensiones de las dos paredes laterales (7) de la pieza central (3) son idénticas, aunque existen realizaciones alternativas en las que las dimensiones de las dos paredes laterales (7) son distintas.

Las dimensiones de la pared intermedia (6) de la pieza central (3) son ligeramente superiores a las dimensiones de las paredes externas (4) de los receptáculos (1, 2), considerando que, en la realización preferente de la invención, las citadas paredes (4, 6) son circulares, el diámetro de la pared intermedia (6) es mayor que el diámetro de las paredes externas (4). Igualmente, en la realización preferente de la invención, la pared perimetral (5) de los receptáculos (1, 2) tiene mayor altura que las paredes laterales (7) de la pieza intermedia.

En la cámara de cultivo objeto de la invención la pieza central (3) encaja y descansa sobre el segundo receptáculo (2) a la vez que permite al primer receptáculo (1) encajar y descansar sobre ella, de modo que la pared perimetral (5) de cada receptáculo (1, 2) encaja en una de las paredes laterales (7) de la pieza central (3), además las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan sobre la pared intermedia (6) de la pieza central (3), de modo que los receptáculos (1, 2) se encuentran enfrentados y generan una cámara interior compuesta por el volumen de ambos receptáculos (1, 2) dispuestos simétricamente y parcialmente comunicados a través del orificio (13) de la pared intermedia (6) de la pieza central (3).

El encaje entre la pieza central (3) y los receptáculos (1, 2) da estabilidad a la cámara de cultivo en conjunto y mantiene unidos los componentes cuando no se está manipulando la cámara de cultivo.

La pared intermedia (6) de la pieza central (3) es paralela a las paredes externas (4) de los receptáculos (1, 2).

En la realización preferente de la invención, la presente cámara de cultivo está fabricada en materiales plásticos transparentes conocidos en la fabricación de otras placas de cultivo. Sin embargo, la cámara de cultivo, en realizaciones alternativas, está realizada en vidrio o cualesquiera otros materiales adecuados a su función.

Aunque en la realización preferente de la invención, las dimensiones de ambos receptáculos (1, 2) son idénticas, tienen forma de revolución y son poco profundos, existen realizaciones alternativas en las que las dimensiones de los receptáculos (1, 2) no son idénticas, y la forma puede variar, ya que tanto los receptáculos (1, 2) como la pieza central (3) pueden tener forma cuadrada, rectangular, etc.

En la realización preferente de la invención tanto la primera cara (8) como la segunda cara (9) de la pared intermedia (6) de la pieza central (3) son lisas, y por tanto se genera un contacto continuo con el extremo de las paredes perimetrales (5) de los receptáculos (1, 2) limitando así el intercambio gaseoso con el exterior de la cámara de cultivo (placas no ventiladas). Sin embargo, en realizaciones alternativas, la primera cara (8) y/o la segunda cara (9) pueden presentar pestañas (12) que impidan un contacto continuo y que, por tanto, formen huecos a través de los cuales se produce un determinado intercambio gaseoso con el exterior (placas ventiladas). Existen realizaciones en las que se combinan caras lisas y caras con pestañas (12) de manera que se puede dar ventilación a cualquiera de los dos receptáculos (1, 2) independientemente. Esto da gran versatilidad a la presente invención, permitiendo variar las condiciones de cultivo y seleccionar aquellas más adecuadas para cada experimento concreto.

Aunque en la realización preferente de la invención, las caras internas de ambos receptáculos (1, 2) son lisas, en realizaciones alternativas pueden aparecer uno o varios tabiques (15) que subdividen en dos o más partes independientes el interior de uno o de ambos receptáculos (1, 2) permitiendo varios cultivos independientes en el mismo receptáculo (1, 2).

La cámara de cultivo objeto de la invención comprende unos medios de apilado, que permiten apilar dos o más placas de cultivo. Dichos medios de apilado, comprenden un primer reborde (16) situado en la pared externa (4) del primer receptáculo (1) y un segundo reborde (17) de diámetro ligeramente diferente al primer reborde (16) situado en la pared externa (4) del segundo receptáculo (2), de modo que enfrentado las paredes externas (4) de un primer receptáculo (1) y un segundo receptáculo (2), el primer reborde (16) encaja en el segundo reborde (17) y se impide el movimiento relativo entre dos placas de cultivo apiladas.

REIVINDICACIONES

1. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles, **caracterizada por** que comprende:

- un primer receptáculo (1),
- un segundo receptáculo (2), y
- una pieza central (3) entre el primer receptáculo (1) y el segundo receptáculo (2),

donde los receptáculos (1, 2) comprenden una pared externa (4) y una pared perimetral (5) perpendicular a la pared externa (4), y donde la pieza central (3) comprende una pared intermedia (6) con al menos un orificio (13), y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos, tal que las paredes laterales (7) de la pieza central (3) rodean las paredes perimetrales (5) de los receptáculos (1, 2) y las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan en la pared intermedia (6) de la pieza central (3).

2. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde la primera cara (8) y la segunda cara (9) son lisas.

3. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde al menos una de las caras (8, 9) comprende una pluralidad de pestañas (12) donde se apoya la pared perimetral (5) de un receptáculo (1, 2), facilitando así el intercambio gaseoso con el exterior. .

4. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 3, **caracterizada por** que la pieza central (3) comprende un reborde interno (10) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde interno (10) y la pared lateral (7) configuran un alojamiento (11) para el extremo de la pared perimetral (5) de uno o ambos receptáculos (1, 2) quedando así enclavados.

5. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 4, **caracterizada por** que comprende una membrana o filtro (14) cubriendo el orificio (13) de la pared intermedia (6) de la pieza central (3), donde la membrana o filtro (14) es porosa permitiendo el paso del aire y compuestos volátiles, y bloqueando el paso de elementos de mayor tamaño.

6. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 5, **caracterizada por** que comprende:

- un primer reborde (16) situado en la pared externa (4) del primer receptáculo (1), y
- un segundo reborde (17) situado en la pared externa (4) del segundo receptáculo (2),

donde enfrentado las paredes externas (4) de un primer receptáculo (1) y un segundo receptáculo (2), el primer reborde (16) encaja en el segundo reborde (17) impidiendo el movimiento relativo entre dos placas de cultivo apiladas.

7. Cámara de cultivo para ensayos microbiológicos de competencia mediante compuestos volátiles según cualquiera de las reivindicaciones 1 a 6, **caracterizada por** que comprende al menos un tabique (15) en al menos un receptáculo (1, 2), tal que el tabique (15) divide en partes independientes el interior de dicho receptáculo (1, 2).

