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HIGHLIGHTS IN PLANT MULTITROPHIC INTERACTIONS: BENEFICIAL MICROORGANISMS FOR SUSTAINABLE AGRICULTURE

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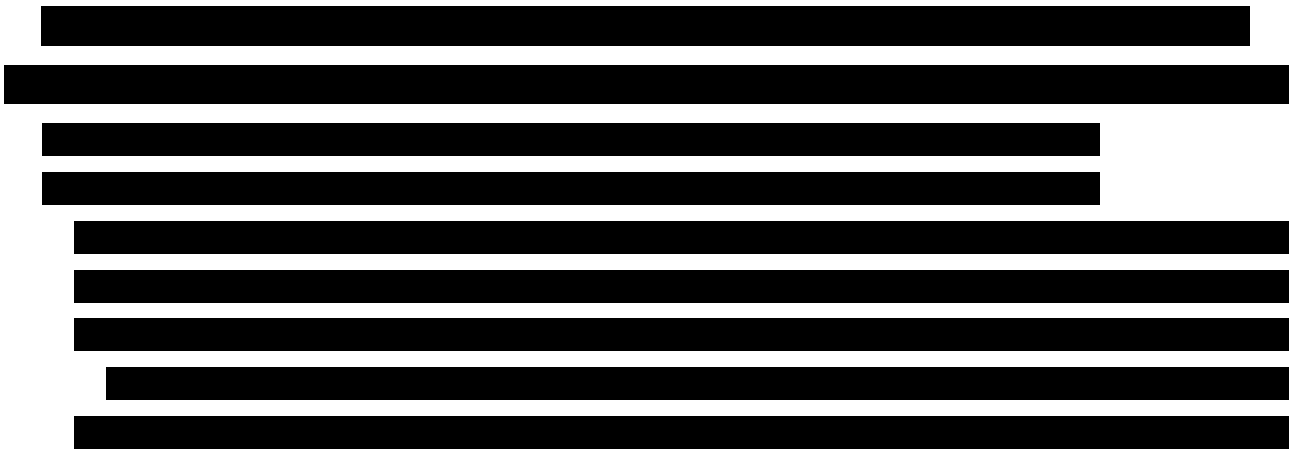

Abstract

Plants are central to complex networks of multitrophic interactions; a few, yet mostly important, interactions seriously affect and compromise crop quality and yield. The growing demand for safer agri-food products, using fewer pesticides and fertilisers, stimulated research on understanding and management of beneficial microorganisms (BMs) for agriculture, and the exploitation of plants and microbial volatile organic compounds (VOCs) that drive such relations between plants, BMs and herbivores, as a solution to enhance both crop defense and production.

Among BMs, microbial endophytes, that are associated with the majority of plant species, and their bioactive products, were identified as environmentally friendly options plant biocontrol agent. We carried out an in-depth study to investigate whether a complex multi-trophic interaction involving tomato, the BM *Beauveria bassiana* and the the pathogenic fungus *Botrytis cinerea* exists.

First, I investigated if plant-BM interactions could be driven by volatile organic compounds (VOCs) released by plants constitutively or induced by abiotic or biotic stresses. Reviewing available literature, evidence was provided that mVOCs emitted by BMs are in some cases perceived by sensing plants driving beneficial effects, mainly promoting growth promotion and enhancing stress resistance (**Chapter 2**).

Then, I focused on deciphering endophyte behaviour of the entomopathogenic fungus *B. bassiana*, known to be able to colonise a wide range of host plants, and to be used as an alternative for the sustainable management of insect pests. We used tomato (*Solanum lycopersicum L.*), as experimental model plant, testing whether plants are colonized by the endophytes, and how plant colonization affects physiological parameters of tomato plants which were grown under control (unstressed) conditions, or simultaneously exposed to the pathogenic fungus *B. cinerea*, a cause of substantial losses in tomato crops worldwide. Results suggested that successful endophytic colonization by *B. bassiana* achieved in tomato plants under fruiting time in seeds from tomato fruits, further proposing *B. bassiana* endophytic strain as plant growth promoter. A comparison with the well-characterised *Trichoderma harzianum* suggested similar behaviour for both BMs resulting in a significant but transient (1-2 day-long) reduction of stomatal conductance and CO_2 assimilation, which in turn indicates priming and rapid activation of defensive (rejection) responses. *B. bassiana* helped tomato plants fight *B. cinerea*, whose symptoms in leaves resulted were reduced by up to 35% with respect to plants not harbouring *B. bassiana*. Lower VOC emission in *B. bassiana*-treated plants confirmed that these plants did not activate defensive responses and suggested that VOCs do not explain protection by *B.bassiana* against *B. cinerea* (**Chapter 3**).

I further tried to assess *in vitro* whether fungal VOCs (fVOCs) are involved on the effect of *B. bassiana* against *B. cinerea*. An airborne and direct communication method between to fungi was

proposed to be used as experimental tool for testing VOCs communication between fungi. Our test confirmed results based on in vivo studies with tomato plants that *B. bassiana* VOCs are unable to control the growth of the pathogenic fungus *B. cinerea*, and are not responsible for its biocontrol, which was positively assessed elsewhere (**Chapter 4**). As shown in Chapter 4, fungi emit a broad spectrum of species-specific VOCs, whose complete knowledge is so far lacking. Volatilomes of four species of *T. harzianum* were characterized, which may help with fungal detection and identification in vivo, as well as for real-time studies on multitrophic interactions between beneficial microorganisms and host plants (**Chapter 5**).

In conclusion, the work carried out contributed to a better understanding on plant-multitrophic interactions and to valorisation and use of BMs to improve plant growth and to minimize the use of pesticides and other unsustainable or noxious agricultural practices.

Chapter 1

1. General introduction

1.1 Plant-pathogens relationships in agro-ecosystems today

Agricultural ecosystems have spread rapidly to face the growing human population (Foley et al. 2011) leading to increasing food needs, to an enormous reduction in plant and animal diversity (Tilman et al. 2001), and to specialization and improvement of few high-profit, high-yield crops in different soil, climate, and weather conditions. Approximately 540 million ha are now planted annually to maize, rice and wheat (FAO Statistics, 2018). This compares with the approximately 600 million ha of tropical rainforests that grew over millions of years (McDonald et al. 2016). Breading is leading to exploitation of genetic diversity of the main food crops, with selection of genes for high productivity, and against genes involved in plant defense, but negative for human use. Genetic uniformity enables mechanization and increases the efficiency of food production. However, new, host-specialized, 'domesticated' crop pathogens, more virulent compared to their 'wild' ancestors, have evolved rapidly (Read 2016). The global food crop production is now threatened daily by these emerging groups of pathogens and pests, viruses, bacteria, fungi, nematodes and parasitic plants, which are a major cause of yield loss and can seriously compromise food security (FAO 2018). Also plants which are less intensively grown but fulfil important nutritional requirements in restricted areas of the globe and are of strong economic interest are subjected to diseases both in the field and in post-harvest (Strange and Scott. 2005). The control of plant-pathogens interaction thus continues to be essential for helathy and productive agroecosystems. To increase food production without further degrading natural ecosystems and with successful outcomes when controlling emerging pathogens, a significant re-engineering of agroecosystems is required. The introduction of transgenic technology may provide a direct approach to improve crops for resistance to biotic stresses in a targeted manner and with minimal effects on beneficial soil microbes and the environment (Kamthan et al. 2016; Ahmar et al. 2020). However, genetic improvement has rather selected against genes coding for resistance to abiotic/biotic stresses, and in favour of genes coding for productivity, which makes plants more available and palatable also for pests and pathogens. The development of biotechnologically-based pest control tools and strategies surely represents a possible new solution for cultivated plants (Barrett et al. 2021), but must be assisted by improved knowledge of plant interactions with the environment, including those organisms that create useful (beneficial) or noxious (pathogenic) interactions with them. These sustainable plant protection strategies will significantly improve and/or induce natural defense barriers, contributing to the reduction of chemical pesticide use in agriculture, enforced by EU legislation (EU Directive 2009/128/CE), with positive impacts on agri-business economy, food safety and the environment.

1.2 Framework of the thesis

This Ph.D. project has been carried out at the University of Naples Federico II (Italy), in collaboration with the Institute for Sustainable Plant Protection of the National Research Council of Italy (IPSP-CNR). I also spent a fully supported seven-month stage abroad as Ph.D. guest at the Environmental Simulation Research Unit of the Helmholtz Zentrum München (EUS-HMGU), Germany, achieving an important requirement to make me eligible for the European Doctorate.

1.3 Aim of the thesis

My thesis activities were supported by the Research Project of National Interest (PRIN) Plant multitROphic interactions for bioinspired Strategies of PEst ConTrol (PROSPECT), that aims to characterize the functional basis and metabolic pathways of the complex network of trophic interactions and signalling between plants, beneficial soil microorganisms (BMs), pests (phytophagous insects and plant pathogens) and their natural antagonists, in order to develop bio-inspired technologies and strategies for sustainable protection of agricultural productions.

We still have a limited view of the functional and ecological aspects of complex multitrophic interactions affecting plant health and food production. In particular, the effect of plant colonization by endophytic entomopathogens is still poorly understood, as we do not know how these organisms alter primary and secondary plant metabolism, affect insects (pests and their natural enemies) on colonized plants, or influence the susceptibility to other entomopathogens in insect hosts feeding on colonized plants. Other soil fungi (e.g. *Trichoderma* spp.) are not entomopathogens; yet they are known as biological control agents (BCA) of plant pathogens, acting via the induction of plant resistance and/or exerting a direct effect thanks to the production of hydrolytic enzymes (Woo et al. 2022). Even in this case much more needs to be known. Improved knowledge on multitrophic interactions will help overcome limits that prevent the development of sustainable strategies of plant protection and will preserve and enhance BMs and the ecological services they provide.

Specific objectives were the evaluation of the impacts of beneficial microorganisms (*Trichoderma harzianum* and *Beauveria bassiana*) on the phenotype and physiology of tomato plants through non-destructive (*in vivo*) measurements of:

- Primary metabolism: including the study of photosynthesis, photorespiration and mitochondrial respiration, and the evaluation of photosynthetic limitations thanks to a portable system of simultaneous analysis of gas exchanges and chlorophyll fluorescence (Heinz Walz GmbH, Effeltrich, Germany).

- Secondary metabolism: studying the blend and amount of emitted volatile organic compounds (VOCs) with Gas chromatography-mass spectrometry (GC-MS) and Proton Transfer Reaction - Time of Flight - Mass Spectrometer (PTR-ToF-MS).

1.3.1 Experimental hypothesis

My experimental hypothesis is that BMs interact with plants affecting many physiological processes. If plants perceive the infection by BMs as a foreign invasion, then a reduction of photosynthesis and stomatal conductance and an activation of plant defense mechanisms (antioxidants and emission of VOCs) is expected. If BMs behave as growth promoters, on the other hand, a stimulation of photosynthesis and a relaxation of plant defences is expected. By considering "the plant side", this study helps covering existing gaps on the mechanisms behind interactions with beneficial microorganisms.

1.4 Overview of the chapters

The Ph.D. thesis is mainly composed of the following sections:

- General Introduction (Chapter 1);
- Scientific papers, published or in preparation, within the scopes of the thesis (Chapters 2,3,4,5). For each article, the references with complete bibliographic details are provided.
- Conclusion and future perspectives (Chapter 6).

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Chapter 2

This chapter consists of one published peer reviewed article.

The beneficial microorganism (BM)-plant interactions have been proposed as an eco-friendly solution to improve plant resistance to stresses and to increase productivity sustainably.

Aim of this chapter is to provide an overview of scientific evidence that this positive interaction is often mediated also by the release of microbial Volatile Organic Compounds (mVOCs).

2. Volatile organic compounds in the interaction between plants and beneficial microorganisms.

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PLANT-MICROORGANISM INTERACTIONS

Volatile organic compounds in the interaction between plants and beneficial microorganisms

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ABSTRACT

A growing population coupled with a higher demand for food is putting pressure on agriculture. The use of synthetic pesticides and chemical fertilizers allowed us to boost agricultural productions, but at a great environmental cost. Exploitation of beneficial microorganism (BM)-plant interactions has been proposed as an eco-friendly solution to improve plant resistance to stresses and to increase productivity sustainably. We provide an overview of scientific evidence that this positive interaction is often mediated also by the release of microbial Volatile Organic Compounds (mVOCs). A few mVOCs are reported to have a double, not mutually exclusive, positive effect on plants, as plant growth promoters, and/or inducers of resistance against biotic and abiotic stress factors. They may also alter plant VOCs indirectly improving plant performances. However, mechanisms and functions of mVOCs need deeper investigation. By understanding mVOC modes of action on plants, further tools for sustainably improving plant productivity in agro-ecosystems may become soon available.

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2.1 Introduction

According to the Food and Agriculture Organization (FAO), the estimated world population for 2050 will be nearly 9.15 billion people (FAO 2018). To meet the food demand of growing population, increasingly intensive agricultural practices, including wide use of chemical pesticides and fertilizers, are currently performed, causing a drastic loss of biodiversity as well as environmental and health problems (Gunnell et al. 2007; Leach and Mumford 2008; Önder, Ceyhan, and Kahraman 2011; Brühl and Zaller 2019). The search for technologies that effectively protect crops and at the same time are respectful of the environment, represents one of the major current challenges for agriculture. Some of these novel tools are based on multitrophic interactions where organisms (often insects) are used to feed on, predate, or parasitize pests.

Multitrophic interactions are driven by volatile organic compounds (VOCs) that are released by plants constitutively, or are induced by abiotic or biotic stresses (Loreto and Schnitzler 2010). Multitrophic interactions have been described for a growing number of plant pathogens and pests (Tack and Dicke 2013; Busby et al. 2015; van Dijk, Ehrlén, and Tack 2020; Noman et al. 2021), and start to be used as a tool for integrated pest management (IPM) also in field conditions (Brilli, Loreto, and Baccelli 2019; Cellini et al. 2021). However, most of these interactions apply to the aboveground environment (plant leaves, fruits, and their hosts). Belowground VOC-driven multitrophic interactions that help plants self-defend have been also described (D'Alessandro et al. 2014), but the soil environment, in its complex richness of microbial life, needs a deeper exploration in order to be able to exploit organisms that may protect plants or stimulate plant growth even under constrained environments. Soils are a major reservoir of life on Earth. Soil microorganisms play a vital role, such that soil has been considered a real living, complex and dynamic organism (Berg et al. 2014; Fierer 2017) that may influence plant functions and orchestrate a wealth of plant metabolic changes and defense reactions (Bardgett and van der Putten 2014; Farrar, Bryant, and Cope-Selby 2014). Indeed, during their entire life, plants interact with soil using their root system (in the rhizosphere, Turner, James, and Poole 2013; Andreote, Gumiere, and Durrer 2014; Fitzpatrick et al. 2020).

A wide range of bacteria and fungi, like nitrogen-fixing bacteria, ecto and endomycorrhizal fungi and other plant growth-promoting microorganisms (PGPMs), has been identified as responsible for the rhizosphere-based beneficial plant-microbe association (see Box 1). It has been shown that some soil microorganisms can improve crop nutrition and promote plant growth by mobilizing nutrients that are not readily available to plants (Jacoby et al. 2017), but even if awareness and knowledge of the plantsoil microorganism interactions has formidably advanced in recent years, the underlying mechanisms appear to be increasingly complex and much more remains to be known.

Box1. Soil microorganisms that are beneficial to plants (BMs).

This box is intended to provide a glossary helping define those BMs that have been found to exert their positive action on plants via mVOCs (Table 1) or, more indirectly, via modification of plant VOC blends (Table 2).

Plant Growth Promoting Microorganisms (PGPMs). PGPMs include all microorganisms colonizing roots, and promoting plant growth directly by facilitating nutrient uptake (as biofertilizers) or modulating plant hormone levels. They may also induce biocontrol of plant pests or pathogens. Main PGPMs are bacteria and fungi.

Plant Growth Promoting Rhizobacteria (PGPRs). This is the most represented beneficial root bacterial association in the rhizosphere. It is defined by Kloepper and Schroth (1981) as 'the soil bacterial community that competitively colonized plant roots and stimulated growth and thereby reducing the incidence of plant diseases'. PGPRs can be divided into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR). Both groups reported as VOC emitters. Among microorganisms listed in Table 1 PGPR are represented by genera: Bacillus, Enterobacter, Proteus, Pseudomonas, Stenotrophomonas, Streptomyces and Paenibacillus.

Plant Growth Promoting Fungi (PGPFs). Include the group of rhizosphere fungi that colonize plant roots and enhance plant growth and disease suppression (Hyakumachi 1994; Pieterse et al. 2014). PGPF classification does not represent any real biological similarity between fungi, as PGPFs may have different taxonomy, habitats, physiology and interaction with plants. In this review PGPFs are symbiotic or symbiotic, biotroph or saprophytic. Among the symbiotic PGPFs, mycorrhizae are an important component of soil communities. In mycorrhizal symbioses, the fungus colonizes the host plants roots, either intracellularly as in arbuscular mycorrhizal fungi (AMF), or extracellularly as in ectomycorrhizal fungi. No VOC emission from mycorrhizal fungi is reported as they have a biotrophic habitus. However, it is challenging to discriminate between VOCs emitted by the fungus from those emitted by the plant. On the contrary, there are many studies confirming that mycorrhizal fungi may induce changes in plant volatilome as reported in Table 2.

Here we will focus on reviewing growing scientific knowledge about VOCs as key messages allowing intercommunication between plants and the main soil microorganisms that are beneficial to plants (BMs). We review both interactions that are mediated directly by microbial VOCs (mVOCs) and those where BMs alter plant VOCs, indirectly affecting plant interactions with the environment and the other organisms. We argue that learning how mVOCs shape the plant-soil microorganism interactions may be a further, precious tool for sustainable agriculture, and also for exploring possible consequences of changing climate on the VOC-driven plants-BMs web (Box 2).

Box 2. BMs and the changing environment

Change in climate can alter the environmental conditions drastically and also impact plant-microbe associations (Fitzpatrick et al. 2020).Rising CO_2 concentrations, warming and drought, are some of the major effects of climate change that may affect the performance of plants-BMs interactions, plant competition and plant community structure (Compant, van der Heijden, and Sessitsch 2010; Sharma et al. 2014; de Vries et al. 2019). For example, rising CO_2 was reported to have different effects on hyphal root biomass of C_3 and C_4 plants (Poorter and Navas 2003; Madhu and Hatfield 2013). Rising atmospheric CO_2 presumably will affect rhizo-competent bacteria and fungi more strongly than typical bulk-soil representatives (Drigo, Kowalchuk, and van Veen 2008, 2009). In summary, the effect of rising CO_2 on plant-BM soil associations seems very variable and difficult to assess without further, possibly large-scale, tests. As for the effect of temperature on plant-BM interactions, the majority of AMF strains respond to higher temperatures with enhanced growth and plant colonization, at least in temperate regions (Heinemeyer and Fitter 2004; Heinemeyer et al. 2006). Finally, drought stress is a negative consequence of global warming that plagues increasing agricultural soils worldwide. Our understanding of the impacts of drought on the interactions between plant and BMs is still too preliminary. Generally, however, drought reduces AMF colonization (Auge 2001), but mechanisms of adaptation of certain AMF strains to drought conditions have been also described (Li et al. 2019), and may change the pattern in the long run.

