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OVERVIEW ON UTILITIES AND ANALYSIS TECHNIQUES OF ORGANIC VOLATILE COMPOUNDS (VOCS) PRODUCED BY FUNGI BELONGING TO *TRICHODERMA* SPP

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Fungi can be found in most habitats, competing with a high number of other organisms. Fungal volatile organic compounds (VOC) can have many effects on these competitors, and can be used for defense, plant growth promotion, plant resistance or as attractants for micro- and macro-organisms. Technological advances with respect to the ability to analyze volatile organic compounds and the capacity to establish their biological activities are important for both the elucidation of the complex mechanisms which underlie communication in biological systems and the desirable field of application. This review focuses on the analytic techniques and potential applications of volatile organic compounds produced by fungi, with special regard to *Trichoderma* spp.

Keywords: Trichoderma spp, volatile compounds, plant growth promotion.

INTRODUCTION

Volatile organic compounds (VOCs) are low molecular weight compounds with high vapor pressure and low polarity which easily evaporate at room temperature and are able to diffuse through the air and soil [1, 2].

Numerous volatile organic compounds are produced by living organisms as part of their metabolic processes. Production of volatile compounds with antimicrobial activity has been reported in bacteria, filamentous fungi, yeast and higher plants[3]. However, VOCs produced by fungi are relatively less studied compared to volatile compound of bacterial and plant origin.

Volatile metabolites from fungi can be intermediate or end products of different metabolic pathways and more than 300 distinct VOCs have been identified as: terpenes, sesquiterpenes, alcohols, ketones, aldehydes, lactones, esters, thiols, furans, short-chain fatty acids, sulphur and nitrogen – containing compounds [4–7].

The composition of the mixture of such volatile compounds may vary during time, being influenced by temperature, substrate, strain age and other environmental factors depending on the species [8–10].

Several reports and reviews have been published on the production of volatile compounds by microorganisms[2,9]. So far, the importance of VOCs in functioning of both atmospheric and soil ecosystems, as well as the many potential biotechnological applications in food, environment, agricultural, medical and pharmaceutical industries have been discussed[11].

Additionally, fungal VOCs could be used as biomarkers for species identification[5,11,12], being useful for differentiation among strains.

A large number of VOCs have been described, and depending on their uses and their structures, different approaches are utilized for their sampling, preparation, separation, identification and quantification. The present review aims to summarize data on the analysis methods and potential applications of volatile organic compounds produced by fungal species, with focus on *Trichoderma* spp.

ANALYTICAL METHODS FOR EXTRACTION, SEPARATION, IDENTIFICATION AND QUANTIFICATION OF FUNGAL VOLATILE ORGANIC COMPOUNDS

EXTRACTION METHODS FOR MICROBIAL VOLATILE ORGANIC COMPOUNDS

Determination of fungal volatile compounds has been described for different fungi and is

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usually done by gas chromatography (GC) due to the method's powerful separation and highly sensitive detection capabilities[5,13]. Different analytical techniques are used for a large number of compounds, but every method has both advantages and disadvantages, and could not be suitable for certain molecules.

For example, volatile compounds produced by fungi cultivated in liquid[6,14] or on solid growth medium[4,10] have to be extracted in different ways.

Essentially, there are two main approaches: the direct sampling of volatiles from the surrounding air (headspace) or solvent extraction from the sample, followed by purification[7].

Initially, fungal VOCs were isolated by steam distillation followed by liquid-liquid extraction and concentration of the organic extract; however, the extraction with organic solvents gives a more complete profile of VOCs, including polar hydrophilic species and flavor compounds. This method is a very time-consuming procedure, and could imply, in some cases, the degradation or loss of the product of interest. The solvent extraction methods offer less sensitivity compared with thermal desorption or solid phase microextraction (SPME) discussed below. Also, VOCs can be extracted using supercritical fluid carbon dioxide; this is considered a nondestructive method [15, 16].

