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Investigating the effect of belowground microbial volatiles on plant nutrient status: perspective and limitations

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ABSTRACT

Volatile organic compounds displayed biological activities on a wide range of organisms, including plants and microbes. Investigating their role in the plant-microbe interaction processes occurring in the soil is challenging. By simulating belowground communication conditions between plant and microbes, in this study, we aimed to investigate the effects of the volatiles emitted by *Serratia plymuthica* and *Fusarium culmorum* on the nutrient status of maize plants. Plants were grown in potting soil and exposed to volatiles emitted by microbes inoculated in Petri dishes at the bottom of a jar. Nutrients content of plant tissues as well as soil volatiles were analyzed by ICP-MS and GC-MS, respectively. Our results showed that volatiles emitted belowground by *Serratia plymuthica* and *Fusarium culmorum*, in monoculture or interaction, differentially impacted on the content of some nutrient in plants, indicating that microbial volatiles-emitted belowground can affect the nutritional status of plants from a distance.

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Belowground communication; microbial volatiles; plant nutrients

1. Introduction

Plants are involved in intimate interactions with microbes during their entire life, and these interactions are essential for plant growth and health. To cope with different environmental stresses (biotic and abiotic), plants have developed sophisticated mechanisms, involving the production and exudation/emission of an arsenal of chemical compounds able to activate defence strategies. Such compounds define the communication processes between plants and other organisms in the surrounding environment both above and belowground. In the soil, plant-microbe interactions mainly occur in the rhizosphere, where a variety of microbes are attracted by root exudates (primary and secondary plant metabolites) (van Dam and Bouwmeester 2016). The rhizosphere microbiome has important effects on plant health including facilitation in nutrient acquisition, improved abiotic stresses tolerance, plant growth promotion and increased defences against pathogens and insects (Mendes et al. 2011; Pieterse et al. 2014). Soil microbes are able as well to produce and emit a wide array of volatile and non-volatile compounds (Caulier et al. 2019). Recent studies reported that volatile compounds displayed significant biological activities on a wide range of organisms, including plants and microbes (Groenhagen et al. 2013; Piechulla et al. 2017). Volatile organic compounds (VOCs) are small molecules (<300 Da) with high vapor pressure, low boiling point and containing a lipophilic moiety (Schulz and Dickschat 2007). These molecules can spread through the air-filled pores of the soil, allowing long-distance communication among organisms. The chemical structure of VOCs ranges from small aliphatic and aromatic molecules to ketones, alkanes, alkenes and terpenes (Garbeva and Weiskopf 2019). The ‘volatilome’ of an


organism consists of a mixture of different chemical compounds which composition and abundance depend on the growth stage of the organism (Kai et al. 2010; Blom et al. 2011a) and, in the case of plants, also on the organ under consideration (e.g. root or leaf).

The different roles of bacterial VOCs on plants have been recently reviewed by Garbeva and Weiskopf (2019). Most studies show a beneficial impact of bacterial VOCs on plant health by promoting a positive effect on plant growth, induce resistance to biotic stresses, and increase tolerance to abiotic stresses. For example, volatiles emitted by some plant-growth-promoting rhizobacteria (PGPR) stimulate plant growth and induce systemic disease resistance against plant pathogens (Ryu et al. 2003; Lee et al. 2012; Park et al. 2015; Raza et al. 2016; Tahir et al. 2017).

Most of the data showing the beneficial effects of bacterial VOCs on plants have been obtained in artificial environments (Bailly and Weiskopf 2012; Liu and Zhang 2015). A dual plate system, which relies on the avoidance of direct contact between plant and microbes, is commonly used for testing the effect of microbial volatiles on plant growth (Liu and Zhang 2015). However, this *in vitro* system largely differs from the belowground communication conditions. To overcome this point, experimental systems were established, consisting of plants growing in potting soil and exposed to volatiles emitted to microbes growing in Petri dishes at the bottom of a jar/box (Park et al. 2015; Tahir et al. 2017; Cordovez et al. 2018).

