The influence of maize (*Zea mays* L.) roots, mycorrhizal fungi and agricultural soil management practices on the emission of soil volatile organic compounds

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Summary

The smell of freshly cut grass is one of the most recognisable examples of the emission of a special type of gaseous molecules by plants during wounding or stress, named “volatile organic compounds” (VOCs). The emission of these VOCs has been well documented in above-ground ecosystems, but not in the soil. This is unfortunate, because VOCs can be used as an important tool to assess the health of the plant-microbe ecological relationships in the soil. We used a direct soil VOC measurement method, where we first sampled air with a syringe from a buried inverted funnel, followed by transferring them to a machine that measures the sampled air VOC content, named “Proton-Transfer-Reaction Mass Spectrometer” (PTR-TOF-MS). This was done for two experiments, with the first experiment determining the difference between soil VOCs emitted by maize plants (*Zea mays* L.) grown with and without mycorrhizae (soil fungi that provide nutrients to plants). In the second experiment, we determined the difference in VOC emissions from both the soil and harvested roots, between maize plants grown in different agricultural soil management practices. In the first experiment, we found a higher emission of VOCs related to decomposition in the soils of maize grown without mycorrhizae. In the second experiment we could not draw a conclusion on the difference between the different management practices, because of a high soil water content that made the measurement of VOCs difficult. However in the same experiment, we also did not find a difference between the VOCs emitted by harvested roots, which could be the result of a low root colonisation by mycorrhizae and other soil organisms. What we concluded is that the direct soil measurement method can be used to measure soil VOCs, that is a basic step into achieving in the future further goals towards describing these emissions.

Keywords: Volatile organic compounds (VOCs), soil, direct soil VOC measurement method, PTR-TOF-MS, maize (*Zea mays* L.), mycorrhizae, agricultural soil management practices, harvested roots, high soil water content

Abstract

Volatile organic compounds (VOCs) are secondary metabolites that play an important role in the intra- and interspecies interactions between plants and soil organisms such as arbuscular mycorrhizal fungi (AMF). Despite their importance, there is no well-established method to measure soil VOCs. We tried to address this problem, by determining if a direct soil VOC measurement method was effective for the non-invasive measurement of soil VOCs. We first sampled air with a syringe from an inverted funnel buried in the soil, before transferring it into a "Proton-Transfer-Reaction Mass Spectrometer" (PTR-TOF-MS) to measure the VOC content in the air sample. This was done in two experiments, with the first one testing if we can distinguish maize (*Zea mays* L.) rhizosphere communities colonised with (AMF+) and without (AMF-) the AM-fungus *Glomus irregulare* based on their VOC emissions. In a second experiment we tried to determine if we could distinguish between agricultural soil management practices, based on the VOCs emitted in the soil and by harvested roots. In the first experiment we were able to distinguish between the AMF+ and AMF- treatments, with a higher emission of four decomposition related VOCs in AMF-. This could indicate that plants grown in the AMF- treatment use priming to acquire nutrients. In the second experiment we were unable to distinguish the agricultural managements presumably due to high soil water content, which complicated the measurement VOCs. We however also found that there was no significant difference between VOC profiles of the agricultural managements in harvested roots, probably due a low colonisation by AMF or other microbes. We concluded that the direct soil measurement method can be effective in measuring VOCs in well-aerated soils, but it still needs several improvements in order to better capture the VOC emissions by roots and AMF.

Keywords: Volatile organic compounds (VOCs), soil, direct soil VOC measurement method, PTR-TOF-MS, *Zea mays* L., rhizosphere, *Glomus irregulare*, agricultural soil management practices, harvested roots, well-aerated soil

Contents

1. Introduction

Plants have an incredible ability to influence their surroundings by creating small, dynamic "ecosystems" around their various organs. The rhizosphere, a narrow zone of nutrient rich soil that surrounds plant roots, is one of those ecosystems. It is a complex biodiverse environment consisting of a very high number of microorganisms and invertebrates whose community structure is heavily influenced by root exudates. These are compounds originating from primary metabolism, such as carbohydrates and amino acids, which are used as substrates to promote the growth of heterotrophic microbes (Barea et al., 2005; Philippot et al., 2013; Venturi and Keel, 2016). However, these primary metabolites are not the only method used by plants to influence the rhizosphere, with secondary metabolites (like alkaloids, terpenoids and benzoates) also playing a very important role. These compounds are primarily used as signal molecules, facilitating the interactions between plants and microorganisms whose interaction plays a key role in multiple soil processes (Barea et al., 2005; Venturi and Keel, 2016). Important soil functions that involve these organisms are the restriction of the growth of pathogens, the fixation of nitrogen (N_2), the acquisition of important nutrients and phytoremediation of soils contaminated with heavy metals (Barea et al., 2005; Philippot et al., 2013; Tkacz and Poole, 2021). Major groups of microorganisms that contribute to these interactions are rhizobium bacteria and mycorrhizal fungi, which are associated with roots and are responsible for major processes that benefit host plants such as the fixation of N_2 and the supply of important nutrients respectively (Barea et al., 2005; Philippot et al., 2013). Additionally, mycorrhizal fungi also establish close associations with endosymbiotic soil bacteria linked with processes such as nitrogen fixation, phosphorus solubilization and pathogen control (Larsen et al., 2015). Due to the positive impact of these processes on host plant species, it is important to further investigate how these microbe-microbe and plant-microbe interactions occur and how they influence these soil processes. This is important in particular when investigating the differences in soil microbe driven processes in different land-use types. A good example of this is the transition from intensive forms of agriculture to sustainable forms of agriculture or nature restoration, where there has to be dealt with negative legacy effects of previous soil land uses on soil processes (Philippot et al., 2013).

There are multiple signal molecules used by both plants and soil microbes to facilitate interactions, like diffusible signal factors, antibiotics, phytohormone-like molecules and volatile organic compounds (VOCs) (Venturi and Keel, 2016). VOCs in particular play an important role in the long-distance plant-microbes and microbe-microbe interactions in the rhizosphere, making them a very important group to investigate (Bouwmeester et al., 2019; Venturi and Keel, 2016). They are molecules with a low boiling point and a high vapour pressure, with a Reid vapor pressure over 10.3 Pa at a temperature of

293.15 K and pressure of 101.325 kPa (Kamal et al., 2016; Schenkel et al., 2018). Due to these characteristics, VOCs evaporate at room temperature (Kamal et al., 2016). Their molecular masses range from 17 Da (in ammonia (NH_4^+)) to 272 Da (in diterpene hydrocarbons) (Weisskopf et al., 2021). The major compound classes where VOCs belong to are fatty acid derived molecules (including alcohols, aldehydes and acetates), terpenoids (such as sesquiterpenoids and monoterpenoids), benzenoids and phenyl-propanoids (Bouwmeester et al., 2019; Dudareva et al., 2013). All organisms are able to produce VOCs for multiple purposes, like plants defending themselves against herbivores and pathogens and also sending stress signals to conspecifics (Delaney et al., 2015; Khashi U Rahman et al., 2019; Weisskopf et al., 2021), plants using VOCs to attract pollinators (Bouwmeester et al., 2019; Jürgens and Bischoff, 2017) and the use of VOCs by animals to mark territory and signal their identity (Portillo-Estrada et al., 2021; Soso and Koziel, 2017). However, despite the important role VOCs play in facilitating interactions in rhizosphere communities, their role in these ecosystems remains underexplored. This is in contrast to the above-ground ecosystems, where the interactions between plants and other organisms with VOCs have been well documented (Peñuelas et al., 2014; Schenkel et al., 2018; Singh et al., 2021). The main reason why soil VOCs are not explored well is due to many difficulties with their diffusion in the soil matrix, which makes it hard to measure them (Insam and Seewald, 2010; Mäki et al., 2019). The bad diffusion rate of these gaseous compounds in the soil is caused by several factors such as a low porosity of the soil caused by the soil texture and the low amount of soil organic matter, the adsorption of volatiles on clay minerals and soil organic matter, the dissolution of VOCs in soil water and the degradation of the emitted soil VOCs by anaerobic soil bacteria (Insam and Seewald, 2010; Mäki et al., 2019; Peñuelas et al., 2014). Because of these issues, there is no well-established method to measure soil VOCs (Mäki et al., 2019). This research project will focus on finding a method to measure these VOCs and to explore if we can distinguish multiple soil rhizosphere communities by their VOC profiles, in order to find out if they are a good indicator for the soil microbial community composition.

In both soil and above-ground ecosystems, the emission of VOCs by plants is either constitutive or induced by abiotic and biotic stressors, which form important indicators for plant metabolism and health at a certain point in time (Holopainen, 2004; Niederbacher et al., 2015). Constitutive emissions are the volatiles emitted by plants during metabolic processes, with only environmental conditions (light and temperature) regulating their emission regardless of environmental stressors. Examples of volatiles that are emitted constitutively are methanol and isoprenoids, the emission of which is an indicator of plant growth and photosynthesis respectively (Niederbacher et al., 2015). Induced VOCs are mostly produced by plants as a result of both abiotic and biotic stressors (Holopainen and Gershenzon, 2010; Niederbacher et al., 2015). The emission of induced VOCs as a result of abiotic

factors is mostly caused by water, salt, heat and photooxidative stresses (García-Plazaola et al., 2017; Holopainen and Gershenzon, 2010). *Arabidopsis thaliana* (L.) Heynh. for example, produces VOCs such as monoterpenes, sesquiterpenes and methanol as a result of salt stress in order to increase resistance against the stressor and to signal neighbouring plants (Lee and Seo, 2014). A major biotic stressor for plants that causes the induced emission of VOCs is herbivory, which causes leaf wounding (Holopainen and Gershenzon, 2010; Portillo-Estrada et al., 2015). The types of VOCs that are released during leaf wounding are green leaf volatiles (GLV's) (hexenals, hexenol and hexenyl acetate) and acetaldehyde, which play an important role in protecting plant tissues against infection and in inducing defence reactions in plants (Brilli et al., 2011; Fall et al., 1999; Portillo-Estrada et al., 2015). Both constitutive and induced VOC emissions can be expected from plant roots and other microbes in the rhizosphere, making it important to investigate how their emission changes during the plants lifetime as a result of changing environmental conditions. However, there is another important source of soil VOCs: the microbial decomposition of plant litter (Mäki et al., 2019; McBride et al., 2020). The emission of VOCs from litter decomposition can be very high, with for example *Pinus* litter having an emission of 3-11 g of VOC-C m^{-2} yr^{-1}. An emission rate that is comparable to the emission coming from *Pinus taeda* L. roots, which have an emission of 9 g of VOC-C m^{-2} yr^{-1} (McBride et al., 2020). Some VOCs that are emitted during the decomposition of litter, are similar to those measured during the leaf senescence of herbaceous plants and broadleaf trees such as methanol, acetic acid, acetaldehyde and formic acid (Portillo-Estrada et al., 2020). So due to the abundance of these VOC sources in the soil we shall expect to measure these VOCs in large concentrations.

It is important to note that despite the difficulties in the measurement of soil VOCs, there has been an increasing interest in researching the role VOCs play in facilitating the interactions between plants, soil bacteria and fungi in recent years (Schenkel et al., 2018; Singh et al., 2021), with multiple studies focusing on the influence of microbial volatiles on the growth of plant roots (Moisan et al., 2021; Schenkel et al., 2018), their role in the defence against root pathogens (Singh et al., 2021; van Agtmaal et al., 2015; Wietse et al., 2019) and in increasing resistance against abiotic factors (Weisskopf et al., 2021; Yasmin et al., 2021). A good example is a study by Yasmin et al. (2021), who found that VOCs emitted by "Plant growth promoting rhizobacteria" (PGPR) increased drought resistance and growth of maize plants during drought stress. Another example is the study by Moisan et al. (2021), who found that VOCs emitted by the pathogenic fungus *Rhizoctonia solani* AG2-2 IIIb caused a significant directional root growth in *Brassica rapa* L. plants towards media inoculated with the pathogen. In both studies the VOCs were measured from cultivated microbes grown on specific media such as MS agar and $1/5^{th}$ PDA medium in Yasmin et al. (2021) and Moisan et al. (2021) respectively. There is however a big problem with this approach due to the fact that the microbes in these media are not exposed to

the VOCs and other secondary metabolites emitted by other microbes and the host plants, compared to the natural rhizosphere community, which increases the probability that the VOCs emitted by the species grown in agar are different compared to the natural conditions (Singh et al., 2021). There are however also some studies that do measure the VOCs from soil samples (in a pot or petri dish), to get a more accurate picture of the VOCs emitted by those soil microbe communities (Schenkel et al., 2018; Singh et al., 2021; van Agtmaal et al., 2015). It is however important to note that this still does not provide a full picture of the VOCs emitted by the rhizosphere, because of the missing influence of plant roots. Which is not the case in direct soil measurements, where their influence on the emission of soil VOCs is taken more into account. This raises the question: why is this method is not used in the above-mentioned studies? The reason why is probably due to the aforementioned difficulties in measuring the VOCs directly from the soil, which makes further research in direct soil VOC measurement methods necessary. For this reason, we will try to find a method to measure VOCs directly from a living rhizosphere.

Research into VOC emissions in the rhizosphere is particularly important to research the mutualistic relationship between plants and mycorrhizal fungi, one of the most well-known symbiotic relationships (Genre et al., 2020; Tedersoo et al., 2020; van der Heijden et al., 2015). A widespread relationship in which the vast majority of vascular plants (85-90% of all angiosperms) partake in (Genre et al., 2020). There are several advantages for plants to form this symbiotic relationship, like the provision of nutrients (mostly nitrogen (N) and phosphorus (P)) in exchange of carbon of the host plant and the protection against biotic and abiotic stressors such as pathogens, drought and heavy metals (Tedersoo et al., 2020; van der Heijden et al., 2015). In this project we will focus on the influence of arbuscular mycorrhizal fungi (AMF), one of the four types of mycorrhizal fungi with the others being ectomycorrhizal fungi (ECM), orchid mycorrhizal fungi (ORM) and ericoid mycorrhizal fungi (ERM). AMF is the most common mycorrhizal fungus type, with ca. 71-72% of all plant species forming an association with them (Brundrett and Tedersoo, 2018; Genre et al., 2020; van der Heijden et al., 2015). There are several features which are unique to each type of mycorrhizal fungi, but only the features of AMF will be discussed. AMF mostly belong to the phylum Mucoromycota, subphylum Glomeromycotina (Bonfante and Venice, 2020; Brundrett and Tedersoo, 2018; Tedersoo et al., 2020) and form an obligate mutualistic relationship with most Angiosperms, ferns and bryophytes (however most mosses are non- mycorrhizal). The most distinguishable structures that they form are arbuscules and vesicles, which are formed inside the cell (figure 1 A and B). They supply host plants with phosphorus (P) and have a low carbon (C) degradation capacity (Brundrett and Tedersoo, 2018; Tedersoo et al., 2020). Currently, there is a lot of uncertainty about how AMF can influence the VOCs emitted by host plants (Meier and Hunter, 2019). Research on the influence of mycorrhizal fungi on

the emission of volatiles has so far focused more on the aerial parts of the host plants than on roots (Sun and Tang, 2013). An example of their influence on aboveground VOCs was discovered by Velásquez et al. (2020), who found that there was an 85% increase in defence-related VOC emissions in the leaf tissue of *Vitis vinifera* L. cv. Sangiovese plants inoculated by the AM-fungus *Funneliformis mosseae*. However, this increased leaf VOC emission is in most cases very species specific as proven by Meier and Hunter (2019), where they found that AMF colonisation increased the constitutive VOC emission of GLVs and methyl-salicylate in *Asclepias curassavica* L*.,* but not in *Asclepias incernata* L. plants. It is however important to note that the increased emission of the induced VOCs caused by aphid herbivory, happened in both species independently of AMF colonisation. Making the relationship between AMF and VOC emissions complicated. An example of the influence of AMF on root VOCs has been found in the study of Sun and Tang (2013), who discovered that AMF inoculated *Sorghum bicolor* (L.) Moench roots emitted more alcohols, ethers, alkenes and acids compared to non-inoculated roots. However, this study did not measure the VOCs emitted by inoculated plants from the soil, but from harvested roots. This will be different in the current experiment, where the measurements are done primarily non-destructively with direct measurements from a living rhizosphere community in order to understand how AMF can influence rhizosphere VOC emissions.

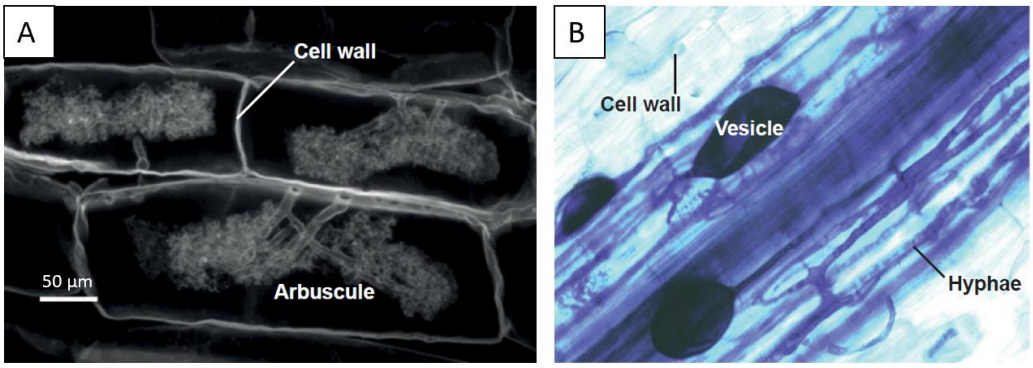

Figure 1: The most distinguishable structures of arbuscular mycorrhizal fungi (AMF). A) Arbuscules, B) Vesicles. A) The arbuscules formed by AM-fungi in *Pisum sativum* L. roots (Figure adapted from Fig. 1 b in van der Heijden et al. (2015)). B) Vesicles of the AM-fungus *Glomus intraradices* in trypan blue stained clover roots (Figure adapted from Fig. 1 a in van der Heijden et al. (2015)).

This research project will investigate how maize (*Zea mays* L.) roots and the AM-fungus *Glomus irregulare* (DAOM198197) are able to affect the emission of VOCs from the soil over time. We will also test for differences in soil VOC emissions between maize plants grown in different agricultural management practices. In addition, we have also tested how the VOC emissions from the soil differed from harvested roots.

In this study, we asked the following questions:

1. Are we able to distinguish a maize rhizosphere community that is inoculated with the AM-fungus *G. irregulare* from a community that is not inoculated with AMF using the measured soil VOC profiles? Can we identify specific VOCs that are produced in higher concentrations in a rhizosphere inoculated with or without AMF?
2. Are we able to measure VOCs emitted by a rhizosphere community reliably with a direct soil VOC measurement method? And how does the emission VOCs change throughout the plant's lifetime depending on treatment?
3. Do we measure a difference in the emitted soil VOCs from the rhizospheres of maize plants grown in different agricultural soil management practices? And how does the soil microbe community in different agricultural soil management practices influence the emission of VOCs by harvested plant roots?

It was hypothesised that there would be a difference between the VOCs profiles of rhizosphere communities inoculated with and without AM fungi, because of the higher emission of fungal VOCs as a result of AMF growth. Additionally, the number of emitted VOCs are predicted to increase over time due to increased root and hyphae activity in the later stages of the plant's life, with the concentration of certain VOCs increasing or decreasing over time. We also hypothesise that there will be some difference between the soil VOC profiles emitted by certain types of agricultural management practices due to a difference between the microbial communities, making it possible to distinguish the treatments. Moreover, it is expected that the VOCs emitted from harvested roots will differ between agricultural soil management practices. This is because of a change of root VOC emissions, caused by the interactions between the plant roots and soil microbes, depending on treatment.

2. Materials and methods

2.1. Experimental design

To test the effect of the soil microbial community on the production of volatile organic compounds, two experiments were carried out between July 23rd 2021 and January 14th 2022. The experiments were set up in building C of the Campus Drie Eiken of the University of Antwerp in Antwerp, Belgium (51° 9' 46.43552" N, 4° 24' 13.25554" E) in a well-lit experimental room. The first experiment investigated how the emission of soil VOCs from a rhizosphere community influenced by maize roots (*Zea mays* L.) and the AM-fungus *Glomus irregulare* (DAOM198197) evolved over a certain period of time, while the second experiment focused on the emission of VOCs from the rhizosphere of maize plants grown in a soil inoculated with multiple agricultural soil management practices.