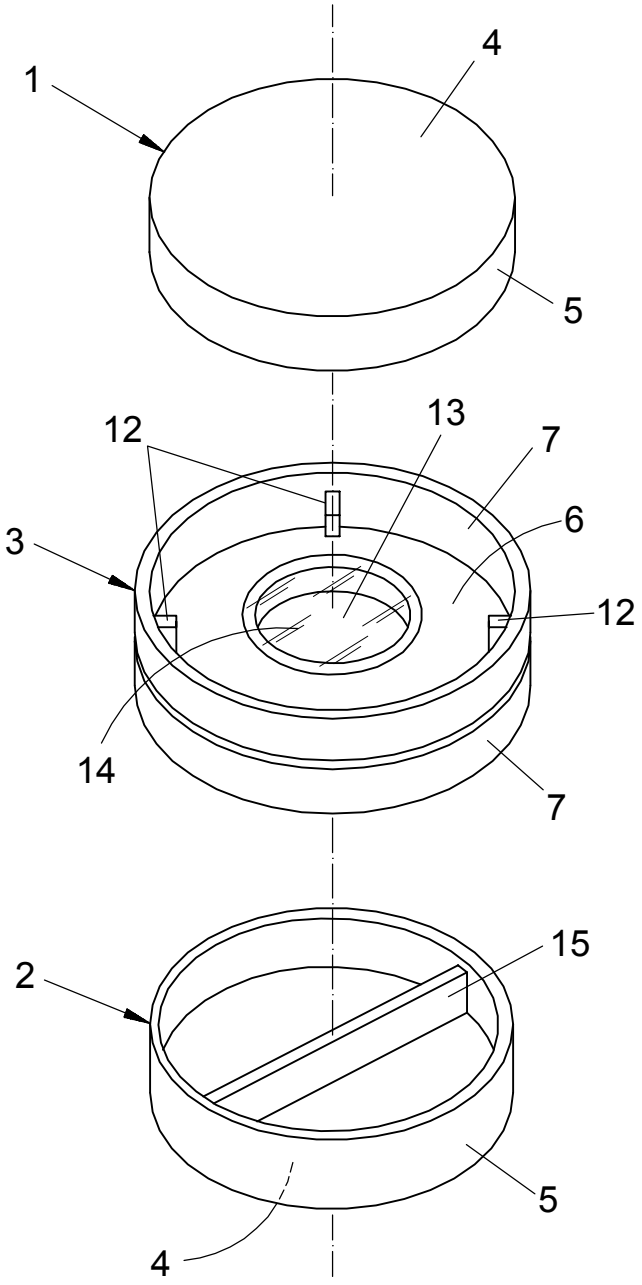

FIG. 1

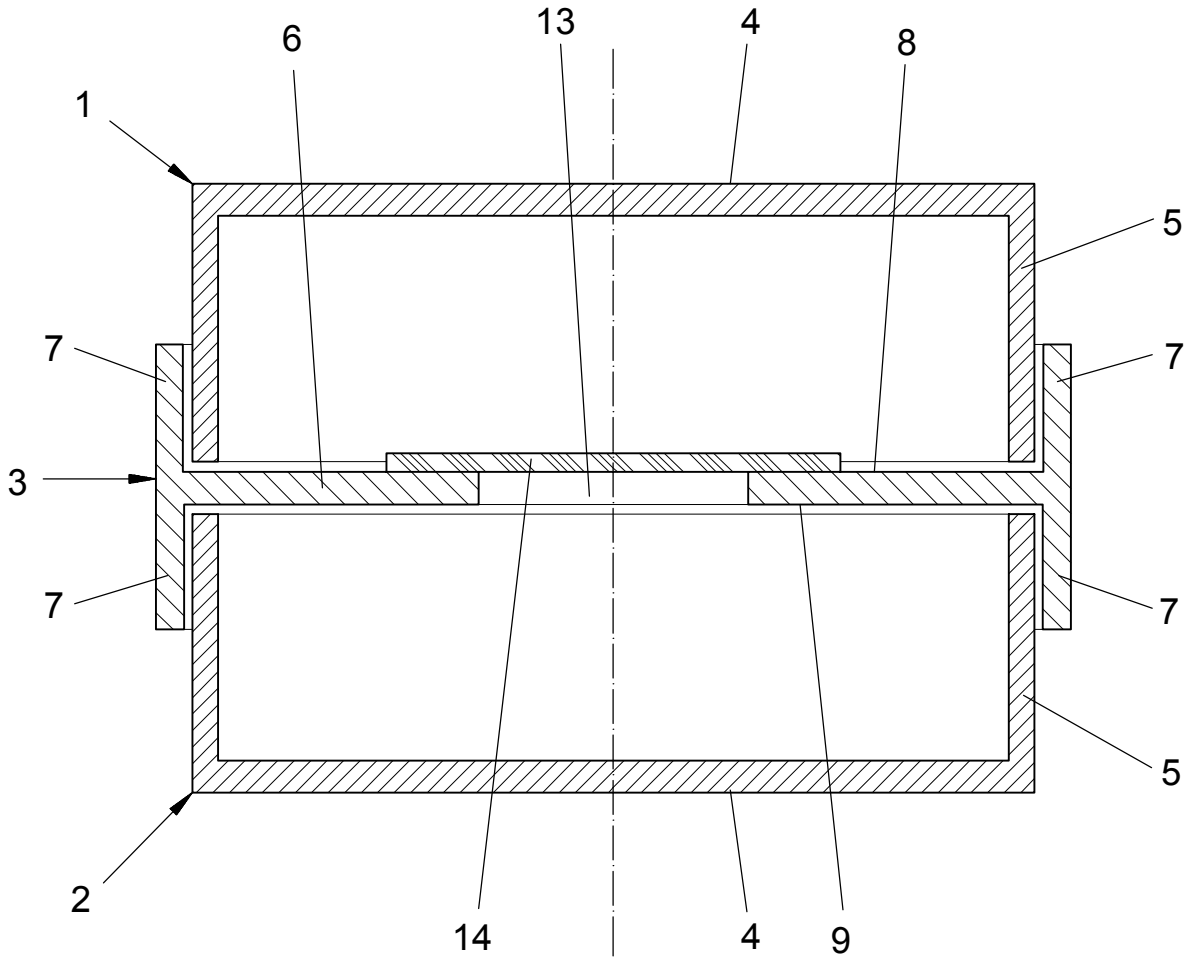

FIG. 2

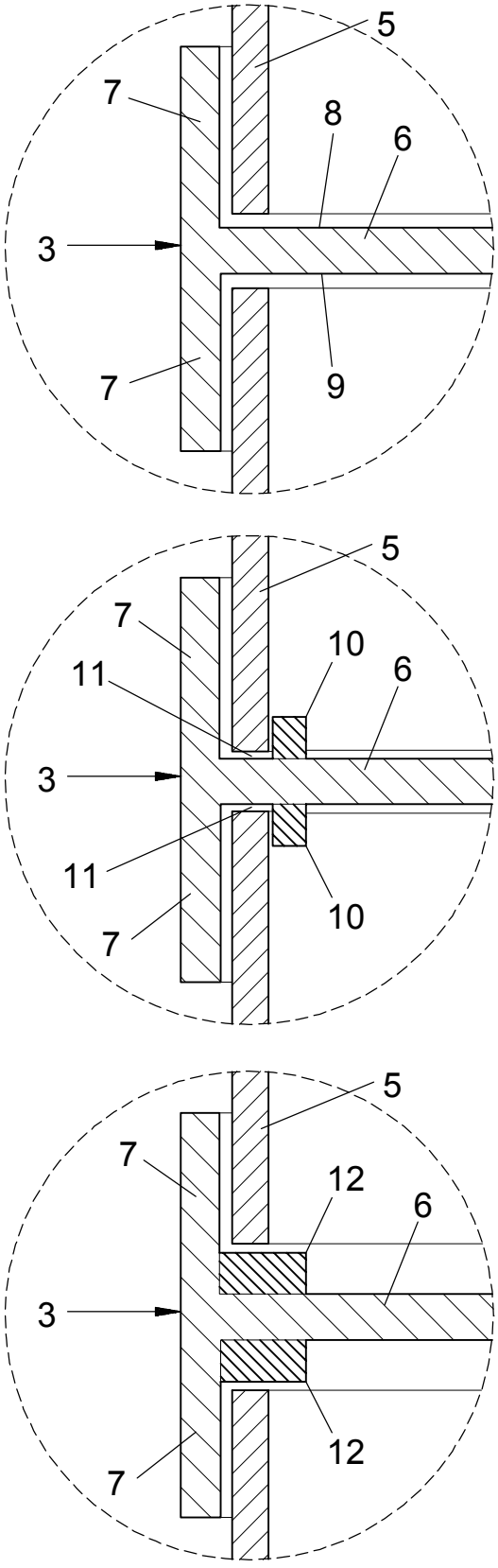

FIG. 3

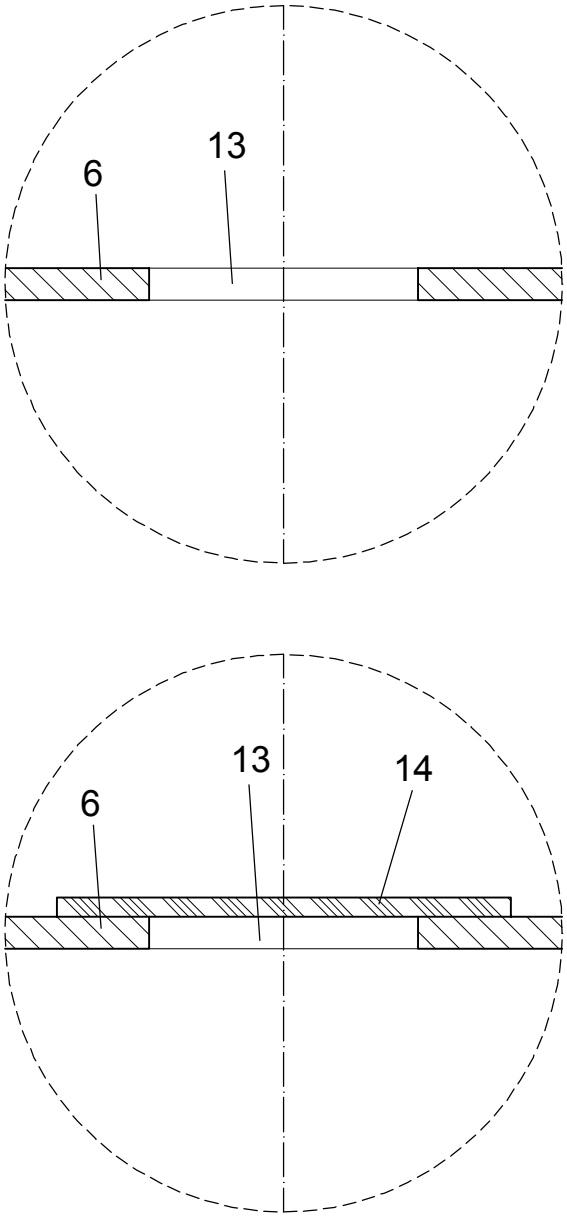

FIG. 4

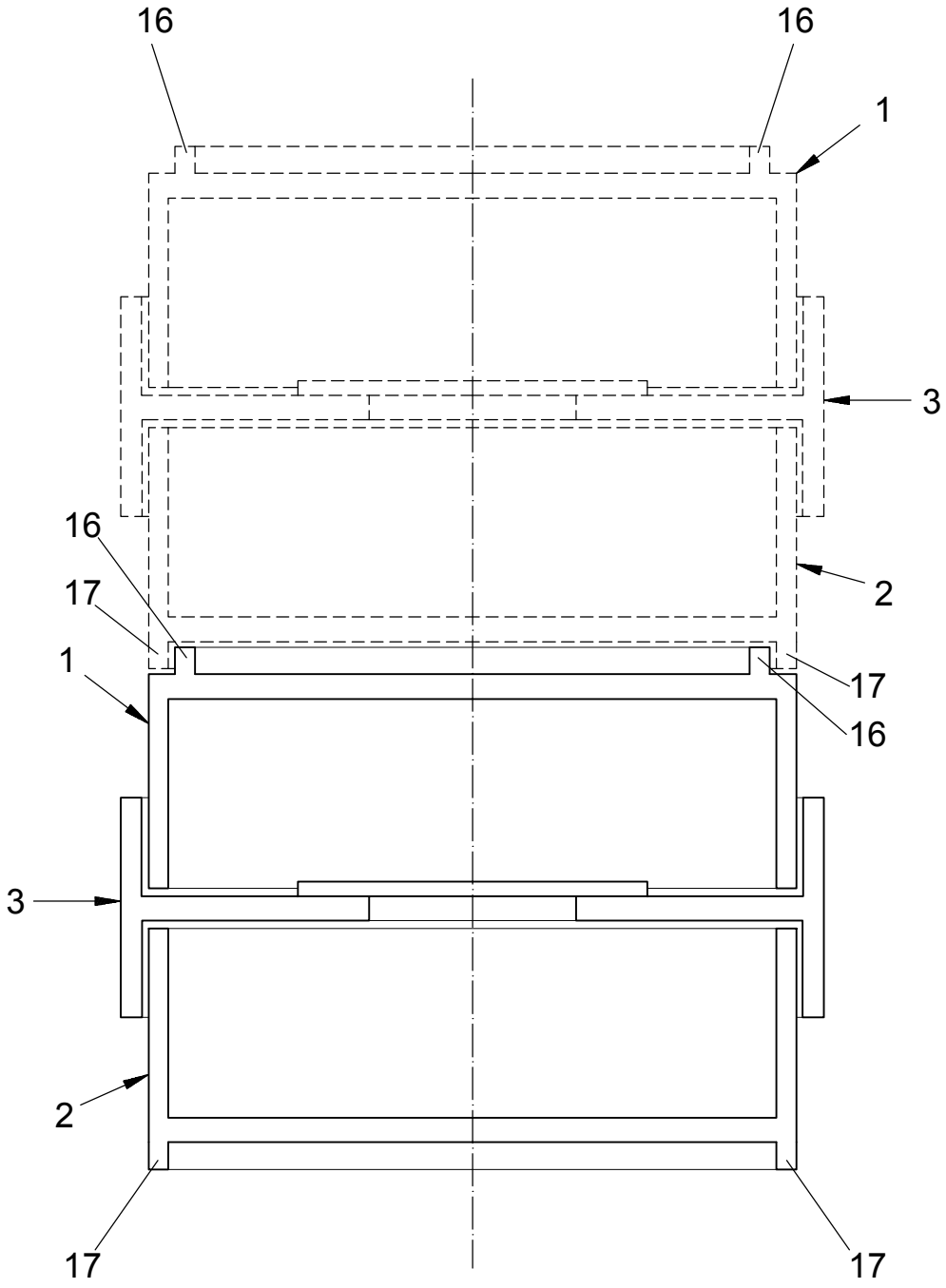

FIG. 5

Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo

MODELO DE UTILIDAD

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Este documento es un justificante de que se ha recibido una solicitud española de modelo de utilidad por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	U202131032	
Fecha de recepción:	20 mayo 2021, 16:29 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	U 2020/50419	
Solicitante:	Universidad de León	
Número de solicitantes:	1	
País:	ES	
Título:	CÁMARA DE CULTIVO PARA EVALUAR LOS EFECTOS DE COMPUESTOS VOLÁTILES EN LAS INTERACCIONES PLANTA-MICROORGANISMO	
Documentos enviados:	Descripcion.pdf (9 p.) Reivindicaciones.pdf (2 p.) Dibujos.pdf (7 p.) OLF-ARCHIVE.zip FEERCPT-1.pdf (1 p.)	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
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CÁMARA DE CULTIVO PARA EVALUAR LOS EFECTOS DE COMPUESTOS VOLÁTILES EN LAS INTERACCIONES PLANTA-MICROORGANISMO

DESCRIPCIÓN

Sector de la técnica

La presente invención se refiere a una cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo. La cámara de cultivo objeto de la invención es de aplicación en el campo de la investigación *in vitro* en botánica, fisiología vegetal y agricultura, y más concretamente en la categoría de recipientes y cámaras para el cultivo *in vitro* de plantas o partes de plantas.

Antecedentes de la invención

El trabajo de laboratorio con órganos y tejidos vegetales, o plantas completas, requiere de una tecnología *in vitro* específica que se adecúe a las características de estos seres vivos. En este contexto, existen numerosas cámaras y recipientes diseñadas para cultivar plantas y realizar experimentación en ambientes controlados. Estas cámaras son de diversa forma, tamaño y material, atendiendo a las especificidades y necesidades de cada investigación. En la actualidad los materiales más habituales en la fabricación de estas cámaras y recipientes son materiales plásticos y vidrio.

Por otro lado, la evaluación de la interacción entre microorganismos y plantas es un campo de creciente interés tanto en ciencia básica como aplicada, especialmente en agricultura y sectores relacionados. Estas interacciones pueden producirse a través del contacto directo entre los organismos o pueden estar mediadas por metabolitos producidos por los mismos. Estos metabolitos pueden ser solubles o volátiles. Existe numerosa bibliografía científica donde se expone la importancia y diversidad de efectos que dichos metabolitos tienen en el desarrollo y crecimiento de especies vegetales, así como en la interacción con microorganismos.