In the present study, we aimed to test the effect of microbial volatiles emitted belowground on the nutritional status of maize plants by using a *pot-in-jar* system. We tested the ability of volatiles to diffuse freely in soil, by trapping the

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volatiles in the soils. As model microbes, we used the beneficial bacteria *Serratia plymuthica* and the plant-pathogenic fungus *Fusarium culmorum*. *Serratia plymuthica* PRI-2C is an enterobacterium isolated from maize rhizosphere that shows strong antimicrobial activity against several fungal plant pathogens (Garbeva et al. 2014). *F. culmorum* is one of the most important fungal soilborne plant pathogens that causes diseases on a wide diversity of cereal crops, including maize. When exposed to the volatiles emitted by this fungus, *S. plymuthica* experienced an increased emission of the volatile terpene sordorifen (Schmidt et al. 2017). Our results showed that volatiles emitted belowground by these two microbes in monoculture or interaction affected the content of some nutrients in the maize plants.

2. Material and Methods

2.1. Strains and growth conditions

The bacterial strain *S. plymuthica* PRI-2C was isolated from rhizosphere of maize plants at different phenological stages and grown in the Wildekamp field, located in Bennekom, The Netherlands (Garbeva et al. 2004). The soil in this field was a loamy sand rich in organic matter (2.5%) with slightly acidic pH (5.5–6.5). The *S. plymuthica* PRI-2C strain was pre-cultured from frozen glycerol stocks on 0.1 Tryptic Soy Broth agar plates (TSB) and grown for three days at 20°C before it was used (Garbeva and De Boer 2009). The fungal strain *F. culmorum* PV was isolated from a sandy dune soil in The Netherlands (De Boer et al. 1998), pre-cultured on 0.5 Potato Dextrose Agar plates (PDA) (Fiddaman and Rossall 1993) and incubated for five days at 20°C before use.

2.2. Seeds priming

Maize seeds (*Zea mays* cultivar PR39F58) were surface sterilized and germinated on Petri dishes with moistening filter paper in the dark and at 25°C for two days. Seedlings were primed via volatiles by exposing them to the volatile compounds produced by *S. plymuthica* PRI-2C, *F. culmorum* PV, or both in interaction. Seedlings were placed on the lid of a petri dish (10 cm diameter) containing 0.5 Murashige & Skoog (M&S) agar. *F. culmorum* PV and *S. plymuthica* PRI-2C were grown on small (3.5 cm) agar plates containing 3 mL 0.5 PDA medium or 1.5% water-agar supplied with artificial root exudates (WA + ARE). The composition of ARE stock solution was as follows: 18.4 mM glucose; 18.4 mM fructose; 9.2 mM saccharose; 4.6 mM citric acid; 9.2 mM lactic acid; 6.9 mM succinic acid; 18.4 mM L-serine; 11 mM L-glutamic acid and 18.4 mM L-alanine (C/N 10.4) according to (Schmidt et al. 2016). *F. culmorum* PV plugs (6 mm in diameter) were inoculated on 0.5 PDA plates. A volume of 150 µl of 10⁷ cells/ml *S. plymuthica* PRI-2C suspension was inoculated on WA+ARE plates (1.5% water agar supplied with artificial root exudates (ARE)) (Schmidt et al. 2016). The seedlings and the small agar plates containing the bacteria and/or the fungus were all placed inside a 120×120 mm square plate. The plate was closed and sealed with parafilm. A control treatment was set up by exposing the seedlings only to agar media. Seeds were primed for 5–6 days in the greenhouse with the following conditions: temperature during the day (16 h): 21°C; temperature during the night: 16°C; an average level of humidity: 60% Rh.