In both experiments, maize plants were grown in individual plastic pots with drainage holes (28 cm width, 28 cm tall, 17 L), which were first disinfected with 1-5% diluted bleach. All pots were then coated in "Fluorinated Ethylene Propylene" (FEP) film of 0.05 mm thickness (200 gauge) (Adtech Polymer Engineering Ltd, Stroud, UK) on both the bottom and sides. FEP is a chemically inert material that does not emit VOCs (Mäki et al., 2019; Zhu et al., 2015), therefore preventing interference in the VOC measurements by emissions from the plastic pots, caused by the chemical degradation by UV-light and heat (Curran and Strlič, 2015; Lomonaco et al., 2020). The bottom FEP layer had holes punched into it to allow the flow of water. The bottom of the pots was also covered in perforated non-weaved polypropylene geotextile of a 2 mm thickness and a mesh size of 90 µm (THRACE NonWovens & Geosynthetics S.A., Athens, Greece), to avoid soil loss from the drainage holes and to increase the water permeability. The bottom FEP layer was placed above the polypropylene geotextile layer to avoid the emission of VOCs by this layer, which like most polymers can also emit VOCs through chemical degradation processes (Lomonaco et al., 2020).

In both experiments, an acid sandy soil (pH = 5.9) with low organic matter was used as a 'background soil', which was adapted for both experiments. This soil was obtained from a local supplier (Arabesk tuinarchitectuur, Zandhoven, Belgium). Before use, this soil was pasteurized in an electric soil steriliser (Pro-Grow Supply Corp model SS-60R, Brookfield, WI, USA) for a period of 6 hours at 82 °C. This was done because prior research found that low temperature steam treatments at around 60-80 °C are effective in killing most fungi and other soil organisms like nematodes (Dietrich et al., 2020; Van Loenen et al., 2003). Each pot in both experiments was initially filled with 15 kg dry weight background soil (weight measured with a 65,000 ± 1 g measuring scale (KERN & SOHN GmbH model fkb 65k1a,

Balingen, Stuttgart, Germany) and moisture with a soil moisture sensor (IMKO micromodultechnik GmbH model HD2, Ettlingen, Germany)), which slightly varied between the experiments.

Experiment 1

Experiment 1 (will be also referred to as the "AMF experiment") had three treatments: 1) Soil inoculated with the AM-fungus, but without maize plants (P-AMF+), 2) soil with maize plants, but not inoculated with an AM-fungus (P+AMF-) and 3) soil with maize plants and inoculated with an AM-fungus (P+AMF+). The main treatments (P+AMF- and P+AMF+) of the experiment both had 5 replicates, while the no plant control (P-AMF+) had three replicates. In total, 16 pots were used in the first experiment, with 13 used for VOC measurements and 3 additional pots used as extra replicates to analyse the AMF colonisation and the composition of the soil microbial community. The treatments of these 3 pots were P+AMF-, P+AMF- and non-sterile soil. The soil mixture in all pots had a dry weight of 15 kg. The soil inoculation protocol is explained in 2.3.

The first experiment was done in two runs, with the first run between the 23rd of July 2021 and the 9th of September 2021 and the second run between 16th of September 2021 and the 12th of January 2022. The first run was done to test the following factors: the right method to measure soil VOCs, to see if the inoculation of the soil with *Glomus irregulare* was successful and if the type of maize (the dwarf maize "TomThumb popcorn" (Le Grainier Sàrl, Bex, Switzerland)) used was suitable in the experimental conditions. To measure VOCs from the soil, a modified glass funnel was placed in the soil, which consisted of a 1/8 inch outer-diameter PTFE tube inserted into a glass funnel (Glasatelier Saillart bv., Meerhout, Belgium) and secured with parafilm. The funnel was made of glass, because it does not emit VOCs (Ahmed et al., 2018; Alapieti et al., 2021). This funnel was used to create an air pocket in the soil where the VOCs from the soil could be collected and later extracted for measurement.

During this first run, we discovered that the initial background soil mixture was not suitable for the measurement of VOCs due to a low soil amount of macropores in the soil, which made the transport of VOCs through the soil matrix difficult (Kai et al., 2016; Pandey Bipin et al., 2021; Schmidt et al., 2019; Tyc et al., 2017). It is however important to note that VOCs can also diffuse through water filled pores, but over shorter distances (Kai et al., 2016; Tyc et al., 2017). Given the unsuitability of the initial soil type, a new 18 kg dry weight mixture was made consisting of 50% river sand (Betonfabriek Coeck, Niel, Belgium), 40% of the inoculated background soil and 10% lava (De Ceuster Meststoffen NV/SA (DCM), Grobbendonk, Belgium). The lava was used because it improves the drainage of heavy wet soils, according to the supplier. This mix had a coarser soil texture, due to the sand, allowing more rapid water and air movement (Pepper and Brusseau, 2019). The background soil was re-used because of

the successful inoculation of maize roots during the previous run. The type of maize was also changed to a bigger variety: highly inbred *Zea mays* L. ssp. *mays* W22 Goodman-Buckler (Accession: PI 693394, USDA ARS Iowa State University, IA, USA), which can create a more extensive root network compared to the dwarf maize, making the measurement of belowground VOCs more likely.

In the second run, two kinds of stainless steel cores were also installed in the soil of each pot (stainless steel (type 304: 72.4% Fe, 18% Cr, 8.2% Ni, 1.1% Mn) 10 O.D. cm × 12 cm depth closed-bottom soil cores (Yuansheng Industrial Co., Ltd, Shantou, China)), creating two compartments: one with a 1 mm mesh where fine plant roots and mycorrhizal hyphae can grow in and one with 30 µm mesh where only mycorrhizal hyphae can grow in. By creating compartments, we can investigate whether certain VOCs are produced only from mycorrhizal fungi (figure 2 B). The cores were only in the pots during in the second run of experiment 1, to determine the origin of the VOC emissions in more detail. The cores were not installed in experiment 2, because in that experiment we did not investigate the origin of the VOC emissions in more detail (figure 2 A). The cores were sterilized with 1-5% diluted bleach before being placed in the pots with the new soil mixture. The cores were made out of stainless steel, because it is an inert surface that does not emit VOCs (Auguste and Miller, 2020; Mäki et al., 2019). The modified funnels were further modified for the second run of experiment 1 by covering them with black isolation tape to avoid algal growth due to light incidence.

Experiment 2

In the second experiment (will also be referred to as the "Soil experiment"), the agricultural soil used to inoculate the background soil (see 2.3 for inoculation procedure) was sampled from the multiyear field trial (BIOPACT), managed by "the Flemish institute for Agricultural and Fisheries research" (ILVO). The trial was located at ILVO plant 9 located in Merelbeke, Belgium (50°59'6.84'' N, 3°46'24.10''E, 24 m above sea level), an area with a fully humid temperate climate with warm summers (a mean annual temperature of 9°C and an annual precipitation of 725 mm) (D'Hose et al., 2016). The soil in this field is sandy loam with a soil particle size distribution of 57.0% sand, 37.7% silt and 5.3% clay (D'Hose et al., 2016). The BIOPACT field trial had a four-crop rotation on a four-year basis at the time of sampling, with the following crops: potato (*Solanum tuberosum* L.), forage maize (*Zea mays* L. ssp. *mays*), spring barley (*Hordeum vulgare* L.) and fodder beet (*Beta vulgaris* L. ssp. *vulgaris* var. *crassa*) (fodder beet replaced leek (*Allium porrum* L.), which was used in the previous rotations by D'Hose et al. (2016)). Three of the four crops in rotation are able to form a symbiotic relationship with mycorrhizal fungi, namely potato, forage maize and spring barley (Genre et al., 2020; Jerbi et al., 2022). The only crop that does not form a mycorrhizal symbiosis is fodder beet (Fernández et al., 2019; Yolcu et al., 2021), the crop which grew on the agricultural field at the time of sampling. However, we still expected that

mycorrhizal symbiosis could be established despite the fact that the non-mycorrhizal crop fodder beet was planted at the time, because of remaining spores in the soil from previous rotations. We sampled 1000 mL topsoil (0 to ±10 cm) from four agricultural management practices (four replicates per treatment) used in the field trial, which were: “ploughing” (P), “ploughing with compost” (PC), “non-inversion tillage” (NI) and “non-inversion tillage with compost” (NIC). The sampled plots were all fertilised with pig slurry (D’Hose et al., 2016).

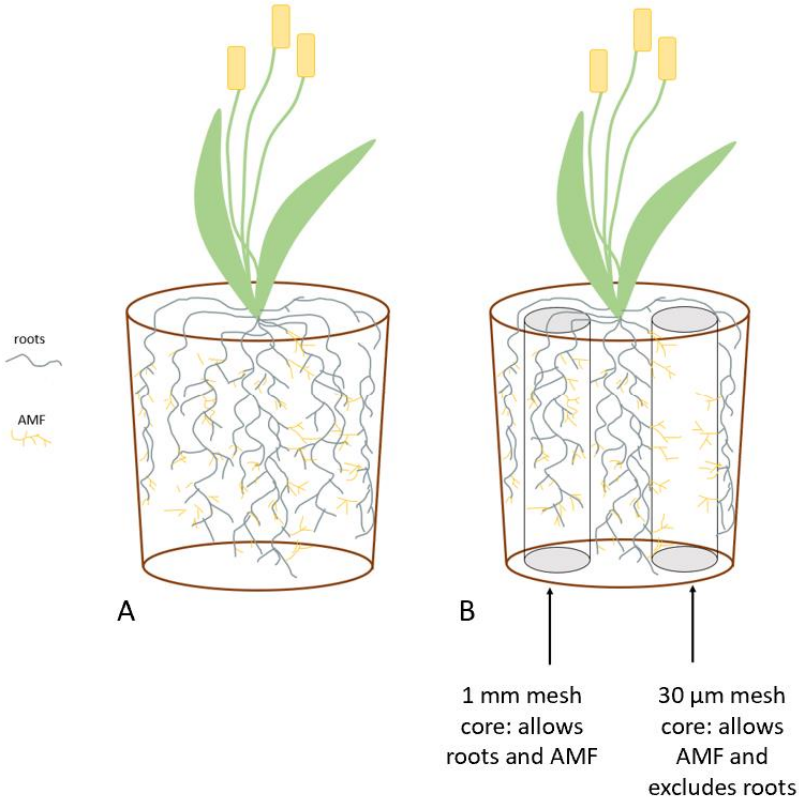

Figure 2: Set-up pots experiment 1 and 2. A) Experiment 1 run 1 and experiment 2. B) Experiment 1 run 2, with the cores included.

2.2.Plant growth conditions

Before sowing, the maize seeds were sterilised using the protocol from Yandeau-Nelson (2010). The first step was to place the needed number of seeds in a 100 mL plastic pot. The seeds were then covered with pure bleach and inverted 3-4 times to get sterilised, followed by rinsing 8 to 10 times with Milli-Q® water. After sterilisation two non-germinated maize seeds were sown in each pot, to increase germination success. In addition several seeds were left to germinate on moistened geotextile (a material that retains and diffuses water well) in a petri dich closed with parafilm, to serve as replacements in case the original seeds in the pots did not germinate. The best germinating seeds for that purpose were visually selected. In most cases, only one seedling throve of the two planted seeds,

with only a minority with two seedlings. In those cases, one of the seedlings was removed in order to have one plant per replicate.

The plants were grown under a light rack specifically built for this experiment (figure 3). The structure had a length of 6 m, a width 1 m wide and height of 2.20 m and was subdivided in four compartments. Inside each compartment, eight pots could be placed. For each experiment, two compartments were used, totalling 16 plants per experiment. Three 80-W full spectrum LED growth lamps, that delivered precision light at 2.5 µmol/J, were installed in each compartment to make the germination and growth of the plants possible (Specifications: 80 W, PF ≥ 0.9, 0.9 A, Max AC, 100-277V, 50/60 Hz, 2.5 µmol/J, 200 µmol/s; Total Grow model Mezzo, Holland, MI, USA). The lights were hung on adjustable cable hangers, initially at a height of 1 m from the floor. The plants were grown under a photoperiod of 16h:8h day:night, with the day period running between 5:47 h in the morning to 21:47 h in the evening (Western European time GTM+1 with summer correction) to match the solar noon at the location (at 13:47) with the midday of the artificial photoperiod of 16h. Additionally, the plants were also exposed to natural light from the window to improve their growth conditions, however, the strength of the growth lamps avoided phototropism towards the natural light of the windows. Each pot was put in the light rack on individual saucers with a diameter of 35 cm, to avoid exchange of leached water among the different pots.

Figure 3: Maize plants growing inside two compartments of the wooden light rack in experiment 1 run 2. Each compartment contains eight pots.

The plants were watered once a week on the saucers, where the water diffused into the soil. This was done to avoid soil compaction caused by the pressure applied by a water stream on the topsoil. However sometimes the plants were gently sprayed from the top to avoid dehydration of the topsoil

layer. The relative humidity of the room was monitored and kept at around 50%. It is important to note that the plants were always watered after the soil VOC measurements, because a high soil water content negatively influences the diffusion of VOCs. This is due a multitude of factors, like the dissolution of polar VOC compounds and the promotion an higher uptake of VOCs by soil microbes (Insam and Seewald, 2010; Mäki et al., 2019; Rinnan and Albers, 2020).

The plants were fertilised with a NPK fertiliser twice during both runs of the experiment 1 and twice in experiment 2 as well. In both experiments 100 mL of NPK fertiliser was added to each pot. This fertiliser consisted of a 10 L mixture of the following macronutrient salts: 6.92 g of 45% P_2O_5 (Scoriethom, Geel, Belgium), 76.08 g of $Ca(NO_3)_2$ and 23.97 g of K_2SO_4 (both from Aquariumbemesting.nl, Eindhoven, The Netherlands). Additionally, we also added 2.72 g of essential micronutrients in the NPK solution ("Tenso sporenelementen"; Aquariumbemesting.nl, Eindhoven, The Netherlands). We made sure to not add too much NPK fertiliser to the pots, because it makes the establishment AMF symbiosis more complicated. This is particularly the case for P, because a high P concentration makes the establishment of the AMF symbiosis more unlikely (Ven et al., 2019).

2.3.Mycorrhizal inoculum extraction and soil inoculation

In both experiments the soil was inoculated with the given treatment before the seeds were planted. There were big differences between the soil inoculation protocols in both experiments, because of the use of differing types of inoculum. In experiment 1 the inoculum consisted of root fragments cut from a maize roots colonised by *Glomus irregulare* (DAOM198197), while in experiment 2 the inoculum was the extracted soil from a field experiment (See 2.1).

For the soil inoculation of experiment 1 (both runs), we received a 50 mL falcon tube with maize roots inoculated with *Glomus irregulare* (DAOM198197). The inoculum was collected on the 15th of July 2021 (run 1) and the 8th of September 2021 (run 2) in differing amounts, 13 g and 23 g in run 1 and 2 respectively. This influenced the amount of inoculum used for soil inoculation, with 0.7 g added in the soil for the first run and 1.0 g in the second run. The first step in the preparation was to cut the maize root in several 2-4 mm root fragments, followed by weighing the amounts wanted for the inoculation for the pots (for each pot separately). The inoculum was then subdivided between treatments. For the treatments P-AMF+ and P+AMF+, the inoculum was put in 9 petri dishes closed with parafilm and preserved in a refrigerator until inoculation. For the treatment P+AMF-, the roots were put in 100 mL Erlenmeyer flask, covered and sterilised in an autoclave at 121 °C and pressure >1 bar for a period of 25 min (Vertical autoclave, VAPOR-Line, VWR part of Avantor, Radnor Township, PA, USA) to kill all the AM-fungus spores. After the subdivision, a certain amount of root fragments (2.23 g in run 1 and 6.5 g in run 2) was preserved to make a microbial wash (300 mL in the first run and 400 mL in the second

run). This is a wash consisting of the root bacteria included in the 'root water' (the fluid where the root network was kept in) and the bacteria on the living maize root fragments. To make the microbial wash, a part of the root fragments was first added to the 50 mL falcon tube containing 'root water'. This was followed by adding milli-Q® water in the falcon tube until it reached a volume of 25 mL. The root fragments and fluid were then mixed well with and filtered through a funnel with a 12-15 µm mesh to filter out the AM fungus spores. This was done until we had a 100 mL filtrate, which was then diluted with milli-Q® water until the wanted volume was reached. This whole process had to be done carefully to avoid contamination of the microbial wash with fungal spores.

After finishing the preparation, the inoculation itself could begin, which differed between runs. In the first run there was a 5-10 cm hole made in the centre of the pot (below the area where the plant grows), where the root fragments were put in. To avoid cross contamination the pots of the P+AMF- treatment were inoculated first, followed by the inoculation of the pots with the P-AMF+ and P+AMF+ treatments with living inoculum. The last step of the inoculation was addition of 20 mL microbial wash to every pot (for both run 1 and 2). The inoculation was slightly adjusted in the second run: first we removed 1 L of topsoil from the layers in-between the two cores (at the very centre of the pot), followed by mixing the root fragments in deeper soil layers. This was done because the soil already had a lot of inoculated maize root fragments in the soil remaining from the first run. Making the role of the newly added root fragments different: serving as an additional source of fungal spores to stimulate the formation of AM-symbiosis.

In the second experiment the pots were inoculated by mixing the agricultural soil samples with the sterile background soil. The agricultural soil made up 5% of the total dry weight of the soil in the pot, which was 789 g of inoculum per pot (making a total dry weight of 15 789 g per pot). The inoculation began with scooping out 1 L sterile topsoil from the pot, which was left on the side to put over the mixed soil after mixing as a protecting layer. In the next step, 2 L of background soil was scooped out from the deeper layers of the same pot. This soil was then mixed with 789 g dry weight of the agricultural soil and put in the pot again and covered with the sterile topsoil. The dry weight of the agricultural soil was determined by drying a part of the sample in a drying oven to determine the moisture level of each soil sample.

2.4. VOC measurements

To identify the VOCs emitted by the plants, microbes and AM-fungi in the rhizosphere a "Proton-Transfer-Reaction Time-Of-Flight Mass Spectrometer" (PTR-TOF-MS 8000, Ionicon Analytik GmbH, Innsbruck, Austria) was used. The functioning principle of the mass spectrometer is explained in Supplement 1. In both experiments the following settings were used: In the drift tube the pressure was kept at 2.3 mbar, the voltage at 600 V and the temperature at 80 °C to reach a field density ratio (E/N) of 140 Td (Td = Townsend; 1 Td = 10−17 V cm2) (see Supplement 1 for a more detailed explanation about its importance). Mass spectra were measured every 32 µs with a mass range of 1-318 m/z ("m/z" = mass over charge). Every week before the measurement began, the mass spectrum was first calibrated in the PTR-TOF-MS with a two-point calibration using the following compounds with known molecular masses (one at the beginning and one at the end of the mass spectrum): a nitrosonium ion (NO^+, *m/z* = 29.99744 Da) and a fragment of 1,3-diiodobenzene ($(C_6H_4I)H^+$, *m/z* = 203.94305 Da). The fragment of 1,3-diiodobenzene is a good calibration compound, because it is constantly permeated into the air sample.

The measurement of VOCs in experiment 1 and 2 involved the use of a FORTUNA® 100 mL all glass syringe (Poulten & Graf GmbH, Wertheim am Main, Germany) with a Luer lock and a stainless steel needle. In the first step of the measurements the syringe needle was inserted into the 1/8 inch O.D. teflon tube, which is part of the modified funnel, to sample 40 mL of air from the air pocket in the funnel (figure 4 A). The sampled air was then inserted in the 1/8 inch PTFE tube, which was attached to the PEEK inlet of the PTR-TOF-MS, followed by a slow and constant administering of air sample to the mass spectrometer to measure the VOCs (figure 4 B). For each measurement, the time ranges (t_{begin}, t_{end}) were written down in seconds using the increase in concentrations of monitoring compounds as a guide to determine between which time range a sample was inserted. These time ranges were later used to average the spectra of each measured sample, in order to determine the average VOC concentrations. It is because of this reason that it is important that the time ranges included an equal representation of both fluid (VOCs measured first by the PTR-TOF-MS) and sticky compounds (VOCs that are measured later) (see 2.6) (figure 4 B). Monitoring compounds used for this purpose were of acetaldehyde ($(C_2H_4O)H^+$, *m/z* = 45.033 Da), ketene ($(C_2H_2O)H^+$, *m/z* = 43.018 Da) and acetic acid ($(C_2H_4O_2)H^+$, *m/z* = 61.028 Da). For each measurement we also wrote down in which spectrum file, generated by the PTR-TOF-MS, the data was saved. This is important for the data analysis phase (see 2.6).