The most commonly used methods to collect volatiles from the air surrounding sample: static head space sampling and dynamic headspace sampling. In the static headspace method, the volatiles in the sample are allowed to equilibrate with the air from an airtight container before a known air volume is collected and analyzed by GC-MS[7].In dynamic headspace sampling, a volume of purified air is passed over the sample, and the airborne volatiles are concentrated onto an adsorbent trapping material such as Tenax, Carbopak B or Silica gel, followed by thermal desorbtion into the GC-MS[3,13].

Another important headspace technique for extraction of VOCs is solid phase microextraction (SPME)[17,18]. Introduced in the early nineties, SPME technique is an efficient and popular analytical tool for assessing VOCs in quantities ranging from ppt to ppm. The solid-phase microextraction (SPME) consists of catching the VOCs contained in the headspace above a sample in an SPME vial containing fiber coated with adapted stationary phases (divinylbenzene-carboxen polydimethylsiloxane). This technique can be automated and used for the direct and non-invasive extraction of volatiles from living fungal cultures. Also, it is suitable for monitoring fungal VOCs profiles over time and under different growth conditions[4]. SPME reduces preparation time by combining extraction, concentration and introduction into one step, while possessing an increased sensitivity compared to other extraction methods[19].

SPME has been broadly applied in the flavor and fragrance industry for monitoring freshness and detecting fungal contamination in stored products and the like[4;19], as well as for investigating minute amounts of VOCs released by some fungal species. SPME was also used by Keszler *et al.* [17] for the extraction of nineteen pyrone and dioxolane derivates and two aliphatic esters produced by *Trichoderma atroviride*: as a result of using this method, they found and identified four new compounds previously unknown in other *Trichoderma* spp. Furthermore, Stoppacher *et al.*[4] and Ramos et al,[20] also applied this method for the extraction of *Trichoderma* VOCs. A method based on SPME coupled with GC-MS was used by Kluger *et al.*[13] for detection and identification of VOCs produced by *Fusarium graminearum* and *Trichoderma atroviride.* Roze *et al.*, identify 24 volatile compounds in the headspace of *Aspergillus parasiticus* cultures using the same extraction method, SPME [5].

The more traditional method of simultaneous distillation extraction (SDE) combines vapor distillation and solvent extraction[3]. Some earlier studies comparing SDE and SPME used for analyzing the VOCs of fungi found that SDE was almost inadequate to determine full volatile profile[15]. The data obtained by Jelen[21] showed that the profile of VOCs strongly depended on the extraction method: in fact, SPME proved to be a more selective, accurate and fast screening method. On the other hand, the distillation extraction technique of volatile metabolites has many advantages: it does not require large amounts of reagents and specialized equipment, and it is fast and inexpensive. In addition, the procedure allows an easy separation of the non-volatile from the volatile metabolites, which is important because in GC-MS analysis the presence of non-volatile compounds can cause damages to the column.

SEPARATION AND DETECTION OF MICROBIAL VOLATILE ORGANIC COMPOUNDS

The large number of VOCs, their different chemical structures, the usually low concentration as well as the fact that they tend to occur in mixtures, make separation and quantification very challenging.

Separation and detection of fungal volatile metabolites are commonly obtained by gas chromatography. The sample is evaporated and carried by an inert carrier gas (often He). The gas moves the sampled compounds through a column where they are separated with respect to size, polarity and other chemical-physical properties depending on the column characteristics.

Constituents of complex mixtures of VOCs can be distinguished by flame ionization detection (FID or most widely used mass spectrometry[22]). Sometimes Infrared Spectroscopy using Fourier transform methods can also be combined with GC-MS, due to their ability to differentiate between isomers.