2.3. Effect of microbial volatiles on maize plants

In order to investigate the response of plants to volatiles emitted by *F. culmorum* and/or *S. plymuthica* PRI-2C, a pot-in-jar system was used, based on the protocol described in Tahir et al. (2017) with a few modifications (Figure 1). Small Petri dishes (diameter: 3.5 cm) inoculated with the bacteria, the fungus or both, were placed in the bottom of the jars. A sterile mesh was used to contain the soil (200 g of gamma-sterilized sand) in the pots. Primed seedlings were transferred to the sandy soil and exposed to the microbial volatiles for two weeks in total. Plants were grown in the greenhouse at the above-mentioned conditions, and 10 ml/pot of 0.5 Hoagland solution was added every two days. After a week, small Petri dishes containing the bacterial suspension and the fungal plugs were removed and replaced with new ones. Six replicates for every treatment were used. For the volatile collection in the soil, traps containing 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd, Llantrisant, UK) were used. After overnight collection, the Tenax traps were stored at 4°C until subsequent analyses. After two weeks, all the plants were harvested, and growth parameters (whole plant weight, shoot weight, root weight and root length) were determined. ImageJ was used to measure root length.

2.4. Volatiles analysis

Volatile organic compounds were desorbed from the traps using an automated thermal desorption unit (Unity TD-100, Markes International Ltd., UK) at 210°C for 12 min (He flow 50 ml/min) and trapped on a cold trap at –10°C. The volatiles were introduced into a GC-MS-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap for 3 min to 280°C. The split ratio was set to 1:5, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424–6850, Bellefonte, PA, USA). The temperature program was as follows: 39°C for 2 min, from 39°C to 95°C at 3.5°C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min, and finally to 300°C at 40°C/min, hold 20 min. The volatiles were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full-scan mode (30–400AMU, four scans/s) and extracted with MassHunter Qualitative Analysis Software VB.06.00 Build 6.0.633.0 (Agilent Technologies, Santa Clara, USA) using the GC-Q-TOF qualitative analysis module. Statistical analyses were performed using Metaboanalyst 4.0.

2.5. Ionome profile

Shoot, and root samples were oven-dried at 70°C for six days. Dry weight was measured. Sampled tissues were dried and then mineralized in HNO₃ by using a Microwave Digestion System (Multiwave ECO). Macronutrients and micronutrients content was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, aurora M90 BRUKER). The data collected are from three independent biological replicates.

Statistical differences between treatments were determined using one-way ANOVA followed by pairwise post hoc Tukey's test, in the case of multiple comparisons. For pairwise