La evaluación de los efectos producidos por compuestos volátiles reviste una especial complejidad debido a las características fisicoquímicas de los mismos, por lo que existen en la actualidad diversas aproximaciones y metodología para llevar a cabo este tipo de experimentos. Estas metodologías pueden dividirse en dos grupos generales:

1- Sistemas pasivos, donde los compuestos volátiles difunden pasivamente de unos organismos a otros. Suelen ser sistemas basados en introducir recipientes con los organismos en recipientes más grandes donde se produce la interacción.

2- Sistemas activos, donde los volátiles son propulsados activamente para que pasen del espacio que ocupa un organismo al del otro. Estos dispositivos son más complejos y suelen requerir bombas, circuitos y una fuente de alimentación.

En la bibliografía científica se han descrito las siguientes metodologías generales para la realización de experimentos de competencia a través de compuestos volátiles:

- Placas Petri subdivididas. Se trata de placas con uno o más tabiques que dividen la superficie de placa en varias partes donde pueden cultivarse microorganismos y plantas sin que entren en contacto directo. Sistemas de este tipo se han utilizado tanto en modo cerrado, empleando parafilm u otro elemento adhesivo para el sellado de las zonas de unión entre elementos, como en modo abierto, omitiendo el uso de parafilm. Estos sistemas presentan un doble problema. En primer lugar, limitan el espacio de crecimiento del microorganismo y de la planta. En el caso particular de la planta es especialmente importante considerar que el cultivo en placa Petri permite un crecimiento en altura de la planta muy moderado (apenas 10 mm). Por otro lado, el eventual contacto de la parte aérea de la planta con la tapa o paredes de la placa Petri puede suponer un factor de estrés mecánico que impediría la correcta interpretación de los resultados. El segundo problema tiene que ver con el riesgo de contaminación de la planta con el microorganismo.
- Introducción de las placas Petri abiertas dentro de recipientes más grandes. Se trata de contenedores plásticos que contienen compartimentos para el cultivo de plantas en sustratos de distinta naturaleza y espacio suficiente para alojar placas Petri abiertas con el cultivo microbiano. Este sistema es engorroso, supone un gasto extra y dificulta la manipulación y la toma de medidas durante el periodo que se desarrolla el experimento. Normalmente estos sistemas se utilizan en modo cerrado mediante sellado del recipiente de mayor volumen. Una variante a este sistema consiste en situar el cultivo microbiano bajo el compartimento destinado al cultivo del material vegetal. De esta manera se consigue que los volátiles producidos por el microorganismo difundan pasivamente en el sustrato utilizado para el crecimiento de las plantas.

La cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención se dirige a solucionar los citados problemas generados en la puesta a punto y desarrollo de los experimentos para identificar compuestos volátiles activos y/o testar el efecto de dichos compuestos sobre el crecimiento

y desarrollo de plantas, así como la evaluación de los efectos producidos por compuestos volátiles emitidos por plantas o alguna de sus partes sobre otros organismos.

La cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención ofrece flexibilidad a los experimentos de competencia a través de compuestos volátiles, permitiendo modificar las condiciones de cultivo de forma eficiente para ajustarse a las necesidades de la investigación, principalmente en los requisitos de espacio para el crecimiento de plantas, la regulación de los intercambios gaseosos con el exterior, y reduciendo las contaminaciones cruzadas.

También existe un número limitado de documentos del estado de la técnica referidos a cámaras de cultivo para evaluar los efectos de compuestos volátiles sobre plantas. Estos documentos comparten una parte importante de la función general de la presente cámara de cultivo objeto de la invención, pero difieren sustancialmente tanto en la estructura del diseño, como en las funcionalidades específicas concretas en lo referente a ventilación, extracción de compuestos volátiles y disposición de los elementos que la forman.

Descripción de la invención

Es objeto de la invención una cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo; así como el efecto de estos mismos compuestos volátiles sobre plagas o enfermedades de plantas cuando se encuentran interaccionando con estas; o, adicionalmente, la evaluación de los efectos producidos por compuestos volátiles emitidos por plantas o alguna de sus partes sobre otros organismos.

La presente cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo comprende: un primer receptáculo, un segundo receptáculo, y una pieza central entre el primer receptáculo y el segundo receptáculo.

Los receptáculos comprenden una pared externa y una pared perimetral perpendicular a la pared externa, y donde la pieza central comprende una pared intermedia con al menos un primer orificio, y dos paredes laterales que se proyectan desde el perímetro de la pared intermedia en sentidos opuestos, tal que las paredes laterales de la pieza central rodean las paredes perimetrales de los receptáculos y las paredes perimetrales de los receptáculos se apoyan en la pared intermedia de la pieza central. La pared perimetral del primer receptáculo tiene una altura diferente a la pared perimetral del segundo receptáculo.

En la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención la pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde la primera cara y la segunda cara son lisas.

En la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención la pared intermedia de la pieza central comprende una primera cara enfrentada con el primer receptáculo y una segunda cara enfrentada con el segundo receptáculo, donde al menos una de las caras puede comprender una pluralidad de pestañas donde se apoya la pared perimetral de un receptáculo, facilitando así el intercambio gaseoso con el exterior.

En la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención, la pieza central puede comprender un reborde interno en al menos una de las caras de la pared intermedia, tal que el reborde interno y la pared lateral configuran un alojamiento para el extremo de la pared perimetral de uno o ambos receptáculos quedando así enclavados.

En la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención la pieza central puede comprender un reborde en al menos una de las caras de la pared intermedia, tal que el reborde y la pared intermedia están configurados para alojar una membrana o filtro y/o para evitar el goteo de líquidos desde la primera cara.

La cámara cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención puede comprender una membrana o filtro cubriendo el primer orificio de la pared intermedia de la pieza central, donde la membrana o filtro es porosa y permite el paso del aire y compuestos volátiles, y bloquea el paso de elementos de mayor tamaño.

En la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención la pared perimetral de al menos un receptáculo puede comprender un segundo orificio cubierto por un septo, diafragma o válvula, tal que el segundo orificio impide la libre salida de gases al exterior a través de

dicho segundo orificio y permite la extracción de los mismos del interior de la cámara mediante punción con elementos de tipo jeringuilla o unión mediante un tubo.

La cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención puede comprender al menos un tabique en al menos un receptáculo, tal que el tabique divide en partes independientes el interior de dicho receptáculo.

Breve descripción de los dibujos

Para complementar la descripción que seguidamente se va a realizar y con objeto de ayudar a una mejor comprensión de las características de la invención, se acompaña a la presente memoria descriptiva de un juego de dibujos en base a los que se comprenderán más fácilmente las innovaciones y ventajas del dispositivo objeto de la invención.

La figura 1 muestra una vista en explosión de la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención.

La figura 2 muestra una vista en explosión de la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención en orientación opuesta a la figura 1.

La figura 3 muestra una vista en sección de la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención montada.

La figura 4 muestra una vista en sección de la cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención montada en orientación opuesta a la figura 3.

La figura 5 muestra secciones de tres formas de realización de los medios de unión entre los dos receptáculos y la pieza central y una tercera sección mostrando las pestañas para proporcionar una cámara de cultivo con aireación.

Las figuras 6a y 6b muestran en detalle el primer orificio central de la pared intermedia horizontal del elemento intermedio, con una variante donde la pared intermedia horizontal es lisa (a), y otra variante con un reborde en torno a dicho primer orificio (b). Ambas

variantes presentan a su vez una variante en la que el primer orificio está abierto, y otra variante donde está cubierto por una membrana o filtro.

Las distintas referencias numéricas que se encuentran reflejadas en las figuras corresponden a los siguientes elementos:

1. primer receptáculo,
2. segundo receptáculo,
3. pieza central,
4. pared externa,
5. pared perimetral,
6. pared intermedia,
7. pared lateral,
8. primera cara,
9. segunda cara,
10. pestañas,
11. primer orificio,
12. segundo orificio,
13. membrana o filtro,
14. reborde,
15. reborde interno,
16. alojamiento.

Descripción detallada de la invención

La cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo objeto de la invención comprende un primer receptáculo (1) y un segundo receptáculo (2) y una pieza central (3) que se sitúa entre el primer receptáculo (1) y el segundo receptáculo (2), donde el segundo receptáculo (2) es de mayor dimensión que el primer receptáculo (1).