Mass spectrometry is a powerful technique used to identify and measure a wide variety of compounds. Basically, the mass spectrometer converts the sampled compounds into gaseous ions, and the most common ionization process for gas phase analysis involves bombardment of molecules with electrons (electron impact ionization – EI). The molecule is given enough energy to eject one of its electrons and become positively charged. The bombardment of electrons also results in fragmentation of the molecule; this gives a number of ions with different mass-to-charge (*m/z*) ratios. The fragmentation of each molecule is unique and is used as a chemical fingerprint to characterize the compounds. Then, compounds are identified using libraries or databases of mass spectra, or by comparison of retention time and spectra with those of authentic standards [4,7,17,21].

Booth *et al.*,[23] described a technique that rapidly traps and collects fungal VOCs: the trapping materials, Carbotrap A and B and bentonite-shale, were placed in a stainless steel column, allowing the recovery of mg quantities of compounds that can be subsequently used for bioassays, separation, various analyses and, potentially, for NMR (Nuclear Magnetic Resonance) spectroscopy in order to identify volatile compounds produced by fungi.

"Electronic nose" is a new-generation sensors technology that promises to revolutionize VOCs detection. The method can be used to detect the presence of fungi in agricultural products and perhaps to identify particularspecies. This system uses metal oxide sensors with various selectivity for different complex VOC profiles[24], providing an impedance response to volatile compounds which is measured and simultaneously displayed. "Electronic nose" appears to be promising in agricultural applications aimed at the determination of mycotoxins producing fungi in fruit, grains and meat. Also, detection of fungal VOCs by "electronic nose" has been used to detect spoilage in stored grains [12].

FACTORS AFFECTING EMISSION OF VOCS

Production of volatile constituents in fungal species is influenced by numerous factors such as strain type, developmental stage, substrate composition, medium moisture, and incubation time. Environmental factors such as temperature, pH of the substrate, light and levels of CO_2 or O_2 probably influence VOCs profile.

The qualitative and quantitative production of volatile compounds in *Trichoderma* spp. is apparently specie-dependent, and changes with substrate composition. It has been reported that different growth conditions lead to distinctive VOCs profiles (both in type and amount). Wheatley *et al.*[8] demonstrated that *Trichoderma* spp. grown on low nutrient media produced less inhibitory volatile compounds in comparison with those grown on high nutrient medium.

Some growth-linked parameters (concentration of inoculum, media, temperature and relative humidity) influence microbial VOCs production by a quantitative rather than qualitative point of view: results provided by Bruce *et al.*[9] showed that VOCs profile of *Trichoderma aureoviride* changes significantly with the culture age. Similarly, Wheatley *et al.*[8] found that both the composition of the medium and the types of isolates influenced the amount and the effectiveness of the volatiles released by *Trichoderma* spp. Polizzi *et al.*[25] studied the production of 6-pentyl-2pyrone and other volatiles by *Trichoderma atroviride* under different growth conditions: the reported data showed that VOCs production by *T. atroviride* was not related to the initial spore concentration. However, the relative humidity level had an effect on VOC production, and also temperature, but to a lesser degree.

UTILITIES OF VOLATILE ORGANIC COMPOUNDS

Although the production of volatile compounds by microorganisms has been known for many years[26], it is only since the last decade that an increasing number of studies on the diversity and potential functions of these compounds have been reported. Microbial VOCs display versatile bioactivities: for example, some of them are able to inhibit both bacterial and fungal growth and spore germination, to promote or inhibit plant growth, to induce plant resistance also by attracting both micro and macroorganisms[7,11, 27–29].

ROLE OF FUNGAL VOLATILE ORGANIC COMPOUNDS IN BIOLOGICAL CONTROL

Fungal volatile organic compounds have been used in agriculture, especially in biological control strategies to prevent plant pathogenesis and thus to reduce the amount of fungicides used. Kanchiswamy *et al.*[30] supported that microbial volatile metabolites could have a strong impact for crops management, and can be exploited as an ecofriendly, cost-effective and sustainable strategy for agricultural practice.