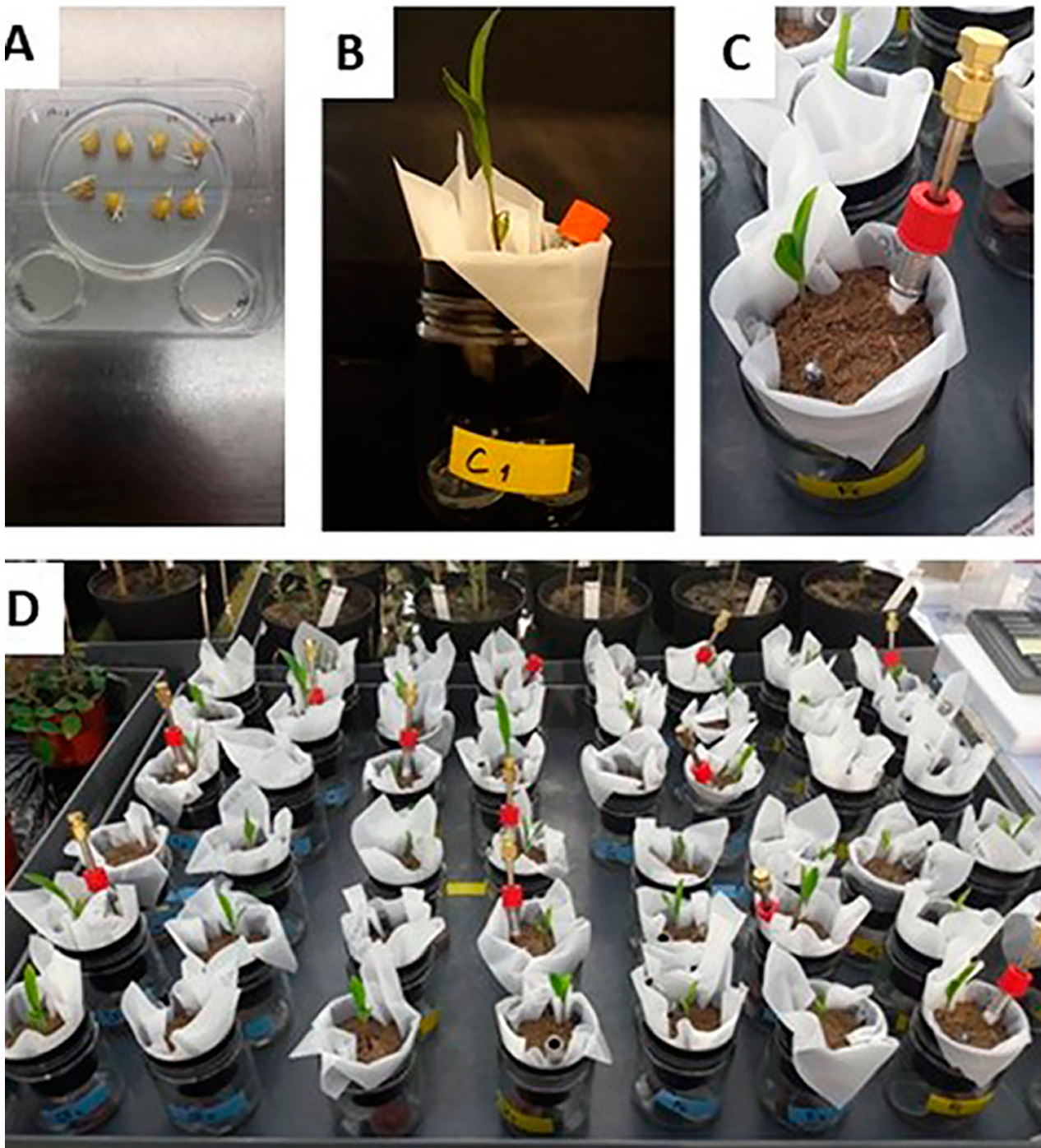


Figure 1. (A) seeds priming; (B) pot-in-jar system; (C) volatiles trapping system application (D) plants growing in the greenhouse.

comparisons T-test has been used. All statistical analysis has been performed using the software Past3.

3. Results and discussion

3.1. Microbial-emitted volatiles belowground affect nutrient content in maize plants

The volatiles emitted by either *S. plymuthica* and *F. culmorum* (in monoculture or in interaction) did not significantly affect plant growth after 14 days, even though the shoot biomass of the plants exposed to *S. plymuthica*'s volatiles slightly increased compared to the control plants (Table S1). However, changes in the nutrient content were detected both in shoot and root of maize plants exposed to the different microbial volatiles.

The PCA performed on the macronutrients content of shoot revealed that the first and second component (PC1 and PC2) explained about 98% of the total variability (PC1:96,5% and PC2: 2.18%) (Figure 2). The separation observed between the control samples from all treatments along the PC1 indicated that the presence of microbe-emitted volatiles had an impact on the content of macronutrients. Additionally, a separation of the *F. culmorum* monoculture from the other treatments was evident along the PC2, suggesting that the volatile blend produced by pathogenic *F. culmorum* affected the macronutrient content in different ways (Figure 2).

At the root level, the PCA performed on macronutrients content identified two principal components, PC1 and PC2, which explained the 97,44% and the 1,62% of the total