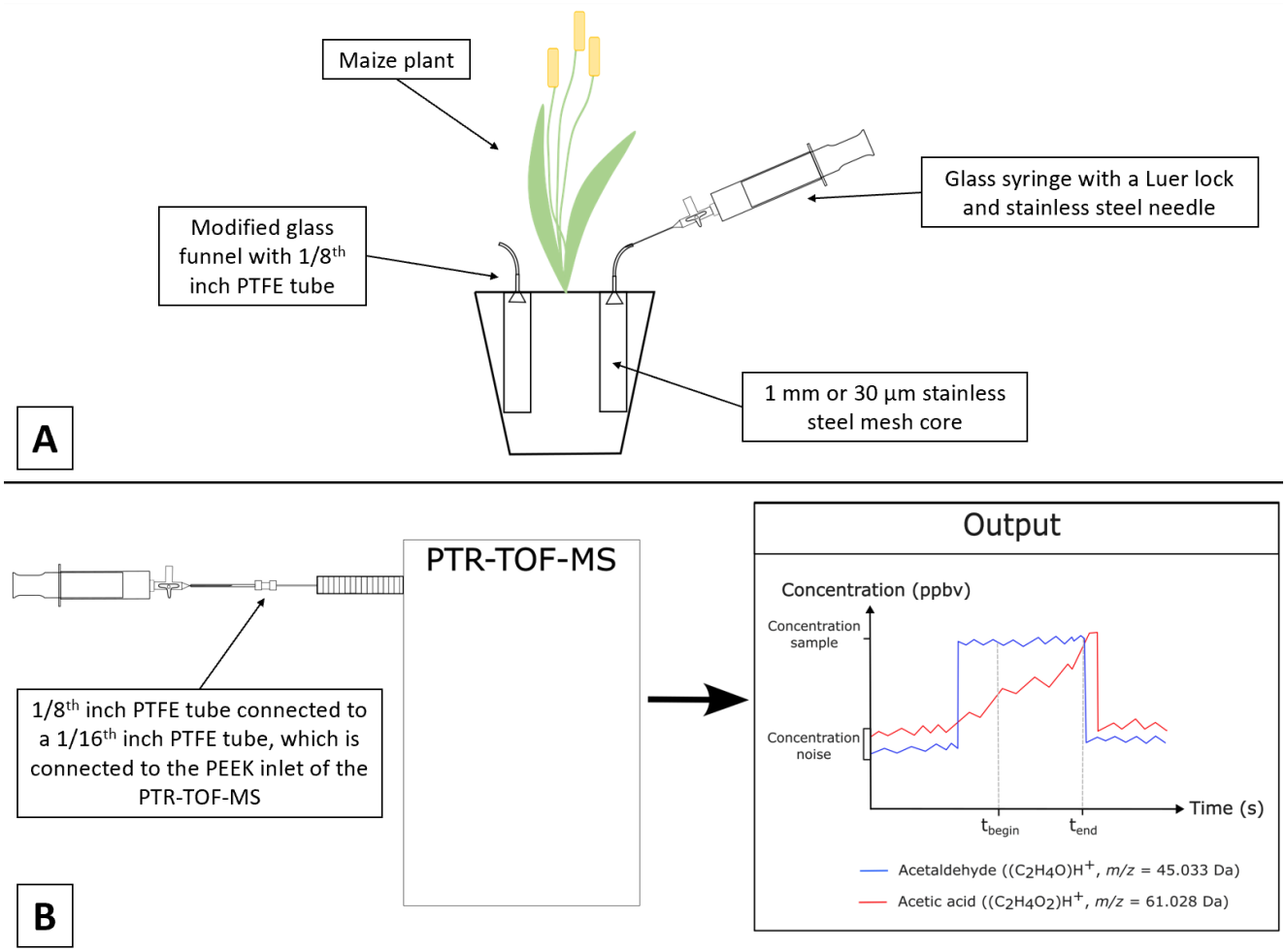

Figure 4: The measurement protocol for soil VOC measurements. A) The sampling of 40 mL air from the inverted funnel with a 100 mL glass syringe for the measurement of VOCs. B) The administering of the 40 mL gas sample in the PTR-TOF-MS to measure the soil VOCs. The measurement is tracked in the output, where the concentration of monitoring compounds such as acetaldehyde ($(C_2H_4O)H^+$, m/z = 45.033) and acetic acid $(C_2H_4O_2)H^+$ increase as a result of the addition of the air sample. In each measurement the time range was measured to average the spectra. This time range (t_{begin}, t_{end}) was selected in such a way that it had a good representation of the concentrations of fluid (acetaldehyde) and sticky compounds (acetic acid).

2.5. Harvest, root VOC measurements and soil DNA analysis

After the soil VOC measuring period ended (in experiment 1 run 2 on December 23rd 2021 and experiment 2 on October 28th 2021), the maize plants were harvested for the following analyses: the VOCs emitted by exposed roots and soil, the dry weight of the plant, the level of mycorrhizal colonisation of the maize roots and the DNA sequencing of the organisms in the rhizosphere and soil. The methods used for the measurement of root VOCs and the determination of the dry weight of the above and belowground biomass will be discussed in this chapter, while the protocols used to determine the level of mycorrhizal colonisation and DNA sequencing are discussed in more detail in Baekelmans (2022).

The first analysis, the VOCs emitted by exposed roots and soil, was only done after the harvesting of experiment 2. This was not done for the second run of experiment 1, because of a defect with the PTR-TOF-MS. The main goal of these measurements is to analyse how the VOCs emitted by exposed roots can differ from when they were in the soil. During this analysis we also used three treatments: 'clean soil' (soil from the pots without root fragments), 'dirty roots' (roots covered in rhizosphere soil) and 'clean roots' (roots without rhizosphere soil). The first step in this analysis was to remove the aboveground biomass of the harvested maize plant. This is followed by harvesting the plant root network from the soil. After harvesting, the root network was split in two: one half of the network was rinsed and dried to make it clean, while the other half was not. The three treatments were put in three separate glass jars closed by perforated lids (each lid had two holes). The holes were then covered with parafilm and incubated for one hour. After one hour the VOCs were measured by puncturing a parafilm covered hole with the needle of the syringe, followed by sampling 40 mL of air from the jar and inserting it in the PTR-TOF-MS for measurements.

In all experiments the dry weights of both the above and below ground biomass was measured to see if there were significant differences between the treatments. For the determination of the dry weight of the above-ground biomass, we measured the biomass of the vegetative (stem and leaves) and reproductive tissues (seeds) in all experiments. We compared the dry weights of the vegetative and reproductive tissues both separately and combined. This is different for the determination of the dry weight of the below ground biomass (the root network), which differs between experiments. In the first run of experiment 1 only the below ground biomass was not measured, because the root network was used for the inoculation of the soil for the second run. In experiment 2 the dry weight of the root network was measured, using the dry weight of the roots used during the root VOC measurements. In experiment 1 run 2 the below ground biomass was also measured, through the analysis the roots in the 1 mm core. We only analysed those roots, because the root that grew in the 1 mm core were the ones analysed during the soil VOC measurements.

2.6.Data analysis VOC measurements

The data analysis of the measured VOCs consisted of two parts: 1) the identification of VOCs and 2) determination of the average measured concentrations per treatment. The first part of the data analysis was performed in the software PTR-MS Viewer version 3.4.2. (Ionicon, Innsbruck, Austria), while the second part of the analysis was performed in Microsoft Office Excel (Microsoft, Albuquerque, NM, USA).

2.6.1. The identification of emitted VOCs

There were several steps in the identification of VOCs. They were the following: 1) mass calibration, 2) defining average spectra per measurement, 3) investigating the presence of the known compounds and 4) the identification of previously unmeasured VOCs. Each step of the protocol will be explained in this chapter.

The first step in the analysis was the mass calibration of the uploaded spectrum files, which included the measurements, in the software. The mass calibration of the spectra is generally always done before the measurement itself starts (see 2.4), but it is important to double-check it before further processing. The same two aforementioned compounds are chosen for the calibration, namely NO^+ (*m/z*= 29.99744 Da) and $(C_6H_4I)H^+$ (*m/z*= 203.94305 Da). This is because they are easily identifiable peaks with known molecular masses.

After the mass calibration, several averaged spectra were defined based upon the time range (t_{begin}, t_{end}) that corresponds to a certain treatment and date. The reason why measured spectra are averaged according to their measurement time range is to reduce the noise, which is lower in an average spectrum. It is important here that each selected range includes both the “fluid” and “sticky” compounds, in order to have an equal representation of both types of compounds (figure 4 B).

Before the identification of unknown compounds was initiated, it was crucial to analyse the already known compounds. To do this the peak table had to be revised first. The peak table is a crucial part in the data analysis, because all the known VOCs, which were registered during previous studies, are listed in that table. The problem is that not all compounds are measured during this study, making it important to revise the table by reorganising the known VOCs in the list and deleting the non-measured volatiles. During the revision it was important to check the presence of each compound over multiple spectra to avoid pre-mature deletions. The revision of the peak table also included the formation of multipeaks, which are peaks formed by two or three compounds with close peak centres

and overlapping peak ranges. Another thing that was done during this step was the registration of the isotope containing molecules (molecules who include isotopes of e.g. carbon and oxygen, like ^{13}C and ^{18}O) of all known compounds, with a lot of attention going to the peaks of isotope containing molecules that overlapped with the peaks of other non-isotope containing molecules at the same m/z (see 2.6.2).

Investigating the peaks of the isotope containing molecules was also important during the investigation of the effects of ^{13}C labelling, in a pilot-experiment in which we tried to see if we were able to distinguish the VOCs emitted by plants from the ones emitted by soil microbes. The details about the ^{13}C-labelling experiment can be found in supplement 2.

The last step in the protocol was the identification of the VOCs that were not measured in previous studies. In this step it was important to select the average spectra with the best signal. In this project, these spectra were found in the measurements of the harvested roots which were incubated in a glass jar for an hour (see 2.5). After this selection it was possible to go through the spectrum to look for interesting peaks. A newly measured peak was registered when it met the following criteria: 1) the compound was not yet known (not already listed in our peak table), 2) it was found in some treatments but not in others and 3) The peak is large enough to cross the intensity threshold, which is an intensity of 1 mV (the intensity is a unit that describes how much of a certain compound is detected by the PTR-TOF-MS, with higher intensities in abundant compounds that are detected more). Above this threshold the peaks were big enough to not be considered as noise. For the identification of the new compounds the "identify" tab was used, a tab which investigates the peak to find out which compound it can be. A compound was registered in the peak table when it met the following conditions: 1) the centre of the recommended peak overlapped perfectly with the measured one, 2) the suggested isotope peaks also overlapped well with the measured peaks and 3) it was a realistic compound that can exists in natural circumstances. An example of this process was the peak seen at *m/z*= 63.026 Da for a 'clean root' measurement of experiment two of a plant grown in mixed agricultural soil which was ploughed and had compost added (PC) (Figure 5). The software recommended multiple compounds at this mass arranged according to their likelihood of being correct. Two recommended compounds were interesting: $(C_2H_6)H^+$ (figure 5 A and B) and $(CH_4NO_2)H^+$ (figure 5 C and D). But only $(C_2H_6)H^+$ was correct, because the peak of the theoretical curve (the green curve in figure 5) overlapped perfectly with the centre of both the measured peak (the purple peak in figure 5 A) and the peaks of the isotope containing molecules (also referred to as 'isotope peak') (figure 5 B). This was not the case for the other recommended compound: $(CH_4NO_2)H^+$, where the peak centres and the isotope peaks did not overlap with the theoretical peaks (figure 5 C and D). After the identification phase the peak table was calculated in each file, followed by the exportation of the measured VOC concentrations (in ppb). The exported files were used for the determination of the average VOC concentrations.

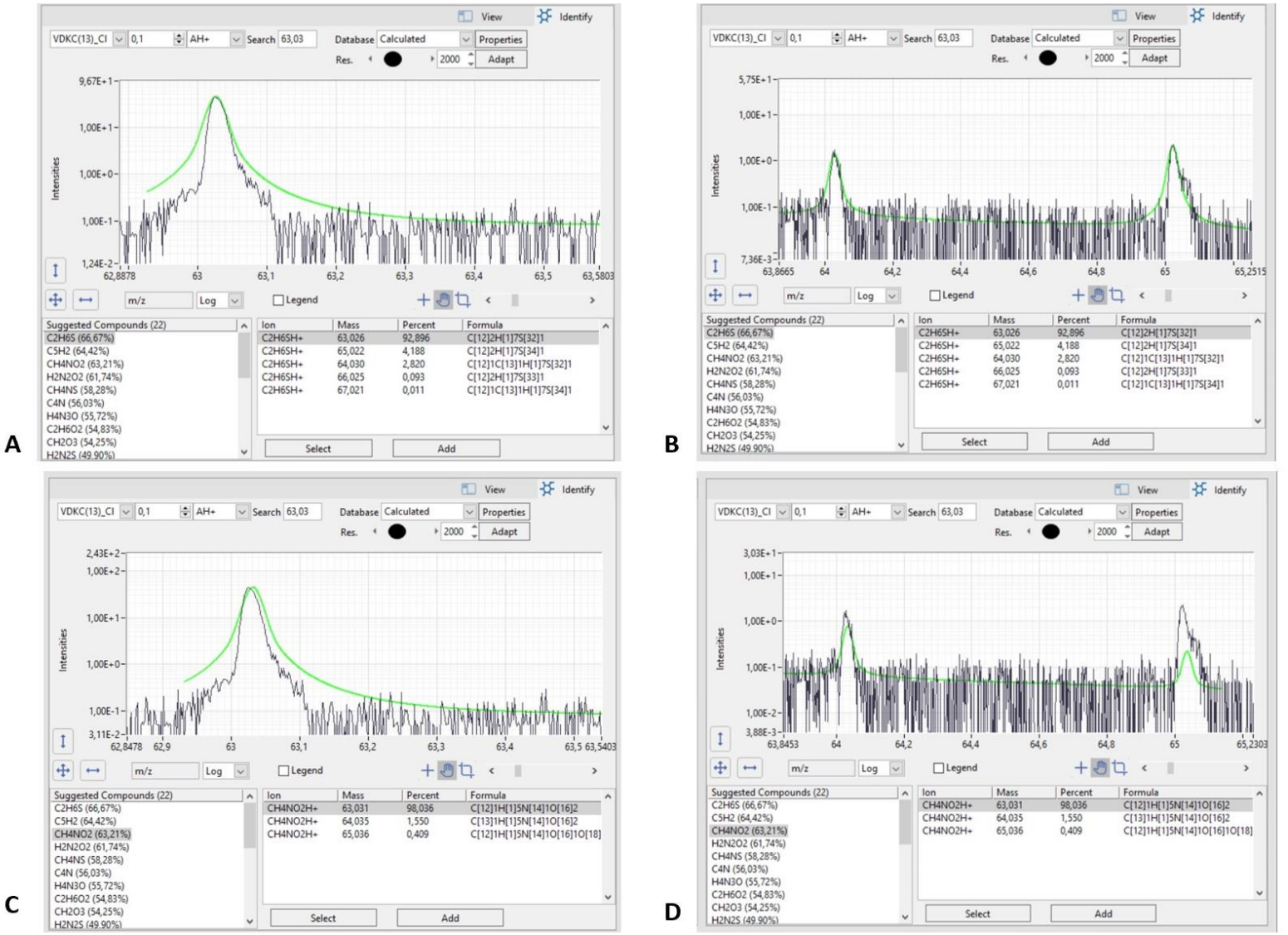

Figure 5: Determination of the correct compound in PTR-MS Viewer for *m/z*= 63.026 Da. A and B are both of the correct compound ($(C_2H_6S)H^+$), because the main peak overlaps with the suggested peak (green peak) (A) and the isotope peaks (B). This is not the case for the compound shown in C and D ($(CH_4NO_2)H^+$), where the main peak does not overlap perfectly (C) and the isotope peaks (D) do not overlap with the suggested peaks. In all graphs, the x-axis represents the molecular mass (m/z) in Da, while the y-axis represents the intensity (in mV), a unit that represents the amount of a certain compound detected by the PTR-TOF-MS. (Figure made in PTR-MS Viewer 3.4.2)

2.6.2. Determination of the VOC concentrations

The first step in the determination of the concentration of the VOCs, was a calculation of the average VOC concentration of all compounds for each sample across all dates of measurement. This was done in a similar way as the averaging of the spectra in the first part of the data analysis, using the time ranges and file info of every sample. The time ranges were either in seconds or cycles (for the measurements where the time ranges were registered later). A cycle corresponds to the time range of the spectrum recordings (every 32 µs) averaged into one spectrum. It was 1 s or 1.6 s during our experiment. Because the times were exported in cycles, the seconds had to be converted into cycles before the time range were able to be used for the averaging of the VOC concentrations. This was done using the averaged spectra, whereby 1 cycle was either 1 s or 1.6 s. After this conversion, all the average concentrations of the samples were calculated using the time ranges.

After the averaging of the concentrations two corrections occurred: an "isotope correction" and a "background emission correction". In the "isotope correction", the concentrations of the overlapping isotope containing molecule (the most common isotope is ^{13}C, followed by ^{18}O) is subtracted from the total concentration (figure 6), known as the artifact concentration ($C_{artifact}$) (see formula 1). This was done for 6 compounds shown in table 1. This correction had to be done to avoid overestimating the concentration of the actual non-isotope containing compound that is located at that m/z value. It is important to note that in general the concentrations of the isotope containing compounds are not accounted for.

$$C_{\text{compound 2}} = C_{\text{artifact}} - \frac{\text{isotope \% of total mass compound 1}}{100} * C_{\text{compound 1}} \qquad (1)$$

Table 1: The VOCs were there are overlaps between the concentrations of isotope containing and non-isotope containing molecules.