Hundreds of volatile compounds are produced by *Trichoderma* spp.; for example, Siddique *et al.*[6] reported the production of 282 VOCs by *Trichoderma harzianum*. Among them, some secondary metabolites showed an *in vitro* antifungal activity against different phytopathogens, mainly through the inhibition of spore germination and mycelium growth[31,32,33], proving to play an important role in the biocontrol mechanism of *Trichoderma* against different pathogens. Volatile compounds produced by *Trichoderma harzianum* decreased the growth of *Colletotrichum capsici* which causes leaf blight on basilic, chickpea and pepper as well as dieback in pigeon pea[34]. Studies by Aarti and Meenu[35] showed that VOCs accumulated by different *Trichoderma* species (such as 1-methyl and cyclopropane pentene as well as some alkanes derivatives and ketones) reduce the mycelium growth of phytopathogens like *Sclerotinia sclerotium* and *Macrophomina phasseolina*. Some authors [36, 37, 38, 39, 40] have shown that the volatile compounds produced by *Trichoderma* strains were also highly effective in suppressing *Fusarium oxysporum f.sp. lycopersici, Rhizoctonia solani, Fusarium graminearum, Pythium ultimum,* and *Phytophthora cinnamom* infection.

Experiments performed in-laboratory by Zhang *et al.*[41] demonstrated that the antifungal effect of VOCs produced by *Trichoderma harzianum* on *Fusarium oxysporum f.sp. cucumerinum* is influenced by the composition of the medium.

Furthermore, volatile compounds from *Trichoderma gamsii* YIM PH30019, whose VOC profile contains 22 molecules including dimethyl disulfide, dibenzofuran, methane thiol and ketones, showed a significant growth suppression on *Epicoccum nigrum, Scytalidium lignicola, Phomaherbarum* and *Fusarium flocciferum*[42]. Similar results were reported by Barakat *et al.*[43], who found a decreasing in the mycelium growth rate of *Botrytis fabae* exposed to *Trichoderma* spp. VOCs.

Crutcher *et al.*[43] produced the first study regarding a gene involved in the biosynthesis of volatile compounds in *Trichoderma virens*, from a gene cluster involved in terpenoids volatile synthesis in filamentous fungi.

Stoppacher *et al.*[4] identified a mixture of VOCs in *Trichoderma atroviride* with antifungal properties including sesquiterpenes like as α-farnesene, γ-curcumene, α-zingiberene, nerolindol and some alcohols and derivatives. Also, three new acorane sesquiterpenes with antifungal activity were isolated by Li *et al.*[45] from *Trichoderma* strain YMF1.02647. Bruce *et al.*[9] shows that volatiles from *Trichoderma* can also produce fungi static and fungicidal effects against selected wood decay fungi.

The ability of *Trichoderma* species to produce a great number of volatile like pyrones and sesquiterpenes with fungi static activity has been reported by Amin *et al.* [33] and Reino *et al.*[31]. Also, Vinale *et al.*[32] demonstrated that VOCs of *Trichoderma* spp. play a key role in mycoparasitism as well as in the interaction with plants. Studies of Pezet *et al.*[46] also, showed that 6PAP produced by *Trichoderma harzianum* is able to inhibit the germination of conidia of the necrotrophic?? fungal pathogen *Botrytis cinerea*, reducing the mycelial growth of the parasite *in vitro*. Some oxylipins such as the 1-octen-3-ol and its analogues 3-octanol and 3-octanone have been detected from cultures of *Trichoderma* spp. and these are able to induce sporulation and conidiation and contribute to enhanced plant resistance to *Botrytis cinerea* by inducing defense signaling cascades. It has been suggested that the specificity of the cell response to particular C8-VOCs involves the membrane receptors that could transmit the VOC signal into the conidiation pathways[10].