Los receptáculos (1, 2), en la realización preferente de la invención, tienen forma de revolución, y sobre ellos se coloca el medio nutritivo o sustrato para el cultivo. La pieza central (3), en la realización preferente de la invención, también tiene forma de revolución y un diámetro ligeramente superior al diámetro de los receptáculos (1, 2).

Los receptáculos (1, 2) comprenden una pared externa (4) que, en la realización preferente de la invención, es de forma circular y lisa en su cara interna, y también comprenden una pared perimetral (5) que surge perpendicular a la pared externa (4).

En la realización preferente de la invención, la pared perimetral (5) del primer receptáculo es de menor dimensión que la pared perimetral (5) del segundo receptáculo (2), de este modo la cámara de cultivo comprende un primer receptáculo (1), con una menor altura de su pared perimetral (5), y un segundo receptáculo (2), con una mayor altura de su pared perimetral, tal y como se expuso antes en esta misma descripción.

En la realización preferente de la invención, la pared perimetral (5) del segundo receptáculo (2) presenta una altura entre 4 y 8 veces superior a la de la pared perimetral (5) del primer receptáculo (1), no limitando esta realización preferente otras posibles realizaciones que presentan alturas diferentes de las paredes perimetrales (5) de los dos receptáculos (1, 2).

Los dos receptáculos (1, 2) pueden colocarse indistintamente en la parte superior o inferior de la cámara, atendiendo a las necesidades concretas de uso en cada caso.

La pieza central (3) comprende una pared intermedia (6) y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos. La pared intermedia (6) comprende una primera cara (8) orientada hacia uno de los receptáculos y una segunda cara (9) orientada hacia el otro receptáculo.

Para fijar la pieza central (3) y los receptáculos (1, 2) la pieza central (3) puede comprender un reborde interno (15) en al menos una de las caras (8, 9) de la pared intermedia (6) de modo que el reborde interno (15) y la pared lateral (7) configuran un alojamiento (16) para el extremo de la pared perimetral (5) de un receptáculo (1, 2) quedando así enclavados ambos elementos. Este reborde interno (15) puede ser sustituido por cualquiera otro sistema de enclavado que sirva al mismo propósito.

Además, la pared intermedia (6) aloja un primer orificio (11), que es circular en la realización preferente de la invención. El diámetro del primer orificio (11) en la realización preferente de la invención, es aproximadamente de un tercio del diámetro de la pared intermedia (6). Sin embargo, es posible que en lugar de alojar un único primer orificio (11) la pared intermedia (6) aloje más de un primer orificio (11) que tengan otras formas y tamaños diferentes al propuesto en la realización preferente. El primer orificio (11) u orificios (11)

están configurados para permitir el libre intercambio gaseoso entre ambos receptáculos (1, 2) de la cámara de cultivo y limitar las posibilidades de contaminación cruzada entre ambos receptáculos (1, 2).

El primer orificio (11) de la cámara de cultivo objeto de la invención, puede estar cubierto por una membrana o un filtro (13), que es poroso, de modo que permite el paso del aire y compuestos volátiles, pero impide el paso de elementos de mayor tamaño, como las esporas fúngicas. Estos elementos, membrana o filtro (13), pueden presentarse adheridos de forma industrial al contorno del primer orificio (11), a una parte o la totalidad de alguna de las caras (8, 9) de la pared intermedia (6) de la pieza central o también puede fijarse por medios mecánicos a cualquiera de las caras (8, 9) de la pared intermedia (6).

En una realización alternativa, la pieza central (3) comprende un reborde (14) en al menos una de las caras de la pared intermedia (6) en torno al primer orificio (11) y separada del borde de este, de modo que el reborde (14) actúa como asiento para la colocación de la membrana o filtro (13) y/o impide el goteo de líquidos. El reborde (14) se alza perpendicular a la pared intermedia (6) y paralelo a las paredes laterales (7) de la pieza central (3) y es de una altura inferior a la altura de la pared perimetral (5).

En la cámara de cultivo objeto de la invención la pieza central (3) encaja y descansa sobre un receptáculo (1, 2) colocado en la parte inferior a la vez que permite al otro receptáculo (1, 2), colocado en la parte superior, encajar y descansar sobre ella, de modo que la pared perimetral (5) de cada receptáculo (1, 2) encaja en una de las paredes laterales (7) de la pieza central (3), además las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan sobre la pared intermedia (6) de la pieza central (3), de modo que los receptáculos (1, 2) se encuentran enfrentados y generan una cámara interior compuesta por el volumen de ambos receptáculos (1, 2) dispuestos simétricamente y parcialmente comunicados a través del primer orificio (11) de la pared intermedia (6) de la pieza central (3).

El encaje entre la pieza central (3) y los receptáculos (1, 2) da estabilidad a la cámara de cultivo en conjunto y mantiene unidos los componentes cuando no se está manipulando la cámara de cultivo.

La pared intermedia (6) de la pieza central (3) es paralela a las paredes externas (4) de los receptáculos (1, 2).

En la realización preferente de la invención, la presente cámara de cultivo está fabricada en vidrio o en materiales plásticos transparentes conocidos en la fabricación de otras cámaras de cultivo. Sin embargo, la cámara de cultivo, en realizaciones alternativas, está realizada en cualesquiera otros materiales adecuados a su función.

Aunque en la realización preferente de la invención, los receptáculos (1, 2) y la pieza central (3) tienen forma de revolución, existen realizaciones alternativas en las que la forma puede variar. Tanto los receptáculos (1, 2) como la pieza central (3) pueden tener forma cuadrada, rectangular, etc.

En la realización preferente de la invención tanto la primera cara (8) como la segunda cara (9) de la pared intermedia (6) de la pieza central (3) son lisas, y por tanto se genera un contacto continuo con el extremo de las paredes perimetrales (5) de los receptáculos (1, 2) limitando así el intercambio gaseoso con el exterior de la cámara de cultivo (cámaras no ventiladas). Sin embargo, en realizaciones alternativas, la primera cara (8) y/o la segunda cara (9) pueden presentar pestañas (10) que impidan un contacto continuo y que, por tanto, formen huecos a través de los cuales se produce un determinado intercambio gaseoso con el exterior (cámaras ventiladas). Existen realizaciones en las que se combinan caras lisas y caras con pestañas (10) de manera que se puede dar ventilación a cualquiera de los dos receptáculos (1, 2) independientemente. Esto da gran versatilidad a la presente invención, permitiendo variar las condiciones de cultivo y seleccionar aquellas más adecuadas para cada experimento concreto.

Aunque en la realización preferente de la invención, las paredes perimetrales (5) de ambos receptáculos (1, 2) son continuas, en realizaciones alternativas, la pared perimetral (5) de uno o ambos receptáculos (1, 2) pueden comprender un segundo orificio (12) configurado para extracción de volátiles, estando dicho segundo orificio (12) cubierto por un septo, diafragma o válvula que impide la libre salida de gases al exterior a través de dicho segundo orificio (12) y permite la extracción de los mismos del interior de la cámara mediante punción con elementos de tipo jeringuilla o unión mediante un tubo.

Aunque en la realización preferente de la invención, las caras internas de ambos receptáculos (1, 2) son lisas, en realizaciones alternativas pueden aparecer uno o varios tabiques que subdividen en dos o más partes independientes el interior de uno o de ambos receptáculos (1, 2) permitiendo varios cultivos independientes en el mismo receptáculo (1, 2).

REIVINDICACIONES

1. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo, que comprende un primer receptáculo (1), un segundo receptáculo (2), y una pieza central (3) entre el primer receptáculo (1) y el segundo receptáculo (2), donde los receptáculos (1, 2) comprenden una pared externa (4) y una pared perimetral (5) perpendicular a la pared externa (4), y donde la pieza central (3) comprende una pared intermedia (6) con al menos un primer orificio (11), y dos paredes laterales (7) que se proyectan desde el perímetro de la pared intermedia (6) en sentidos opuestos, tal que las paredes laterales (7) de la pieza central (3) rodean las paredes perimetrales (5) de los receptáculos (1, 2) y las paredes perimetrales (5) de los receptáculos (1, 2) se apoyan en la pared intermedia (6) de la pieza central (3),
caracterizado por que la pared perimetral (5) del primer receptáculo (1) tiene una altura diferente a la pared perimetral (5) del segundo receptáculo (2).

2. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde la primera cara (8) y la segunda cara (9) son lisas.

3. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según la reivindicación 1, **caracterizada por** que la pared intermedia (6) de la pieza central (3) comprende una primera cara (8) enfrentada con el primer receptáculo (1) y una segunda cara (9) enfrentada con el segundo receptáculo (2), donde al menos una de las caras (8, 9) comprende una pluralidad de pestañas (10) para apoyo de la pared perimetral (5) de un receptáculo (1, 2) para intercambio de gases con el exterior.

4. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 3, **caracterizada por** que la pieza central (3) comprende un reborde interno (15) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde interno (15) y la pared lateral (7) configuran un alojamiento (16) para el extremo de la pared perimetral (5) de uno o ambos receptáculos (1, 2) quedando así enclavados.

5. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 4, **caracterizada por** que la pieza central (3) comprende un reborde (14) en al menos una de las caras (8, 9) de la pared intermedia (6), tal que el reborde (14) y la pared intermedia (6) están configurados para alojar una membrana o filtro (13).

6. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 5, **caracterizada por** que comprende una membrana o filtro (13) cubriendo el primer orificio (11) de la pared intermedia (6) de la pieza central (3).

7. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 6, **caracterizada por** que la pared perimetral (5) de al menos un receptáculo (1, 2) comprende un segundo orificio (12) cubierto por un septo, diafragma o válvula, configurado para extraer volátiles.

8. Cámara de cultivo para evaluar los efectos de compuestos volátiles en las interacciones planta-microorganismo según cualquiera de las reivindicaciones 1 a 7, **caracterizada por** que comprende al menos un tabique en al menos un receptáculo (1, 2), tal que el tabique divide en partes independientes el interior de dicho receptáculo (1, 2).