Molecular Mass (Da)	Non-isotope containing compound with overlap (Compound 2)	The molecule of which the isotope containing compound overlaps with the actual non-isotope containing compound located at that mass. (Compound 1)	Percentage of total molecule mass represented by an isotope containing compound (%)
46.029	$(CH_3NO)H^+$	$(C_2H_4O)H^+$ acetaldehyde	2.204
48.021	$(CH_3O_2)H^+$	$(CH_2O_2)H^+$ formic acid	1.174
58.041	$(C_3H_5O)H^+$	$(C_3H_4O)H^+$ hexenal (frag)	3.225
62.035	$(CH_3NO_2)H^+$	$(C_2H_4O_2)H^+$ acetic acid	2.236
63.026	$(C_2H_6S)H^+$	$(C_2H_4O_2)H^+$ acetic acid	0.415
94.041	$(C_6H_5O)H^+$ phenoxy radical	$(C_7H_8)H^+$ toluene + para-cymene	7.111

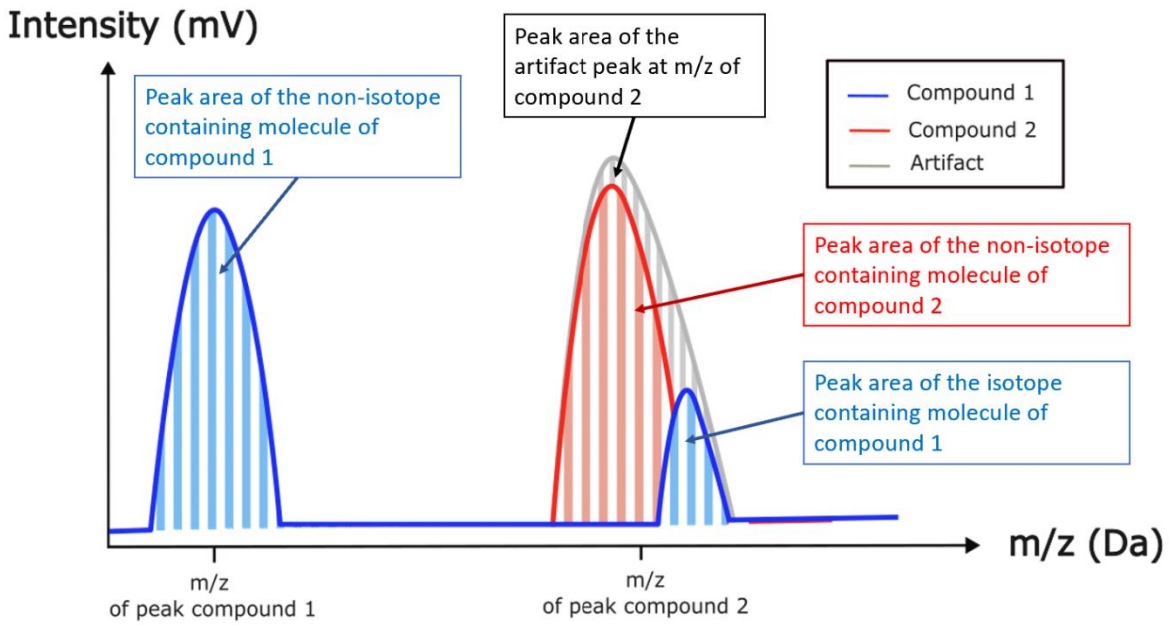

Figure 6: The principle behind the isotope correction. In order to calculate the correct peak area of "compound 2" (the peak area of the red peak), the peak area of overlapping "isotope containing molecule of compound 1" (the peak area of the smaller blue peak) has to be deducted from the peak area of the "artifact peak" (the peak area of the grey peak). The artifact peak area represents the combined peak areas of the "isotope containing molecule of compound 1" and "compound 2" at m/z of peak compound 2. The calculated peak area from the non-isotope containing molecule of compound 2 can then be used to calculate the concentration of compound 2, see supplement 1 for more information about this calculation. It is important to note that the concentrations of all peaks was already calculated by PTR-MS viewer 3.4.2 before the isotope correction was done, this is why the concentrations were deducted in formula 1. X-axis: m/z (Da)= the molecular mass, Y-axis: Intensity (mV)= unit that represents the abundance of a certain compound detected by the PTR-TOF-MS.

The last correction that was done was the “background emission correction”, where the emissions of a control pot were subtracted from the concentrations of a treatment to filter out the background variation (which differed between measurement dates). The concentrations that were used for the background emission correction were from the soil without plants (P-AMF+) for both experiment 1 run 2 and experiment 2. We always took the lowest concentrations for this purpose, because a control should not emit much. This means that we did not use all P-AMF+ replicates. The reason why the ones with a higher emission were ignored is because a high emission could point to a peak in microbial activity or something else in the soil or environment and can skew the data of the corrected concentrations. In case of the VOCs from the harvested roots (see 2.5), we used the clean soil of the pot as a control. Because every soil of each pot in experiment 2 was different, the VOC concentrations from the clean soil of the same pot as the roots was subtracted from the VOC concentrations emitted by the roots. After these corrections, two datasets were made for the statistical analysis: an isotope corrected dataset and a background emission corrected dataset.

2.7. Statistical analysis of the data

Both a one-way “Analysis of Variance” (ANOVA) and a TUKEY multiple comparisons of means post-hoc tests (only when there was a significant difference in the ANOVA test) were used to test if there was a significant difference between the dry weights of the maize plants grown under different treatments in experiment 1 (run 1 and 2) and experiment 2. The difference in dry weights was considered significant when $p < 0.05$. The dry weights of vegetative aboveground biomass (stem and leaves), the total fruit biomass, the total above-ground biomass (sum of the stem and leave biomass and total fruit biomass), the roots, and the total fruit mass were compared separately in experiment 1 run 2 and experiment 2. The root-shoot ratios were also compared. In experiment 2 the root biomass of the roots without rhizosphere soil was compared (known as ‘clean soil’), because in the ‘dirty root’ treatment the masses can vary based on the amount of rhizosphere soil remaining on the roots (see 2.5).

For the VOC measurements several tests were used to distinguish rhizosphere communities based upon their emitted soil VOC profiles. This was done for both the second run of the AMF experiment and the Soil experiment. Additionally, we investigated if we were able to distinguish the VOC profiles emitted by the harvested roots of experiment 2 (see 2.5) in context of both the state of the harvested root types (roots with and without rhizosphere soil) and the original agricultural soil management type (the treatments of experiment 2). A “Principle Component Analysis” (PCA) was done first for the VOC data in all experiments for both the data that was isotope and background emission corrected (will be referred as ‘corrected data’) and the data was only isotope corrected (will be referred as ‘non-

corrected data') (see 2.6). The main goal of this analysis was to investigate the impact of different potential sources of the variation in the soil VOC profiles, which are the experimental treatment (mycorrhizal colonisation in the AMF experiment and the agricultural management practices in the Soil experiment), the date of measurement (not a factor in the VOCs from harvested roots), the cores (only for the second run of the AMF experiment) and the harvested root type (only for the VOCs from harvested roots). For the PCA the non-corrected data was "composed", to only focus on how the proportions of the VOCs differed between treatments. This was done to prevent skewing the PCA towards the replicates with the highest total VOC emission. The main goal of the PCA of the non-corrected data was to test how much the time effect (which can be mostly caused by environmental factors) and treatment effects differed from the background emission corrected data. A PCA of the non-corrected data was not done for the harvested roots, because we are only interested in the difference between the VOC emissions of the root types and the agricultural management practices, not how it relates to the VOCs emitted from the clean soil. Additionally, time was not a source of variation in the harvested roots due to the fact that each replicate was measured once. The second test that was used in addition to the PCA is a "Permutational Multivariate Analysis of Variance using distance matrices" (PERMANOVA) using Euclidian distances, which investigated if there were any significant differences between the VOC profiles based on the main sources of variation and how much each source contributed to the total variation. The impact of a source was considered significant when $p < 0.05$ and strength of the contribution of each source was determined based upon the R^2 value, where the source with the highest R^2 value has the largest contribution on the total variation between the VOC profiles.

In the further analysis only the corrected data was used, because the time effect has less impact on the corrected data compared to the non-corrected data (see 3.2 and 3.3). This is probably due to the VOC emissions caused by changes in the background soil microbial community (not part of the rhizosphere) and environmental influences (e.g.: humidity and temperature), both factors that can change significantly between dates of measurement. After the PCA and PERMANOVA analyses, a "sparse Partial Least Squares Discriminant Analysis" (sPLSDA) was performed to test how well the experimental treatments were able to be distinguished based on their VOC profiles and to identify which volatiles played the most important role in this distinction. After the identification of the most important VOCs in experiment 1 run 2 and experiment 2, their emission was plotted over the lifetime of the maize plant using a scatterplot to see how the emission of these compounds changes. For the VOC emissions of the harvested roots, boxplots were made for the most important VOCs which were identified by the sPLSDA. In order to compare the VOC concentrations of those VOCs between the different treatments. In addition one-way ANOVA and TUKEY multiple comparisons of means post-hoc

tests were performed on the VOCs emitted by the harvested roots, to test if the concentrations of the important VOCs differed significantly between both maize root types and agricultural soil management practices.

To compare how well the VOC emission profiles were correlated with the bacterial and fungal community composition, a Procrustes test was performed for the AMF and agricultural soil experiments. This test compared the sPLSDA dimensions for the soil VOC emission profiles of the last day of measurement with the "Non-Metric Multidimensional Scaling" (NMDS) genetic sequence data for bacterial and fungal communities, collected for the same experiments by Baekelmans (2022). The reason why only the VOC profiles of the last day were compared with the NMDS of the genetic sequences, is because these represent the volatiles emitted by the rhizosphere close to or at the time of harvesting. This is important, because the genetic sequencing only happens once after that last VOC measurement. To test if there was a significant correlation between the VOC emissions and the microbial community composition a "permutational test of the significance of the Procrustes results" was done.

All statistical analyses were done in R version 4.1.3 (R Core Team, 2022).

3. Results

3.1. Above and below ground biomass

Mycorrhizal colonisation had no significant effect on plant biomass in both runs of experiment 1, with similar results in the ANOVA tests in all plant organs. For the shoot biomass, there were no significant differences between the shoot dry weights of inoculated and non-inoculated plants in run 1 (*F = 1.13, p = 0.32*) and run 2 (*F = 0.4, p = 0.54*). This is also the case for the total fruit dry weights *(F = 0.0028, p = 0.96*), the total above-ground dry weights (sum total fruit and shoot dry weights) (*F = 0.34, p = 0.57*) (supplement 3 figure S.3 A), the root dry weights (*F = 0.033*, *p = 0.86*) (supplement 3 figure S.3 B) and the root to shoot ratios (*F = 0.24, p = 0.65*) of experiment 1 run 2.

The results were different between the plant organ dry weights of the agricultural management practices in experiment 2. First there were no significant differences between above-ground dry weights of the plant shoots (*F = 1.53, p = 0.26*) and total fruit mass (*F = 0.76, p = 0.54*) separately. However, there was a significant difference between the total aboveground biomasses, in particular between the "ploughing" (P) and "non-inversion tillage with compost" (NIC) treatments (ANOVA, *F = 3.97, p = 0.035*, TUKEY $HSD_{P\text{-}NIC}$, *p = 0.042*). The "non-inversion tillage" (NI) and "non-inversion tillage with compost" (NIC) treatments also generally had a higher total shoot dry weights compared to the "ploughing" (P) and "ploughing with compost" (PC) treatments, with the lowest emissions in the P treatment (supplement 3 figure S.4 A). This was not the case for the root biomass (*F = 0.44, p = 0.73*) (supplement 3 figure S.4 B) and the root shoot ratio's (*F= 0.45, p = 0.72*), who both did not differ significantly between the agricultural soil management practices.

3.2.VOC profiles experiment 1: Mycorrhizal colonisation

In the AMF experiment, the first thing investigated, using PCA and PERMANOVA tests, was how the VOC profiles differed between the mycorrhizal colonisation, the dates of measurement and the mesh cores. What we found is that we are able to make a distinction between the dates of measurement and mycorrhizal colonisation with the VOC profiles, with date of measurement being the most important source of variation. It is hereby important to note that the impact of the dates of measurement was stronger in the non-corrected data (*F = 452.99, **R^2 = 0.94**, p = 0.001*) compared to the corrected data (*F = 9.24, **R^2 = 0.30**, p = 0.001*) (figure 7 B and D), proving the effectiveness of the background soil correction in the reduction of the time effect. It is because of this fact that the

background soil correction will be used for further analysis (see 2.6.2 and 2.7). The interaction between the mycorrhizal colonisation and the measurement date is the second most important source of variation in both the non-corrected data (*F = 2.39* R^2 *= 0.0090, p = 0.001*) and corrected data (*F = 2.19,* R^2 *= 0.072, p = 0.001*), followed by the mycorrhizal colonisation in both corrected (*F = 8.11,* R^2 *= 0.027, p = 0.001*) and non-corrected data (*F = 16.92,* R^2 *= 0.0064, p = 0.001*). However, the difference between the treatments is not visible in the PCA biplot for both the non-corrected and corrected data (figure 7 A and C), because of a high overlap between the ellipses of each treatment. This makes a sPLSDA necessary, in order to make a more reliable separation between the AMF colonisation treatments based on their VOC profiles. The soil cores were not a significant source of variation of the VOC profiles in both the corrected (*F = 0.37,* R^2 *= 0.0012, p = 0.76*) and the non-corrected data (*F = 0.87,* R^2 *= 0.00016, p = 0.44*).

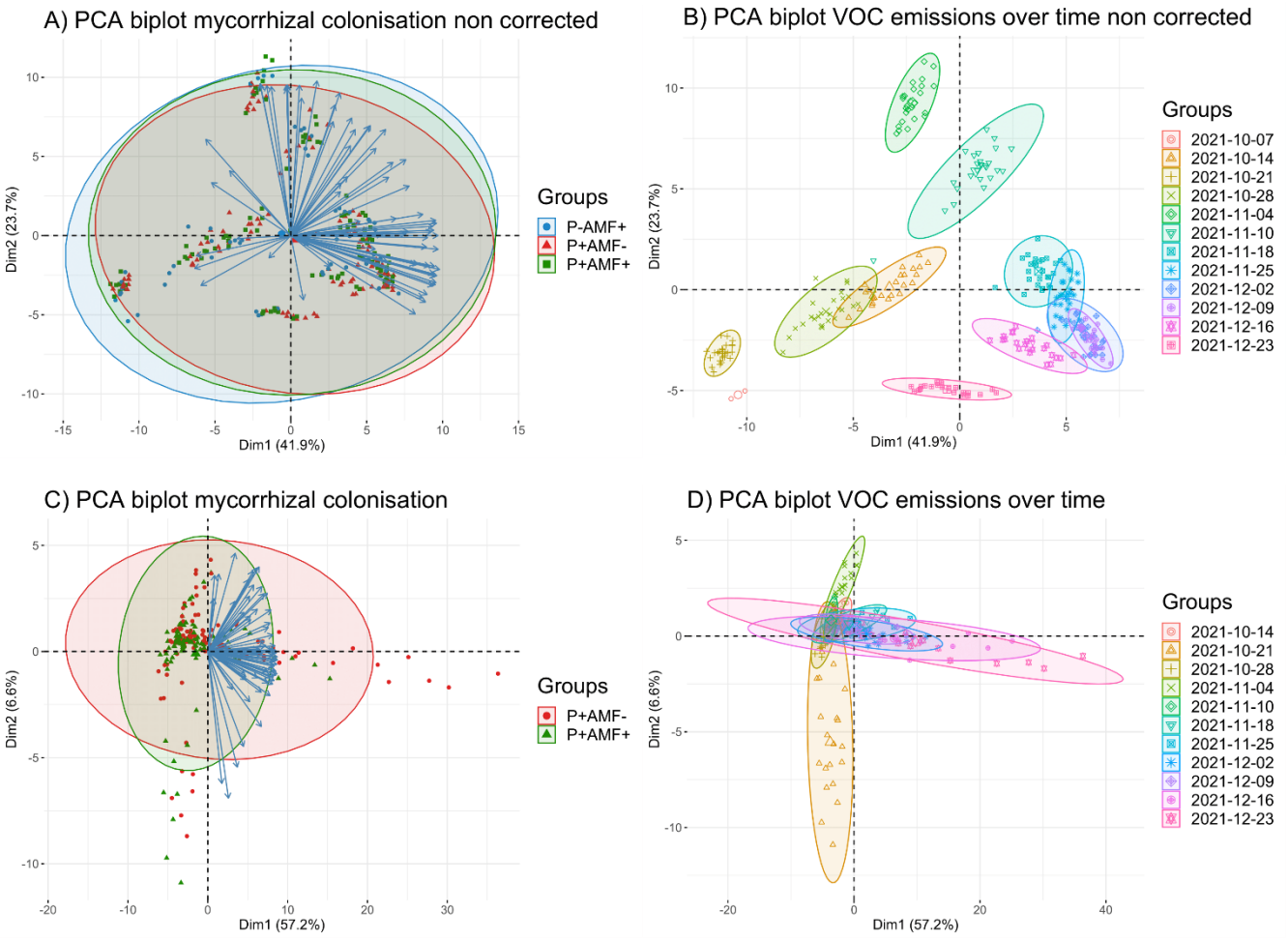

Figure 7: PCR analysis experiment 1 for both non-corrected data and corrected data investigating the difference in VOC profiles based upon mycorrhizal colonisation and dates of measurement. A) VOC profiles grouped according to mycorrhizal colonisation, non-corrected data. B) VOC profiles grouped according to dates of measurement, non-corrected data. The VOC vectors are the same as A C) VOC profiles grouped according mycorrhizal colonisation, corrected data. D) VOC profiles grouped according to date of measurement, corrected data. The VOC vectors are the same as C. The vectors in A and C represent VOCs, but the labels of the compound names are left out. The rate of mycorrhizal colonisation: P+AMF+ = colonised, P+AMF- = not colonised

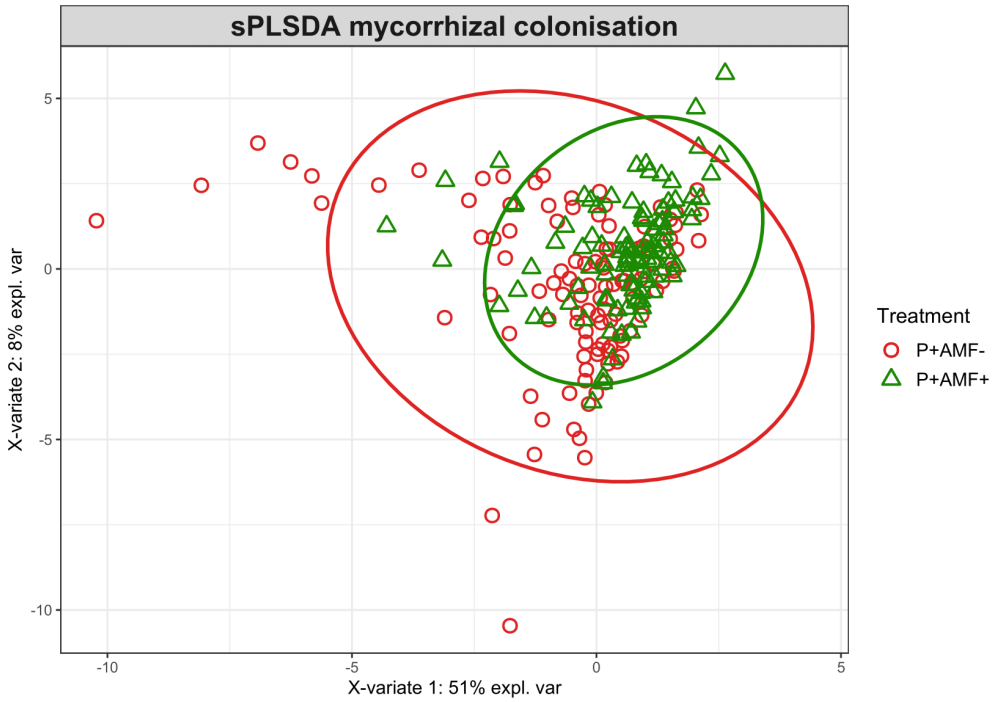

Figure 8: sPLSDA graph of the mycorrhizal colonisation. The X-variate 1 is the variable component that explains the most variation (51%) between plants inoculated with (P+AMF+) and without AMF (P+AMF-).

In the sPLSDA of the corrected VOC concentrations, we can see that there is a minimal difference between the VOC profiles of plants inoculated with (P+AMF+) and without AMF (P+AMF-) (figure 8). This means that the VOC profiles between both groups did not differ much, however P+AMF- does have multiple measurements that are located further away from the "central cluster" on the "X-variate 1" axis that represents 51% of the explained variation between the VOC profiles (the "central cluster" is the area where both P+AMF- and P+AMF- overlap in figure 8). In the same test, we found that this is due to several VOCs that were emitted in higher concentration by the rhizosphere communities of non-AMF inoculated plants throughout the plants lifetime. These VOCs were the following: propene ($(C_3H_6)H^+$, *m/z*= 43.060), acetone ($(C_3H_6O)H^+$, *m/z*= 59.053), acetic acid ($(C_2H_4O_2)H^+$, *m/z*= 61.036), methyl-acetate ($(C_3H_6O_2)H^+$, *m/z*= 75.047), toluene ($(C_7H_8)H^+$, *m/z*= 93.077) and hexenal ($(C_6H_{10}O)H^+$, *m/z*= 99.080). The emission of all these compounds follows a highly similar pattern, so only propene ($(C_3H_6)H^+$) and acetone ($(C_3H_6O)H^+$), are shown here in figure 9. What we can see is that during the plants' lifetime, their emission increases in a similar pattern in both the rhizosphere communities of P+AMF+ and P+AMF-, making the influence of time clear in the VOC profiles. However, the plants in the P+AMF- communities emit more of these volatiles at all timepoints than the plants grown in the P+AMF+ treatment, with the biggest difference in adult plants (figure 9). There were no specific VOCs found in the sPLSDA that were emitted more by a soil community inoculated with arbuscular mycorrhizal fungi (P+AMF+).