2.2 Plant-environment communication alphabet: the volatile organic compounds.

All organisms can and often do emit VOCs. Here we will focus on plant VOCs whereas in the following section, emission of VOCs by soil microorganisms will also be addressed. It is well known that plant VOCs mediate plant interactions with the surrounding environment (Erb and Kliebensteinb 2020). VOC signaling role is deeply investigated to understand plants multi-trophic interactions (Volpe et al. 2018). Emission of VOCs by plants is ubiquitous and can take place from their above-ground (Mofikoya et al. 2019) and below-ground parts (Massalha et al. 2017). Some plant VOCs are constitutively emitted. This is the case of isoprene, the most abundant VOC on Earth being released in detectable amounts by about 20% of the plant species worldwide (Loreto and Fineschi 2015). Many other VOCs are instead induced by abiotic (Loreto et al. 2006; Loreto and Schnitzler 2010) or biotic stresses (Dicke and Loreto 2010). VOCs are generally directly released into the atmosphere from flower petals (Kolosova et al. 2001) where they are produced in epidermal cells (Adebesin et al. 2017), or through the stomata or cuticle of leaves after biosynthesis in mesophyll cells (Loreto and Schnitzler 2010). VOCs can also be released by plant roots (Delory et al. 2016; Rizaludin et al. 2021). In this case the site of synthesis is not known, but for example a high isoprene synthase activity was observed in vascular tissues, root hairs, root caps and developing lateral roots, suggesting that

isoprene characterizes all rapidly developing root regions (Miloradovic van Doorn et al. 2020). Some families of plants have developed specialized structures where VOCs can be concentrated in large permanent pools; this is the case of resin ducts and glandular trichomes of leaves, stems and buds (Tissier, Morgan, and Dudareva 2017). Many VOCs are toxic to organisms, and permanent pools are sealed to avoid autotoxicity. VOC spreading from permanent pools typically occurs after damage to the specialized structure. When emitted, these VOCs are rapidly oxidized in the air and powerfully seal the wounds avoiding microorganism colonization (Pasqua et al. 2002) or deterring incoming herbivores with their stinky odor or because they are toxic when ingested. This applies to terpenes (e.g. Song et al. 2021) that also deter oviposition (Holopainen and Gershenzon 2010); phenylpropanoids volatile derivatives such as methyl salicylate (Glinwood and Pettersson 2000); and glucosinolate volatiles such as isothiocyanates (Agrawal and Kurashige 2003). While VOCs are often used by plants to self-defend, they may also be part of a more sophisticated communication system at higher trophic levels (Bouwmeester et al. 2019). The best known among these VOC-driven communications is floral VOC-mediated plant-pollinator chemical communication, where compounds are detected by antennae of pollinators in a very specific way improving pollen transfer efficiency (Proffit et al. 2020).

Table 1. *Microbial Volatile Organic Compounds (mVOCs) in plant-beneficial soil microorganism (BM) communication. mVOC beneficial effect on plants is shown as Plant Growth Promotion (PGP) or Induced Resistance (IR). Signalling pathways, many of which indicating onset of Induced Systemic Resistance (ISR) in plants, are indicated as mevalonate (MVA) salicylic acid (SA), jasmonic acid (JA), cytokinins (CK), ethylene (ET), auxins (AUX), strigolactones (STR), brassinosteroids (BR), sensor kinase (GacS), quorum-sensing (QS), or unknown (U). Main VOC detection systems are Gas Chromatography-Mass Spectrometry (GC-MS), Electron Ionization Mass Spectrometer (EI-MS), GC with Flame Ionization Detection (GC-FID), Solid Phase Microextraction (SPME), Headspace-solid phase microextraction (HS-SPME), Proton-Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS). BM names are shown as mentioned in the original papers.*

Main VOC emitted by the BMs	BM Producer	Sensing plant	VOC effect on plant		Signaling pathway	VOC detection	Reference
			PGP	ISR			
Sesquiterpenes above all (-)Thujopsene	L*accaria bicolor*	*Populus*; *Arabidopsis thaliana*	x		U	Twisters-GC-MS	Ditengou et al. 2015
Geosmin (trans-1,10-dimethyl-trans-9-decalol)	*Tricholoma vaccinum*	*Picea abies*	x		MVA	SPME-GC/MS	Abdulsalam et al. 2021
2-methyl-butanal; 3-methyl-butanal	*Cladosporium halotolerans* (strain NGPF1)	*Nicotiana benthamiana*	x		U	HS-SPME-GC/MS	Jiang et al. 2021
3-hydroxy-2-butanone (acetoin)	*Bacillus amyloliquefaciens; Bacillus mojavensis*	*A.thaliana; Lactuca sativa; Mentha piperita*	x	x	CK, ET	GC-MS	Ryu et al. 2003; Rudrappa et al. 2010; Asari et al. 2016; Fincheira et al. 2018; Rath et al. 2018; Cappellari and Banchio 2019; Silva Dias et al. 2021
" "	*Bacillus vallismortis* (strain EXTN1)	*Nicotiana tabacum*	x	x	U	SPME-GC/MS	Ann et al. 2013

2, 3-butanediol	*B. amyloliquefaciens;* *B. mojavensis;* *Bacillus subtilis*	*A. thaliana* *Ocimum basilicum;* *Mentha piperita;* *Cucumis sativus*	x	x	SA-ET, JA	GC-MS	Ryu et al. 2003, 2004; Farag et al. 2006; Zhang et al. 2008b; Santoro et al. 2011; Bitas et al. 2013; Asari et al. 2016; Hao et al. 2016; Gao et al. 2018; Song et al. 2019; Yang and Zhang 2019
" "	*Enterobacter aerogenes*	*Zea mays*		x	U	GC-MS (GC-FID)	D'Alessandro et al. 2014
" "	*Pseudomonas chlororaphis*	*N. tabacum*; *A. thaliana*		x	GacS	GC/EI/MS	Han et al. 2006; Cho et al. 2008
tetrahydrofuran-3-ol; 2-heptanone; 2-ethyl-1-hexanol	*Bacillus sp.*	*A. thaliana;* *Solanum lycopersicum*	x		AUX, STR	SPME-GC/MS	Zhang et al. 2007; Jiang et al. 2019
6,10,14-Trimethyl-2-pentadecanone; Benzaldehyde	*Bacillus ssp.*	*A.thaliana*	x		QS	SPME-GC/MS	Gutiérrez-Luna et al. 2010
3-pentanol	*B. amyloliquefaciens*	*Capsicum annuum*		x	SA, JA	-	Choi et al. 2014
Albuterol; 1,3-Propanediole	*B. subtilis*	*S. lycopersicum*	x		CK-ET	SPME-GC/MS	Tahir et al. 2017
α-pinene, (−)-trans caryophyllene; tetrahydro-2,2,5,5-tetramethylfuran; dehydroaromadendrene; (+)-sativene	*Cladosporium cladosporioides*	*N. tabacum*		x	U	SPME-GC-MS	Paul and Park 2013
Indole	*Proteus vulgaris*	*A. thaliana*	x		AUX, CK, BR	-	Bhattacharyya et al. 2015

3-nonene; 1-undecene	*Pseudomonas fluorescens*	*A. thaliana*	x	x	GacS	GC-Q-TOF-MS	Cheng et al. 2016
Dimethyl disulphide	*Bacillus cereus C1L*	*N. tabacum*; *Z. mays*		x	U	SPME-GC-MS	Huang et al. 2012
" "	*Bacillus sp*	*Nicotiana attenuata*	x		U	SPME-GC-MS	Meldau et al. 2013
" "	*Pseudomonas simiae* *Stenotrophomonas maltophilia*	*S. lycopersicum*	x		U	SPME-GC-MS	Rojas-Solís et al. 2018.
1,3,5-Trichloro-2-methoxy benzene	*Streptomyces spp.*	*A. thaliana*	x		U	SPME-GC-MS	Cordovez et al. 2015.
2-Methyl propanol; 3-methyl-butanol	*Phoma sp*	*N. tabacum*	x		U	SPME-GC-MS	Naznin et al. 2013.
Tridecane (C13)	*Paenibacillus polymyxa*	*A. thaliana*	x		U	GC-MS	Lee et al. 2012.
C2-pentylfuran	*Bacillus megaterium*	*A. thaliana*	x		U	SPME-GC-MS	Zou et al. 2010.
β-caryophyllene	*Talaromyces sp*	*Brassica campestris*	x	x	U	GC–MS/MS	Yamagiwa et al. 2011
4-Heptanone; Nonane; 2-Octanone; Limonene; 3-Methyl-1-butanol 1-Decene; 2-Heptylfuran; Butane-2,3-dione; 2-Methylpropan-1-ol; 3-Methylbutan-1-ol; Limonene; Undecane; Camphor; Benzoic acid;	*Trichoderma spp*	*A.thaliana;* *S. lycopersicum*	x		U	SPME-GC-MS PTR-TOF-MS	Lee et al 2016; Nieto-Jacobo et al. 2017; Guo et al. 2019, 2020.

Nonanoic acid; β-Acoradiene							
Isobutyl alcohol; Isopentyl alcohol; 3-Methylbutanal	*Trichoderma viride*	*A.thaliana*	x		U	SPME-GC-MS	Hung et al. 2013.
Isopropanol; Acrylonitrile; 2-Pentanone; 1-Butanol; Propyl acetate; Butyl acetate; Pentyl acetate; Styrene; Furfural; Heptanal; 2-Heptanol; Furfuryl alcohol; 2-Acetylfuran; 2-Ethylhexyl alcohol; Nonyl alcohol; Methyl salicylate; Undecanal; 1,2,3,4-Tetrahydro-5-methylnaphthalene; 2-Phenoxyethanol; Sesquiterpene; Isocaryophyllene; n-Heptadecane; Methyl dihydrojasmonate; Isopropyl laurate; Octanal-2-(phenylmethylene)	*Trichoderma atroviride*	*A.thaliana*	x		U	GC-MS	Lee et al. 2015.
6-pentyl-2H-pyran-2-one (6-PP)	*T. atroviride*	*A.thaliana*		x	AUX	SPME-GC-MS	Hung et al. 2013; Garnica-Vergara et al. 2016.

Sesquiterpenes; Monoterpenes	*T. virens*	*A.thaliana*	x	x	JA	SPME-GC-MS	Contreras-Cornejo et al. 2014.

Table 2. *Changing volatilome in plants (plant VOCs) treated with beneficial microorganisms (BMs). Main BMs involved are Arbuscular Mycorrhizal Fungi (AMF), Entomopathogenic Fungi (EF), Plant Growth Promoting Fungi (PGPFs) and Plant Growth Promoting Rhizobacteria (PGPRs). + / - indicate increase/decrease of plant VOCs caused by BMs (second column), or positive/negative effect in plant defense against pests or pathogens (third column). U indicates unknown effect against other organisms. VOCs are detected by Gas Chromatography-Mass Spectrometry (GC-MS), Electron Ionization Mass Spectrometer (EI-MS), Headspace-solid phase microextraction (HS-SPME), Proton-Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS). BM names are shown as mentioned in the original papers.*

Plant + BM treatment	Changing plant volatilome		VOC detection	Reference
	Main plant VOCs involved	Tested effects on other organisms		
Plant + AMF *Phaseolus vulgaris + Glomus mosseae*	(+) Monoterpenes (β-ocimene) and sesquiterpenes (β-caryophyllene)	(-). Predators strongly attracted to mycorrhized plants, which are also more attacked by the mites *Tetranychus urticae*	PTR-TOF-MS	Schausberger et al. 2012
Plant + AMF *Sorghum bicolor* + ● *Glomus mosseae or* ● *Glomus intraradices*	(+) Alcohols, alkenes, ethers and acids - linear-alkanes	U	HS-SPME/GC-MS	Sun and Tang 2013

Plant + AMF *Vicia faba + Glomus ssp.*	(-) Sesquiterpenes ((E)-β-caryophyllene , (E)-β-farnesene)	(-) Mycorrhized plants more attractive to the pea phytophagous aphid *Acyrthosiphon pisum*	GC-MS	Babikova et al. 2014
Plant + PGPF *Solanum lycopersicum L. + Trichoderma longibrachiatum*	(+) Cis-3-hexen-1-ol, α-pinene, methyl salicy-late, longifolene, and β-caryophyllene	(+) Colonized plants more attractive to *Aphidius ervi* (parasitoid of the aphid *Macrosiphum euphorbia)*	GC-MS	Battaglia et al. 2013
Plant + PGPF *Solanum lycopersicum L. + Trichoderma asperellum* (strain M2RT4)	(+) Methyl salicylate, (Z)-jasmone	(+) Methyl salicylate repellent effect on *Tuta absoluta*. (Z)-jasmone reduces larval activity of *Tuta absoluta*	GC-MS	Agbessenou et al. 2022
Plant + PGPR *Vitis vinifera* + ● *Bacillus licheniformis* ● *Pseudomonas fluorescens*	(+) Monoterpenes (α-pinene, terpinolene, 4-carene, limonene, ocimeneeucalyptol and lilac aldehyde A), and the sesquiterpenes (α-bergamotene, α-farnesene, nerolidol and farnesol)	U	GC-EI-MS	Salomon et al. 2014, 2017
Plant + PGPR *Vitis vinifera* + ● *Microbacterium imperiale* or ● *Kocuria erythromyxa* or ● *Terribacillus saccharophilus*	(+) Monoterpenes (α-pinene, terpinolene, 4-carene), and sesquiterpenes (nerolidol)	(+) Bacterized plants show reduction in lesion size when infected by the necrotrophic fungus *Botrytis cinerea*	GC-EI-MS	Salomon et al. 2016
Plant + PGPR *Zea mays + Bacillus sp.*	(+) Linalool - methyl-2-methylene-2-hexen-1-ol, α-Copaene	(+) Significantly fewer eggs laid by the moth *Ostrinia nubilalis*	GC-MS	Disi et al.2018
Plant + AMF/PGPB ● *Fragaria x ananassa + Septoglomus viscosum + Pseudomonas sp* ● *Fragaria x ananassa + Funneliformis mosseae + Pseudomonas sp*	(+) Esters and alcohols, 3-methyl-1-butyl acetate, 1,6-heptadien-4-ol	U	HS-SPME/GC-MS	Todeschini et al. 2018

Plant + EF *Vitis vinifera* + *Beauveria bassiana*	(+) γ-Terpinene, geranylacetone, m-cymene, and naphthelene.	(-) Inoculated grape plants infested by the mealybug *Planococcus ficus*	GC-MS	Moloinyane and Nchu 2019
Plant + AMF *Vitis vinifera* + *Funneliformis mosseae*	(+) Monoterpene alcohols (myrtenol, p-cymen-7-ol, p-mentha-1,8-dien-7-ol) and 3-hexenal, benzaldehyde, geraniol, MeSA, trans-2-hexenal	U	HS-SPME/GC-MS	Velásquez et al. 2020

Not only pollinators but also phytophagous insects and their antagonists are attracted by the volatile blend produced by plants, and use plant VOCs for host identification to identify the host. While constitutive blends mainly have been evolutionary used to attract herbivores and pollinators, the herbivore-induced plant volatiles (HIPVs), mostly green leaf volatiles (GLVs), terpenes and aromatic compounds, drive the attraction of natural enemies of herbivores such as parasitoids and predators (Dicke and Baldwin 2010; Gasmi et al. 2018; Turlings and Erb 2018).

VOCs may also mediate dispersal of pathogens (both fungi and bacteria) as both fungi and bacteria may be vectored by pollinators that visit infected VOC-emitting plants, leading to unexpected risks for the plants (e.g. Cellini et al. 2019).

VOC emission also plays an intriguing signaling function between plants. Monoterpene biosynthesis and emission elicited by pathogen bacteria have been demonstrated to activate plant defense and Systemic Acquired Resistance (SAR) in neighbor plants (Riedlmeier et al. 2017), and classic experiments show that plants may sniff neighbors, being attracted (Runyon, Mescher, and De Moraes 2006) or repelled (Santonja et al. 2019). Despite growing evidence that VOCs are able to mediate above-ground plant-plant interactions, we do not know how plants can detect VOCs as they do not have a neuronal system where the odor is processed to activate the response of the receiving plants. The 'olfactory system' of plants must be more rudimental. However, a few odorant binding proteins (OBPs) transporting VOCs to receptors (such as in animals) have been retrieved in plants (Loreto and D'Auria 2022), and at least some OBPs originally described to bind specifically methyl salicylate and methyl jasmonate, may indeed not be very selective, as many VOCs may dock into their tertiary structure (Giordano et al. 2021).

Ethylene, a very important volatile hormone, has receptors (Bleecker et al. 1988; Lacey and Binder 2014) but is not an odorant VOC and its binding is not through OBPs. While VOCs are a cornerstone element of plant communication with animals and airborne pathogens and pests and perhaps also among the aerial parts of different plants, are they also effective belowground? If the release of aboveground VOCs is well documented, less is known about their impact on the under-ground world. As seen earlier, roots are also able to release VOCs. Much alike aboveground plant parts, roots can detect foreign chemical signals from their neighbors, and release VOCs involved in biotic interactions belowground, playing important ecological functions in the soil ecosystem (Delory et al. 2016). However, the roles played by VOCs emitted by roots in signaling between and within plants, are still poorly documented.

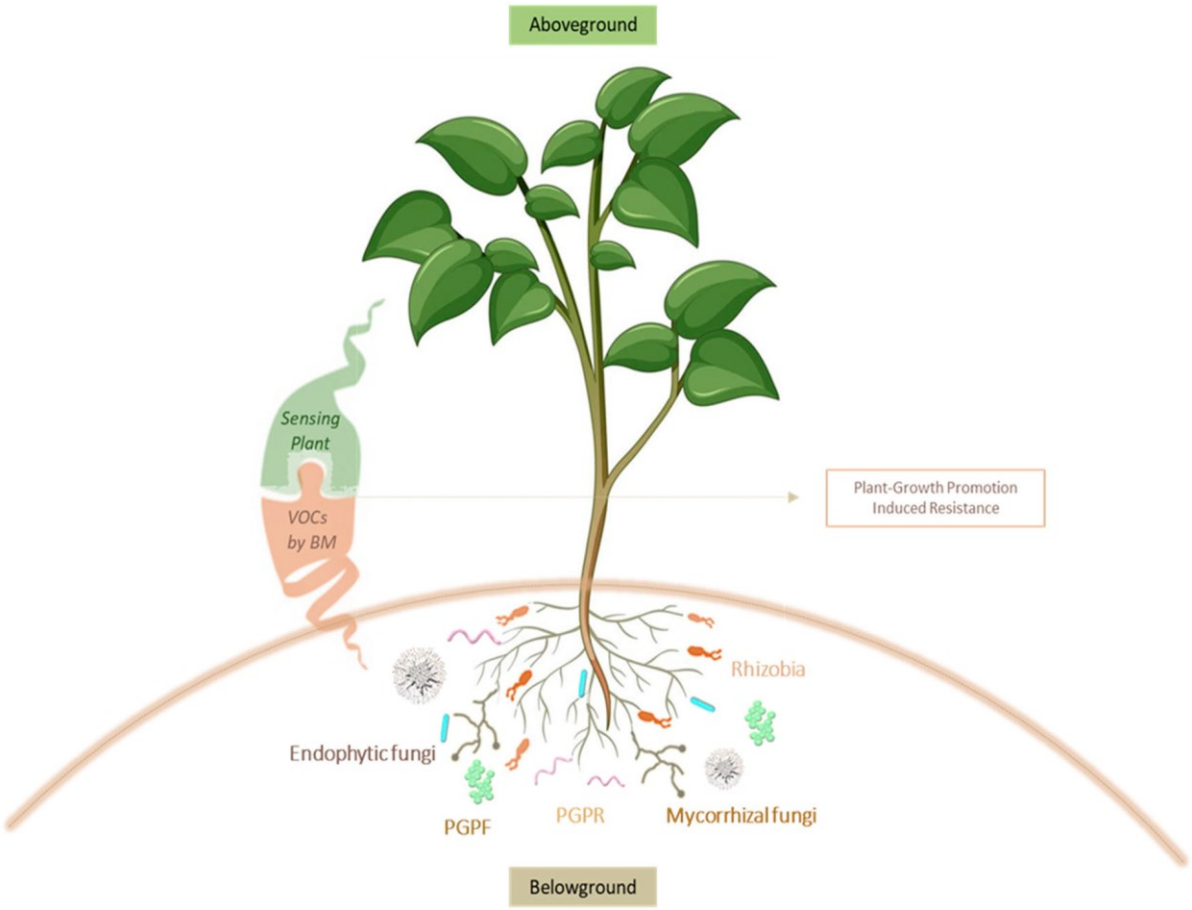

Figure 1. *Volatile organic compounds (VOCs) can mediate plant/beneficial soil microorganism (BM) communication. Soil beneficial microorganisms (mycorrhizal fungi, rhizobia, endophytic fungi, plant growth-promoting fungi (PGPF) and rhizobacteria (PGPR)) interact in multiple ways with plants. VOCs, interpreted as bidirectionally sent and perceived signals, play a role in this communication. Here we review cases where specific mVOCs emitted by BMs are perceived by sensing plants driving beneficial effects, mainly promoting growth promotion and enhancing stress resistance.*

2.3 Microbial VOCs in the communication between plants and soil microorganisms.

The rhizosphere is the soil area where VOCs, interpreted as bi-directionally sent and perceived signals, likely drive plant soil microorganism communication (Shulz-Bohm et al. 2018). Microbial VOCs (mVOCs) include alcohols, benzenoids, aldehydes, alkenes, acids, esters, terpenoids and ketones (Morath, Hung, and Bennett 2012; Lemfack et al. 2018; Misztal et al. 2018; Guo et al. 2020). The high chemical diversity of these compounds, and the involvement of different biochemical pathways, make the wide spectrum of mVOC-induced responses less explored, especially in terms of how these signals are processed by the receiver organisms.