The most abundant VOC produced by fungi, 1-octen-3-ol, functions as a developmental signal for many species. It is produced through the enzymatic breakdown of linoleic acid. These compounds inhibit mycelia growth of *Penicilium expansum* at low concentration[2]. Cultures of *Trichoderma* grown in the dark produce very low concentration of 1-octen-3-ol, 3-octanol and 3-octanone which induce conidiation; however, the highest concentration of these compounds inhibited growth and conidiation[10,11,47]. Studies from

Stoppacher *et al.*[4] reported that the above mentioned compounds reached their maximum accumulation during the conidiation process.

Due to the previously discussed biocontrol properties, microbial volatile compounds are used in the food industry as agents for prevention against postharvest fungal spread[47]. Information on the possible use of *Trichoderma* derived VOCs on mycotoxigenic fungus is scarce and limited to few studies; the first report on the inhibition effect produced by the VOC of *Trichoderma harzianum* on *Aspergilus flavus* proliferation in stored maize is provided by Aguero *et al.*[48]: Authors demonstrated that these VOCs were effective in preventing the proliferation of *Aspergilus flavus*, significantly reducing the level of aflatoxin B_1.

ROLE OF FUNGAL VOLATILE ORGANIC COMPOUNDS AS AROMA CONSTITUENTS IN FOOD

Microorganisms represent an important source of natural compounds, especially used in the food industries for aroma. Because of the interest on coconut aroma (δ- lactone 6 penthyl-α-pyrone, 6PP) as a food additive, some authors investigated the production of this compound by different *Trichoderma* species[20, 49, 50, 51]. Later, its production was correlated with the effectiveness of *Trichoderma harzianum* isolates against *Botrytis cinerea*, suggesting potential fungicidal properties of 6PP. The results obtained by Pezet *et al.*[45] supported that *Trichoderma pseudokoningii*, producing increased amounts of 6PP, strongly reduced *in vitro* mycelial growth of the pathogen. Experiments from Simon *et al.*[52] showed that same *Trichoderma pseudokoningii* showed the ability to also inhibit the growth of *Gaeumannomyces graminis;* moreover, studies of El-Hasan *et al.*[37], described for the first time the efficacy of 6PP produced by *Trichoderma* isolates in suppressing the biosynthesis of fusaric acid in *Fusarium moniliforme*.

Vinale *et al.*[32], evaluated the effect of 6PP isolated from *Trichoderma atroviride* in tomato and canola seedlings inoculated with *Botrytis cinerea*, observing a reduction of disease symptoms which correlated with an induced expression of the salicylic acid responsive gene *Pathogenesis Related Proteine 1* (PR-1). In comparison to the control, tomatoes and canola plants treated with 6PP were more vigorous and showed a more extended root system. VOCs produced by *Trichoderma* spp. were also effective against *Fusarium solani* and *Fusarium oxysporum radici lycopersici*[40].

ROLE OF FUNGAL VOLATILE ORGANIC COMPOUNDS IN PLANT GROWTH PROMOTION

Recent data indicates that VOCs may regulate plant growth and morphogenesis[27; 29]. The most abundant VOCs involved in plant growth promotion were isobutyl alcohol, isopenthylalcohol, farnesene, 3 methyl butanol and geranyl acetone[27].

Some literature data show that 6PP isolated from *Trichoderma harzianum* seems to act as plant growth regulator, being able to significantly inhibit the growth of etiolated wheat coleoptiles at a relatively high concentration (10^{-3} M) while not effective at lower doses. Thus, it is hypothesized that 6PP may act as auxin-like compound, whose effect can be exerted between at 10^{-5} M and 10^{-6} M concentration.

Windham *et al.*[53] proved that volatile and/or cellophane-diffusible metabolites of *Trichoderma harzianum* have a stimulating effect on seed germination of corn, tomato and tobacco. They confirm that, in contrast to control, the stimulation of germination occurred in the presence of VOCs even though isolates of *Trichoderma* were not placed in contact with the seed.