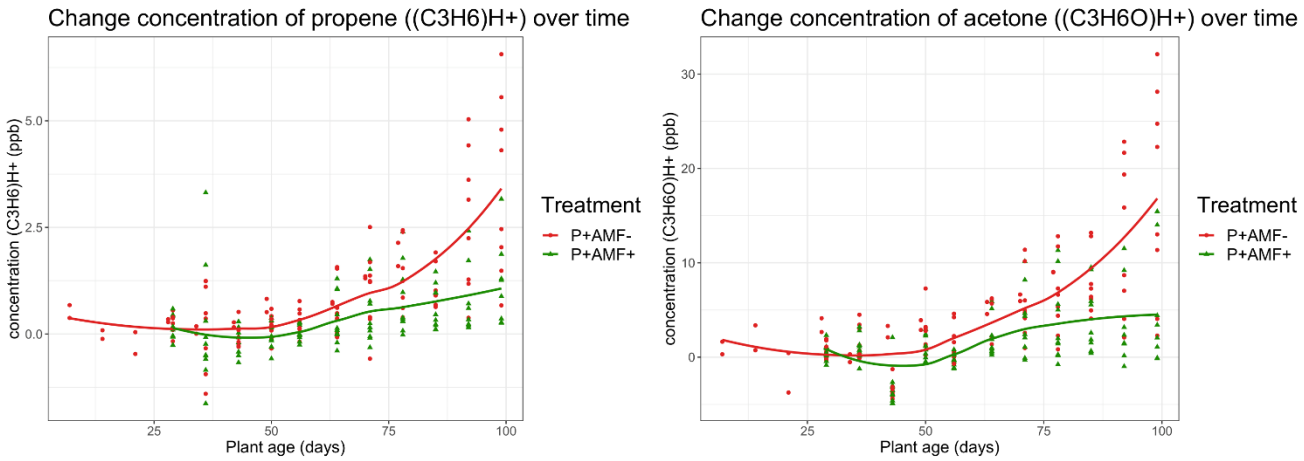

Figure 9: The change in concentration of propene ($(C_3H_6)H^+$, *m/z*= 43.060) (left) and acetone ($(C_3H_6O)H^+$, *m/z*= 59.053) (right) over the lifetime of the maize plant inoculated with (P+AMF+) and without (P+AMF-) *G. irregulare*.

In the Procrustes analysis, we found that only the bacterial community composition is significantly correlated with the emitted VOCs (*sum of squares = 0.69, correlation = 0.56, p = 0.005*). This is visible in figure 10 A, where we can see that the bacterial community composition is matched closely to the VOC emission profile (visible with the short arrows). This is not the case for fungal community composition, with a non-significant correlation between the fungal community and the VOC emission profiles (*sum of squares = 0.91, correlation = 0.30, p = 0.32*). This is also visible in figure 10 B, where the fungal community composition and the VOC profile do not match closely, in the P+AMF- treatment in particular.

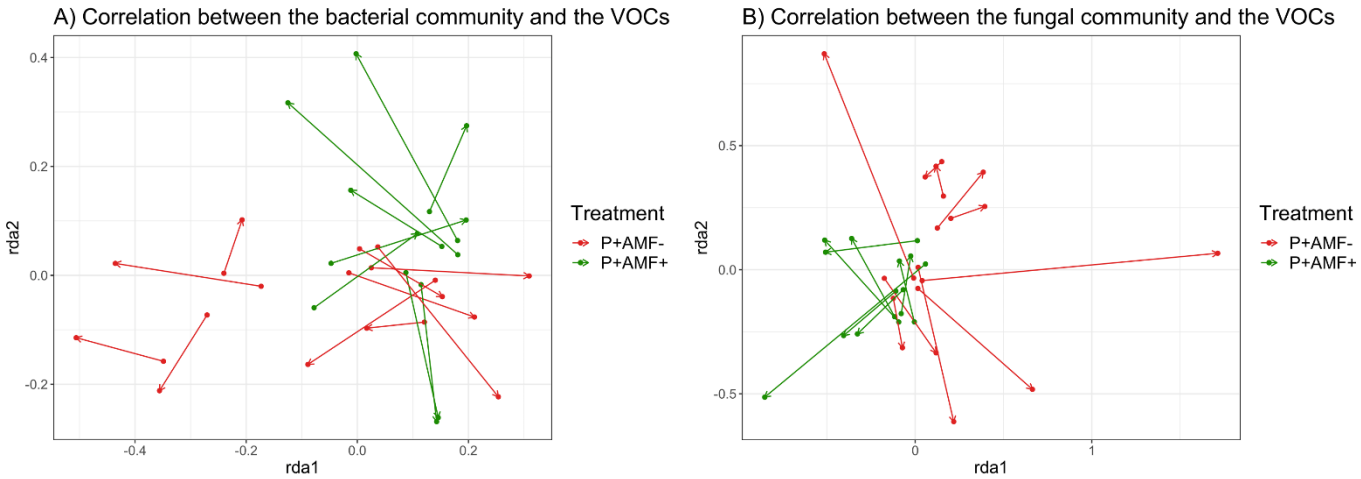

Figure 10: Procrustes analysis of the AMF experiment. A) The correlation between the bacterial community composition (beginning of the arrow) and the VOC emission profiles (end of the arrow). B) The correlation between the fungal community composition (beginning of the arrow) and the VOC emission profiles (end of the arrow). Colonisation by AM-fungi: P+AMF+ = Colonised, P+AMF-: Not colonised.

3.3. VOC profiles experiment 2: Agricultural management practices

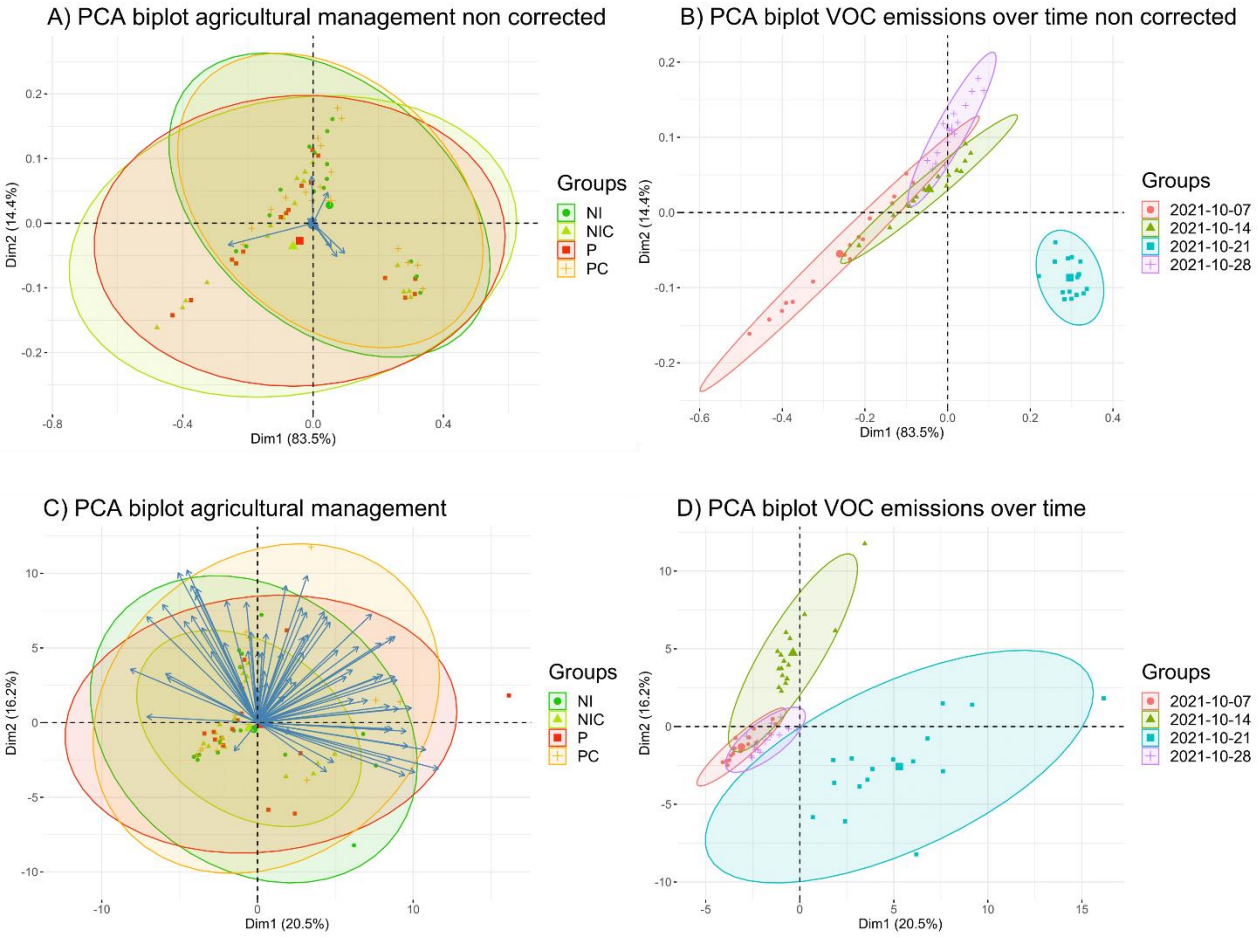

Figure 11: PCA analysis experiment 2 for corrected and non-corrected data visualising the differences in VOC profiles between the agricultural soil management strategies and the dates of measurement. A) Difference in VOC profiles between the agricultural management practices, non-corrected data. B) Difference in VOC profiles between dates of measurement, non-corrected data. The VOC vectors are the same as A C) VOC profiles of the agricultural management practices, corrected data and D) VOC profiles of the dates of measurement, corrected data. The VOC vectors are the same as C. The vectors in A and C represent VOCs, but the labels of the compound names are left out. The different agricultural soil managements: NI = Non-inversion tillage, NIC = Non-inversion tillage with compost added, P = ploughing and PC = Ploughing with compost added.

In this experiment, the PCR and PERMANOVA tests were also used to make a distinguishment between the agricultural management practices and dates of measurement, based on the VOCs emitted in the soil. What we found is that the dates are also the most important source of variation between the VOC profiles in this experiment, with a higher impact in the non-corrected data (*F = 302.83,* $\mathbf{R^2 = 0.85}$*, p = 0.001*) compared to corrected data (*F = 34.45,* $\mathbf{R^2 = 0.53}$*, p = 0.001*) (figure 11 B and D). This makes it, just as in experiment 1, necessary to use the corrected data for further analyses. The agricultural management practice is the second most significant source of variation between the VOC profiles in both the non-corrected data (*F = 25.05,* R^2 *= 0.07, p = 0.001*) and corrected data (*F = 8.73,* R^2 *= 0.14, p = 0.001*). The difference between the VOC profiles is more visible in the PCA of the non-corrected data,

with “non-inversion tillage” (NI) and “ploughing with compost” (PC) having a more similar VOC profile, similar for “non-inversion tillage with compost” (NIC) and “ploughing” (P) (figure 11 A). This is however not visible in the corrected data, making a sPLSDA necessary to accurately distinguish the agricultural management practices based on their emitted VOC profiles (figure 11 C). The interaction between the soil types and measurement dates in experiment 2 is not significant in the corrected data (*F = 1.74, R^2 = 0.081, p = 0.075*), while it is significant in the non-corrected data (*F = 3.83, R^2 = 0.032, p = 0.001*).

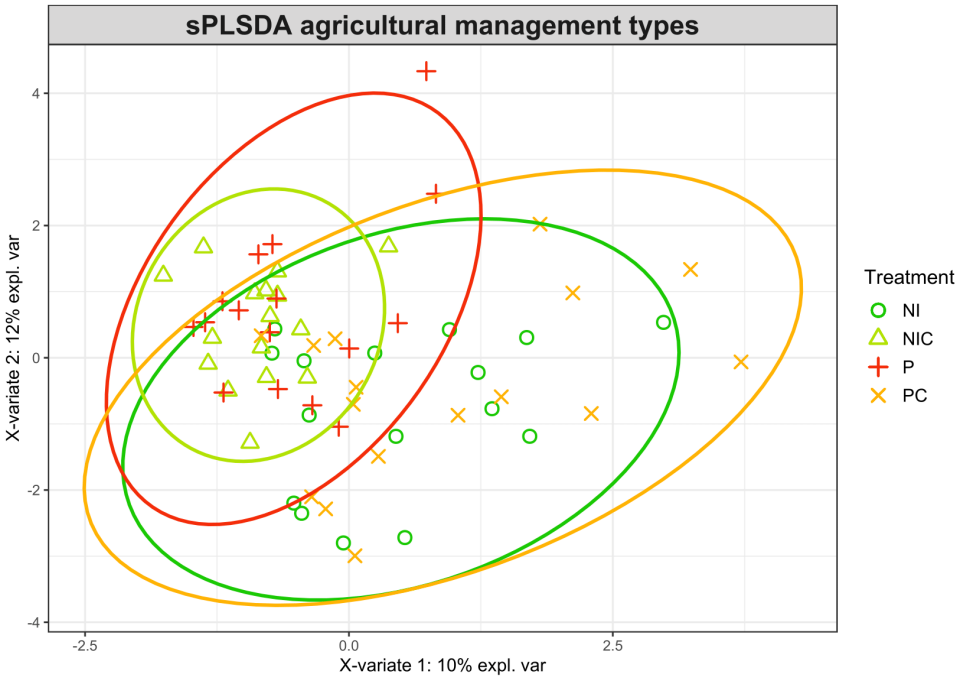

Figure 12: sPLSDA of the soil VOC profiles of maize plants grown in different agricultural soil management practices. The X variate 1 and 2 explain the most variation between the VOC profiles, representing 10% and 12% of the variation respectively. The agricultural managements were the following: NI = Non-inversion tillage, NIC= Non-inversion tillage with compost, P = Ploughing and PC = Ploughing with compost.

In the sPLSDA of the corrected VOC concentrations (figure 12), there are similar patterns as seen in the PCA of the non-corrected data (figure 11 A). The VOC profiles of the agricultural soil managements “non-inversion tillage” (NI) and “ploughing with compost” (PC) are very similar and are differentiated from the other agricultural management with “X variate 1” that represents 10% of the explained variation (figure 12). The rhizosphere communities of maize plants grown in the agricultural management practices “non-inversion tillage” (NIC) and “ploughing” (P) also produce similar VOC profiles, which are separated from the other treatments with the “X-variate 2” that represents 12% of the explained variation (figure 12). The difference between these VOC profiles is caused by the emission of certain volatiles. The volatiles found in the sPLSDA that are emitted in higher concentrations by communities grown in NIC and P are acetaldehyde ($(C_2H_4O)H^+$, *m/z*= 45.033) (figure 13), ethanol ($(C_2H_6O)H^+$, *m/z*= 47.049), acetone ($(C_3H_6O)H^+$, *m/z*= 59.053) and methyl vinyl ketone (MVK) or methacrolein (MACR) ($(C_4H_6O)H^+$, *m/z*= 71.049). It is also important to note that acetaldehyde

($(C_2H_4O)H^+$) was also negatively associated with the agricultural soil managements NI and PC, meaning that this compound is emitted in lower concentrations by these treatments. This is visible in figure 13, where we can see that the treatments NI and PC have a negative emission throughout most of the plants lifetime while the other do have long moments with positive emissions. For treatments NI and PC, the sPLSDA found two VOCs that were emitted in higher concentrations: acetic acid ($(C_2H_4O_2)H^+$, *m/z*= 61.036) and methyl acetate ($(C_3H_6O_2)H^+$, *m/z*= 75.047). For both volatiles this was also visible when analysing the change in concentrations over the plant's lifetime, which is shown for acetic acid ($(C_2H_4O_2)H^+$) in figure 13.

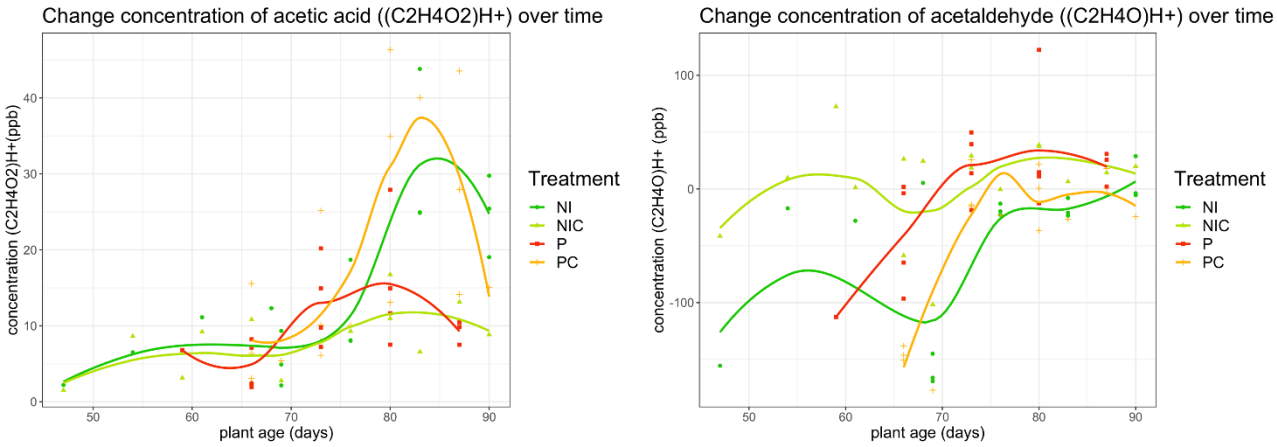

Figure 13: Change in concentration of acetic acid ($(C_2H_4O_2)H^+$, *m/z*= 61.036) (left) and acetaldehyde ($(C_2H_4O)H^+$, *m/z*= 45.033) (right) throughout the lifetime (plant age) of maize plants grown in different agricultural management practices. The treatments are NI = non-inversion tillage, NIC= non-inversion tillage with compost, P = ploughing and PC = ploughing with compost.

In the Procrustes analysis we see that there is no significant correlation between both the bacterial (*sum of squares = 0.96, correlation = 0.20, p = 0.75*) and the fungal (*sum of squares = 0.95, correlation = 0.23, p = 0.65*) community compositions and the VOC emission profiles on the last day of measurement. This is in stark contrast with the results in experiment 1, where there was a significant correlation between the bacterial community composition and the VOC emissions. The non-significant correlation is also visible in figure 14, where the graph shows that the microbe community composition and the VOC emissions do not match well (long arrows). This means that there is a big difference between the composition of the communities and the VOCs that are emitted in the soil.

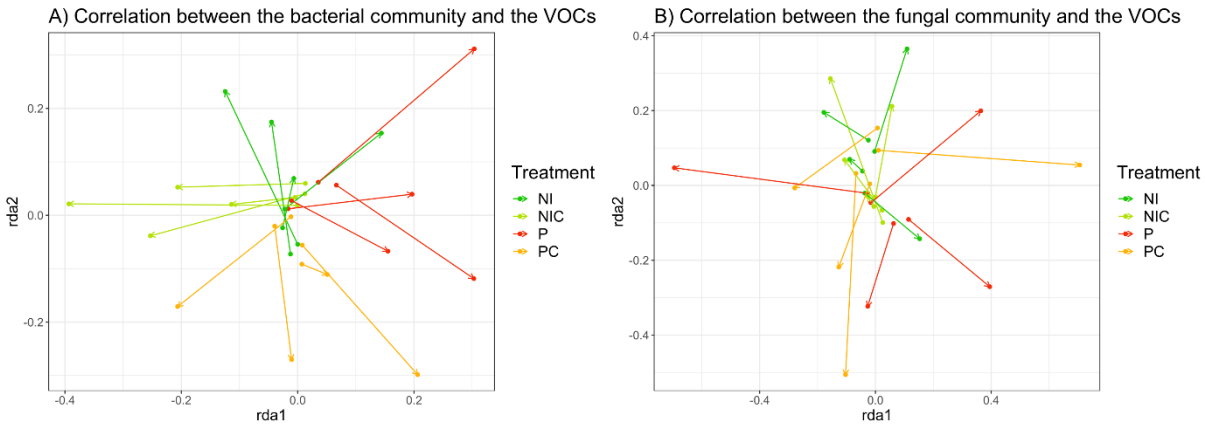

Figure 14: Procrustes analysis agricultural soil experiment. A) The correlation between the bacterial community composition (beginning of the arrow) and the VOC emission profiles (end of the arrow). B) The correlation between the fungal community composition (beginning of the arrow) and the VOC emission profiles (end of the arrow). The agricultural management practices: NI = Non-inversion tillage, NIC= Non-inversion tillage with compost, P = Ploughing and PC = Ploughing with compost.