We know, however, that some mVOCs are main actors in plant beneficial associations in the ecosystem (Hung, Lee, and Bennett 2015; Mhlongo et al. 2018), and in the biocontrol of phytopathogenic attack (Tilocca, Cao, and Migheli 2020), and drive additional microbe-microbe interactions (Vasseur-Coronado et al. 2021), also towards other soil inhabiting organisms (Werner, Polle, and Brinkmann 2016).

Soil BMs produce mVOCs that act as signaling molecules resulting in positive effects on plants in a direct or indirect way (Bailly and Weisskopf 2012; Bitas et al. 2013; Poveda 2021). The direct way is when mVOCs are sensed by the plants, and induce growth promotion (Fincheira and Quiroz 2018) and/or stress resistance (Liu and Zhang 2015) (Figure 1). The indirect way is when mVOCs are received by pests or pathogens, and act as repellents, or by friendly insects and microorganisms, and act as attractants, in all cases resulting in a benefit for the plant.

Main features of BMs-mVOCs that are received by plants (main mVOCs emitted, BM emitter, sensing plant, effect on plant, suggested signaling pathway, mVOC detection system) are shown in Table 1, where VOC physiological effects for the plants are classified as (i) plant growth promotion or (ii) induction of stress resistance. These two main effects will be further reviewed in the following two sub-headings of this paper. It is important, however, to observe that: first, some mVOCs are able to exert both effects (and indeed as noted earlier many soil microorganisms are both growth promoters and stress resistance inducers, see Box 1) and, second, to our knowledge only some BM strains are able to produce the specific VOCs that elicit plant responses, whereas VOC profiles of other VOC-emitting strains (e.g. of *Trichoderma harzianum*, *T. hamatum*; *T. velutinum* when looking at *Trichoderma* species that are often used as BMs) may be different and not yet assessed for their impact on plants (Guo et al. 2019).

2.3.1 Plant growth promotion

Plant growth promotion can be stimulated by mVOCs emitted by both bacteria and fungi (Table 1). mVOC sensing resulted in a two-fold increase in the fresh weight of Arabidopsis (Zou, Li, and Yu 2010; Asari et al. 2016; Hao et al. 2016) and increased 5–9 fold the fresh weight of tobacco (Ann et al. 2013; Meldau et al. 2013). mVOCs also increased length and numbers of lateral roots (Gutiérrez-Luna et al. 2010; Meldau et al. 2013) as well as roots dry weight of plants (Santoro et al. 2011).

Among mVOCs emitted by bacteria, 2,3-butanediol (2,3-B) and 3-hydroxy-2-butanone (acetoin), the precursor of 2.3-B, seem to be able to induce both types of physiological effects: a general plant growth promotion and an increased stress resistance activity, whereas other compounds seem to more specifically induce singular patterns (Table 1). 2,3-B and acetoin are produced by different genera of bacteria (e.g. *Bacillus* spp., *Serratia* spp., *Enterobacter* spp., *Pseudomonas* spp.) (Xiao and Xu 2007;

Silva Dias et al. 2021) where they have important functions, such as electron donor, carbon feedstock, pH neutralizer of the surrounding environment (Yang and Zhang 2019). In interaction with plants, however, the functions of these compounds could be completely different. Out of the three stereoisomers of 2,3-B present in nature and produced by microorganisms (Sabra, Groeger, and Zeng 2016; Gao et al. 2018), only the isomer 2R,3R-B seems to have plant growth promotion as well as stress inducing resistance activity (Ryu et al. 2003; Farag et al. 2006; Han et al. 2006). Plants respond to this compound in a dose-dependent way, increasing leaf sizes and root biomass up to a threshold which depends on the experimental conditions (Ryu et al. 2003, 2004; Song, Riu, and Ryu 2019). *Bacillus subtilis* and *B. amyloliquefaciens* both induce plant growth promotion on *Arabidopsis* (Ryu et al. 2003) and both strains emit 2,3-B, acetoin, and other VOC compounds, even if with different concentrations. However, only the mVOCs produced by *B. amyloliquefaciens* were able to promote plant growth also in *Arabidopsis* mutants ein2 (cytokinin and ethylene insensitive) and cre1 (cytokinin receptor deficient). Based on these results, the cytokinin signaling pathway appears to play a role in growth promotion following exposure to *B. subtilis* VOCs, although further investigation is needed. This same interaction was studied at transcriptomic level revealing a differential expression of genes involved in cell wall synthesis, primary and secondary metabolism, stress responses and hormones in *Arabidopsis* seedlings exposed to mVOCs produced by *B. subtilis* (Zhang et al. 2007). In these experiments mVOCs seemed to promote plant growth through a different regulation of auxin. Zhang et al. (2008a) also reported higher photosynthetic efficiency and chlorophyll content due to a different modulation of endogenous sugar/ABA signaling in *Arabidopsis* plants after exposure to *B. subtilis* mVOCs. mVOCs produced by some fungi, like *Trichoderma* spp, *Alternaria alternata*, *Penicillium charlesi* and *P. aurantiogriseum*, also have shown plant growth promotion activity (Ezquer et al. 2010; Li et al. 2011; Hung, Lee, and Bennett 2013; Sánchez-López et al. 2016). Finally, it should be said that, despite evidence of plant growth promotion by mVOCs being firmly established, some mVOCs may also act as plant growth-inhibitors (Kanchiswamy, Malnoy, and Maffei 2015; Song et al. 2022; Vlot and Rosenkranz 2022). The blend of VOCs emitted by some bacteria may contain both growth promoting and inhibiting compounds, at varying concentration ratios. Elements such as nutrient richness and bacterial population size, as well as increasing distance from the plant (which reduces mVOC concentration reaching the plant) (Lee et al. 2012), could also heavily influence the ratio of growth promoting and inhibiting compounds and the consequent response of receiver plants (Song et al. 2022).

2.3.2 Induced resistance (IR)

Many studies have focused on mVOCs from BMs for their effects on plant growth and disease control (Table 1), but little is known about the molecular basis of Induced Resistance (IR) elicited by mVOCs (Naznin et al. 2014; Pescador et al. 2022). Same bacterial mVOCs that promote plant growth (e.g. 2, 3-B and acetoin) are able to induce plant resistance against abiotic stresses like salinity (Zhang et al. 2008b; Vaishnav et al. 2015; Cappellari and Banchio 2019) and osmotic/drought stress (Cho et al. 2008, 2013; Zhang et al. 2010). In particular, Zhang et al. (2008b) showed that mVOCs emitted by *B. subtilis* GB03 were able to protect *Arabidopsis* plants against salt stress, reducing Na+ accumulation by 50% and increasing biomass in comparison to plants not exposed to mVOCs. However, Na+ accumulation was not reduced in a athkt1 mutant, where a xylem parenchyma Na+ transporter was not expressed (Sunarpi et al. 2005), and was reduced by only 15% in a sos3 mutant where SOS3 protein, required for the activation of the H+/Na+ antiporter SOS1, was not present (Shi et al. 2003). Acetoin emitted by *B. amyloliquefaciens* was also able to increase salt tolerance in *Mentha piperita* by increasing the level of salicylic acid (Cappellari and Banchio 2019). Salicylic acid concentration increased also in water stressed *Arabidopsis* plants treated with *Pseudomonas chlororaphis* O6 (a strain emitting 2,3-B) or with direct application of 2,3-B. Plants treated with *P. chlororaphis* O6 or 2,3-B exhibited a higher tolerance to drought, compared to control (unstressed) plants, due to a reduction of water loss directly correlated with stomatal closure (Cho et al. 2008). Furthermore, it has been shown that 2,3-B can increase the contents of nitrogen oxide and hydrogen peroxide which, at moderate concentrations, prime antioxidant plant defenses and enhance drought tolerance (Cho et al. 2013).

BMs interacting with plants induce several metabolic changes, many of them driving to Induced Systemic Resistance (ISR) which we consider as a main, but not unique, feature of IR (Table 1). Bacterial mVOCs are able to induce plant defense against different pathogens, mainly activating salicylic or jasmonic acid signaling pathways (Naznin et al. 2014; Li and Kang 2018). This is usually associated with elicited plant cellular defense responses, such as the deposition of callose-rich papillae at the sites of pathogen infection (Segarra et al. 2009). Acetoin produced by *B. subtilis* was able to induce systemic resistance in *Arabidopsis* against *Pseudomonas syringae* through a stimulation of the SA-signaling pathway (Rudrappa et al. 2010). Acetoin produced by *B. vallismortis* also induced plant growth promotion, as well as systemic resistance against *Pectobacterium carotovorum* subsp. carotovorum strain SCC1 in *Nicotiana tabacum* (Ann et al. 2013). Also 2,3-B produced by *B. subtilis* and *B. amyloliquefaciens* was able to induce systemic resistance against *Erwinia carotovora* in Arabidopsis (Ryu et al. 2004) and tobacco (Han et al. 2006). mVOCs produced by fungi may defend plants from pathogens and pests, and in fact there is a growing amount of literature showing that fungi

are prolific emitters of VOCs involved in inducing biotic stress resistance (Weisskopf, Schulz, and Garbeva 2021). Naznin et al. (2014) reported that *Arabidopsis* plants, challenged with the pathogen *P. syringae* pv. tomato, were protected against infection if pre-treated with PGPFs *Cladosporium* sp. (Dc4) and *Ampelomyces* sp. (Fa3). The authors identified methyl benzoate (MeBA, emitted by *Cladosporium*) and m-cresol (emitted by *Ampelomyces*), as the VOCs responsible for disease suppression. Analysis of *Arabidopsis* defense-related genes by real-time qRT-PCR showed that the two VOCs act through different signaling pathways: MeBA mainly involves the JA-signaling pathway with partial involvement of SA as well, whereas m-cresol activates both SA-and JA pathways. *Arabidopsis* plants treated with 1-octen-3-ol, a mVOC commonly produced by fungi, were more resistant to *Botrytis cinerea* and several genes of the JA/ethylene-dependent pathogen or wound defense signaling pathways were stimulated (Kishimoto et al. 2007, not shown in Table 1, as the treatment was performed with a synthetic compound). mVOCs produced by *T. virens* increased the resistance of *Arabidopsis* against *Botrytis cinerea* increasing JA levels (Contreras-Cornejo et al. 2014). Tomato and oilseed rape seedlings inoculated with spores of *B. cinerea* or *Leptosphaeria maculans,* respectively, and treated with 6PP (a VOC emitted and isolated from different strains of *T. harzianum* and *T. atroviridae*) showed a reduction of disease symptoms, and over-expression of pathogenesis-related proteins was detected in treated plants. We do not report this information in Table 1, as also this VOC was provided exogenously and not directly delivered by the BM. However, this result, associated with plant growth experiments, indicates that 6PP may be both an inducer of plant growth, acting as an auxin-like compound and/ or as auxin inducer, and an activator of plant defense responses (Vinale et al. 2008). Accumulation of callose, similar to that reported after exposure to bacterial mVOCs (see above), and upregulation of defense-related genes was detected also in grapevine plants treated with 6PP and with 2-pentylfuran after *Plasmopara viticola* infection (Lazazzara et al. 2022).

2.4 BM-induced changes of plant VOCs may influence higher trophic levels.

Whether plant exposure to BMs indirectly influences plant VOCs and the subsequent VOC-driven communication of plants with other organisms (Loreto et al. 2014) has only been studied in few cases (Table 2). Plant VOC profiles were deeply altered in sorghum plants by arbuscular mycorrhizae (AMF, see Table 1). Mycorrhized plants, emitted more alcohols, alkenes, ethers and acids but fewer linear-alkanes than controls (Sun and Tang 2013). Plant-AMF interaction enhanced terpenoid biosynthesis and influenced the production of root VOCs in grapevine plants, increasing monoterpene alcohols that play a role in plant defense (Velásquez et al. 2020). Mycorrhizal symbiosis significantly changed the HIPV composition in bean plants attacked by spider mites *Tetranychus urticae*. The higher emission

of β-ocimene and β-caryophyllene increased attractiveness of the predatory mite *Phytoseiulus persimilis* to mycorrhized bean plants with respect to the non-mycorrhized ones (Schausberger et al. 2012). Furthermore, in inoculated strawberry plants, the combined action of both AMF and plant growth-promoting rhizobacteria (PGPRs) affected plant VOC profile and improved chemical composition of strawberry fruits (Todeschini et al. 2018). Analysis of headspace volatiles of maize plants showed quali-quantitative differences when plants were treated with PGPRs. In this case fewer compounds were detected, negatively affecting the host location by herbivorous insects (Disi et al. 2018). Altered VOC production by PGPR-treated plants is surely plant species-specific, and perhaps differences are at individual level, but the benefits for plants are unambiguous.

For example, when grapevine plants infected with the pathogen *B. cinerea* were treated with three PGPRs (*Microbacterium imperiale, Kocuria erythromyxa* and *Terribacillus saccharophilus*) previously isolated from grapevine roots and adjacent soil, a reduced size of pathogenic lesions was shown. It was suggested that increasing plant VOCs (monoterpenes, sesquiterpenes) and induced synthesis of other defensive metabolites (tocopherols and membrane sterols) make stronger the antioxidant capacity of leaf tissues helping PGPR-treated grapevine plants cope with the stress induced by the pathogen (Salomon et al. 2016). The increase of plant VOCs (monoterpenes, sesquiterpenes) was also confirmed in Salomon et al. (2014, 2017) in grapevine plants treated with strains of *Pseudomonas fluorescens* and *Bacillus licheniformis* as PGPRs. Even if the influence of some plant growth-promoting fungi (PGPFs) on plant–insect response has received little attention, novel strategies of PGPF-induced, plant VOC based integrated control of aphids have been developed. Battaglia et al. (2013) showed that root colonization of tomato plants by *T. longibrachiatum* strain MK1 increased the release of some plant VOCs (methyl salicylate, (Z)-3-hexen-1-ol, and β-caryophyllene), thus explaining a significantly higher attractiveness (in terms of in-flight orientation) of these plants to *Aphidius ervi*, the parasitoid of the aphid *Macrosiphum euphorbiae*, compared to uncolonized plants. Similarly, colonization of tomato plants by the endophytic fungus *T. asperellum* mediated induced plant resistance against the lepidopteran pest *Tuta absoluta* by activating in plants both SA and JA defense pathways and the production of methyl salicylate and (Z)-jasmone (Agbessenou et al. 2022). Also in the case of entomopathogenic endophytic fungi (mainly studied for controlling phytophagous insects), plant colonization by the fungus may change the VOC profile of the plants. This is the case of grapevine plants treated with *Beauveria bassiana* where anti-insect plant VOCs were elicited (Moloinyane and Nchu 2019). However, treatment with *Beauveria* did not inhibit the scale insect *Planococcus ficus* infestation, and further investigations are needed to understand the fungus-plant-insect relationship and to improve phytophagous insect management. As already shown, BM-induced changes of plant VOCs often also influence higher trophic levels, but the effect may not always be

beneficial for plants. For example, Babikova et al. (2014) reported that in broad bean mycorrhized plants emission of sesquiterpenes (E)-β caryophyllene and (E)-β-farnesene was suppressed compared to non-mycorrhized ones, increasing the attractiveness on the aphid *Acyrthosiphon pisum*.

2.5 Technical problems when assessing the effect of mVOCs on plants.

Most of the studies about mVOC release by soil microorganisms were performed using artificial environments, like petri dishes, where plants and bacteria were separated by a central septum allowing mVOCs to selectively diffuse throughout the plate. With this set up, the concentration of mVOCs is generally higher than in the natural environment (Liu and Zhang 2015). Moreover, in such experimental set-ups, mVOCs are sniffed directly by above ground plant organs (e.g. pieces of leaves), whereas, in a natural environment, mVOCs are generally 'sensed' by roots, activating subsequent signal at whole plant level. In order to better understand the mechanisms by which mVOCs are able to influence plants, more experiments with improved simulation of the real environment should be performed. Another problem is that BMs (alike plants) do not emit just one specific VOC but a blend of VOCs, and only few studies (Splivallo et al. 2007; Sun and Tang 2013) have tested whether the entire blend or specific VOCs of the blend induce the response in plants. As seen for the plant growth promotion effect, the concentration of mVOCs is another important factor to consider, and this often depends on BM density. Blom et al. (2011), showed that the growth medium and the population density of PGPRs both affect mVOC emissions in terms of quality and quantity. These and other urgent open questions have not been specifically addressed so far, and this is a very promising field of research to further exploit relationships of plants with soil BMs for improving plant growth and defense.

2.6 Conclusions, open questions, and future perspectives.

Plants are central to complex networks of above-ground and below-ground multitrophic interactions. On the other hand, some microorganisms play a vital role as biofertilizer, biocontrol and bioremediation agents in sustainable agriculture. Thus, exploiting beneficial microbe-plant interaction could lead to sustainable strategies of crop production. However, many open questions remain to be tested. BMs emit mVOCs that are sensed by plants, and can promote plant growth and/or improve resistance to induced stresses. As shown above, a few mVOCs have been clearly identified as active compounds in the emitted mVOC blends. However, not all microbial species (or even strains) emit those mVOCs. Are there interspecific/intraspecific differences that explain differences of plant responses? Which species/strains are more effective in actively eliciting plant responses? Plant VOCs can also be singularly responsible for eliciting physiological responses to stresses, or for driving

attraction or repulsion of friend and foe organisms (Loreto and Schnitzler 2010). However, more often visiting organisms sense plant VOC blends more than single VOCs (Holopainen and Gershenzon 2010). Alike plant VOCs, are the other mVOCs of the blends also active, and what is their overall role, as single compounds or as part of a complete blend? If single mVOCs or a more complex mVOC blend induce plant responses, artificially treating the plants with the same VOCs or VOC blend would have the same positive effects (see, e.g. Vinale et al. 2008)? It has also been noted that treating plants with BMs can cause a change of plant VOCs, which in turn can mediate an interaction of the plants with other trophic levels. But, as seen above, mVOCs not always elicit a change of plant VOCs that can be considered beneficial for the plants. When mVOC-induced changes of plant VOCs are beneficial to plants? Is the BM-induced change of plant VOCs more pronounced in the case of microorganisms that systemically move into the plants reaching aerial parts and often have an entomopathogenic action? And, if so, how changing plant VOC blend affect trophic relationships of plants with other airborne organisms, either useful or noxious, and communication with neighbor plants? Understanding the routes of VOC synthesis by microorganisms, plants and other organisms of the ecosystems could be a turning point in deciphering the multi-trophic interactions of plants, filling gaps or opening new questions. We still have a limited view of the functional and ecological aspects of complex multitrophic interactions affecting plant health and food production. The molecular mechanisms underlying genes function and signal transduction during beneficial interactions are not sufficiently known, and there is a critical gap in the knowledge of interactions with plant microbiomes by other partners in the same environment. Indeed, plant productivity and stress resistance in agro-ecosystems depend on diverse microbial communities and on the interplays among the different microorganisms in these communities. From an application point of view, it is necessary to start thinking about how to exploit new knowledge on mVOCs to improve action of beneficial microorganisms, and for the purposes of Integrated Pest Management (IPM), in the perspective of a biological and sustainable agriculture in a changing environment. By deciphering communication between plants and microorganisms via VOCs not only knowledge eabout agro-ecosystem functioning will be enhanced, but also new sustainable defense practices against biotic and abiotic stresses will be developed.