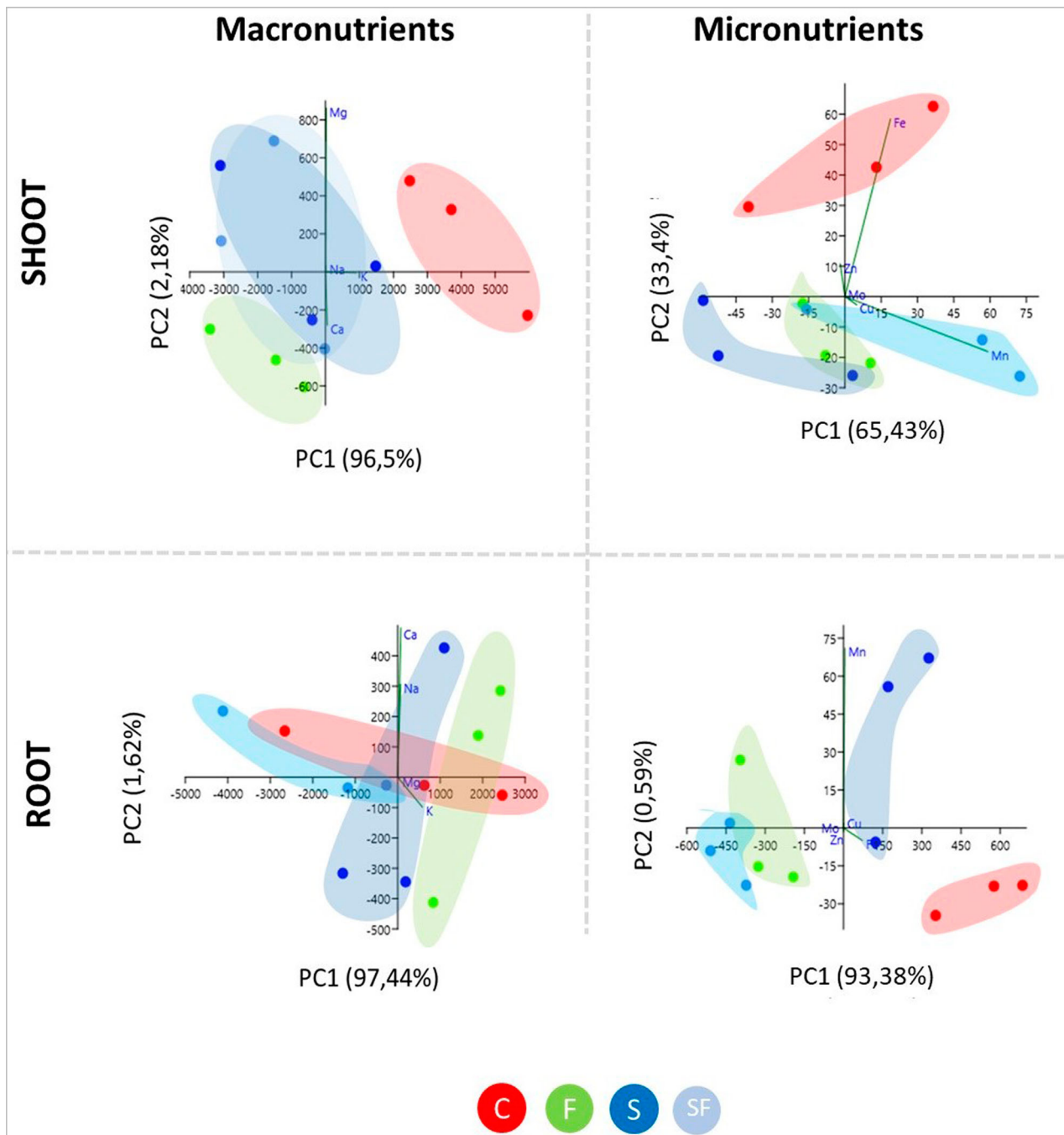


Figure 2. Principal component analysis (PCA) of macronutrients and micronutrients content in both shoot (upper panels) and root (lower panels) tissues of maize plant grown under treatments: C, control; F, *F. culmorum* in monoculture; S, *S. plymuthica* in monoculture; SF, both *S. plymuthica* and *F. culmorum* in interaction.

observed variance, respectively. However, great variability was observed for each treatment (Figure 2).

The PCA performed on the micronutrient content in shoot identified two principal components, PC1 and PC2, which explained the 65,43% and 33,4% of the total variance observed, respectively (Figure 2). The PCA scatterplot revealed a clear separation between the control and the treatments along the PC2, indicating that microbial volatiles affect the micronutrient content in shoot tissues. Furthermore, a separation between the treatments was observed along the PC1, indicating that the volatiles emitted during the interaction of *S. plymuthica* and *F. culmorum* provide a different effect compared to the volatiles produced in monoculture. The PCA performed on the micronutrient content of root samples identified two principal components, PC1 and PC2, which explained 99,38% and 0,59% of the observed variance,

respectively. The scatterplot revealed a clear separation among all the treatments along the PC1, indicating that the volatiles produced by the different microbes in monoculture and interaction, differentially affected micronutrients content in maize root (Figure 2).