3.4. VOC profiles harvested roots experiment 2

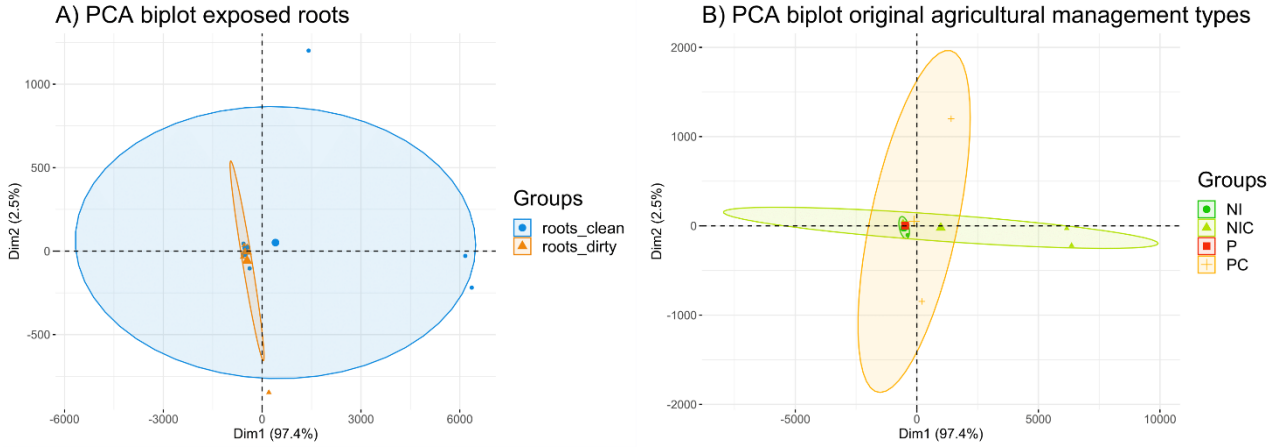

Figure 15: PCA biplots of the VOC profiles emitted by the harvested roots for the corrected data. The VOC profiles were according to the state of the sample type and original agricultural soil management type. A) Comparison of the VOC profiles sampled from exposed clean roots ('roots clean') and exposed roots with rhizosphere soil ('roots dirty') for corrected data. B) The VOC profiles compared according to the original agricultural soil management type of experiment 2 for corrected data. The management types were NI = non-inversion tillage, NIC = non-inversion tillage with compost, P = ploughing and PC = ploughing with compost. The VOC vectors are not shown because they were too big.

The difference between VOC profiles emitted by harvested plant roots was investigated first, with a PCR and PERMANOVA test, for two sources of variation: the harvested root type and the agricultural soil managements of experiment 2. Of the two factors, the agricultural soil management practice had the most influence in the variation between the VOC profiles, but its impact was not significant ($F = 1.62$, $R^2 = 0.14$, $p = 0.19$). The root type also has no significant impact on the variation between the VOC profiles ($F = 2.40$, $R^2 = 0.067$, $p = 0.067$). There is also no significant interaction ($F = 1.55$, $R^2 = 0.13$, $p = 0.17$). This non-significant difference is also visible in the PCA, where it is not clear how much the

VOC profiles differ between the root types (figure 15 A) and the agricultural management practices (figure 15 B).

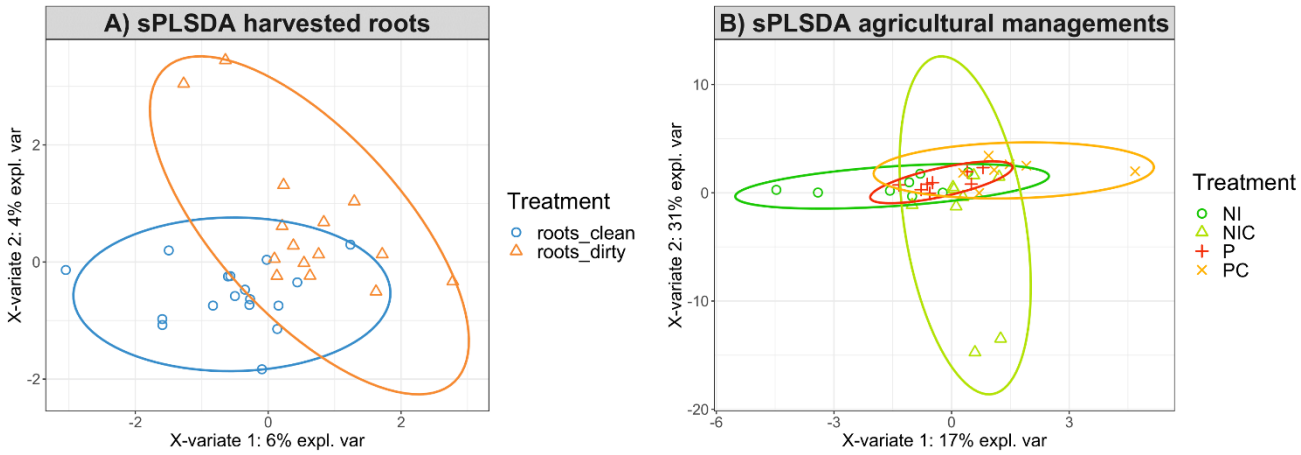

Figure 16: sPLSDA harvested roots experiment 2. A) The differentiation between clean roots (clean soil) and roots with rhizosphere soil (dirty soil). The X-variate is the variate that explains the most variation between the management types (6%). B) The differentiation between the different agricultural management types. X-variate 2 explains most variation between the soil types (31% variation). The management types are NI = non-inversion tillage, NIC = non-inversion tillage with compost, P = ploughing and PC = ploughing with compost.

Despite the non-significant difference between the harvested root types and the agricultural management practices, there still are some noticeable differences in the sPLSDA. When looking at the root types there is some separation between the VOC profiles on mostly the "X-variate 1" (figure 16 A), there were two VOCs that were measured more in the clean roots, which are methanol ($(CH_4O)H^+$, *m/z*= 33.045) and dimethyl sulphide (DMS) ($(C_2H_6S)H^+$, *m/z*= 63.026). When comparing the VOC concentrations, with an ANOVA test, we can see that the concentrations of both methanol ($(CH_4O)H^+$) (*F = 5.75, p = 0.023*) and DMS ($(C_2H_6S)H^+$) (*F = 7.19, p = 0.012*) differ significantly between clean and dirty roots, with a higher emission by the clean roots compared to the dirty roots (figure 17 A and B). In the agricultural management practices, the differentiation between the VOC profiles mostly occurs in the "X-variate 1", which separates the NI, P and PC treatments. This is different for NIC, where the emissions are separated by the "X-variate 2" (figure 16 B). The volatiles that are emitted more by plants roots grown in PC, according to the sPLSDA, are isoprene ($(C_5H_8)H^+$, *m/z*= 69.073), hexanols or hexanal ($(C_6H_{10})H^+$, *m/z*= 83.089), methylenecyclohexane ($(C_7H_{12})H^+$, *m/z*= 97.101), xylene ($(C_8H_{10})H^+$, *m/z*= 107.086), an unknown compound ($(C_8H_{14})H^+$, *m/z*= 111.117) and phenylacetic acid ($(C_8H_8O_2)H^+$, *m/z*= 137.067). There were no specific volatiles for NI and P. However, not all these volatiles differ significantly between PC and the other treatments when investigating the concentrations with ANOVA and TUKEY tests, with only the concentrations of methylenecyclohexane ($(C_7H_{12})H^+$) (ANOVA, *F = 4.24, p = 0.014*, TUKEY $HSD_{PC\text{-}NI}$, *p = 0.0076*) and xylene ($(C_8H_{10})H^+$) (ANOVA, *F = 4.66, p = 0.0092*, TUKEY $HSD_{PC\text{-}NI}$, *p = 0.006*) differing significantly between PC and NI (figure 17 C and D). The volatiles that were emitted in high concentrations by NIC are shown in table S.1 in supplement 4. However of all these

volatiles, salicylic acid ($(C_7H_6O_3)H^+$, *m/z*= 139.039) (ANOVA, *F = 3.39, p = 0.032*, TUKEY HSD_{NIC-PC}, *p = 0.03*) was the only one that differed significantly between NIC and the other treatments, with the largest difference in concentrations between the PC and NIC treatments (figure 17 F). It also was the compound with the highest value on the X-variate 2 axis with -0.34, making it the most strongly correlated with the NIC treatment. For the other volatiles like methanol ($(CH_4O)H^+$) (*F = 1.70, p = 0.19*), the differentiation was not strong enough to be significant (figure 17 E).

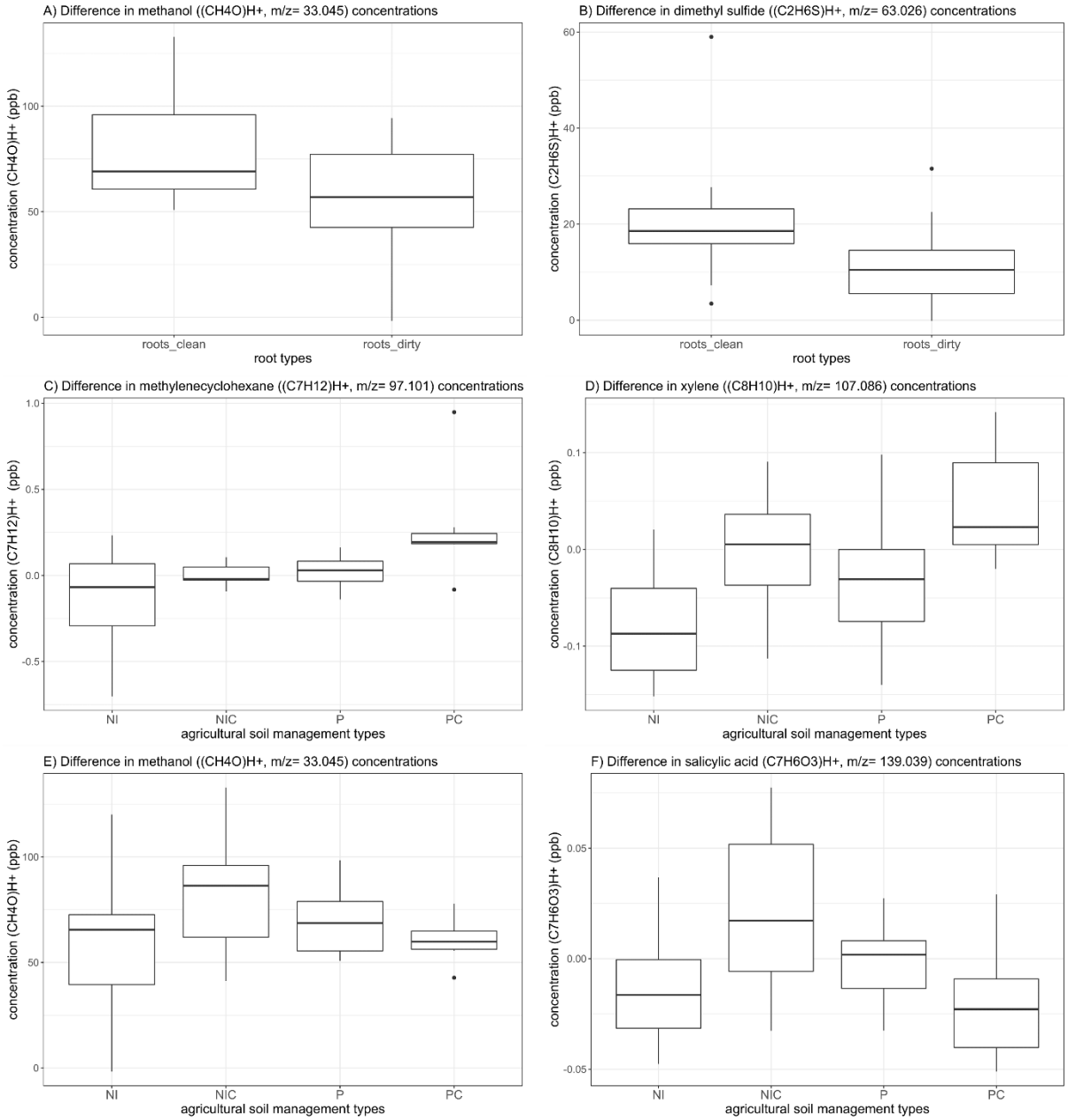

Figure 17: Boxplots of the differences in VOC concentrations of the most important VOCs between the root types and agricultural soil management practices. A and B are the most important volatiles that differentiate the VOC profiles of the exposed clean roots (clean roots) and the roots with rhizosphere soil (dirty roots). C and D are the two VOCs that have the most impact in the differentiation between the agricultural soil managements "ploughing with compost" (PC) and the other treatments. E represents a VOC that is slightly more emitted by roots from the agricultural management "non-inversion tillage with compost" (NIC), but not significant. F is the VOC that has the most impact in the differentiation between the VOC profiles of NIC and the other treatments. The agricultural soil management types in graphs C, D, E and F are "non-inversion tillage" (NI) and "ploughing" (P).

4. Discussion

Despite the important role VOCs play in the facilitation of interactions in the rhizosphere, the measurement of soil VOCs remains very challenging. In this study we tried to find out if we were able to distinguish between different soil communities, using their emitted soil VOC profiles. The effectiveness of the direct soil VOC measurement was tested in two experiments: the "AMF experiment" (experiment 1) and the "Soil experiment" (experiment 2). In addition, we also tested if it is possible to distinguish the agricultural management practices, using the emitted VOC profiles from harvested roots.

4.1. The influence of AMF colonisation on plant biomass and rhizosphere VOC emissions

There was no effect of AM-fungi inoculation on plant biomass. This is a surprising result, because previous studies have shown that crop yields mostly increase with increasing mycorrhizal inoculation due to several advantages AM-fungi provide such as an increased in P-uptake and an increased protection against pathogens and abiotic stressors such as dry soils (Begum et al., 2019; Hu et al., 2009; Ryan and Graham, 2002). Although there are some exceptions, like wheat and field pea, where AMF colonisation does not significantly influence crop yield (Ryan and Graham, 2002), this is however not the case in maize, where yield normally does increase with increasing AMF colonisation (Begum et al., 2019; Ryan and Graham, 2002). This non-significant difference may have been caused by contamination, which did occur during the running of the experiment and was discovered after harvesting, with the P+AMF- pot being contaminated with *G. irregulare*, possibly caused by factors such as the spread of spores by dust or during watering and 'fungus gnat' (Sciaridae) activity (who are known to spread spores of parasitic fungi) (El-Hamalawi, 2008; Scarlett et al., 2014), which became widespread on the 14th of December 2021. However, it is important to note that despite the contamination, the colonisation rate in the P+AMF+ treatment was still significantly higher (ANOVA, $F = 41.04$, $p = 0.0002$) than in P+AMF-, with an average arbuscule abundance of 61 ± 12 % in P+AMF+ and 15 ± 11 % in P+AMF- (data collected by Baekelmans (2022)). It is however unclear that contamination is the only reason why the above-ground biomass does not differ significantly between treatments, with other options being for example the height of the lamps hindering vertical growth or that the nutrients provided by fertilisation were enough nutrients for plant growth. The latter seems unlikely, because the plants inoculated with AMF still developed a good hyphal network despite the

added P during fertilisation. This indicates that plants still received a benefit from the AMF in terms of acquiring nutrients, meaning that the P concentrations were not high enough to negatively influence AMF development (Chen et al., 2021; Ven et al., 2019; Verbruggen et al., 2013).

Despite this contamination, the rhizosphere communities could still be distinguished based on their emitted VOC profiles, with a higher VOC emission in the rhizosphere of plants in the P+AMF- treatment compared to the P+AMF+ treatment. This is evidenced by the fact that there were six volatiles found in the sPLSDA that were emitted more in the P+AMF- treatment throughout the plants lifetime, but no volatile that was emitted more by P+AMF+. We can also see this in the PCA analysis (figure 7 C), where most vectors (that represent VOCs) are oriented towards P+AMF- samples, despite AMF contamination. This is the opposite of what we expected, because we thought that there would be an increase in P+AMF+ plants due to increased VOC emissions by AM-fungi. This is probably due to an already well-known effect of AMF on root exudates: that there is a decrease in root exudates in plants that have a high AMF inoculation (Hussain et al., 2021; Ryan et al., 2012; Wen et al., 2022). More specifically, AMF can decrease the emission of exudates such as carboxylates (organic acids such as citric acids, malonic acids and malic acid), compounds that are used for the mobilisation of inorganic phosphate by decreasing the soil pH (a process called chelation). The use of carboxylates for chelation is costly for plants, leading to a decrease in their emission in inoculated plants in exchange for the 'cheaper' symbiotic relationship (Dakora and Phillips, 2002; Hage-Ahmed et al., 2013; Hussain et al., 2021; Monther and Kamaruzaman, 2012; Ryan et al., 2012; Wen et al., 2022). Another possible reason for the decrease in root exudates is the carbon consumption by AMF, which leads to a decrease in C-availability for the biosynthesis of root exudates. This has as a consequence that there will be a reduction in root exudate emissions by plants, leading to a decrease in their use for nutrient acquisition processes (Wen et al., 2022). One of the most important processes affected by a lower root exudation is priming, which is the stimulation of soil microbes to decompose soil organic matter (SOM) after the input of easily decomposable N and C sources from root exudates by plants (Bengtson et al., 2012; Zhou et al., 2020). A decrease in priming, as a result of AMF colonisation, has been proven by Zhou et al. (2020), who also confirmed decreasing SOM decomposition by free-living soil microbes in soils influenced by AMF colonised plants. This increase in carbon consumption as a result of AM-symbiosis can also have a negative effect on the emission of soil VOCs, because of two reasons: the first reason is a decrease in soil VOC production by plants as a result of carbon allocation to the fungus, just as with root exudates. There is a high chance that this is the case, because the production of VOCs is also dependant on carbon availability (Dudareva et al., 2013). The second reason behind this decrease in soil VOC emissions, is a reduction of decomposition related VOCs from free-living soil as a result of a decrease in priming, caused by a lower emission of root exudates.