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Chapter 3

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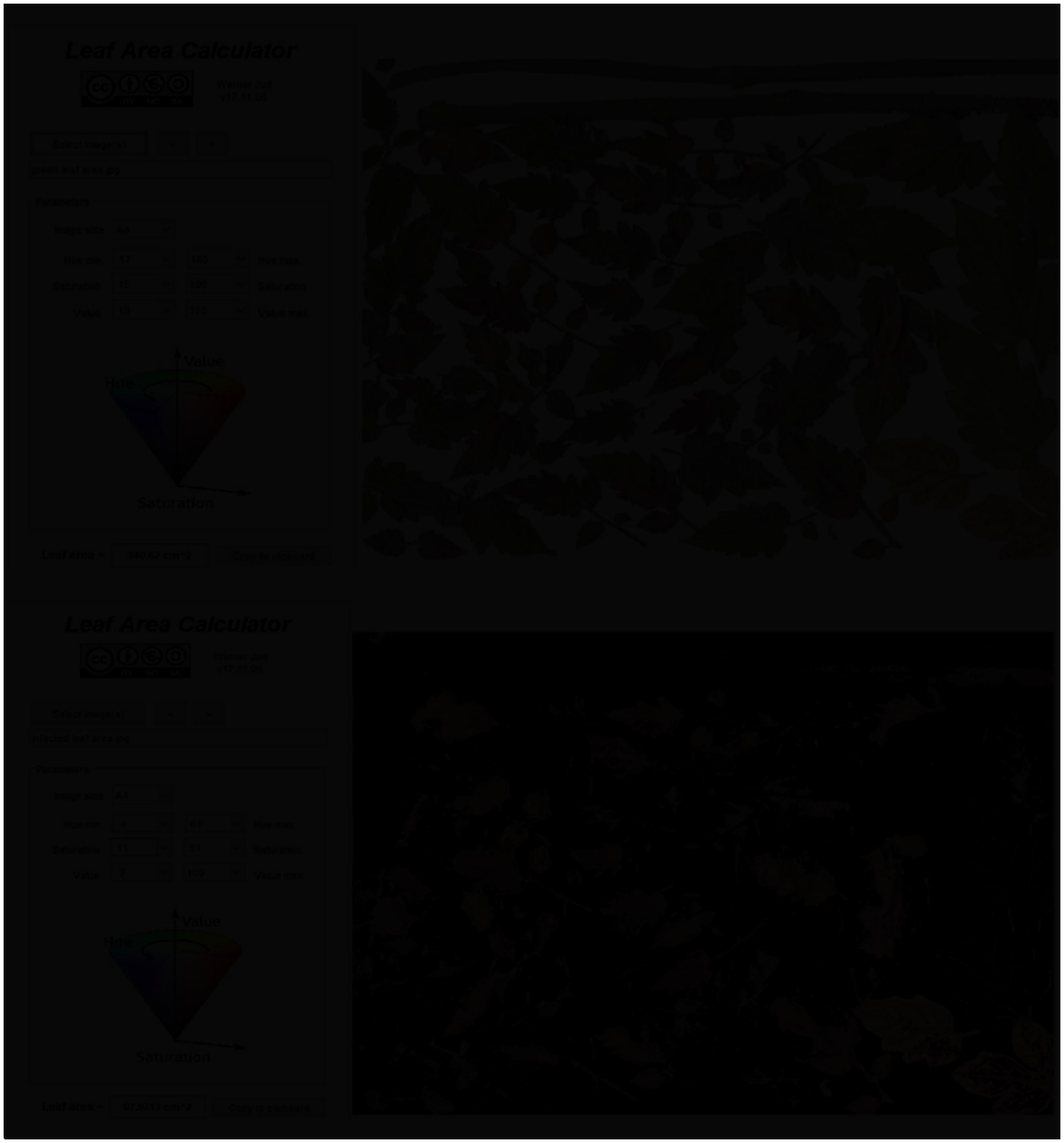
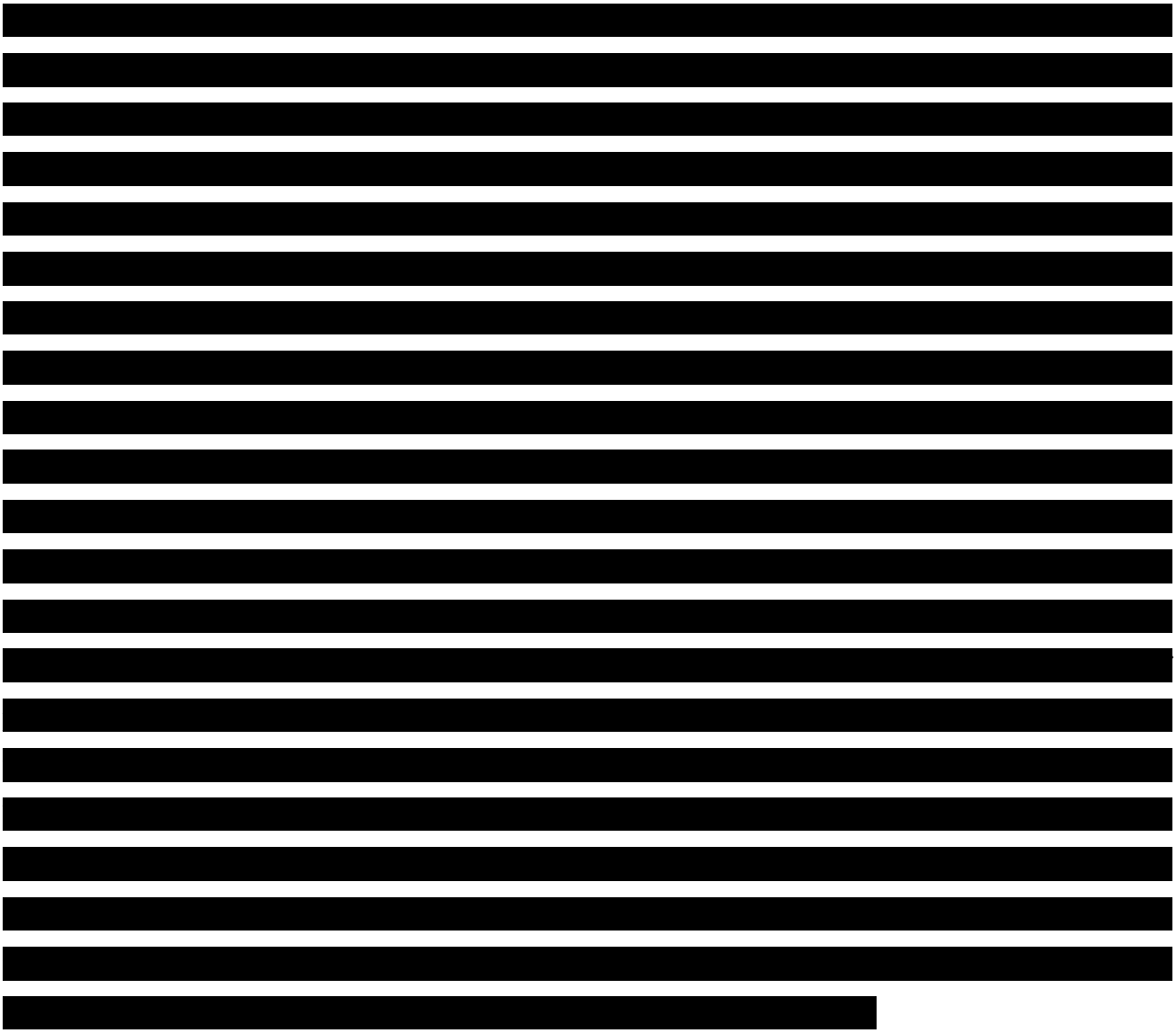

Leaf Area Calculator
Value
Saturation
Leaf Area Calculator
Value
Saturation




a
b

[redacted]

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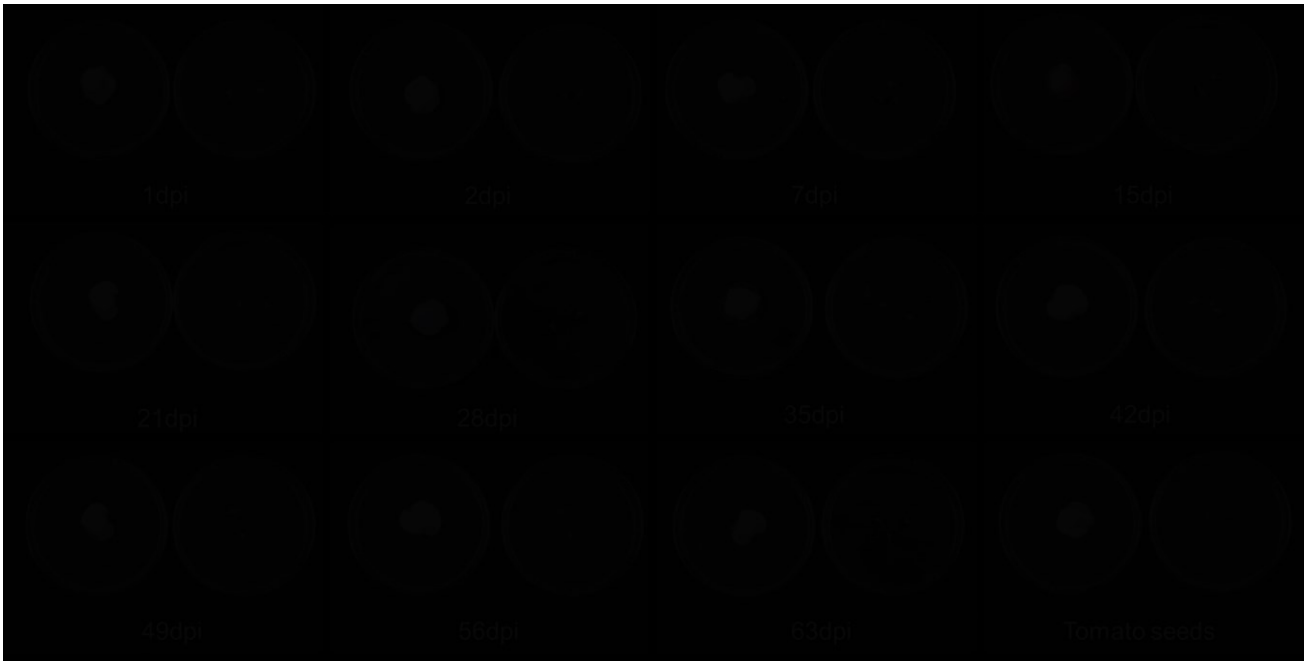
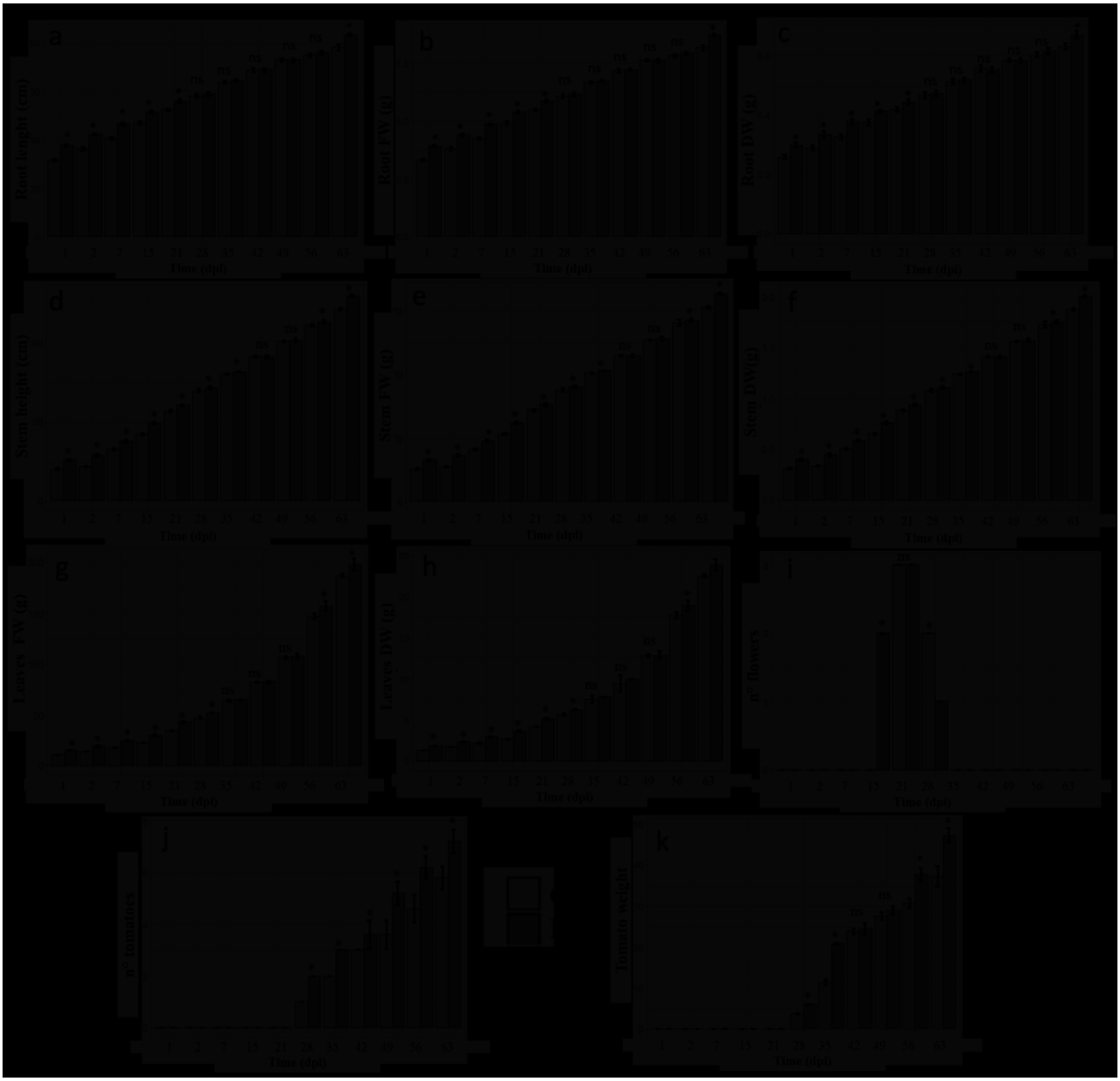
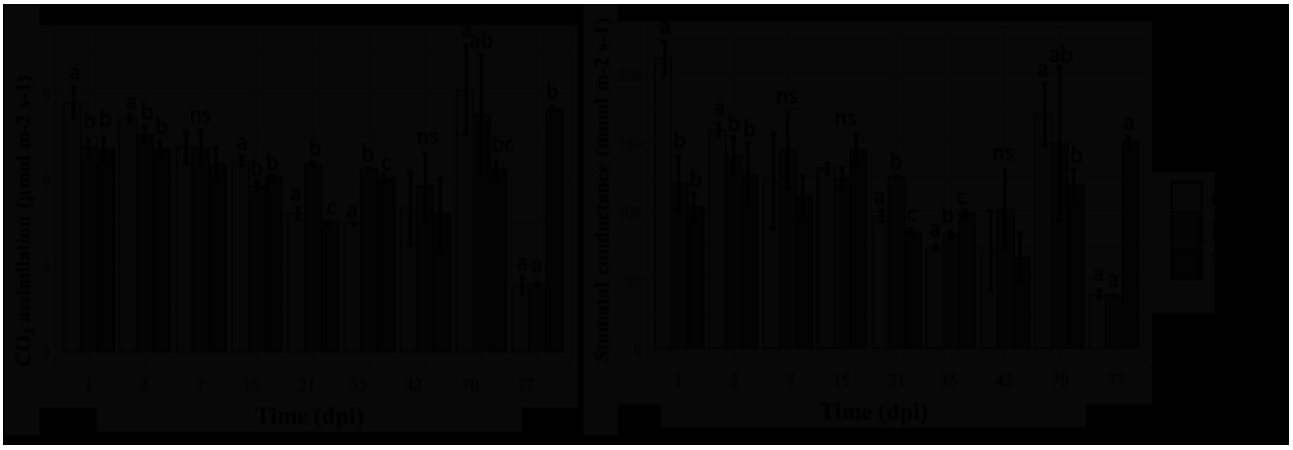
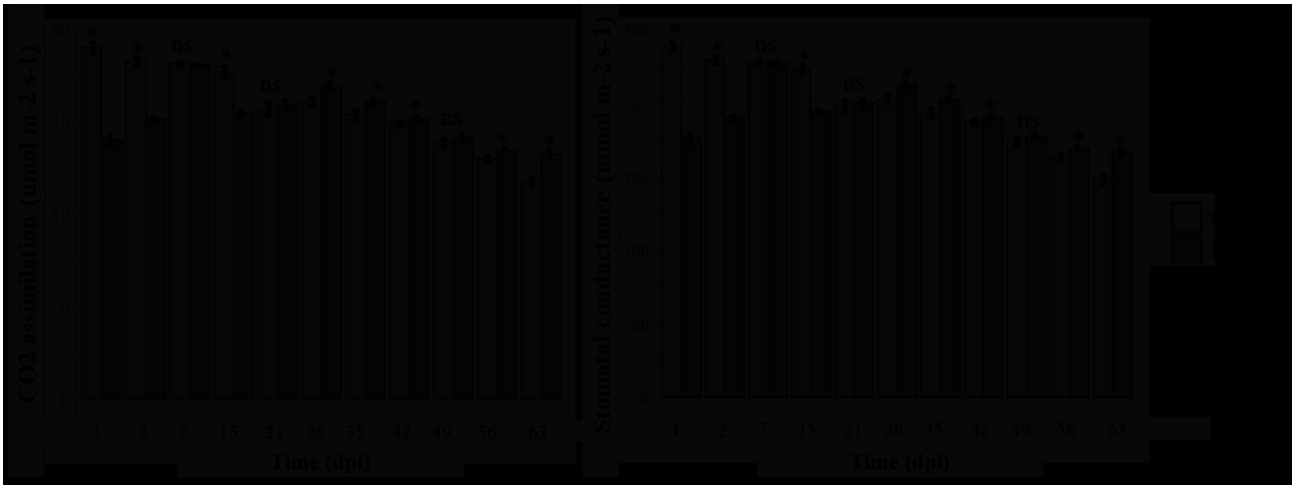

1dpi
2dpi
7dpi
15dpi
21dpi
28dpi
35dpi
42dpi
49dpi
56dpi
63dpi
Tomato seeds


a
b
c
d
e
f
g
h
i
j
k
Time (dpt)


Maximum quantum yield of photosystem II
0.8
0.6
0.4
0.2
0.0
1
2
7
15
21
35
42
70
77
Time (dpi)

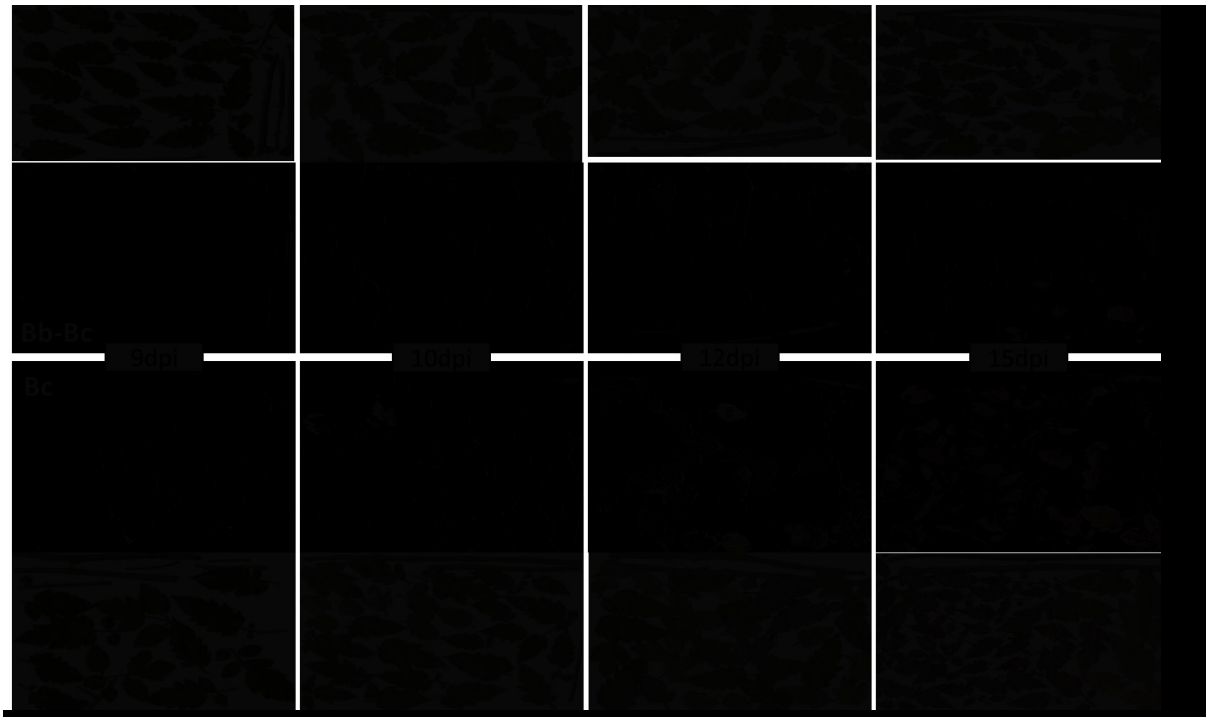

Time (dpi)
Time (dpi)