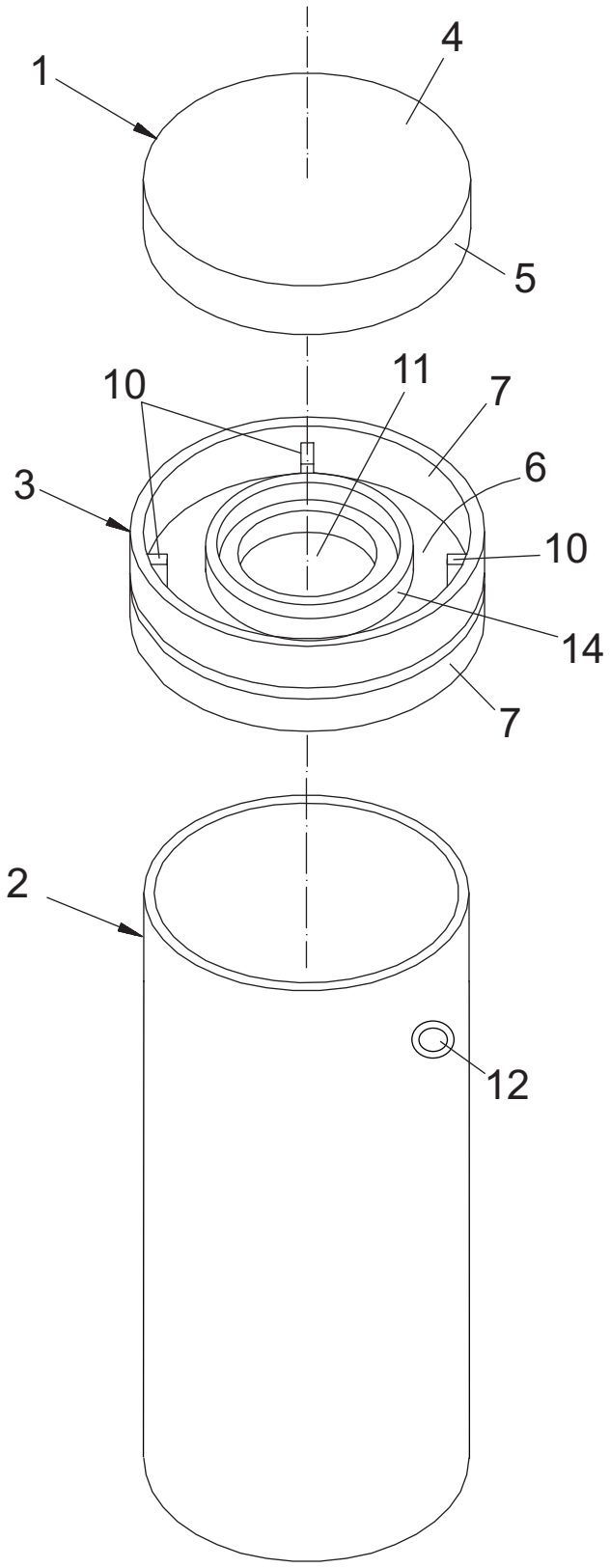

Fig. 1

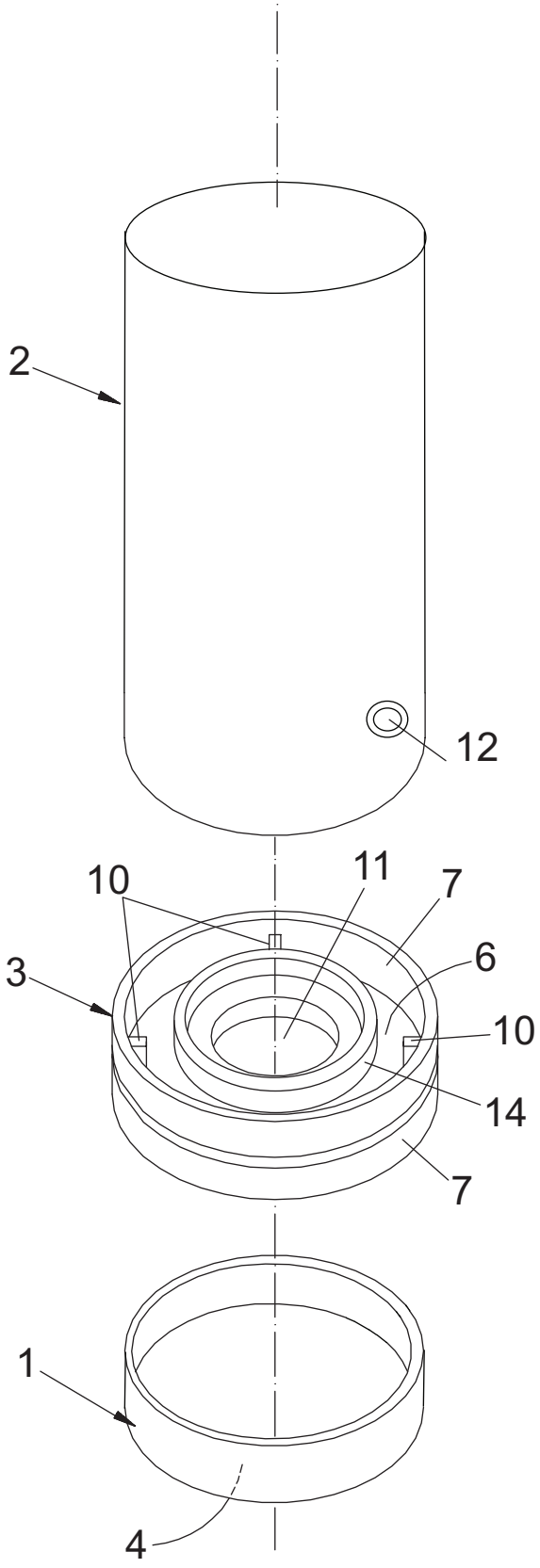

Fig. 2

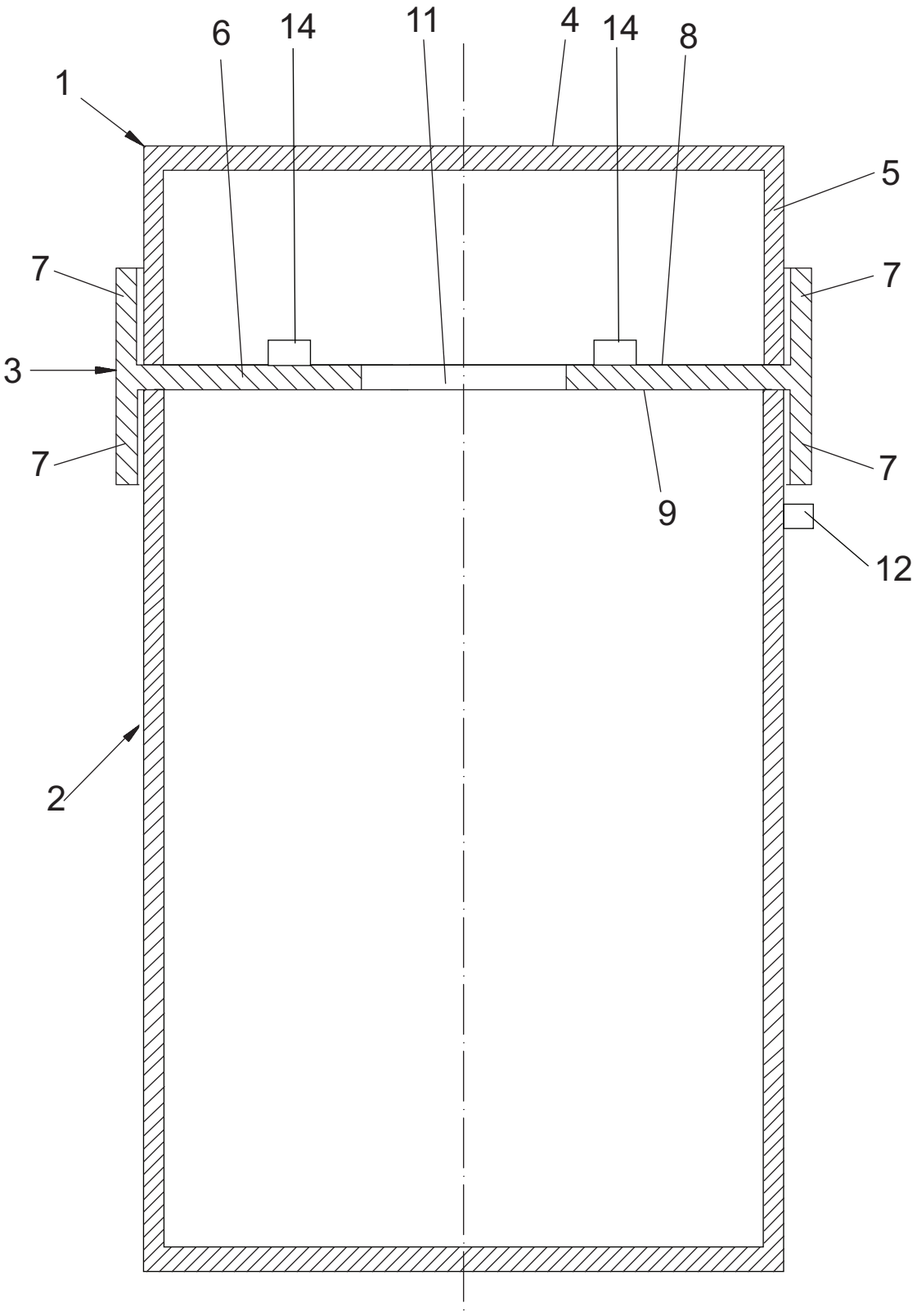

Fig. 3

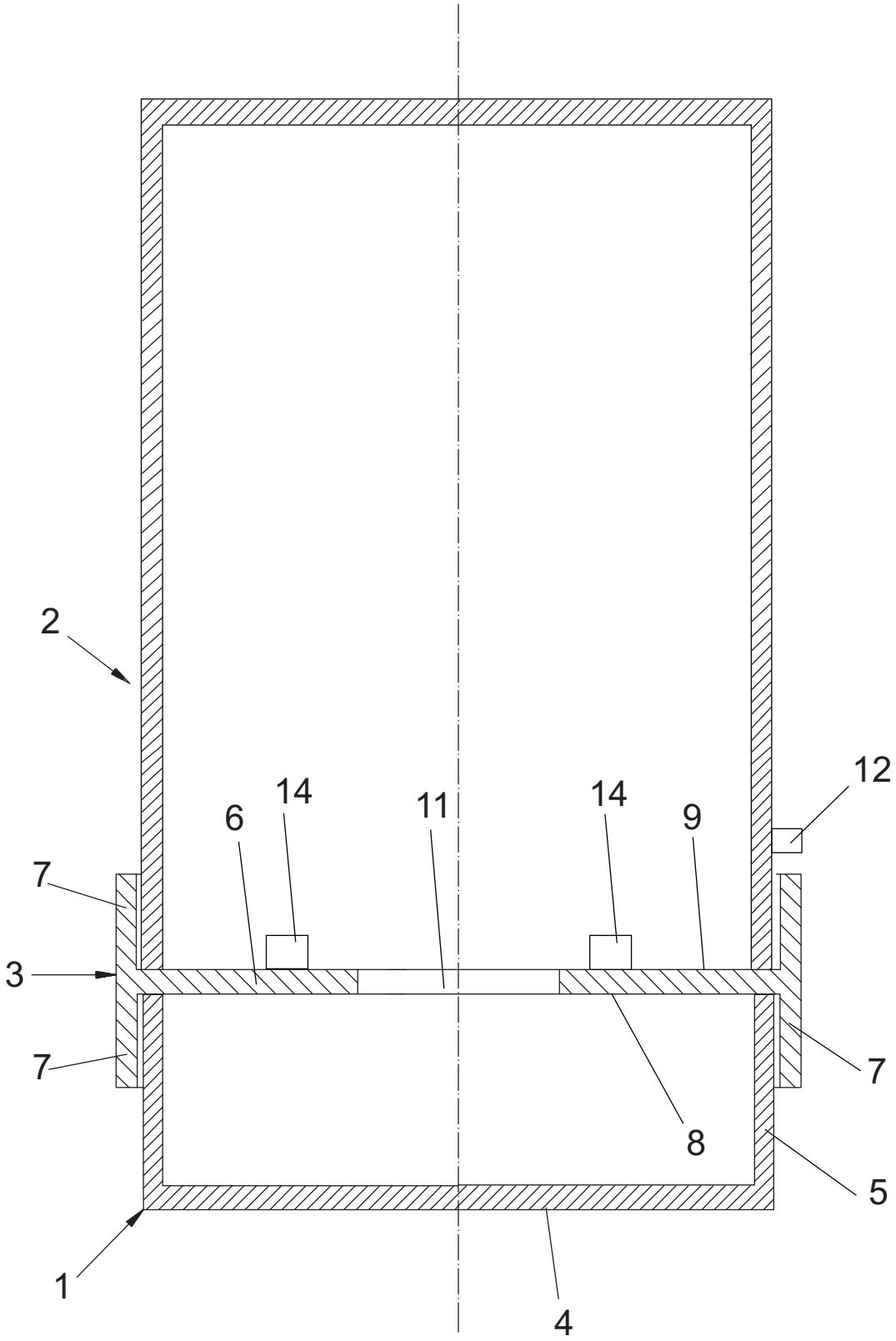