A good indicator of the influence of AMF could be found in the sources of emission of the six specific volatiles that were emitted at a higher rate by the P+AMF- treatment. The first interesting fact about these volatiles is that four of them are emitted during litter decomposition, which included propene ($(C_3H_6)H^+$) (Portillo-Estrada et al., 2020), acetone ($(C_3H_6O)H^+$) (Mozaffar et al., 2018; Niinemets et al., 2014; Portillo-Estrada et al., 2020), acetic acid ($(C_2H_4O_2)H^+$) (Mozaffar et al., 2018; Portillo-Estrada et al., 2020; Portillo-Estrada et al., 2018) and hexenal ($(C_6H_{10}O)H^+$) (Holopainen et al., 2010; Mozaffar et al., 2018; Portillo-Estrada et al., 2020). It is important to note that these volatiles are also produced as a result of different processes or stressors, like propene that is emitted by dry leaf litter (increasing with temperature) (Rhew et al., 2017). Acetone on the other hand is also emitted after leaf wounding and oxidative stress (Portillo-Estrada et al., 2021; Seco et al., 2007). This is also the case for the green leaf volatile hexanal, that is additionally also emitted during drought stress (in the form of trans-2-hexenal) and light dark transitions (Brilli et al., 2011; Catola et al., 2016; Fall et al., 1999; Portillo-Estrada et al., 2021). The other two volatiles that were emitted at a higher rate by rhizosphere communities with a low mycorrhizal colonisation rate were methyl-acetate ($(C_3H_6O_2)H^+$) and toluene ($(C_7H_8)H^+$). Methyl-acetate is mostly emitted as a result of fruit and flower fermentation and leaf transpiration during photosynthesis (Jardine et al., 2014) and toluene is probably produced by photosynthetic activity, with increasing emission under heat stress (Misztal et al., 2015). Due to this higher emission of decomposition-related VOCs by the P+AMF- treatment, there can be assumed that there is a higher litter decomposition by soil microbes in that rhizosphere community as a result of priming. This can also explain why there is increase of their emission during the plants lifetime (as seen in figure 9), with the highest difference in particular being between adult plants of the P+AMF- and P+AMF+ treatments. The reason why this is probably the case is because adult plants have a high root biomass and fast growth rate, which leads to a high nutrient demand (Zhao et al., 2021). To meet those demands, it is possible that the maize plants of the P+AMF- treatment produced a higher quantity of root exudates in order to increase the supply of nutrients by both changing the microbial community composition and stimulating microbial litter decomposition (Zhao et al., 2021; Zhu et al., 2014). This probably did not occur that much in P+AMF+ plants, which meet most of their nutrient demands as a result of their high AMF colonisation. Another factor that also requires a lot of nutrients in adult plants is seed formation (Martínez-Ballesta et al., 2020), which can also cause a higher emission of root exudates by P+AMF- plants in order to acquire the needed nutrients. The stimulation of the soil microbial community by P+AMF- plants could also explain the high correlation between the bacterial community composition and the VOC emission in the Procrustes analysis (figure 10). It however remains difficult to say that for sure without further investigating the correlation between root exudates and the emission of decomposition related VOCs, in order to see if the emission of those VOCs is really linked to root exudation.

It is also important to note that the emission of some of these VOCs could indicate other factors about both the plant growth conditions and the environmental circumstances, like for example the emission of hexenal as a result of drought stress. This was a stressor that the plants in this experiment may have experienced due to dryer soil conditions at the time of measurement. This relates to the fact that we kept the soil dry on purpose at the time of measurement in order to promote the diffusion of VOCs, which diffuse better in dryer conditions (see 2.2) (Insam and Seewald, 2010; Mäki et al., 2019; Rinnan and Albers, 2020). So there can be also assumed that the higher emission of hexenal by P+AMF- plants, in comparison to P+AMF+ plants, could also have been caused by drought stress. This is because plants with a higher AMF colonisation are more resistant to dry conditions (van der Heijden et al., 2015). For toluene and methyl acetate it is difficult to say why it was produced in the rhizosphere, making further research necessary.

What is also interesting in the AMF experiment is that the VOC emission profiles between the mesh cores did not differ significantly, with similar emissions between a core with roots and AMF and the core with only AMF (see 2.1). There can be multiple reasons why this is the case, such as the inability to measure VOCs produced by the AM-fungus, because of a low VOC production. It can however also be the case that we did measure VOCs produced by AMF, with similar concentrations in both mesh cores and in different concentrations between treatments. If that is the case, then there can be expected that there is a higher concentration of VOCs emitted by AMF in the P+AMF+ treatment compared to P+AMF- treatment, due to a higher colonisation rate in P+AMF+. The problem is that we were unable to distinguish the VOCs emitted by AMF from the VOCs emitted other microbes, which makes it difficult to make any conclusions about their emission without further research.

4.2. Differences in soil VOC emissions and plant biomass between the agricultural management practices

Compared to the AMF experiment, we did see a difference between the total above-ground biomasses between the agricultural management practices. The most important finding was that the “Non-inversion tillage” (NI) and the “Non-inversion tillage with compost” (NIC) treatments had a higher (but non-significant) above-ground biomass compared to the “Ploughing” (P) and “Ploughing with compost” (PC) treatments, with the difference in biomass between NIC and P being the largest (supplement 3 figure S.4 A). These findings did differ from in-situ biomass results from the sampled BIOPACT field trial by ILVO, described by D’Hose et al. (2016). In that field trial, they found where they found that fodder maize yield decreased by 5% in plants grown in non-inversion tillage soil, which was

probably caused by a higher penetration resistance that impended the roots from entering the soil (D'Hose et al., 2016). The original field trial is not the only study that contradicts with our results, with most studies on this subject finding a small decrease in crop yields. This is because in most cases a deep non-inversion tillage (tillage at a depth of ≥ 25 cm), which is what they did in D'Hose et al. (2016), leads to a small decrease in crop yields due to soil compaction (Cooper et al., 2016; Morris et al., 2010; Van den Putte et al., 2010). However, there are some exceptions like the research of Peralta et al. (2021), who did find a 6% increase in maize crop yield as a result of deep non-inversion tillage. According to that study, this was caused by soil compaction alleviation in clayey soils, which helped the maize plants to reach the resources in the subsoil. It is however unclear how much this had an effect in this experiment, because the sampled soil was mixed with background soil (see 2.3). This makes it difficult to conclude that these differences in biomass are caused by non-inversion tillage related soil compaction. What can be a better explanation for this difference in biomass is the effects of tillage methods on the soil microbial community and other soil quality indicators. A study on the impact of tillage methods on the soil microbial community and soil quality has been performed by Willekens et al. (2014). In that field trial the researchers found that the topsoil (0-10 cm) quality was higher in non-inversion tilled soils compared to ploughed soils, because a multitude of factors such as an higher diversity in soil microbes such as gram positive bacteria, fungi and AMF, a higher soil carbon concentration and a higher K and Mg content. This is important, because this topsoil layer was harvested for use as an inoculum (see 2.1). Other research on the effect of tillage on the AMF colonisation rate by Bowles et al. (2017), found that non-inversion tillage increased AMF colonisation by an average of 11.2% compared to ploughing. It is however important to note that this increase was lower in maize than in other crops like cotton. So there can be assumed that it can be the case that the higher above-ground biomass for NI and NIC, compared to P and PC, is the result of the better soil quality of the topsoil used during the inoculation, caused by a combined effect of a more diverse soil microbial community, a higher soil carbon content and a higher amount soil nutrients. In case of the addition of compost, the field trial by D'Hose et al. (2016) found that compost addition did not have any effect on the dry matter yields of all investigated crops which were part of the four-crop rotation, including maize. This is despite an increase in soil quality caused by the compost addition, including an increase in soil organic carbon and soil pH. This is, in contrast to the tillage methods, also the case in our experiment, with differences in dry weights between P and PC on one hand and NIC and NI being non-significant (supplement 3 figure S.4 A).

In the sPLSDA analysis (figure 12), we have seen that the NIC and P treatments and the PC and NI treatments have similar VOC emission profiles respectively. These similarities between the VOC emission profiles can suggest that there is a similar microbial community composition between NIC

and P on the one hand and PC and NI on the other hand. However, this is not the case, with microbial community composition data, collected by Baekelmans (2022), suggesting that each treatment differs to a similar extent in terms of their community composition. This could mean that the VOC emission profiles of the treatments are not really connected to the bacterial and fungal community composition, which can be a reason why there was no correlation between the VOC emissions and the fungal and bacterial community composition in the Procrustes analysis (figure 14). This of course creates the question of what the reason is why these particular treatments have a similar VOC emission profile. A possible hypothesis is the location of the pots inside the wooden growth structure (figure 3), because inside this structure the plants grown in the NIC and P treatments were located on the side of the window while the plants grown in the PC and NI treatments were grown on the other side. This is the result of a mistake made during the set-up of the trial, whereby only the ploughing treatments (P and PC) and non-inversion tillage treatments (NI and NIC) were separated without taking the window effect into account. This had as a consequence that the window effect was visible in the VOC emission profiles, with a high emission of certain VOCs by plants or microbes in the rhizosphere that are more of an indicator for the environmental conditions on the side where the maize was grown.

The most important evidence for this, is the emission of VOCs by the NIC and P treatments that could indicate anoxic conditions, caused by a high soil water content. A high soil water content is generally the result of both soil compaction and bad soil drainage (Barickman et al., 2019; Kreuzwieser and Rennenberg, 2014), which was the case in this experiment. These VOCs were acetaldehyde ($(C_2H_4O)H^+$), ethanol ($(C_2H_6O)H^+$) and acetone ($(C_3H_6O)H^+$). The combination of a high emission of both acetaldehyde and ethanol is very interesting, because both VOCs are emitted simultaneously by plant leaves in anoxic conditions (Kesselmeier et al., 2009; Loreto et al., 2006; Portillo-Estrada et al., 2018). This is because in anoxic conditions plant roots and bacteria ferment pyruvate in order to acquire energy, with the production of ethanol as a result. After its biosynthesis, a big amount of the produced ethanol is transported through the plant xylem to the leaves, where it is emitted in the atmosphere. In the leaves ethanol can also be oxidised to acetaldehyde and acetic acid by enzymes such as aldehyde dehydrogenase (Kesselmeier et al., 2009; Loreto et al., 2006; Portillo-Estrada et al., 2018). It is important to note that acetaldehyde is formed as well during the fermentation process, as the final precursor for ethanol (Boamfa et al., 2005; Kesselmeier et al., 2009). Acetaldehyde however is not only used as a precursor for the production of ethanol, but can also be formed during the catalysis of ethanol to remove H_2O_2 which was formed during oxidative stress resulting from a high soil water content (Boamfa et al., 2005; Tang et al., 2010). So because of the fact that ethanol is produced by plant roots and soil bacteria, it can be the case that we can measure it in the soil as well during anoxic conditions and not only in leaves. With acetaldehyde also being produced from ethanol in the

rhizosphere as a result of the oxidative stress or as part of the fermentation process. Acetone is, as previously mentioned, a known volatile that is emitted during litter decomposition (Mozaffar et al., 2018; Niinemets et al., 2014; Portillo-Estrada et al., 2020), but it is also produced by inundated tree roots as a result of anaerobic fermentation of pyruvate or fatty acids (Birami et al., 2021; Grote et al., 2019). This makes it possible that we can measure it in maize roots too, as a result of a high soil water content. It is important to note that acetone is also emitted by plants who experience oxidative stress, caused by ROS accumulation or O_3 exposure (Grote et al., 2019; Niinemets et al., 2014; Seco et al., 2007). Oxidative stress can also be caused by a high soil water content (Tang et al., 2010), which means that it can be possible that the emission of acetone in the rhizosphere was the result of this stressor. In the NIC and P pots we also measured methyl vinyl ketone (MVK) or methacrolein (MACR) ($(C_4H_6O)H^+$). Both MVK and MACR are formed by the oxidation of the VOC isoprene by OH radicals in the atmosphere (Stroud et al., 2001; Tani et al., 2020). Isoprene is a volatile that is produced in plant chloroplasts and emitted by plant leaves during photosynthesis and during thermal and oxidative stress (Velikova, 2008). It is due to this fact, that it is unlikely that plant roots produced isoprene, because they do not have chloroplasts (Kobayashi et al., 2017). There can, however, also be other causes for the emission of isoprene in this experiment, like air pollution affecting the pots caused by human breath or the presence of the soil bacteria *Bacillus subtilis*, which is known for its production of isoprene (Kuzma et al., 1995; Sivy et al., 2002).

The emission of anoxia related volatiles is not the case for the plants grown in the NI and PC treatments, where these compounds were not emitted in high concentrations. In these treatments acetic acid ($(C_2H_4O_2)H^+$) was mostly emitted, which is a VOC emitted during litter decomposition (Mozaffar et al., 2018; Portillo-Estrada et al., 2020; Portillo-Estrada et al., 2018). They also emitted methyl acetate ($(C_3H_6O_2)H^+$), but it is unclear why it is produced in the soil because it is related to fruit and flower fermentation and photosynthesis (Jardine et al., 2014). This could mean that the pots of the NI and PC were not that water saturated. It is however unclear why this is the case, but it can be that there was more evaporation in these pots compared to the NIC and P pots due to additional sunlight exposure on those pots.

The overall conclusion from this experiment is that it is difficult to derive any assumption about how the microbial community influences the rhizosphere VOC emission profiles due to the bad quality of the inoculated background soil in this experiment, which was both compressed and had a high water saturation. This limited both the diffusion of oxygen and VOCs in the soil matrix (Kai et al., 2016; Pandey Bipin et al., 2021; Tang et al., 2010). In the case of soil compression, there is less diffusion because of a decrease in macropores (diameter > 10 μm), which play a crucial role in the facilitation of long-distance VOC diffusion in the soil (Kai et al., 2016; Pandey Bipin et al., 2021), making it very difficult to

measure the VOCs emitted by plant roots and soil microbes with the PTR-TOF-MS. A high soil water saturation on the other hand is also very problematic for the diffusion of VOCs, because of a higher dissolution of water-soluble VOCs and an increased microbial VOC uptake (Insam and Seewald, 2010; Mäki et al., 2019; Rinnan and Albers, 2020).

4.3. VOC emissions from harvested roots

There was no significant difference between the VOC profiles of the harvested roots between both the agricultural management practices and the harvested root types (roots with and without rhizosphere soil) in either the PCA or PERMANOVA analyses (figure 15). This is an interesting result, because we expected some changes in root VOC emissions due to the influence of mycorrhizal fungi or other soil organisms like pathogenic fungi, two groups where it has been proven that they can influence the emission of root VOCs (Dreher et al., 2019; Schulz-Bohm et al., 2018; Sun and Tang, 2013). For example, research by Dreher et al. (2019) found that *Medicago truncatula* Gaertn. roots emit limonene after contact with spores of the AM-fungus *Rhizosphagus irregularis*. The emission of VOCs by *M. truncatula* Gaertn. roots also changed after the roots were inoculated with the parasitic oomycete *Aphanomyces euteiches*, which led to the emission of sesquiterpenes such as nerolidol, a known stress compound plants emit after leaf wounding by herbivores. This probably means that the roots in the different agricultural management treatments were either not colonised by AMF or other pathogens or were not colonised enough to have a significant impact on the root VOC emissions, resulting in similar VOC profiles between the different treatments. This was probably caused by the intensive land use at the sampling sites of the BIOPACT field trial by ILVO, where the soil is both fertilised and disturbed by tillage, both actors that negatively affect the AMF diversity and abundance (Edlinger et al., 2022).

However, even when the there was no significant difference between the VOC profiles of both the agricultural soil management types and harvested root types, there were still some volatiles found in the sPLSDA that differed significantly between the treatments. In the case of the harvested root types, we found methanol ($(CH_4O)H^+$) and dimethyl sulphide (DMS) ($(C_2H_6S)H^+$). VOCs that were emitted in significantly higher concentrations in roots without rhizosphere soil (known as “clean roots”) than in roots with soil (known as “dirty roots”) (figure 17 B). This is interesting, because these are both volatiles that are known to be emitted by roots, with methanol in particular being emitted constitutively during root growth, induced during leaf wounding and during senescence (Loreto et al., 2006; Niederbacher et al., 2015; Portillo-Estrada et al., 2020). This means that we can assume that harvested roots emit this volatile as a result of both wounding and senescence, which brings up the question: why is it

emitted more by clean roots? This is because, both root types experience wounding, as a result of cutting, and senescence. There can however be a few reasons why there is a low methanol concentrations measured in the dirty roots, like the high water-solubility of methanol which can lead to its dissolution in the water filled pores of the soil surrounding the roots. Another reason why there could be a higher methanol emission by clean roots, is an increased wounding as a result of cleaning. It can however also be the case is the higher uptake of methanol by soil microbes in the rhizosphere soil, known as methanol-oxidising microorganisms (Kolb, 2009), which decreases its emission in the 'dirty roots'. The other VOC emitted more by clean roots was DMS, a volatile that can be emitted by maize and by microbes which decompose sulphur containing amino acids in the soil. It is also a compound that is not soluble in water and can be degassed quickly, with an exception being in saturated soils where diffusion is more difficult (Jardine et al., 2015). Since it was mostly emitted by clean maize roots, clearly maize roots are the main source of DMS, not soil microbes. The emission of this compound by dirty roots is probably hindered by the highly water saturated rhizosphere soil which encapsulated the roots.

For the agricultural management practices, there were also a few VOCs that were emitted more by some treatments, despite that there was no significant difference between the VOC profiles of the agricultural management types. With some VOCs being emitted more by either the PC treatment or the NIC treatment. For the PC treatment we can see that maize roots emit significantly more methylenecyclohexane ($(C_7H_{12})H^+$) and xylene ($(C_8H_{10})H^+$) compared to the other treatments, NI in particular (figure 17 C and D). It is difficult to state that xylene is emitted by maize roots, because it is a known air pollutant (Treesubsuntorn and Thiravetyan, 2018), which means that it could be due to contamination of the background atmosphere air in the glass jar. Methylenecyclohexane on the other hand is an interesting VOC, because it is known to be emitted by fungi of the genus *Trichoderma,* a fungal genus known for their ability to enhance plant growth due to their ability to reduce plant diseases, making them useful biofungicides in agricultural soils (Lee et al., 2016; Ortega et al., 2021). So, the emission of this volatile could be an indication of their presence. In the NIC treatment, salicylic acid ($(C_7H_6O_3)H^+$) was the most emitted VOC. This is a stress-related plant hormone (Dani and Loreto, 2022) that can also be emitted as a result of leaf wounding in the form of methyl salicylate (Portillo-Estrada et al., 2021). However, it is not clear why it was emitted more by this treatment in particular. So what can be concluded is that despite these significant differences in concentrations of some VOCs between certain agricultural management types and harvested root types, that it not enough to make the VOC profiles significantly different. This is probably because there are not enough VOCs (only 1 or 2 in most treatments) that have a significantly higher emission or that have high relative abundance in

a certain treatment, in order to make the whole VOC profile of that treatment differ significantly from the other treatments.

4.4. Effectiveness of the direct soil measuring technique

The effectiveness of the direct soil VOC measurement was mixed, with its success depending on a multitude of factors. The first factor is the soil texture, where we saw that that it plays a crucial role in the ability to measure soil VOCs. This is really visible when we compare the second run of the AMF experiment with the results of the soil experiment, where the soil was compacted and water saturated. Issues relating to VOC collection and measurement were solved in the second run of the AMF experiment, where the soil was not water saturated and not compressed, leading to more representative VOC measurements. This however makes the use of the direct soil measurement technique impossible in naturally water saturated soils. The second factor that we had to consider was the sampling method, with the syringe proving the most effective method to sample VOCs from the soil in this experiment. This is mainly for one reason: traveling distance between the pot and the PTR-TOF-MS. The first method used in this experiment was a long teflon tube, that connected the measuring instrument and the funnel in the pot, which proved to be ineffective in sampling VOCs due to a high dilution of the VOC concentrations before entering the mass spectrometer. As a consequence, this created a very bad signal, with a concentration lower than the background noise. The syringe solved that problem by keeping the VOCs more concentrated, before they were administrated to the PTR-TOF-MS.

However, despite successfully correcting a lot of these issues, the direct soil VOC measurement method is not yet able to clearly determine which organisms emitted the VOCs. We tried solving this problem by doing ^{13}C-labelling pilot-experiment (see supplement 2), to determine which VOCs could be emitted by plants. The main goal of that experiment was to use the increase in concentration of the ^{13}C-containing molecule of the VOC of interest as an indicator for the emission of the VOC by plant roots. This unfortunately failed, probably due to factors such as a low $^{13}CO_2$ concentration in the cuvette (we for example added 10% $^{13}CO_2$), low exposure time or the wrong way of adding ^{13}C. In other studies using this technique, other methods were used to label plants, such as the addition ^{13}C-glucose in xylem or cut shoots (used in Ghirardo et al. (2011)) and the addition 99% ^{13}C enriched pyruvate to cut plant shoots at ether the C1 and C2 position dissolved in deionised water, that was used in Fasbender et al. (2018) and Werner et al. (2020). These methods were not available in time for measurement, making it difficult to test their effectiveness in our experiment. Moreover the pilot-experiment was not continued after the failed test, due to time constraints. However, it could also be

the case we were just unable to detect the VOCs originating from plants or mycorrhizae, because we did not measure deep enough in the soil. This is because for a lot of VOCs the detectability decreases drastically with distance depending on the unique diffusion ability of each VOC, as proven by Schulz-Bohm et al. (2018). So it could be that we were too far from the root or hyphae, to measure the VOCs emitted by them. This could again be a reason to assume that we mostly measured VOCs emitted during decomposition by bacteria, as the Procrustes analysis of the AMF experiment suggests. This makes it interesting to investigate how the VOCs could differ between the upper part of the pot, where the funnel was located, and the deeper part of the pot, which was closer to the roots. So what can be concluded is that the direct soil VOC measurement technique is promising, but it still needs significant improvements in order to capture the VOCs in the rhizosphere more effectively.