Time (dpi)
Time (dpi)


CO2 assimilation (umol m-2 s-1)
Stomatal conductance (mmol m-2 s-1)
Time (dpi)
1 2 7 15 21 28 35 42 49 56 63
ns
*


CO2 assimilation (umol m-2 s-1)
Stomatal conductance (mmol m-2 s-1)
Time (dpi)
C
B

[redacted]



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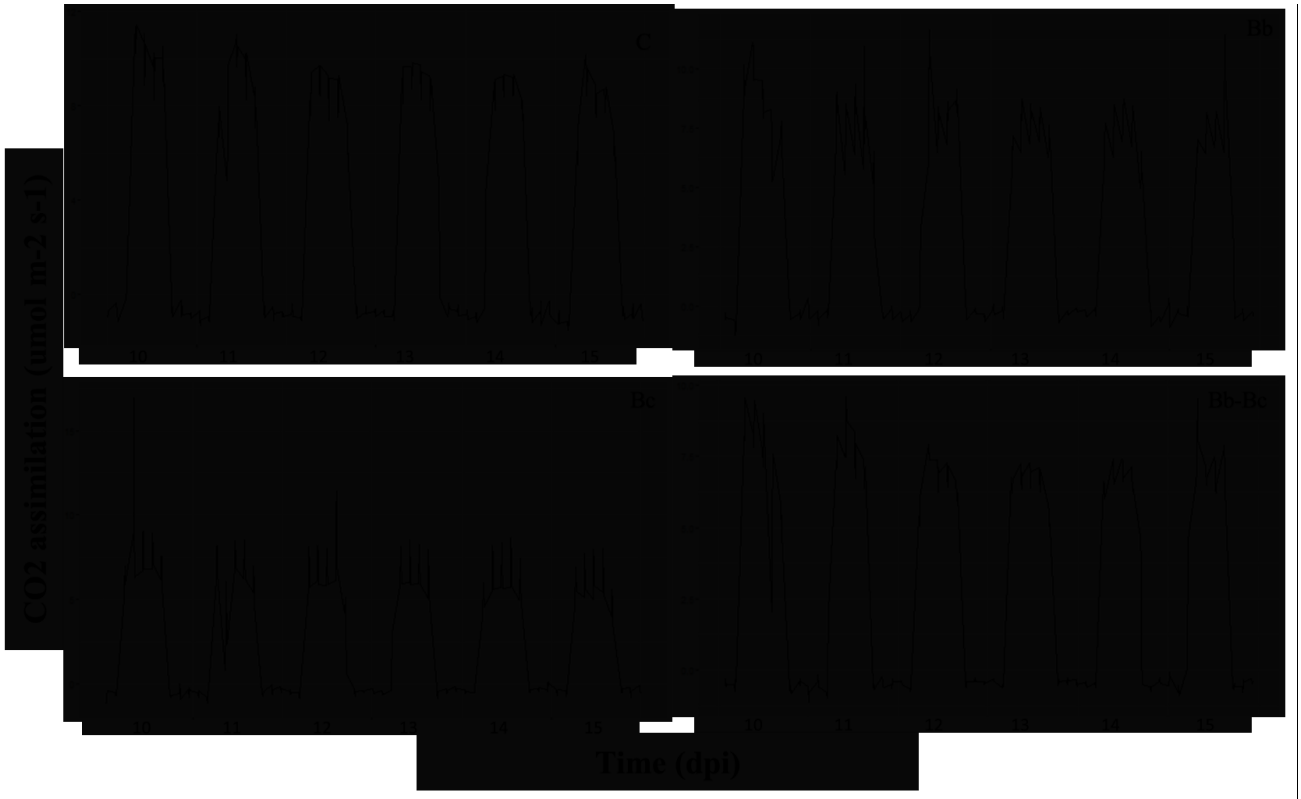
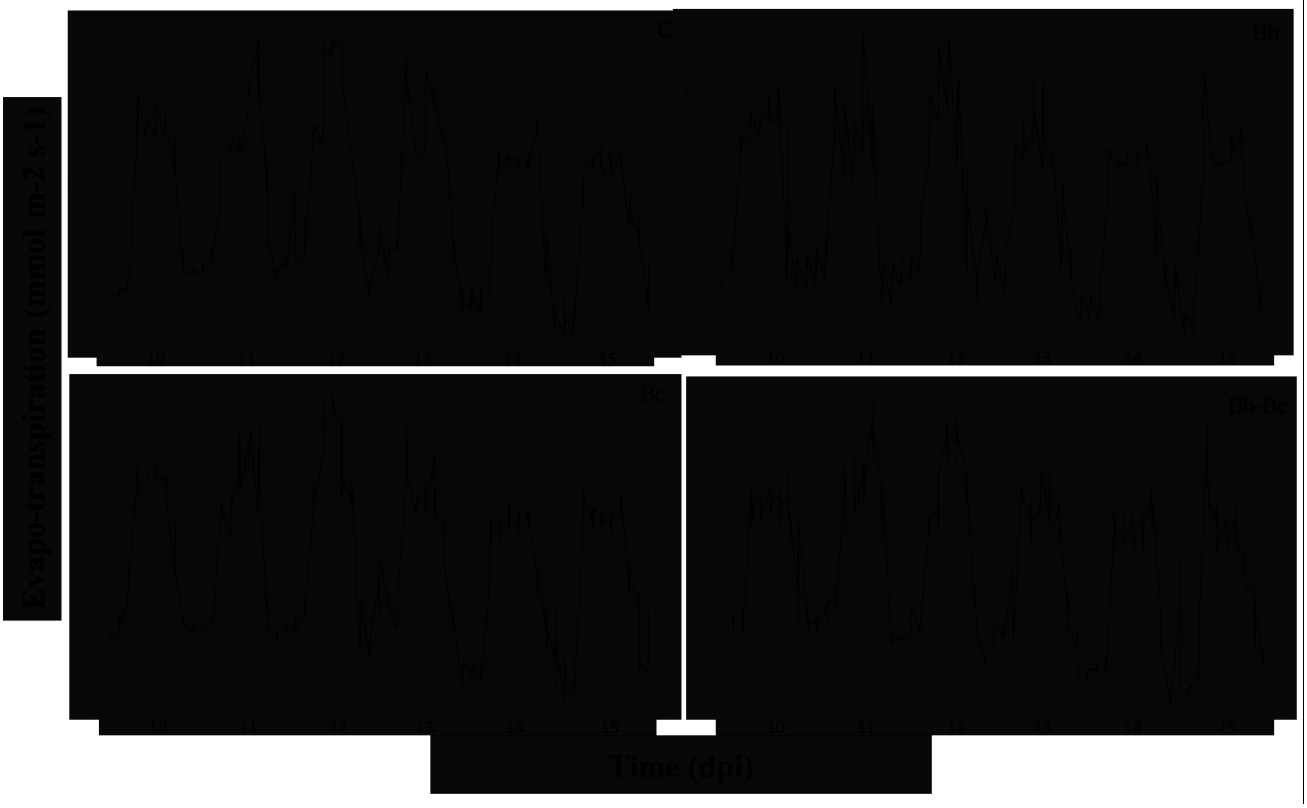
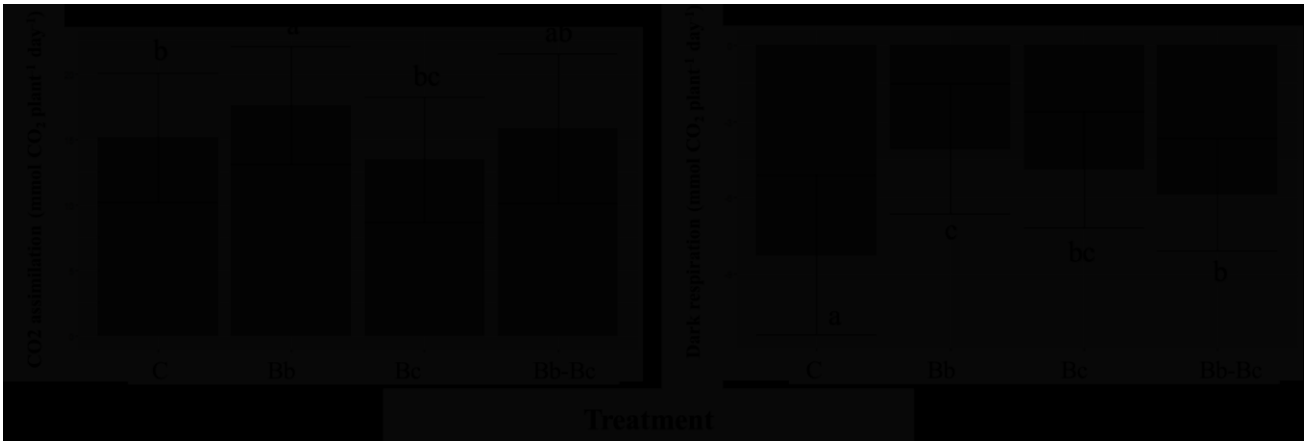
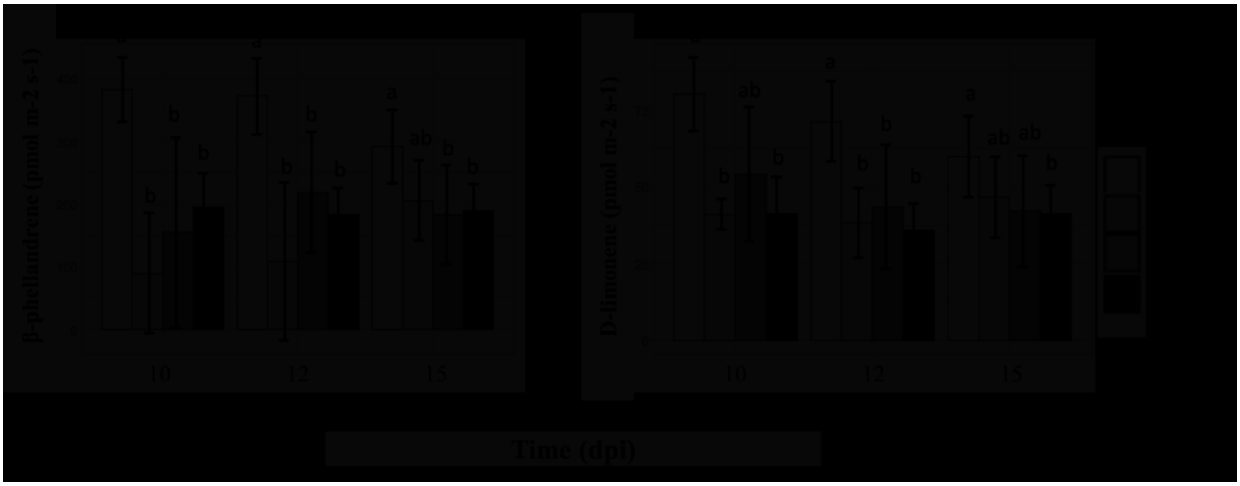

C
Bb
Bc
Bb-Bc
CO2 assimilation (mmol m-2 s-1)
Time (dpi)


C
Bb
Bc
Bb-Bc
Evapo-transpiration (mmol m-2 s-1)
Time (dpi)


b
a
ab
bc
c
bc
b
a
C
Bb
Bc
Bb-Bc
C
Bb
Bc
Bb-Bc
Treatment

Timepoint	Total pmol m^{-2} s^{-1} (a)	Total pmol m^{-2} s^{-1} partioned for treatment (b)		% [2]	β-Phellandrene pmol m^{-2} s^{-1} (c)	% [3]	D-limonene pmol m^{-2} s^{-1} (d)	% [4]
10dpi	**1175.67**							
		C:	511.22	43.48%	382.11	74.74%	80.13	15.67%
		Bb:	147.72	12.56%	89.79	60.78%	41.06	27.80%
		Bc:	232.89	19.81%	154.82	66.48%	54.07	23.22%
		Bb-Bc:	283.84	24.14%	195.28	68.80%	41.26	14.54%
12dpi	**1191.25**							
		C:	479.93	40.29%	371.57	77.42%	71.28	14.85%
		Bb:	186.98	15.70%	108.69	58.13%	41.33	22.10%
		Bc:	284.55	23.89%	218.51	76.79%	43.48	15.28%
		Bb-Bc:	239.78	20.13%	183.59	76.57%	35.80	14.93%
15dpi	**1159.22**							
		C:	378.71	32.67%	291.11	76.87%	59.90	15.82%
		Bb:	265.77	22.93%	205.85	77.45%	46.62	17.54%
		Bc:	243.55	21.01%	183.29	75.26%	42.14	17.30%
		Bb-Bc:	271.18	23.39%	188.53	69.52%	41.14	15.17%

[redacted]


β-phellandrene (nmol m-2 s-1)
D-limonene (nmol m-2 s-1)
10
12
15
Time (dpi)

[redacted]

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[redacted]

[redacted]

[redacted]

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Chapter 4

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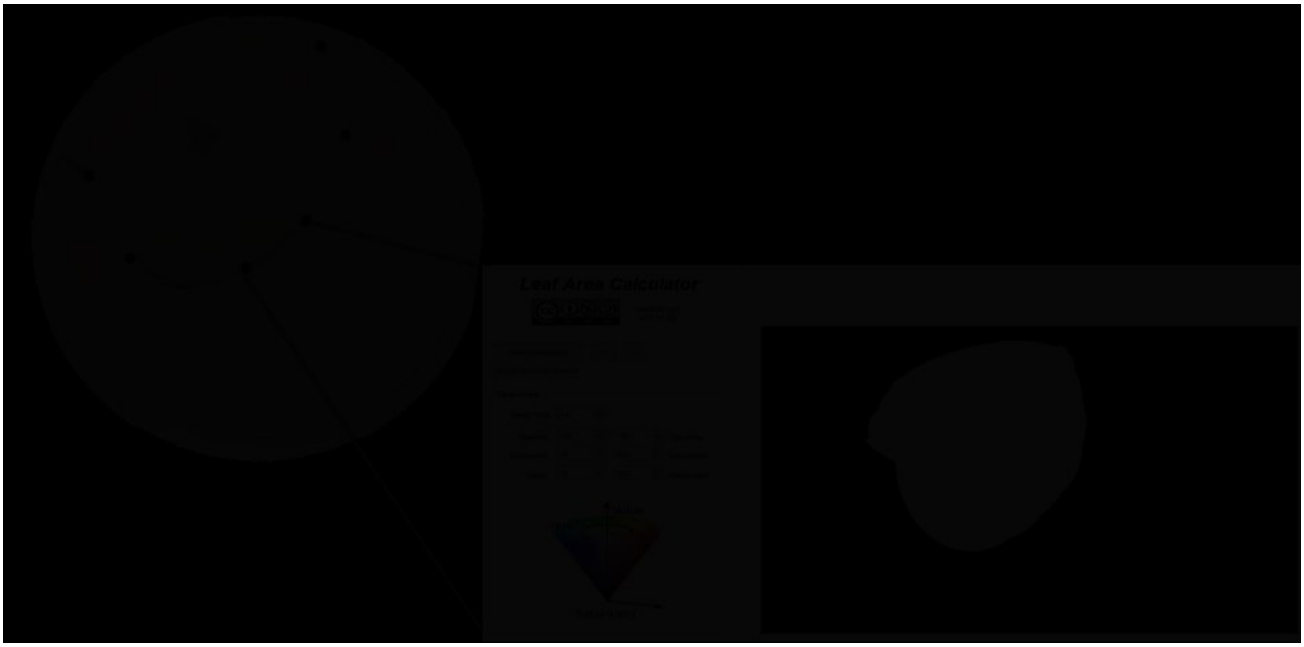
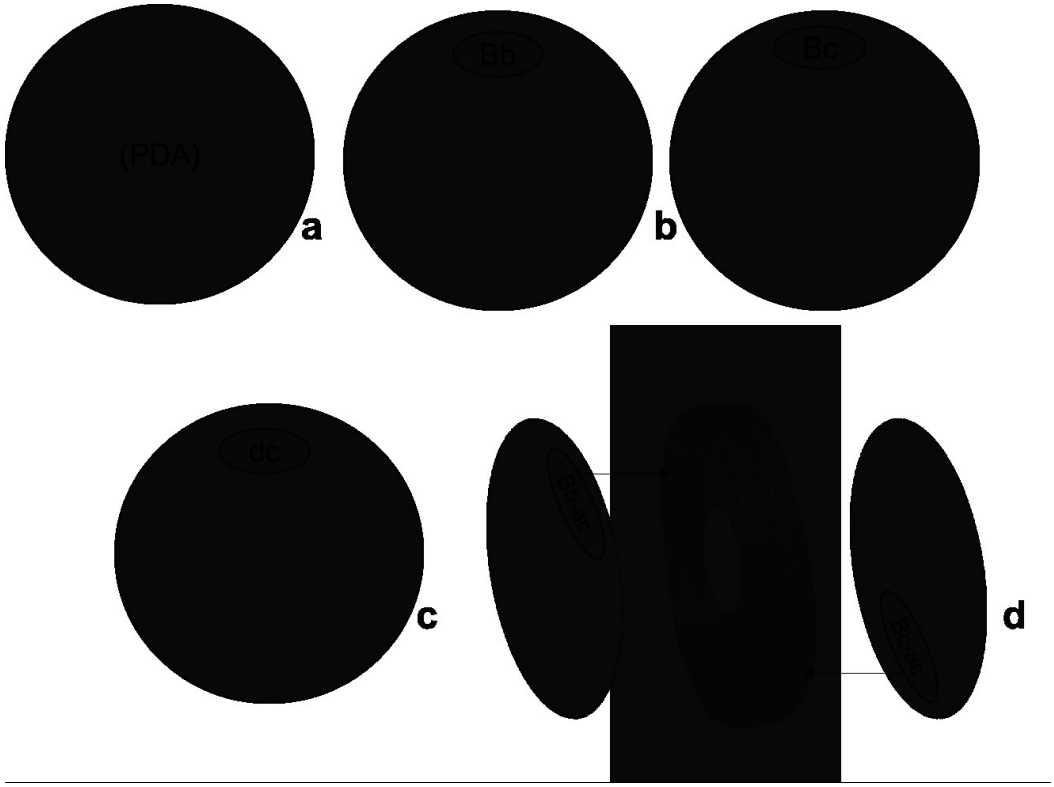
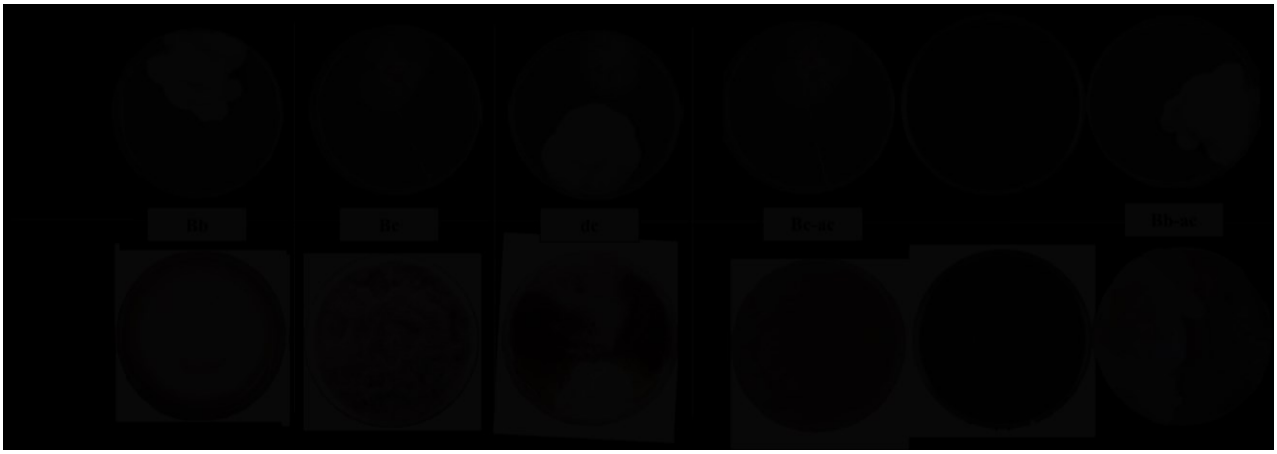
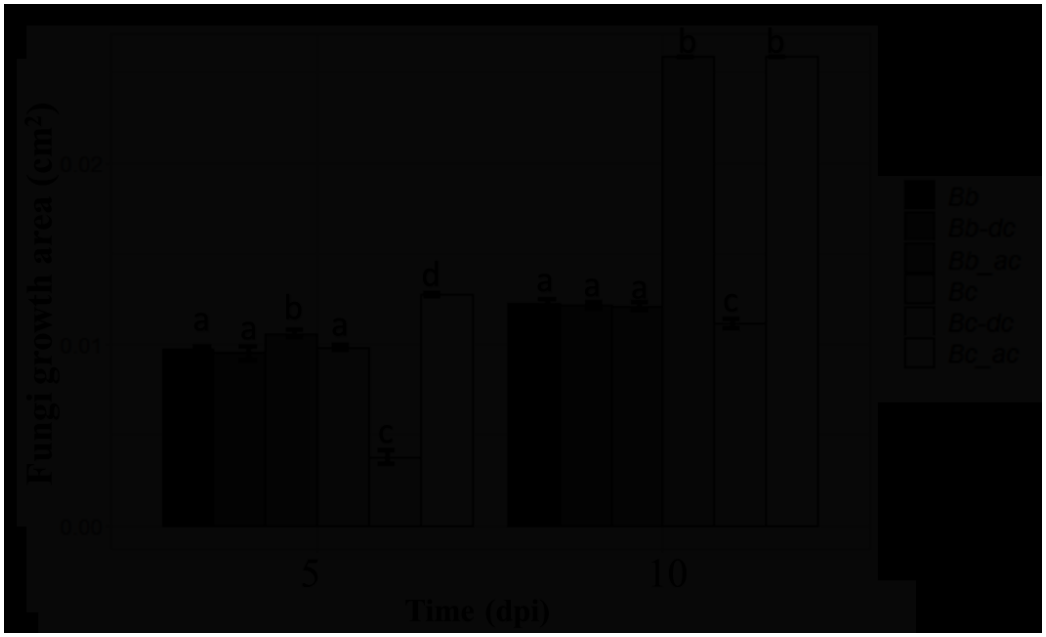
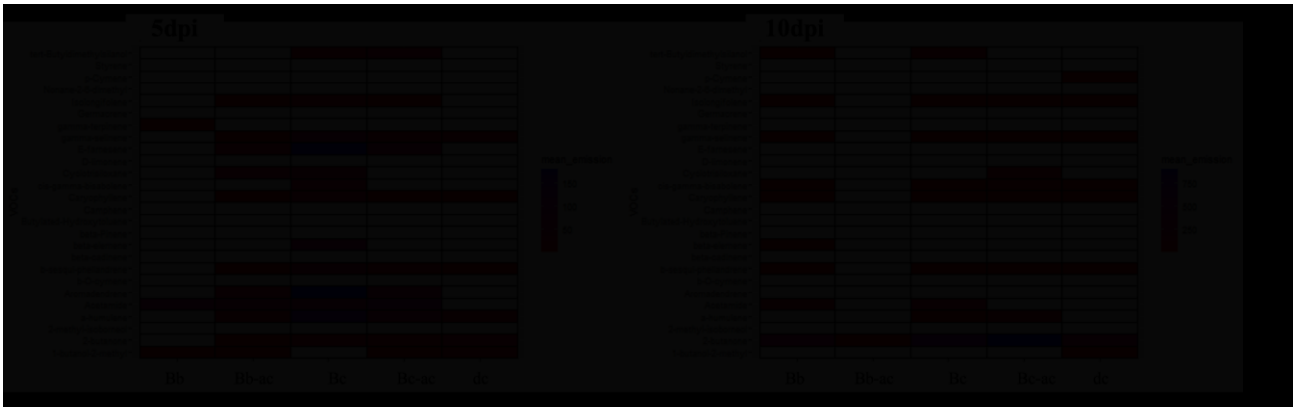