The role of VOC emitted from the biocontrol fungal agent *Trichoderma viride* in the promotion of plant growth has been studied in *Arabidopsis* plants[27]. It has been observed that *T. viride* promotes growth through increasing lateral roots formation, root biomass, plant height, flowering and chlorophyll content in leaves**.** Additionally, Contreras-Cornejo *et al.*[29] demonstrated that VOCs of *Trichoderma virens* stimulate *Arabidopsis* growth and development: their results indicated a modification of root system architecture by increasing formation, number and length of lateral roots without affecting primary root growth. Compounds 6PP and acetoin, which were produced at highest concentration by 5 day old and 14 day old cultures of *Trichoderma,* have been shown to induce plant growth [32]. Lee *et al.*[54] evidenced that both the age of the fungal culture and the exposed plant material are very important for the growth modulation effects. However, these authors reveal the presence of 6PP in VOC profile of *Trichoderma viride,* while in previous studies Hung *et al.*[27] did not find the compound in experiments regarding growth promotion of *Arabidopsis* in relation with *Trichoderma*.

Thus, fungal volatile mediate growth promotion and/or inhibition may be attributed to a difference in exposure time parameters or to the age of culture of *Trichoderma viride.*

NEMATICIDAL ROLE OF FUNGAL VOLATILE ORGANIC COMPOUNDS

Affokpon *et al.*[55] evaluated seventeen isolates of west-african *Trichoderma sp*., establishing that five of these provided a promising root nematodes Meloidogyne *incognita* control. Field assessment also demonstrated a significant inhibition of nematode reproduction, suppression of root galling and an increase of tomato yield compared to the non-fungal control treatments. Other authors investigated the abilities of VOCs from *Trichoderma* strains to kill nematodes[56]: they isolated and identified three metabolites among which 6PP has a strong nematicidal activity. In this work, 6PP killed >90% of *Panagrellus redivivus* and *Caenorhabditis elegans*, suggesting that the nematicide VOCs could be used to develop nematicidal bioagents. Gliotoxin possessing nematicidal activity and was also obtained from *Trichoderma virens*[7].

Tripathi *et al.*[57] presents *Trichoderma* as a potential bioremediation agent for environmental cleanup in their review. Some fungal volatiles, particularly ketones and alcohols, function in insect attraction or deterrence. 47 volatile substances which caused immobility and mortality to *M. incognita* were identified by Freire *et al.* 2012 from *Fusarium oxysporum* isolate 21.

Some authors studied microbial volatiles-signaling compounds that are active in extremely low concentrationcalled" semiochemicals"[2]. Furthermore, VOCs have proved to be functional as signaling molecules in inter- and intraspecific interactions and cell-to cell communication. Chemotrophic interactions, growth coordination and growth inhibition of other fungi are mediated by volatile signaling metabolites.

ROLE OF FUNGAL VOLATILE ORGANIC COMPOUNDS IN PLANT IMMUNITY

Recent information indicates that microbial VOCs may modulate plant immunity.

Activation of plant defense mechanisms by different *Trichoderma* strains involves the production of defense related metabolites such as phytoalexins, or the induction of pathogenesis related (PR) proteins[29]. Vinale *et al.*[32] evaluated the ISR(induced systemic resistance) inducing ability of volatile compound 6PP isolated from *Trichoderma atroviride* filtrates, in order to investigate the involvement of this compound in the induction of ISR during the *Trichoderma*-plant interaction.

CONCLUSIONS

Trichoderma spp. produce a wide number of VOCs (comprising hydrocarbons, esters, ketones, aldehydes and sesquiterpenes) proven to play an important role in fungal development, defense, protection against stress, activating plant immunity and enhancing plant growth. Substrates, nutrient condition and strain type can influence the variability of volatile constituents produced by *Trichoderma* sp. and thus their potential as biocontrol agents.

Volatile metabolites can be used as part of an ecofriendly, cost effective and sustainable strategy for agriculture practices, replacing chemical fungicides and fertilizers which are expensive and affect environment. In addition, VOCs produced by fungi can also be used as biomarkers and aids in the identification and classification of fungal strains.

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