5. Conclusion

VOCs play a crucial role in inter- and intraspecies interactions responsible for a multitude of ecosystem services in the rhizosphere, such as the fixation of nitrogen and the acquisition of nutrients. For this reason, it is important to investigate how VOCs can be used to analyse the important soil processes and interspecies interactions in a non-destructive manner. In order to understand how these processes can change depending on certain land use types. The measurement of these VOCs however remains difficult, with the main complicating factor for their measurement being their diffusion in the soil matrix. A problem caused by both the soil texture and the water saturation rate. Mainly because of this difficulty, there is currently still no well-established method to measure them. An additional complicating factor could also be the representativeness of the measurements, due to changing soil VOC emissions over time caused by altering soil processes. This makes multiple measurements over time necessary. A lot of these problems were solved in this experiment, by keeping the soil well-aerated, by not diluting the VOCs more during sampling and by measuring soil VOCs multiple times throughout the plants lifetime. By solving these problems, we were able to determine that the direct soil VOC measurement method can be effective in well-aerated soils. However, it is important to note that there are still several unresolved issues in the direct soil VOC measurement method. The first one is that we are unable to distinguish the VOCs that are emitted by roots, AMF and other microorganisms. This is a problem, because in order to further investigate the role VOCs play in soil processes we have to know which organisms emit them. The second big issue is that we probably did not measure deep enough in the soil, which made us unable to for example measure the VOCs emitted by roots that do not diffuse over longer distances in the soil matrix. This also increased the likeliness that we only measured the VOCs emitted by bacteria that inhabit the higher soil layers. Solving these issues in future research is crucial in order to further explore the role VOCs play in the rhizosphere.

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Supplementary material

1. Principle of the PTR-TOF-MS

The PTR-TOF-MS 8000 is a measuring instrument consisting out of four main parts (figure S.1): 1) the hollow cathode, 2) a drift tube reaction chamber, 3) a transfer lens and 4) the time-of-flight (TOF) region (Kari et al., 2018). In the hollow cathode (or discharge cathode) H_3O^+ ions are generated from water vapor, which is introduced as a reagent gas from a liquid water sample holder that contains pure water (figure S.1) (Jordan et al., 2009; Kari et al., 2018). The generated H_3O^+ ions are transported to the drift tube where they meet the sample air which contains VOCs (Kari et al., 2018). This sample air is continuously injected in the drift tube via a gas inlet (Jordan et al., 2009). In the drift tube a proton transfer reaction takes place where a H^+- ion is transferred from H_3O^+ to the VOC according to formula S1 (Jordan et al., 2009; Portillo-Estrada et al., 2021).

$$H_3O^+ + VOC \longrightarrow H_2O + VOCH^+ \quad \text{(S1)}$$

It is important to note that this reaction only occurs when VOCs have a higher proton affinity than H_2O (697 kJ/mol). This transfer reaction does not occur with VOCs that have a lower proton affinity than water (Portillo-Estrada et al., 2021). The protons are then driven through the drift tube by means of voltage acceleration until they reach the lens system, located between the drift tube and the TOF region (Jordan et al., 2009; Portillo-Estrada et al., 2021). During this experiment we maintain a pressure of 2.3 mbar, a voltage of 600 V and a temperature of 80°C to reach a field density ratio (E/N) of 140 Td (Td=Townsend; 1 Td=10^{-17} V cm^2). "E" is the electric field and "N" is the gas density (Portillo-Estrada et al., 2021). However, we experienced problems in the PTR-TOF-MS during the later stages of the experiment, being that the pressure of the drift tube was unable to reach 2.3 mbar. Forcing us to change the voltage to the drift tube to values between 390 and 560 V to maintain an E/N of 140 Td, the wanted conditions. Maintaining this condition is crucial, because the E/N determines the level of fragmentation of the VOCs (Kari et al., 2018; Portillo-Estrada et al., 2021). With higher E/N leading to a higher number of energetic conditions inside the drift tube, resulting in a higher degree of VOC fragmentation (Kari et al., 2018). After the protonated VOCs exit the drift tube, they enter the lens system, where the protonated VOCs will be 'guided' towards the TOF region with a typical repetition rate up to 80 kHz (Jordan et al., 2009; Kari et al., 2018). In the TOF region the VOCs will be exposed to a similar kinetic energy, while they all follow a similar distance and have the same charge (Portillo-Estrada et al., 2021). Which means that their 'time of flight' through the TOF region is dependent on their mass (Kari et al., 2018; Portillo-Estrada et al., 2021). The time of flight will follow a pattern determined by formula S2 (Portillo-Estrada et al., 2021).

$$t_{\text{flight}} = \frac{d}{\sqrt{2U}}\sqrt{\frac{m}{q}} \quad \text{(S2)}$$

With t_{flight}= time of flight, d=distance, U= kinetic energy, m= mass and q= charge

As clearly seen in formula S2, the t_{flight} increases as a result of mass, meaning that the smaller molecules will arrive first on the multichannel plate (MCP) at the end of the TOF region before the larger molecules (figure S.1) (Kari et al., 2018; Portillo-Estrada et al., 2021). This time difference is crucial for to determine what the mass of the VOC ion is. The MCP multiplies the signal received by a single hit of a charged molecules (with H^+). The signal is amplified to the level of being registered by the detector, coupled to a time to digital converter (TDC). The latter converts registers the time of flight at a high precision. The time of flight and signal intensities are plotted on a spectrum, whose x-axis will be transformed into molecular mass after the mass calibration (see 2.6.1.), creating a spectrum peak (Portillo-Estrada et al., 2021). For this experiment the protonated VOCs were pulsed through the TOF region every 32µs, generating 31250 spectra every second with a mass range of 1-318 *m/z* (Portillo-Estrada et al., 2021).

Before the experiment began, the PTR-TOF-MS was calibrated for the different selectivity that this instrument has for compound masses, being the smaller compounds detected more poorly than compounds of higher mass (ca. 100% at mass above 160 Da). A calibration curve (known as the transmission curve) was generated using a gas mixture containing eight pure VOCs with known concentrations, with molecular masses ranging from m/z 33 to 137 Da, was inserted in the mass spectrometer, these compounds were methanol (*m/z*= 33.033), acetaldehyde (*m/z*= 45.033), acetone (*m/z*= 59.049), isoprene (*m/z*= 69.070), MVK + MACR (*m/z*= 71.049), t-2-hexen-1-al (*m/z*= 99.081), c-3-hexen-1-ol (*m/z*= 101.061) and α-pinene (*m/z*= 137.133) at nominal concentration of 1 ppmv each (Apel Riemer, USA) (Portillo-Estrada et al., 2021). By inserting these compounds, the PTR-TOF-MS is able to determine how the transmission values of the VOCs at different molecular masses. The correlation between these transmission values and molecular masses are then used to create the transmission curve, which can then be used to inter- and extrapolate the transmission values of the unknown VOCs measured during the experiment (Portillo-Estrada et al., 2018; Portillo-Estrada et al., 2021). The concentration of the VOCs was determined in the PTR-TOF-MS by the peak area of each compound, the concentration of the ^{18}O-isotope containing molecule of H_3O^+ (500 times less abundant then the non-isotope containing compound), the transmission value of the VOC and the reaction rate coefficient of each compound. The reaction rate coefficient of each compound can be determined in three ways: it can be calculated via direct calibration from the same eight compound VOC mixture used for the construction of the transmission curve, it could be assumed as 2×10^{-9} cm^3 s^{-1} or could be

retrieved from literature (Portillo-Estrada et al., 2018; Portillo-Estrada et al., 2021). In this experiment the coefficient of reaction was assumed as 2×10^{-9} cm^3 s^{-1}.

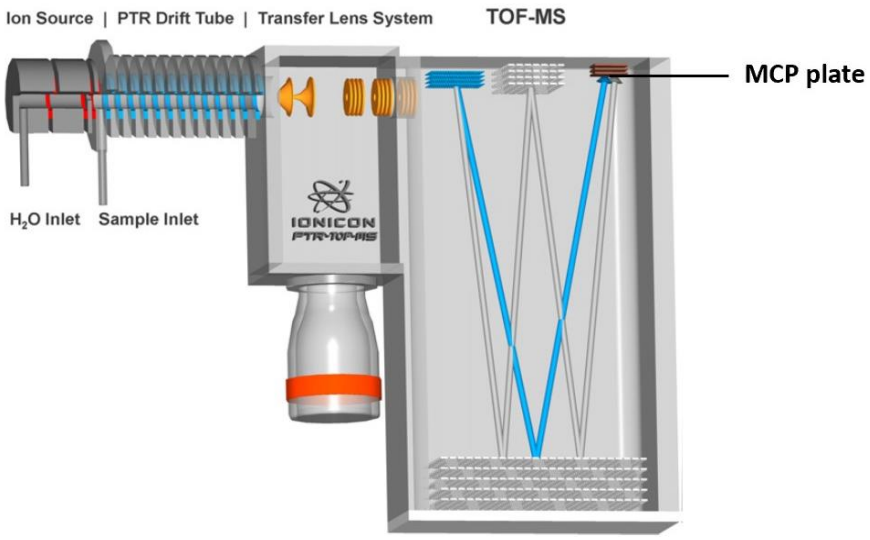

Figure S.1: General build-up of the PTR-TOF-MS, with the four parts: 1) the hollow cathode (= ion source), 2) the drift tube, 3) the transfer lens system and 4) the time-of-flight (TOF) region. In the PTR-TOF-MS that we used, the protons travelled in a V shape towards the MCP plate in the TOF region. (Figure adapted from fig. 1 in Jordan et al. (2009))

2. ^{13}C labelling of maize plants

We tested a setup to label the maize plants with ^{13}C. The goal of this labelling is to help distinguish between the VOCs emitted by the plant or by microorganisms in the soil. By fumigating the aerial part of the plant with $^{13}CO_2$, we hoped to pinpoint VOCs in their isotopic form, thus distinguishing the emissions from the plants and symbionts from the other sources in the soil. Furthermore, we could track the time-course of the transport of ^{13}C from the leaves to the soil, and even potentially distinguish when mycorrhiza would have exchanged the labelled sugars with the roots after detecting mycorrhizal emissions labelled too.

This separate experiment was prepared in the growing room in the basement of building C. In that room a glass cuvette was prepared attached to a Picarro (Picarro inc., Santa Clara, CA, USA) (figure S.2), which monitors the $^{12}CO_2$ and $^{13}CO_2$ concentrations in the cuvette. We tested the capacity of the cuvette to hold a given concentration over time.

A day before the plant was put in the cuvette (figure S.2), that was filled by 2 gas bottles of 99% $^{13}CO_2$ (Sigma-Aldrich, Saint-Louis, MO, USA) in a concentration of 1100 ppb $^{13}CO_2$. This was done the day before, because there had to be tested if the Picarro was able to quantify the increase in $^{13}CO_2$, which it did. The day after a P+AMF+ plant was put in the cuvette for a period for a period of 4 hours. To be sure that the measured soil VOCs were from plant roots, we covered the soil of the plant with

aluminium foil in order to avoid the take up of $^{13}CO_2$ by soil bacteria (figure S.2). Immediately the $^{13}CO_2$ concentration declined to 486 ppb because of there had to be tampered with the cuvette to put the plant in. During this period the $^{13}CO_2$ ran low, so it was supplemented with $^{13}CO_2$ from a gas bottle with a concentration of 10% $^{13}CO_2$ to supplement it. After this fumigation period the plant was removed, followed by soil VOCs measurements with the PTR-TOF-MS. The pot was measured directly, after 2 hours, 21 hours and 25 hours.

To evaluate whether there was an effect of the $^{13}CO_2$ visible we analysed the measured spectra on the software PTR-MS Viewer version 3.4.2. (Ionicon Analytik GmbH, Innsbruck, Austria). After uploading the files in the software, the measured spectra were first mass calibrated again (see 2.6 for more explanation). Followed by defining the spectra, using the monitoring compounds (see 2.6). After the spectra were defined, the peaks of multiple ^{13}C-isotope containing molecules were investigated in order to see if they were higher than during the measurements before ^{13}C labelling. If that is the case, then we could conclude that that volatile was emitted by the plant.

Figure S.2: Set-up ^{13}C labelling. The plant (experiment 1 treatment P+AMF+) was placed in the glass cuvette for labelling with $^{13}CO_2$. To measure the $^{12}CO_2$ and $^{13}CO_2$ concentrations in the cuvette, a Picarro was connected. The soil of the plant was covered by aluminium in order to avoid take up of $^{13}CO_2$ by soil bacteria.

3. Above and below ground biomasses of experiment 1 and 2

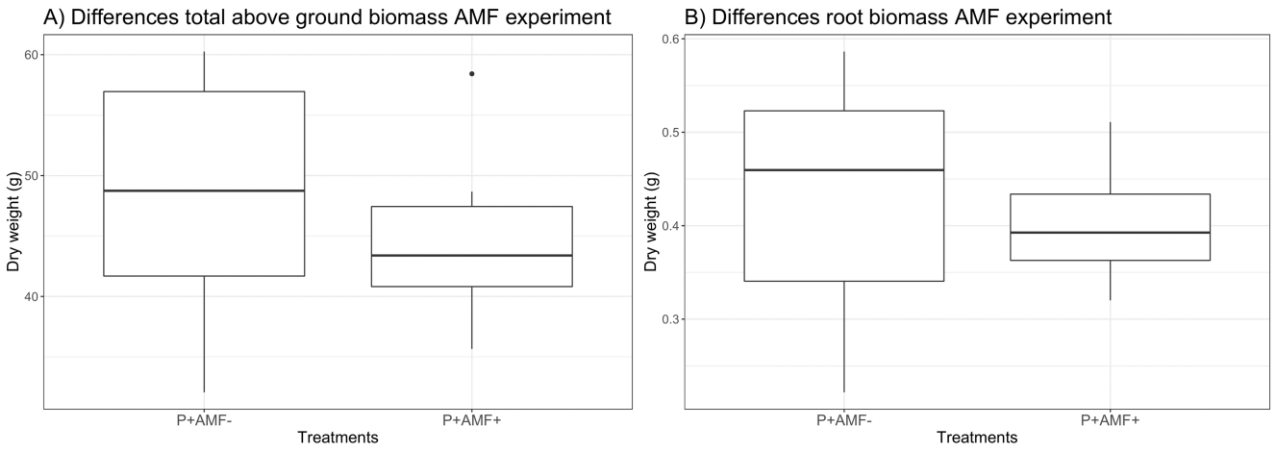

Figure S.3: Differences between the above and below ground (root) biomasses of experiment 1 run 2. A) Difference between the total above-ground dry weights (stem and leaf biomass + fruit biomass) of a plant inoculated with and without AMF. B) Difference between the below ground root biomasses between a plant inoculated with and without AMF. Rate of AMF inoculation: P+AMF-: low AMF inoculation, P+AMF+: high AMF inoculation.

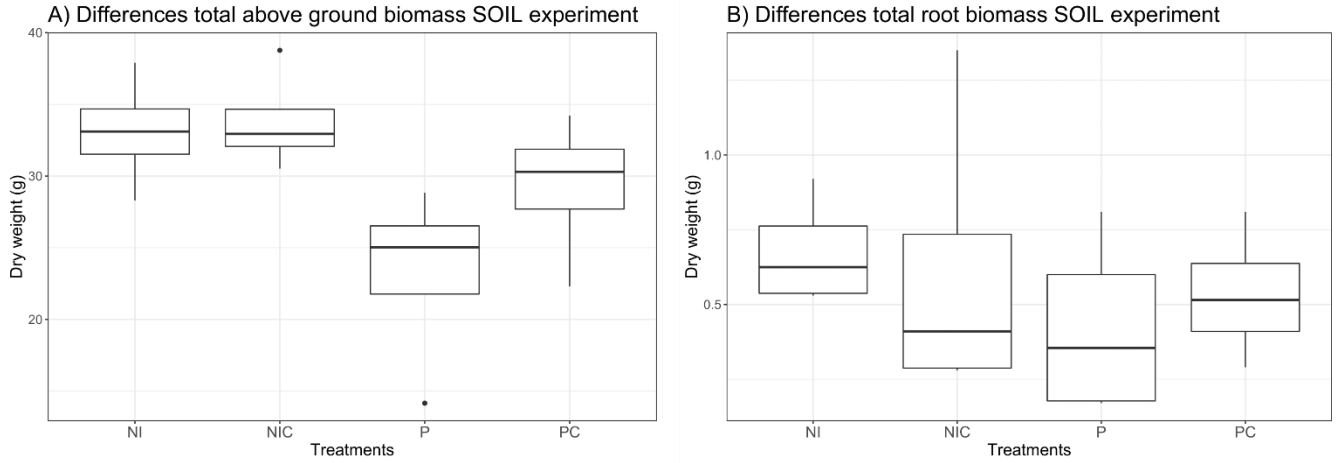

Figure S.4: Difference between the above and below ground dry weights between the agricultural management practices in experiment 2. A) Difference between the total above-ground biomasses (stem and leaf biomass + fruit biomass) of the agricultural managements. B) Difference between the below ground dry weights of the agricultural managements. The agricultural management practices: NI = Non-inversion tillage, NIC= Non-inversion tillage with compost, P = Ploughing and PC = Ploughing with compost.

4. VOCs emitted more by the NIC treatment in the harvested roots of the Soil experiment

Table S.1: The VOCs that are emitted more in the "non-inversion tillage with compost" (NIC) treatment by the harvested roots of experiment 2 (also known as the "Soil experiment"), found by the sPLSDA. Of all these volatiles, only salicylic acid ((C7H6O3)H+, *m/z* = 139.039) was the most positively associated with the NIC treatment, compared to the other agricultural soil management types. This compound is highlighted in bold. When molecule name is shown as "Unknown", it means that the molecular structure is unclear.

Molecular mass (*m/z*) in Da	Molecule formula	Molecule name
29.039	$(C_2H_4)H^+$	Ethene
31.024	$(CH_2O)H^+$	Formaldehyde
33.045	$(CH_4O)H^+$	Methanol
41.043	$(C_3H4)H^+$	Propyne
42.01	$(C_2HO)H^+$	Unknown
42.039	$(C_2H_3N)H^+$	Acetonitrile
43.02	$(C_2H_2O)H^+$	Ketene
43.06	$(C_3H_6)H^+$	Propene
45.033	$(C_2H_4O)H^+$	Acetaldehyde
46.029	$(CH_3NO)H^+$	Formamide
47.012	$(CH_2O_2)H^+$	Formic acid
47.049	$(C_2H_6O)H^+$	Ethanol
48.021	$(CH_3O_2)H^+$	Methyldioxy radical
55.054	$(C_4H_6)H^+$	Butadiene
57.034	$(C_3H_4O)H^+$	Hexenal
57.07	$(C_4H_8)H^+$	Butene
59.053	$(C_3H_6O)H^+$	Acetone
60.056	$(C_3H_7O)H^+$	Unknown
71.013	$(C_3H_2O_2)H^+$	Propiolic acid
73.065	$(C_4H_8O)H^+$	Butanal
75.047	$(C_3H_6O_2)H^+$	Hydroxyacetone or Propionic acid or Methyl acetate
79.054	$(C_6H_6)H^+$	Benzene
81.073	$(C_6H_8)H^+$	Hexenal or Monoterpenes
87.008	$(C_3H_2O_3)H^+$	Unknown
89.06	$(C_4H_8O_2)H^+$	Ethylacetate
93.077	$(C_7H_8)H^+$	Toluene or Para-cymene
113.023	$(C_5H_4O_3)H^+$	2-Furoic acid
135.08	$(C_9H_{10}O)H^+$	Unknown
139.039	**$(C_7H_6O_3)H^+$**	**Salicylic acid**
167.07	$(C_9H_{10}O_3)H^+$	Ethyl salicylate
191.11	$(C_9H_{19}O_2S)H^+$	2-Ethylheptane-1-sulfinate