Leaf Area Calculator


(PDA)
a
Bb
b
Bc
dc
c
d

[redacted]




Fungi growth area (cm²)
0.02
0.01
0.00
a
a
b
a
c
d
a
a
a
c
b
b
5
10
Time (dpi)
Bb
Bb-dc
Bb_ac
Bc
Bc-dc
Bc_ac



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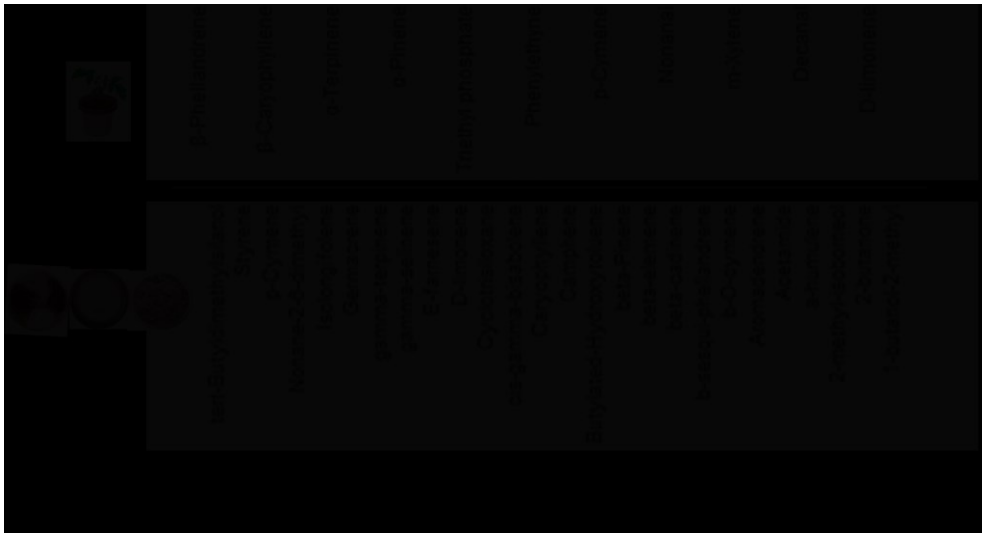

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Chapter 5

This chapter consists of one peer reviewed published article on a topic that represents an important line of research at the Institute for Sustainable Plant Protection of the National Research Council of Italy (IPSP-CNR), were I have developed my research activities.

Plant volatilomes (the blend of volatile organic compounds emitted by plants) have received over the last decade increasing attention in ecological, environmental and agricultural studies due to their potential to be used in the biocontrol of plant pathogens and pests and as plant growth-promoting factors.

Trichoderma spp. are largely used as biocontrol agents (BCAs) and plant growth promoters and was used as a benchmark in our study to compare effects of *Beauveria bassiana* in plants (see Ch. 3). However, a complete knowledge of the volatile profiles of the different *Trichoderma* species is so far lacking. Volatilomes of four *Trichoderma* species were characterized in this study, which may help with fungal detection and identification in vivo, as well as for real-time studies on multitrophic interactions between beneficial microorganisms and host plants.

5. Volatile Organic Compound (VOC) Profiles of Different *Trichoderma* Species and Their Potential Application

Journal of *Fungi*

MDPI

Article

Volatile Organic Compound (VOC) Profiles of Different *Trichoderma* Species and Their Potential Application

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Abstract: Fungi emit a broad spectrum of volatile organic compounds (VOCs), sometimes producing species-specific volatile profiles. Volatilomes have received over the last decade increasing attention in ecological, environmental and agricultural studies due to their potential to be used in the biocontrol of plant pathogens and pests and as plant growth-promoting factors. In the present study, we characterised and compared the volatilomes from four different *Trichoderma* species: *T. asperellum* B6; *T. atroviride* P1; *T. afroharzianum* T22; and *T. longibrachiatum* MK1. VOCs were collected from each strain grown both on PDA and in soil and analysed using proton transfer reaction quadrupole interface time-of-flight mass spectrometry (PTR-Qi-TOF-MS). Analysis of the detected volatiles highlighted a clear separation of the volatilomes of all the four species grown on PDA whereas the volatilomes of the soil-grown fungi could be only partially separated. Moreover, a limited number of species-specific peaks were found and putatively identified. In particular, each of the four *Trichoderma* species over-emitted somevolatiles involved in resistance induction, promotion of plant seed germination and seedling development and antimicrobial activity, as 2-pentyl-furan, 6PP, acetophenone and *p*-cymene by *T. asperellum* B6, *T. atroviride* P1, *T. afroharzianum* T22 and *T. longibrachiatum* MK1, respectively. Their potential role in interspecific interactions from the perspective of biological control is briefly discussed.

Keywords: volatilome; *Trichoderma*; soil–microbe interactions; volatile organic compounds (VOCs); PTR-Qi-TOF-MS

https://doi.org/10.3390/jof8100989

5.1 Introduction

Volatile organic compounds (VOCs) represent a small, but vital portion of the total metabolites produced by living beings derived from both primary and secondary metabolism and characterised by a low molecular weight, low boiling point and high vapor pressure. Their unique properties enable them to mediate important ecological multi-organism interactions both below and above ground, inducing a wide spectrum of responses [1]. VOCs in general, and those emitted by fungi in particular, consist of molecules from different classes. Fungal VOCs belong to several chemical groups with different biochemical origins such as monoterpenes, sesquiterpenes, alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons and ketones as well as nitrogen- and sulphur-containing compounds [2,3]. These compounds may play important roles in inter-and intra-individual communication involving plants, antagonists and mutualistic symbionts [4]. In plants, fungal VOCs are involved in the biocontrol of phytopathogens and pests [5–7], acting as attractants or deterrents for insects and other invertebrates or activating plant defence responses against a pathogen attack as well as providing growth promotion [8–11]. To date, a complete picture of the role of fungal VOCs is still lacking; therefore, the characterisation of species-specific volatile profiles would be helpful to unravel their ecological functions [12–15]. Fungal species belonging to *Trichoderma* spp. are common soil-borne fungi and important opportunistic avirulent plant symbionts and parasites of other fungi as well as beneficial microorganisms in the agro-ecosystem; they are able to influence the soil health and crop performance [16]. The genus *Trichoderma* includes 254 identified species [17] ubiquitously present in forest and agricultural soils, where they are highly interactive with plant roots and rhizospheric microorganisms [18]. Thanks to this wide range of effects, *Trichoderma* spp. are largely used as biocontrol agents (BCAs) and plant growth promoters. In particular, the VOCs emitted by *Trichoderma* spp. have a strong effect against plant pathogenic fungi such as *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Ganoderma sp*., *Penicillium oxalicum*, *Stagonosporopsis cucurbitacearum*, *Alternaria panax*, *Botrytis cinerea*, *Cylindrocarpon destructans* and *Sclerotinia nivalis* [19–21].

The volatile profile emitted by *Trichoderma* species can considerably change [12–14], not only depending on the species, but also as a consequence of the interaction with other organisms [15,22], suggesting important ecological functions of VOCs [8,23,24]. Among the VOCs reported to be emitted by *Trichoderma* [14], hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols and thioesters and their derivatives [25] are the most represented. C8 compounds such as 1-octen-3-ol and 3-octanone (both of them responsible for the mushroom flavour) that are end-products of fatty acid metabolism, have been shown to play a role in the biocontrol activity of *Trichoderma* spp.

[8,23,24] and are already used in biological control practices as fungistatic and fungicidal molecules [26,27]. 6-pentyl-alpha-pyrone (6PP) (a lactone with a coconut-like aroma), which is produced by different *Trichoderma* species such as *T. atroviride* [28,29], *T. asperellum* [30], *T. viride* [31], *T. harzianum* [32], *T. koningii* [33], *T. citrinoviride* and *T. hamatum* [34], has been reported to be able to increase root branching and root hair development [30,35], and to have an effect on plant growth and health at different concentrations [13].

GC-MS is one of the most utilised techniques for VOC detection, even though it has limitations such as its cost-effective separation, identification and quantification of substances combined with its destructive protocols and gas concentration steps [8,12,36]. Despite these limitations, the GC-MS technique has been utilised for VOC profile analyses such as for the "white truffle" fungus Tuber magnatum [37] and in studies on VOC-mediated interspecific interactions in the soil, in particular from different species belonging to the *Fusarium* genus [38] and bacteria [39].

Proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS) has recently been introduced to measure volatile emissions from plants [40,41], soils [42,43], yeasts [44] and bacteria and fungi [14,37,45–49], allowing researchers to overcome a few of the GC-MS limitations. This modern technique for the real-time monitoring of VOCs is highly sensitive and can detect low concentrations of VOCs (parts per trillion volume (pptv)) in air and gas samples, providing a rapid, non-invasive fingerprinting of VOC profiles [50].

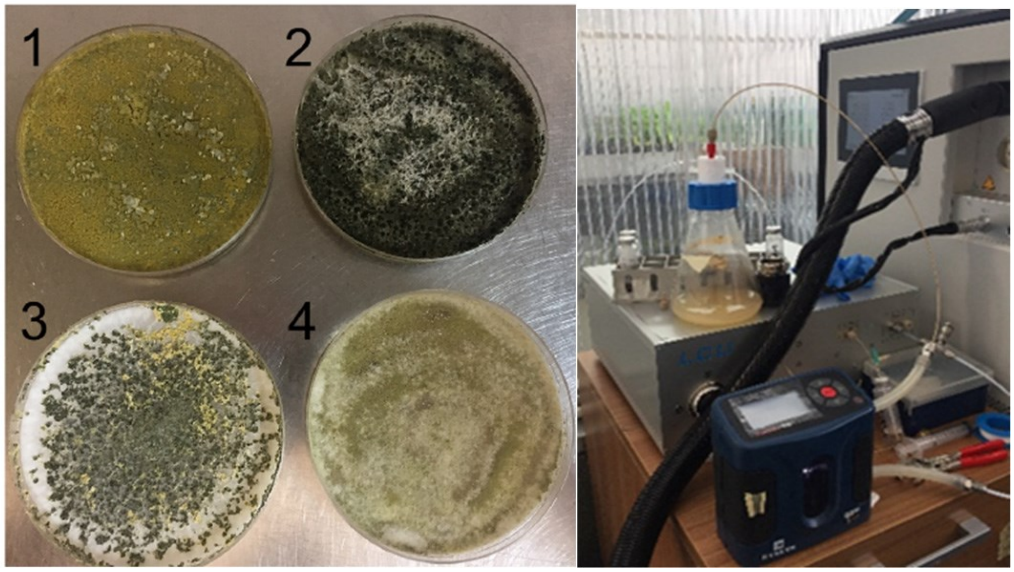
In the present work, we characterised the volatile profiles of four different *Trichoderma* species - *T. longibrachiatum* MK1, *T. atroviride* P1, *T. asperellum* B6 and *T. afroharzianum* T22 - grown both on PDA and in soil by a PTR-Qi-TOF-MS analysis in order to define the VOC molecular markers for each species useful to detect their presence. Microbial volatile detection and characterisation may be used as a diagnostic tool. Moreover, characterising the VOC profile emitted by each different *Trichoderma* species and their role in plant interactions could greatly increase the potential of *Trichoderma* use in agriculture, becoming a tool for the screening and identification of other beneficial microorganisms.

5.2 Materials and Methods

5.2.1 Fungal and Soil Sampling

Four different *Trichoderma* species - *T. asperellum* B6 [51], *T. atroviride* P1 [52], *T. afroharzianum* T22 [53] and *T. longibrachiatum* MK1 [54] - were analysed (Figure 1a). Each *Trichoderma* species was grown on potato dextrose agar (PDA) in 100 mm Petri dishes and maintained at 25°C in the dark with >80% humidity starting from stocks in 20% glycerol stored at -80°C. All isolates were part of the

CNR-IPSP collection. For the VOC measurements, the fungal species were inoculated both on PDA and in soil. The PDA inoculation was performed with 0.5 cm mycelium fungal plugs and transferred into a 1 L Erlenmeyer conical glass flask that contained 100 mL of PDA and equipped with a GL45 3-valve screw cap (Figure 1b). The soil inoculation was carried out with 5 mL of a spore suspension (1 108 spores/mL) of each fungal species in 100 mL of non-sterile commercial soil (Universal potting soil-Floragard Vertriebs-GmbH Oldenburg) contained in a 1 L Erlenmeyer conical glass flask equipped with a GL45 3-valve screw cap. The VOCs from PDA and non-sterile commercial soil contained in the same type of flasks without an inoculum were used as control. All samples were incubated at 25 C in darkness conditions.



(a) **(b)**

Figure 1. *a) Petri dishes containing Trichoderma isolates grown at 25 ± 1 °C in the dark for 14 days: 1: T. longibrachiatum MK1; 2: T. asperellum B6; 3: T. afroharzianum T22; 4: T. atroviride P1; (b) Erlenmeyer conicalglass flask equipped with a GL45 3-valve screw cap containing 100 mL of potato dextrose agar (PDA) medium, connected online with PTR-Qi-TOF-MS.*

5.2.2 VOC Analyses

5.2.2.1 Mass Spectrometer Analysis of the VOCs Produced by Trichoderma spp. Growing on PDA and in Soil

The VOCs emitted by the *Trichoderma* species were measured using PTR-Qi-TOF-MS equipment (Ionicon Analytik GmbH, Innsbruck, Austria) in an air-conditioned room with a constant temperature of 25 + 1 °C. The protonation of VOCs was carried out using H_3O^+ as a proton donor in the transfer reaction and was effective for VOCs with a proton affinity higher than that of H_2O (691.7 kJ mol^{-}1).

The headspace VOC profiles accumulated in the flasks described in Section 5.2.1 were measured by a direct injection of the volatile mixture into the PTR-Qi-TOF-MS drift tube via a heated (80°C) PEEK inlet tube connected to a valve of the GL45 3-valve screw cap. A flow rate of 100 sccm (standard cubic centimetres per minute) in a range of 20–300 m/z for 600 s with an acquisition rate of one spectrum per second was used. The drift tube conditions were 3.8 mbar of pressure, 80°C temperature and 1000 V drift voltage, resulting in a field density ratio of E/N (with E corresponding with the electric field strength and N with the gas number density) of 141 Td (Townsend: 1 Td = 10^{-17} V cm^2). A total of three different biological replicates for each sample were analysed; the measurements were taken eight days post-inoculum, when all four fungal species grown on PDA were at the beginning of exponential hyphal growth, a stage during which most secondary metabolites of fungi are produced [55]. The same timing was applied to the soil samples.

5.2.2.2 PTR-Qi-TOF-MS Data Analyses

The raw data were acquired by TOFDAQ Viewer® software (Tofwerk AG, Thun, Switzerland) and the mass spectra and temporal ion signal profiles were extracted using PTR-MS Viewer software (Ionicon Analytik version 3.3.8) with a custom modified Gaussian function fit for each peak. The data acquisition and peak quantification were expressed as normalised parts per billion by volume (ppbv). To guarantee a high mass accuracy, the calibration of the PTR spectra was performed offline at three calibration points: m/z = 21.022 (H_3O^+); m/z = 203.943 (a fragment of the internal gas standard 1,3-diiodobenzene); and m/z = 330.848 (the internal gas standard 1,3-diiodobenzene). The peaks associated with the PTR-MS ion source — including those ascribed to water chemistry or other interfering ions, e.g., m/z = 31.022 (NO^+), m/z = 32.990 (O_2^+), m/z = 21.022, m/z = 37.028 and m/z = 39.033 (corresponding with H3 18O+ and water cluster ions H_2O-H_3O+ and $H_2OH_3{}^{18}O$+, respectively) — were eliminated. The m/z signals were background-corrected by subtracting the signal obtained from the glass flasks containing only the PDA or the commercial soil. Most of the mass peaks were tentatively identified based on the available literature or by comparisons with genuine standards.