Fig. 4

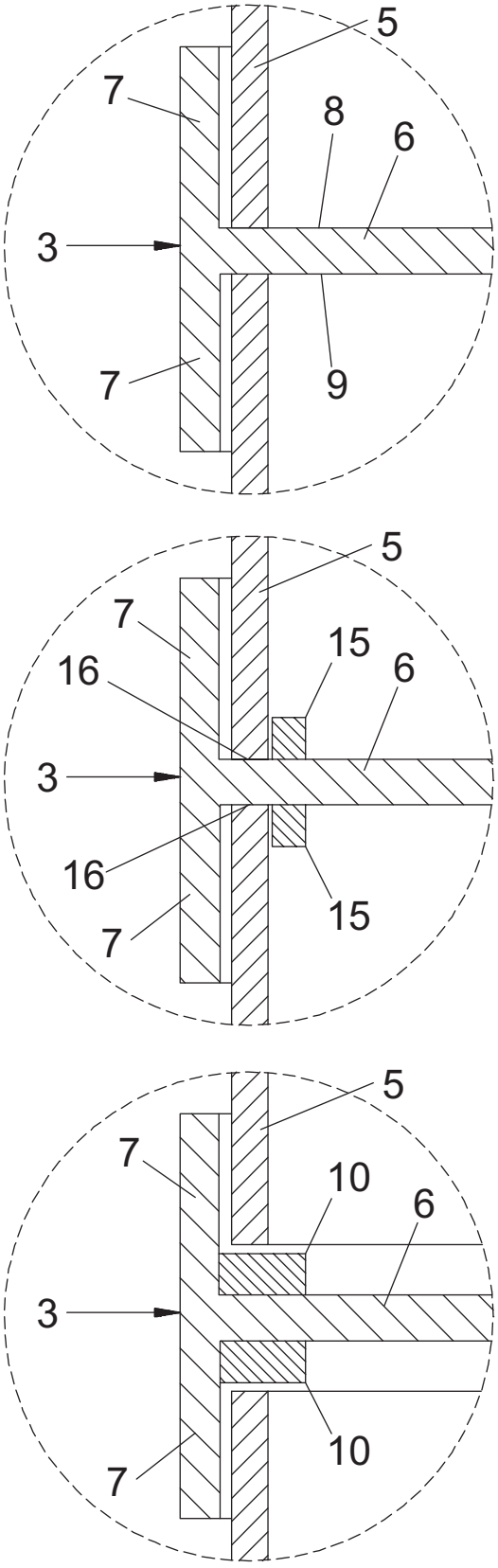

Fig. 5

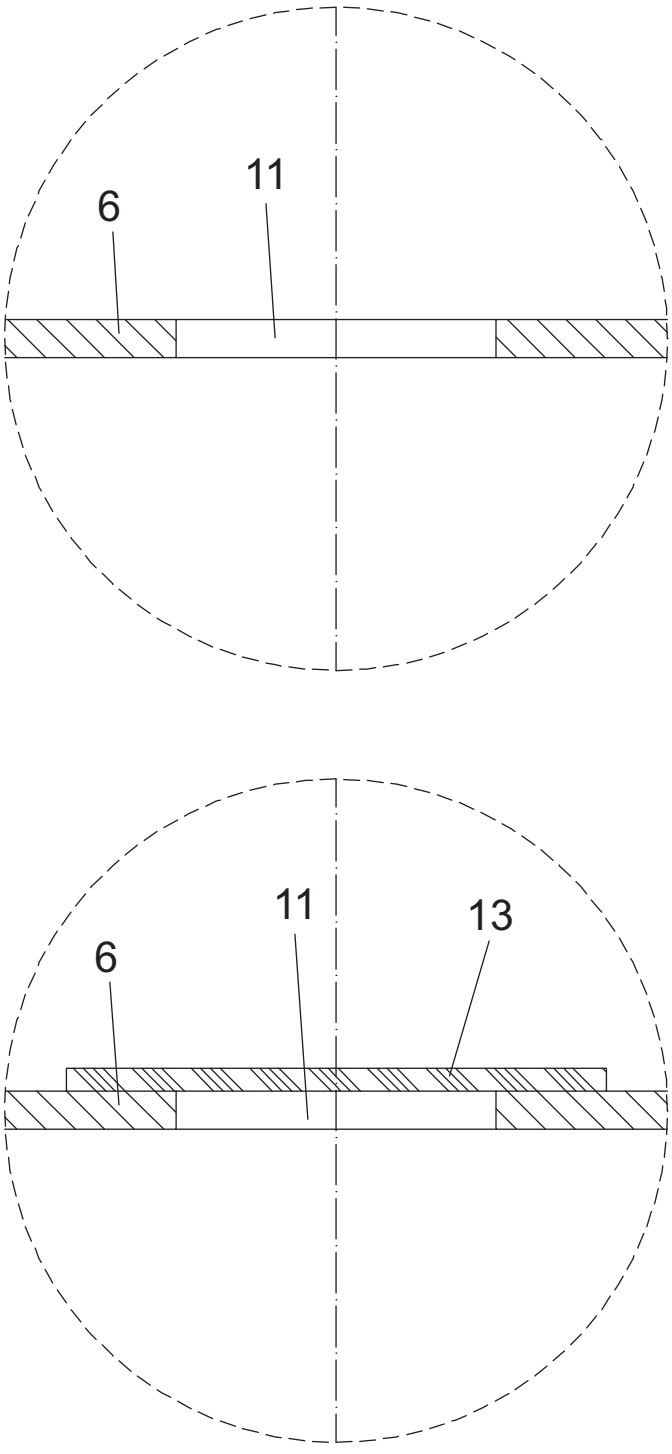

Fig. 6a

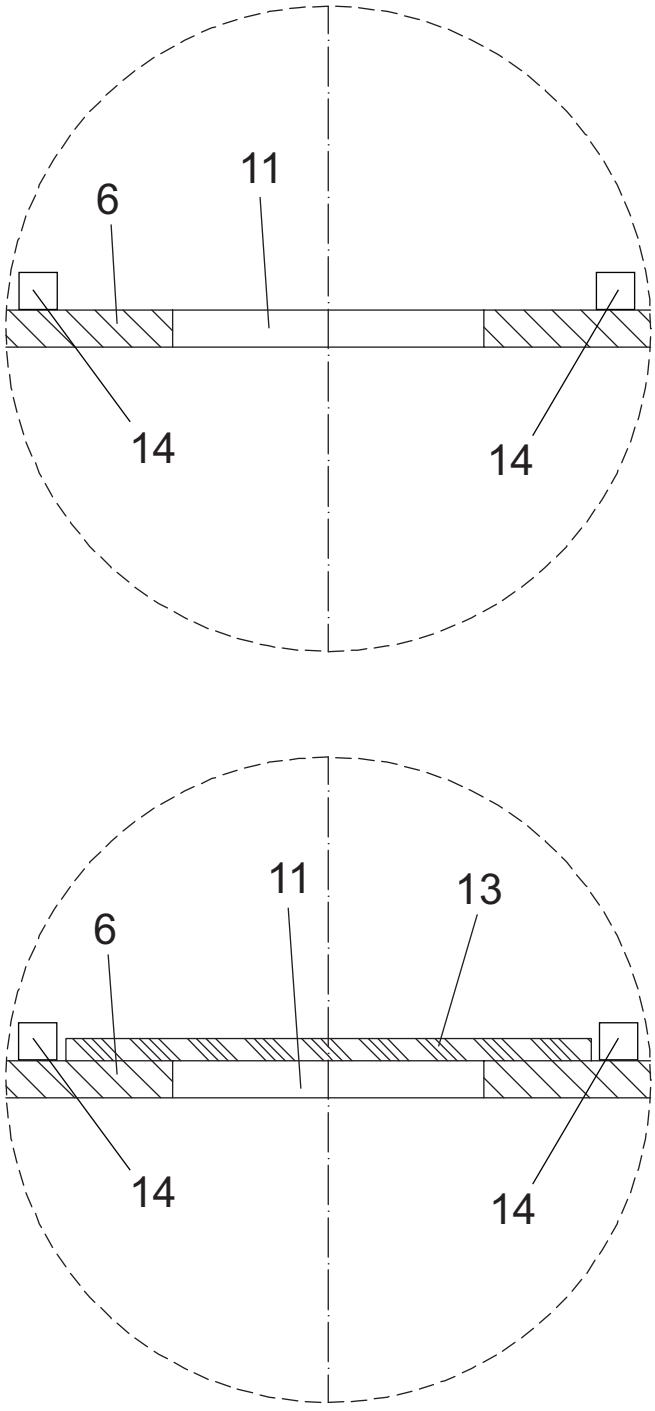

Fig. 6b