5.2.3 Statistical Analyses

All statistical analyses were carried out by using the Metaboanalyst platform (https: //www.metaboanalyst.ca accessed on 20 July 2022) [56]. The data were normalised and auto-scaled (mean-centred and divided by the standard deviation of each variable) prior to each analysis. Principal component analysis (PCA) was carried out on both the whole dataset and the two datasets separately (PDA and soil) as an unsupervised method to highlight the underlying data structure. One-way analysis of variance (ANOVA) was performed coupled with Tukey's HSD test to discover the significantly

different means in the multiple comparisons. The ANOVA results were presented by heat maps and hierarchical clustering in order to provide a more intuitive visualisation of the VOC patterns. The rows and columns were reordered so that rows (and columns) with similar profiles were closer to one another, with each entry displayed as a colour related to its signal intensity. Moreover, dendrograms were created using Pearson correlation-based distances and Ward's method of agglomeration.

5.3 Results

5.3.1 VOC Analyses

A total of 69 VOCs were detected in the range of the measured masses (mass protonated range m/z = 20–300) after the subtraction of the peaks associated with the PTR-MS ion source and their isotopes (Table S1). The putative identification of the VOCs is reported in Table S2. When comparing the total amount of VOCs emitted by the four species on PDA, it was found that, in our conditions, *T. asperellum* B6 emitted the lowest quantity of VOCs (4.06 x 10^2 ppbv), followed by *T. afroharzianum* T22, *T. atroviride* P1 (both 4.46 x 10^2 ppbv) and *T. longibrachiatum* MK1 (4.61 x 10^2 ppbv). The total VOC emission of *T. asperellum* B6 was significantly lower than in all other species ($p < 0.05$). In the soil samples, the total VOC emission profiles were very similar among all *Trichoderma* species, with 3.76 x 10^2 ppbv emitted from *T. asperellum* B6, 3.80 x 10^2 ppbv from *T. longibrachiatum* MK1, 3.81 x 10^2 ppbv from *T. afroharzianum* T22 and 3.88 x 10^2 ppbv from *T. atroviride* P1; the differences were statistically not significant ($p < 0.05$). A preliminary statistical analysis of the samples from both datasets (PDA and soil) was carried out via principal component analysis (PCA) in order to detect patterns in the measured data without any a priori assumption of a particular distribution of the data. This unsupervised method clearly highlighted the separation of VOCs emitted from *Trichoderma* spp. grown in soil and those grown on PDA (Figure 2), with the three first principal components accounting for 87.0% of the variation in the dataset. It was evident how soil samples formed a more compact group compared with the PDA samples (all data PCA loadings are reported in Table S3). Among the PDA samples, *T. longibrachiatum* MK1 and *T. asperellum* B6 were clearly separated by the first two PCs whereas *T. afroharzianum* T22 and T. *atroviride* P1, even if partially overlapping with PC1 and PC2, could be completely distinguished with PC1 and PC3. All soil samples overlapped and the *Trichoderma* species could not be distinguished based on the total VOC emission.

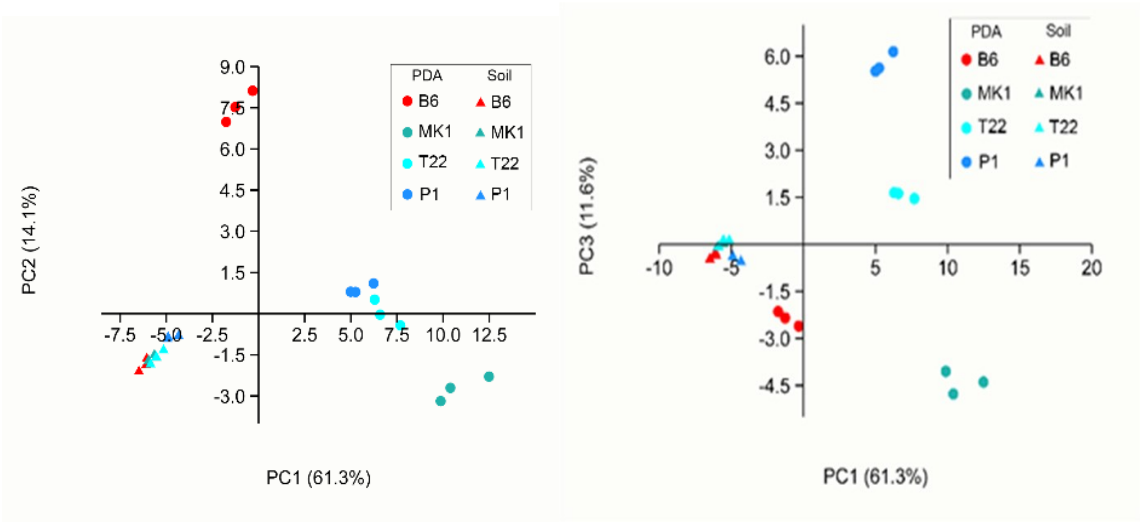
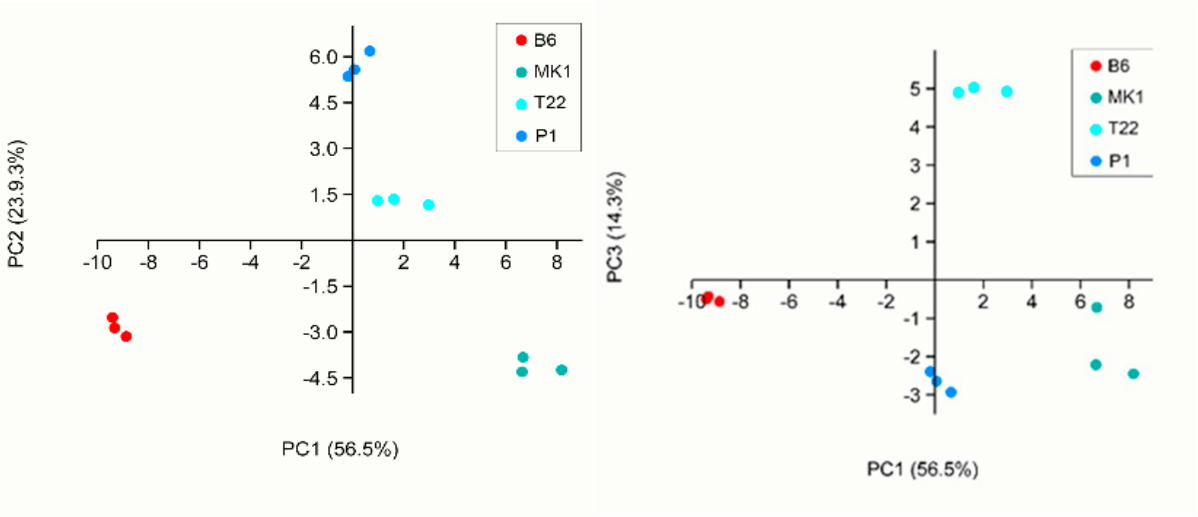


Figure 2. *First three components of the PCA analysis of the PTR-Qi-TOF-MS data of all VOC samples: (a) PC1 and PC2 plot; (b) PC1 and PC3 plot. The variance explained by each component is reported in brackets.*

The VOC emission of the four *Trichoderma* species grown on PDA or in soil were then analysed as a separate dataset in order to have a deeper comprehension of the VOC patterns. The PCA of the *Trichoderma* species grown on PDA clearly highlighted the separation into four different clusters, confirming the existence of a distinct volatile blend profile for each species (Figure 3). The first two principal components explained 80.4% of the total variance; with the third component, the total variance explained was 94.7%. In the score plot of the first two principal components (Figure 3a), *T. asperellum* B6 and *T. longibrachiatum* MK1 were plotted in separate quadrants; *T. afroharzianum* T22 and *T. atroviride* P1 shared the same one. The separation between *T. afroharzianum* T22 and *T. atroviride* P1 occurred with the third component (Figure 3b). The PDA PCA loadings are reported in Table S4.

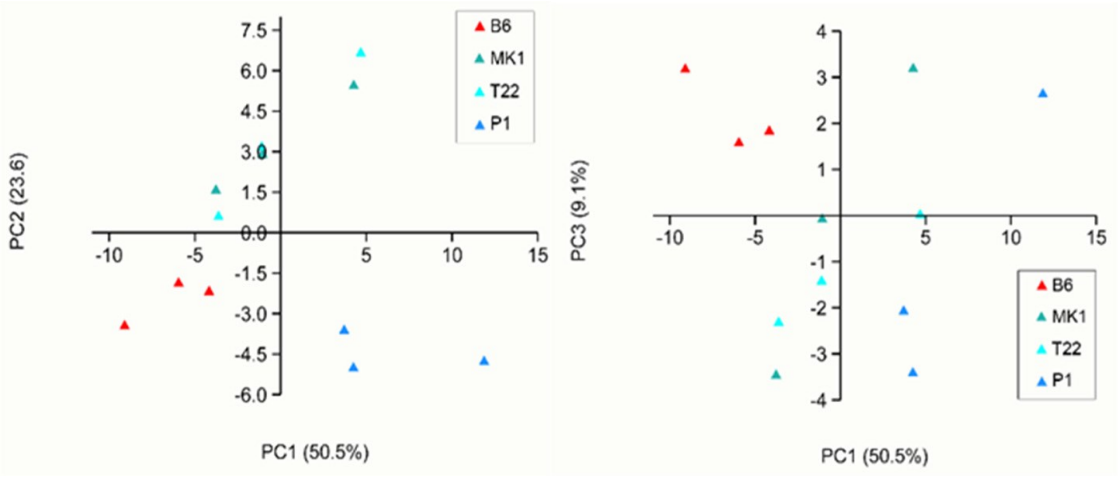


(a) **(b)**

Figure 3. *First three components of the PCA analysis of the PTR-Qi-TOF-MS data of VOCs emitted by samples grown on PDA: (a) PC1 and PC2 plot; (b) PC1 and PC3 plot. The variance explained by each component is reported in brackets.*

From the PCA of the *Trichoderma* species grown in soil, a separation of *T. atroviride* P1 and *T. asperellum* B6 was evident. *T. afroharzianum* T22 and T. *longibrachiatu*m MK1 were superposed both in the PC1–PC2 and PC1–PC3 plot, with the first three principal components explaining 82.6% of the total variance (Figure 4). The soil PCA loadings are reported in Table S5.

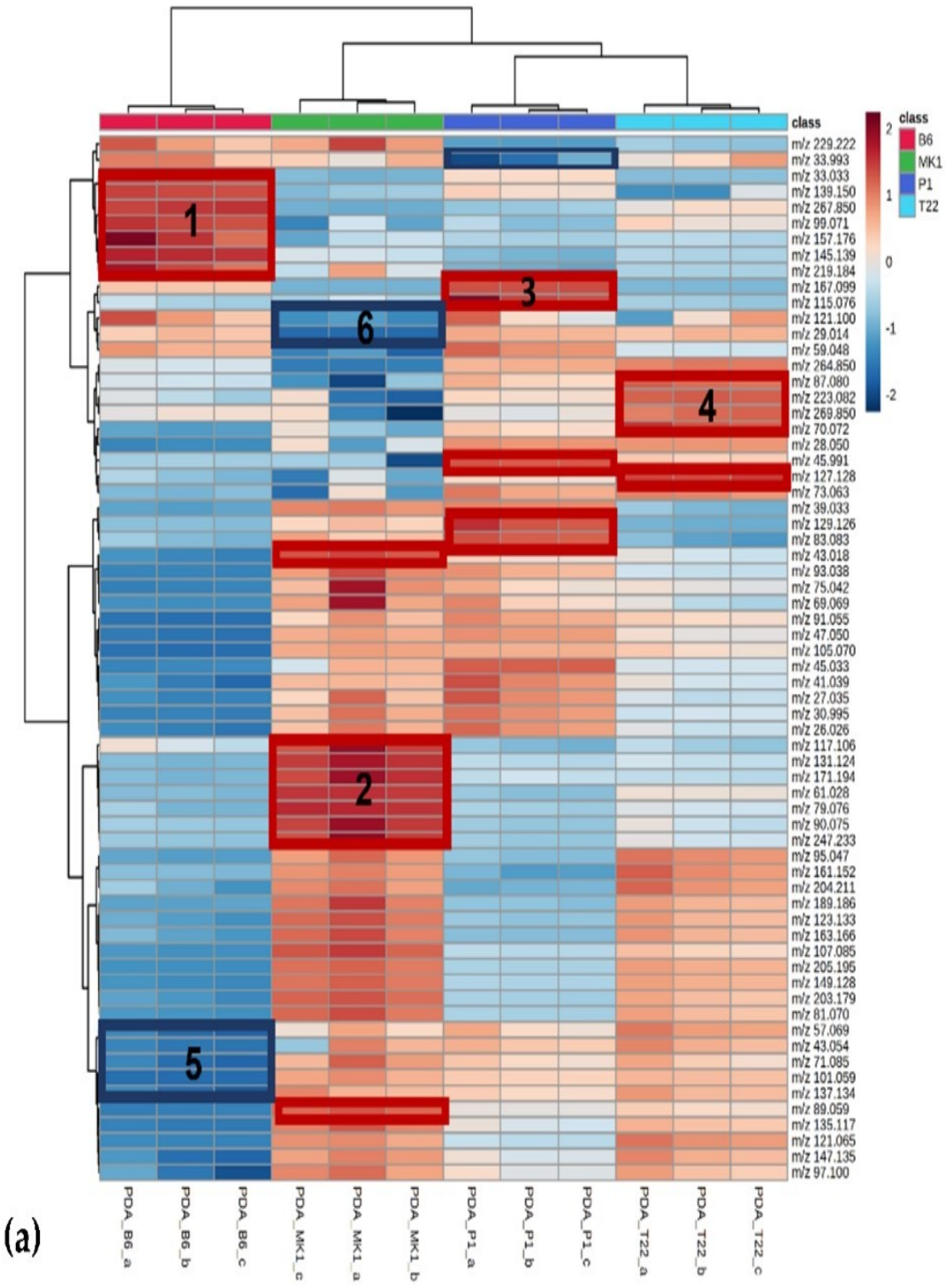
One-way ANOVA and post hoc test identified 66 out of 68 significant peaks ($p < 0.05$) in the PDA data (Table S6) and 25 out of 68 significant peaks ($p < 0.05$) in the soil data (Table S7). The hierarchical heat map clusters of the ANOVA-significant volatiles are presented in Figure 5.



(a) **(b)**

***Figure 4**. First three components of the PCA analysis of the PTR− Qi− TOF− MS data of VOCs emitted by samples grown in soil: (a) PC1 and PC2 plot; (b) PC1 and PC3 plot. The variance explained by each component is reported in brackets.*

The PDA heat map showed well-defined clusters of volatiles characteristically over- or under-emitted among the four different fungal species. In particular, among the over-emitted clusters, *T. asperellum* B6 over-emitted a specific group of volatiles, including m/z: 33.03, 139.150, 267.850, 99.071, 157.176, 145.139 and 219.184 (cluster 1 in Figure 5a). *T. longibrachiatum* MK1 over-emitted a specific group of volatiles, including m/z: 117.105, 131.124, 171.194, 61.028, 79.075, 90.075 and 247.233 (cluster 2 in Figure 5a) as well as m/z 89.05. *T. atroviride* P1 over-emitted m/z 167.099 and 115.076 (cluster 3 in Figure 5a) as well as m/z 45.99 and m/z 59.049. *T. afroharzianum* T22 over-emitted a group of volatiles, including m/z 87.080, 223.082, 269.850 and 70.072 (cluster 4 in Figure 5a) as well as m/z 127.128. Among the under-emitted VOCs, different clusters could be identified in *T. asperellum* B6 with m/z 57.069, 43.054, 71.085, 101.159 and 137.134 (cluster 5 in Figure 5a) and *T. longibrachiatum* MK1 with m/z 121.100 and 29.014 (cluster 6 in Figure 5a); in *T. atroviride* P1, we could only distinguish m/z 33.993 and in *T. afroharzianum* T22, there were no characteristic underemitted VOC clusters.


class
B6
MK1
P1
T22
2
1
0
-1
-2
1
2
3
4
5
6
m/z 229.222
m/z 33.993
m/z 33.033
m/z 139.150
m/z 267.850
m/z 99.071
m/z 157.176
m/z 145.139
m/z 219.184
m/z 167.099
m/z 115.076
m/z 121.100
m/z 29.014
m/z 59.048
m/z 264.850
m/z 87.080
m/z 223.082
m/z 269.850
m/z 70.072
m/z 28.050
m/z 45.991
m/z 127.128
m/z 73.063
m/z 39.033
m/z 129.126
m/z 83.083
m/z 43.018
m/z 93.038
m/z 75.042
m/z 69.069
m/z 91.055
m/z 47.050
m/z 105.070
m/z 45.033
m/z 41.039
m/z 27.035
m/z 30.995
m/z 26.026
m/z 117.106
m/z 131.124
m/z 171.194
m/z 61.028
m/z 79.076
m/z 90.075
m/z 247.233
m/z 95.047
m/z 161.152
m/z 204.211
m/z 189.186
m/z 123.133
m/z 163.166
m/z 107.085
m/z 205.195
m/z 149.128
m/z 203.179
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m/z 57.069
m/z 43.054
m/z 71.085
m/z 101.059
m/z 137.134
m/z 89.059
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m/z 121.065
m/z 147.135
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PDA_B6_c
PDA_MK1_c
PDA_MK1_a
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PDA_P1_a
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PDA_T22_a
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PDA_T22_c

(a)

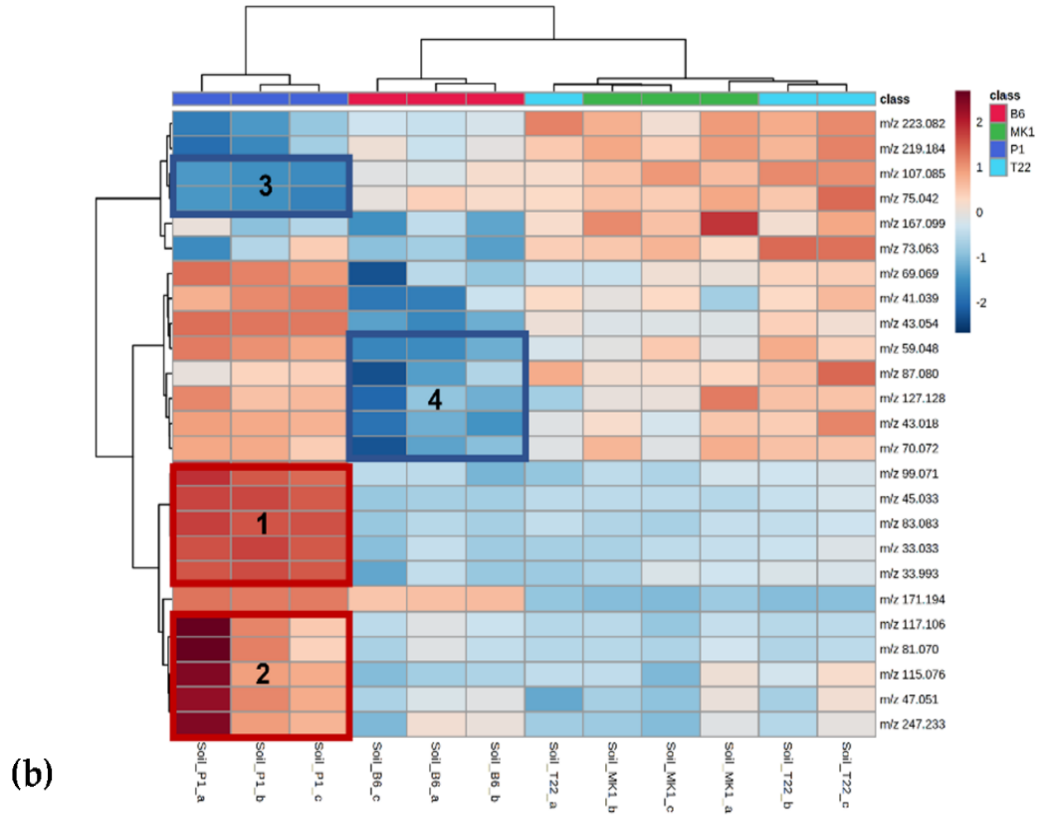

Figure 5. *Heat maps and two-dimensional hierarchical dendrograms of VOCs emitted during fungal growth on PDA (a) and soil (b). Replicates are in columns and variables are in rows. Each coloured cell on the map corresponds with a concentration value following a blue/red chromatic scale from -2 value (very low expression) to 2 (extremely high expression). Numbered boxes represent well defined VOC clusters characteristically over (red)- or under (blue)- emitted. Only ANOVA-significant peaks are used ($p < 0.05$). The Pearson distance and Ward's clustering algorithm were used for the dendrograms. The probable identification and chemical group of the VOCs are detailed in Table S2.*

The VOCs detected from the soil samples treated with the four different *Trichoderma* species allowed a good discrimination between *T. atroviride* P1 and *T. asperellum* B6 whereas *T. afroharzianum* T22 and *T. longibrachiatum* MK1 showed very similar VOC blends. In particular, two VOC clusters were over-emitted in *T. atroviride* P1: m/z 99.071, 45.033, 83.083, 33.033 and 33.093 (cluster 1 in Figure 5b) and m/z 117.106, 81.070, 115.076, 47.051 and 247.233 (cluster 2 in Figure 5b). Among the under-emitted VOC clusters, we could distinguish m/z 107.085 and 75.042 in *T. atroviride* P1 (cluster 3 in Figure 5b,) and m/z 59.048, 87.080, 127.128, 43.018 and 70.072 in *T. asperellum* B6 (cluster 4 in Figure 5b). Tables 1 and 2 report the top twenty most abundant compounds (Top 20) detected in the VOC blends emitted by the four different species when grown in PDA and soil, respectively.

Table 1. *Emission of Top 20 VOCs of Trichoderma isolates grown on PDA.*

B6 [1]	%VOCs [2]	MK1 [1]	%VOCs [2]	P1 [1]	%VOCs [2]	T22 [1]	%VOCs [2]
29.014	2.23	43.018	2.13	47.051	2.21	29.014	2.04
59.048	2.07	47.051	2.10	45.033	2.15	47.051	2.01
33.033	2.02	61.028	2.06	29.014	2.05	205.195	1.93
41.039	1.87	89.059	2.01	41.039	1.98	57.069	1.91
43.018	1.82	205.195	2.00	43.018	1.97	43.018	1.90
33.993	1.81	93.038	1.88	59.048	1.95	41.039	1.85
57.069	1.79	41.039	1.86	93.038	1.85	43.054	1.77
145.139	1.78	45.033	1.83	57.069	1.85	61.028	1.75
47.051	1.77	29.014	1.81	131.124	1.77	45.033	1.75
139.150	1.73	57.069	1.79	26.026	1.76	89.059	1.72
27.035	1.68	149.128	1.75	27.035	1.75	71.085	1.71
219.184	1.67	71.085	1.73	167.099	1.74	59.048	1.70
61.028	1.66	131.124	1.71	105.070	1.73	73.063	1.69
111.046	1.64	135.117	1.69	45.991	1.73	95.047	1.68
26.026	1.64	105.070	1.69	43.054	1.73	149.128	1.68
73.063	1.63	90.075	1.68	39.033	1.70	121.065	1.67
69.069	1.62	26.026	1.67	71.085	1.68	33.993	1.64
39.033	1.60	27.035	1.66	73.063	1.67	27.035	1.63
157.176	1.58	39.033	1.65	89.059	1.62	105.070	1.63
45.991	1.57	81.070	1.64	33.993	1.61	26.026	1.63

[1] Protonated measured (m/z) VOCs of different *Trichoderma* species.
[2] The emission percentage (%) calculated as VOCs emitted/Total VOCs.

Table 2. *Emission of Top 20 VOCs of Trichoderma species grown in soil.*

B6 [1]	%VOCs [2]	MK1 [1]	%VOCs [2]	P1 [1]	%VOCs [2]	T22 [1]	%VOCs [2]
29.014	2.41	29.014	2.39	29.014	2.33	27.035	2.24
33.033	1.99	33.033	1.97	33.033	1.99	28.050	1.97
39.033	1.93	39.033	1.92	33.993	1.91	26.026	1.95
33.993	1.89	33.993	1.88	39.033	1.85	29.014	1.91
28.050	1.87	28.050	1.85	28.050	1.81	30.995	1.87
27.034	1.82	43.018	1.82	99.071	1.81	33.033	1.80
43.017	1.78	27.035	1.79	43.018	1.80	39.033	1.79
26.026	1.76	26.026	1.75	43.054	1.80	41.039	1.75
61.034	1.72	43.054	1.74	41.039	1.77	43.018	1.74
59.048	1.70	61.028	1.73	27.035	1.76	43.054	1.73
41.039	1.69	41.039	1.73	45.033	1.75	45.033	1.72
264.850	1.68	59.048	1.72	26.026	1.71	45.991	1.68
45.991	1.68	264.850	1.67	61.028	1.70	47.051	1.67
43.054	1.67	45.991	1.64	45.991	1.70	57.069	1.64
47.050	1.66	47.051	1.63	59.048	1.70	59.048	1.60
30.995	1.63	30.995	1.63	264.850	1.67	61.028	1.58
204.211	1.59	73.063	1.60	47.051	1.65	69.069	1.58
45.033	1.57	45.033	1.58	30.995	1.59	70.071	1.54
73.063	1.57	204.211	1.58	81.070	1.58	73.063	1.52
69.068	1.54	69.068	1.54	204.211	1.54	81.070	1.46

[1] Protonated measured (m/z) VOC of different *Trichoderma* species.
[2] The emission percentage (%) calculated as VOCs emitted/Total VOCs

A high number of the 20 most abundant VOCs emitted was shared among *T. longibrachiatum* MK1, *T. atroviride* P1 and *T. afroharzianum* T22, with few exceptions. In the Top 20 *T. longibrachiatum* MK1 VOCs, three compounds (m/z 135.117, 90.075 and 81.070) were specific; in the Top 20 *T. atroviride* P1 VOCs, only m/z 167.099 was specific; and in the Top 20 *T. afroharzianum* T22 VOCs, two compounds (m/z 95.046 and 121.064) were specific. A different behaviour was shown by *T. asperellum* B6, which had 7 specific compounds out of 20 (m/z 33.033, 145.139, 139.150, 219.184, 111.046, 69.069 and 157.176). There were no significant differences between the Top 20 VOCs emitted by the four different species in the soil samples. The five most abundant compounds were the same for all soil samples analysed: m/z 29.014, 33.033, 39.033, 33.993 and 28.050. Among them, the mass m/z 29.014 had the highest emission percentage for all VOC blends analysed.

5.4 Discussion

In the present study, the PTR-Qi-TOF-MS technique was utilised to discriminate different species of Trichoderma. Our case study was represented by *T. longibrachiatum* MK1, *T. atroviride* P1, *T. asperellum* B6 and *T. afroharzianum* T22 grown on PDA or in soil, looking for a valid strategy of high-throughput screening for *Trichoderma* spp. identification. This technique was chosen because it is highly sensitive and can detect in real-time low concentrations of VOCs in air and gas samples without sample preparation, derivatisation or concentration; it is excellent for the detection of low molecular weight, oxygenated and polar compounds. The major downside of PTR-Qi-TOF-MS is the identification of compounds, as each detected mass can either be associated with parent molecules or possible fragments from other molecules. Thus, the identification of compounds measured by PTR-Qi-TOF-MS is either putative and based on literature references or determined by a comparison with standards. This mass spectrometry technique has successfully been used to characterise the VOCs of fungi such as *Fusarium* spp. [46], *Muscodor albus* [57], *Tuber magnatum* [37] and different *Mortierella species* [49]. VOCs are involved in various biological processes, including communication among organisms such as plants [22] and microbes as well as in self-signalling [58]. The potential of fungal volatiles is receiving growing attention in agricultural, environmental and ecological studies. Fungal VOCs induce both positive and negative effects on plant growth [13] and have often been used to suppress pathogenic bacteria and fungi [19,59]. Recent studies have reported that microbial VOC compositions are variable, depending on intra- and interspecific interactions [39,60,61]. Previous studies on *Trichoderma* spp. VOCs have shown that the emission profile is species- and even strain-specific as well as substrate composition- and cultivation environment-dependent [15,29,62]. The soil effect on fungal VOC emissions has been previously investigated utilising the solid phase

microextraction (SPME) technique coupled with the GC-MS technique [38]; the authors reported deep differences in the VOC profiles of the same fungal species grown in soil or a malt extract medium. In the present study, we characterised *Trichoderma* VOC emissions both in soil and on PDA in order to develop a fast method that reproduced natural field conditions and that could be used as a diagnostic tool for the identification of *Trichoderma* spp. in vivo. When analysing the PTR-Qi-TOF-MS dataset, we were able to detect 68 VOCs, 66 and 25 of which were differentially produced by the four *Trichoderma* species grown on PDA or in soil, respectively. It is interesting to note that in our experimental condition, *T. asperellum* B6 was significantly the weakest VOC emitter in terms of total ppbv when grown on PDA, whereas the other three species emitted a very similar total amount of VOCs. To the best of our knowledge, this is the first time that such a difference in the total VOC emission has been reported. Even if among *T. longibrachiatum* MK1, *T. atroviride* P1 and *T. afroharzianum* T22 there were no differences in terms of the total VOC emission, their blends of volatiles were diverse in terms of composition. The PCA of the PDA samples (Figure 3) showed a clear separation of four different clades that corresponded with the tested fungal species, allowing us to conclude that different types and quantities of VOCs are produced depending on the fungal species when grown on PDA. The PCA carried out on the whole dataset (both data from PDA and from soil) (Figure 2) clearly highlighted the separation of the volatile profiles of the soil samples from the PDA samples, indicating a significant role of the fungal growth environment on VOC emissions. However, it should be noted that although the profiles of the VOCs obtained from *Trichoderma* spp. grown on PDA were always clearly distinct from each other, those obtained from the fungi grown in soil (Figure 4) permitted us to discriminate well only *T. atroviride* P1 and *T. asperellum* B6 in contrast *T. afroharzianum* T22 and *T. longibrachiatum* MK1 were superposed. In general, all soil samples emitted a minor total VOC amount compared with the PDA samples, probably due to VOC entrapment in the soil or because a large fraction of the compounds produced by the *Trichoderma* species were metabolised by the autochthonous microbiota of the soil. This result was in accordance with Asensio et al. [63] who proved, in a study on the behaviour of Mediterranean soil, that there was an overall VOC uptake. It is known that the soil microbiota and VOC retention in soils are influenced by many environmental factors such as pH, temperature and moisture content [64]. The pH of soils determines the charge of VOCs and modifies their evaporation pressure, which also depends on temperature [65]. Most microbial VOCs are produced in the cell or are released from substrates that are digested by extracellular enzymes, so they are produced in the liquid phase and their emission depends on humidity; in particular, polar compounds are retained more strongly than aromatic and aliphatic molecules [66]. In a few cases, the soil texture can lead to an absorption of VOCs [66] by microorganisms that can utilise them as a carbon source, impacting on the global soil volatilome

[67,68]. Moreover, as soil is sugar-poor and rich in amino acids and lipids [69] compared with PDA, which is a sugar-rich medium, the differences in the total volatile amount among the same species grown in soil or on PDA may be related to the different fungal growth rates on the two substrates [70]. The PCA results matched the heat map results where the three replicates of each treatment lay close together, but the four treatment classes were separated. Each species, in particular in the PDA samples, strongly over- or under-emitted characteristic clusters of volatiles, but the relationships among the four VOC blends did not correspond with the phylogenetics of the *Trichoderma* species [71]. Several VOCs produced by the four *Trichoderma* species were shared among them, making the use of a single molecule as a diagnostic tool unfeasible. More useful for this purpose could be the relative abundance of the different volatiles, with the use of the complete profile instead of a selected ion as a fingerprint of a species [72]. From a general analysis of the VOCs emitted by the four *Trichoderma* species, it is interesting to note that many of the identified molecules have been previously reported to play a role during fungi interkingdom communication. Among them, several have been related to resistance induction, promotion of plant seed germination and seedling development and antimicrobial activity, as 2-pentyl-furan [73], 6PP [74], acetone [75], octanol, 1-octen-3-ol and trans-2-octenal [13,23]. A volatilome analysis of each single species was also performed by ranking the Top 20 emitted volatiles in terms of ppbv expressed as percentage of the total emitted VOCs. This approach confirmed the results obtained with the PCA and heat map analyses, detecting *T. asperellum* B6 as the most different in terms of VOC emissions compared with *T. longibrachiatum* MK1, *T. atroviride* P1 and *T. afroharzianum* T22, which had a more similar Top 20 VOC composition. From Table 1, it was evident that the VOC profiles of the four different *Trichoderma* spp. differed more in quantitative than in qualitative terms. The species-specific mass peaks could not be defined as being exclusively produced by a species, rather, as those with significantly higher concentrations. In the Top 20 emitted compounds, we noticed a few masses shared among all PDA samples such as an alkyl fragment (m/z 41.039), acetic acid fragment (m/z 43.017) and ethanol (m/z 47.050). *T. asperellum* B6 in our experimental condition (PDA) emitted seven compounds not present in the Top 20 of the other three species; in particular, m/z 33.033 (corresponding with methanol) was the most abundant (2.02%). Methanol production has been proven for several Fusarium species grown on PDA [46]; however, up to now, it has never been reported for Trichoderma species. It is interesting to note that in the soil experiments, methanol was present as one of the Top 20 VOCs for all four species, probably being produced by the fungi growth on the plant decay material present in the soil samples [43,76]. Another interesting molecule among the Top 20 emitted VOCs of *T. asperellum* B6 was the compound with m/z 139.150, putatively identified as 2-pentylfuran (Table S2). 2-pentylfuran has been correlated with a reduction in downy mildew severity on grapevines when applied without physical contact with the

leaf tissues [73]. Among the most emitted Top 20 VOCs, p-cymene (m/z 135.117) is T. longibrachiatum MK1-specific. Similar results were obtained by Guo et al. [14], who compared T. hamatum with three other Trichoderma species. p-Cymene is a monoterpene known for its antibacterial, antiviral and antifungal activities [77]. Characteristic of the Top 20 *T. atroviride* P1 VOCs was 6-pentyl-alpha-pyrone (6PP) (m/z 167.099). This volatile is responsible for the coconut-like scent of *T. atroviride* isolates growing on PDA [78] and has been correlated with antimicrobial activity and plant defence induction as well as plant growth promotion [74,79,80]. Among the *T. afroharzianum* T22 Top 20 emitted VOCs, there was m/z 121.064, putatively identified as acetophenone, a molecule reported to show antifungal activity in vitro against *Penicillium italicum* [81]. The four *Trichoderma* species grown on PDA, even if sharing a few compounds among the Top 20 emitted VOCs, showed characteristic molecules that could all be reconducted to the broad-spectrum activities and multiple modes of action of *Trichoderma* spp. In all Top 20 soil sample-emitted VOCs, the four volatile profiles showed a similar amount of emitted compounds; the most abundant VOCs were mainly represented by low m/z VOCs, led by methanol (m/z 33.033). This phenomenon could be related to the metabolic activities of the soil microbial biomass as respiration and enzyme activities which are responsible for the generation of short-chain VOCs and fragments.

5.5 Conclusions

A complete knowledge of the volatile profiles of the different *Trichoderma* species and the relative quantities of each VOC are fundamental prerequisites for the characterisation of the different species, for their detection in vivo as well as real-time studies on their multitrophic interactions. Our study revealed significant quantitative volatilome differences among the four analysed *Trichoderma* spp. grown on PDA, allowing the characterisation of the different volatile blends. The PTR-Qi-TOF-MS technique was found to be suitable for quickly surveying in vivo the different fungal species within the samples growing on PDA, but it failed to discriminate among them when grown in soil and to identify volatile molecular markers. The potential of using PTR-Qi-TOF-MS for the in vivo characterisation of soil microbial communities could be fruitfully exploited, integrating data from other different sources to train artificial intelligence-based systems, resulting in the definition of precise and accurate models.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof8100989/s1, Table S1: Volatiles, expressed as ppbv, detected by PTR-Qi-TOF-MS analyses from soil and PDA samples inoculated with *T. longibrachiatum* MK1, *T. atroviride* P1, *T. asperellum* B6 and *T. afroharzianum* T22; Table S2: Putative identification of VOCs identified through PTR-Qi-TOF-MS analysis; Table S3: All data of PCA loadings; Table S4: PDA PCA loadings; Table S5: Soil PCA loadings; Table S6: VOCs identified by ANOVA analysis ($p < 0.05$) in *Trichoderma*-inoculated PDA; Table S7: VOCs identified by ANOVA analysis ($p < 0.05$) in *Trichoderma*-inoculated soil.

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Chapter 6

6. Conclusions and future perspectives

6.1 Conclusions

Information currently available on the interaction between plants and beneficial microorganisms shows that extensive progress has been made, with the aim to evaluate the potential role in the selection of new beneficial endophytes useful for crop improvement. Many doubts still remain about the mechanisms regulating this interaction. With this in mind, an attempt was made to exploit the language that plants use for their communication and interaction with the surrounding environment. The focus is mainly on the agronomic potential of volatile organic compounds (VOCs) as a solution to defend plants from stresses and to enhance crop production. Further investigations are also needed here.

This PhD thesis was designed to meet the demand for a more sustainable agriculture (adapted to future climate challenges), intensification of productivity and food security. The aim was to unravel the mechanisms underlying plant responses, in terms of primary and secondary metabolism, in the interaction with beneficial endophytic microorganisms, in order to develop bioinspired plant protection tools and strategies.

VOCs play a role in this communication. Some VOCs are emitted by beneficial microorganisms and may have a beneficial effect on plants, priming metabolites that induce growth promotion or induced resistance. In other cases, where plants are exposed to BMs, influences on the VOCs of plants may alter VOC-driven communication of plants with other organisms, involving other trophic levels (**Chapter 2**). Changes of VOCs profiles and other physiological parameters were evaluated in tomato plants exposed to the entomopathogenic endophytic fungus *Beauveria bassiana* and to a following infection by the pathogenic fungus *Botrytis cinerea*. Plants reacted to the recognition of the fungus with a significant but transient (1-2 day-long) reduction of stomatal conductance and CO_2 assimilation indicating rapid activation of defensive (rejection) responses and possible (not investigated) priming of defense metabolites. After successful colonization of tomato plant during a long time-course of analysis until the fruiting time, *B. bassiana* increased plant growth in terms of root length, stem hight and leaf growth, early flowering and increased fruiting. Moreover, when plants were exposed to *B. cinerea* infection, *B bassiana* helped the plant fight the pathogen, reducing necrotic damage by up to 35%. VOC emission did not explain how *B. bassiana* controlled this action towards the pathogen, and the reduction of VOC emission might be interpreted as mirroring an improved plant health status. (**Chapter 3**). Based on results obtained when testing *B. bassiana* effect on plants in Chapter 3, we also tried to assess *in vitro* whether an antifungal effect of *B. bassiana* against *B. cinerea* could be hypothesized, based on volatilome traits. An airborne and direct communication method between to

fungi was proposed to be used as experimental tool for testing VOCs communication between fungi. The method showed promising pre-results which could be extended to other pathosystems, and was important to open to new perspectives for future work. Our test confirmed results based on in vivo studies with tomato plants that *B. bassiana* VOCs are unable to control the growth of the pathogenic fungus *B. cinerea*, and are not responsible for its biocontrol, which was positively assessed elsewhere. (**Chapter 4**). A systematic research on the global VOC profile of fungi is still lacking. To further develop a fast method that reproduced natural field conditions and that could be used as a diagnostic tool for the identification of fungi in vivo, a complete knowledge of the volatile profiles of four different *Trichoderma* species, a known BCA and growth inducer, was provided (**Chapter 5**).

6.2 Future perspectives

There will certainly be an increasing use of beneficial microorganisms as biocontrol and plant growth promoting agents, supporting sustainable use of resources and agro-ecological applications. It will be important to diversify the potential applications of BMs and also select those BMs more effective under a wide range of stress conditions exacerbated by climate change.

Searches for climate-proof BMs, must be assisted by a complete understanding of the mechanisms underlying the soil-plant-microbe interactions, which will require interdisciplinary cooperation among microbiologists, plant physiologists and soil scientists. This will allow us to develop bio-inspired plant protection tools and strategies, which will significantly improve the sustainability of integrated pest management plans, and contribute to future reduction of chemical pesticide use with relevant economic and environmental benefits.