One-way ANOVA analysis revealed significant differences among the treatments ($F, p < 0,05$) in K, Fe, Zn and Mo content in shoot samples, while significant differences in Mn, Fe and Cu were observed at the root level (Table 1). Among the essential macronutrient, K contents decreased in the plants exposed to microbial volatiles (monoculture and interaction) when compared to the control (Tukey's test, $p < 0,05$) (Table 1). Furthermore the content of non-essential nutrients, like Na, was affected in shoot samples of plants exposed to microbial volatiles (monoculture and interaction) when compared to the control ($F, p < 0,05$) (Table 1).

Table 1. Mineral nutrients content (expressed as $\mu\text{g g}^{-1}$ DW) in shoot and root tissues of maize plants grown in pot-in-jar system under the following treatments: C, control (plant not exposed to volatiles); S, plant exposed to *Serratia*-emitted volatiles; F, plant exposed to *Fusarium*-emitted volatiles; SF, plant exposed to both *Serratia*-emitted and *Fusarium* – emitted volatiles. Data are shown as the mean (\pm SE) from three independent biological replicates.

	shoot				root			
	C	S	F	SF	C	S	F	SF
Na	174.41 ± 11.55 b	92.69 ± 8.93 a	96.44 ± 5.69 a	101.62 ± 7.93 a	999.22 ± 149.33 a	977.41 ± 269.63 a	1072.35 ± 158.19 a	751.95 ± 27.14 a
Mg	4115.1 ± 121.24 a	3894.61 ± 235.1 a	3366.42 ± 103.9 a	4055.19 ± 302.6 a	1011.65 ± 159.8 a	957.91 ± 51.35 a	1111.41 ± 85.93 a	838.5 ± 67.48 a
K	13625.92 ± 1003.27 b	8925.23 ± 1327.54 a	7763.34 ± 27.63 a	8030.71 ± 865.24 a	8659.13 ± 1479.2 a	8534.42 ± 651.98 a	10215.23 ± 422.15 a	6697.43 ± 371.66 a
Ca	3817.66 ± 311.15 a	3470.44 ± 124.06 a	3708.97 ± 128.82 a	3862.79 ± 318.37 a	1964.1 ± 152.58 a	1832.26 ± 247.46 a	2206.61 ± 274.05 a	1762.03 ± 60.72 a
Mn	275.29 ± 18.7 a	255.55 ± 20.44 a	284.55 ± 9.41 a	325.16 ± 27.58 a	141.72 ± 10.45 ab	185.02 ± 25.91 b	108.83 ± 11.71 a	92.48 ± 6.06 a
Fe	164.06 ± 16.17 b	95.72 ± 4.98 a	106.35 ± 4.10 a	118.75 ± 3.28 a	1293.55 ± 97.37 c	958.37 ± 60.20 b	451.09 ± 60.32 a	317.64 ± 39.72 a
Cu	8.28 ± 0.78 a	7.87 ± 0.09 a	9.47 ± 0.95 a	14.13 ± 3.76 a	23.36 ± 1.62 c	16.51 ± 2.97 c	12.32 ± 0.89 b	2.92 ± 1.86 a
Zn	51.83 ± 4.37 b	40.53 ± 3.12 ab	38.07 ± 0.56 a	37.83 ± 1.49 a	26.02 ± 1.24 a	25.89 ± 2.66 a	35.6 ± 1.33 a	29.03 ± 4.12 a
Mo	0.86 ± 0.06 c	0.33 ± 0.03 b	0.04 ± 0.004 a	0.28 ± 0.009 b	0.61 ± 0.02 a	0.14 ± 0.06 a	0.54 ± 0.32 a	0.41 ± 0.07 a

Note: Different letters (in bold) mean significant difference ($p < 0.05$).

Among the micronutrients, Fe content decreased in the shoot of all maize plants exposed to the microbial volatiles when compared to the control, while plants exposed to *F. culmorum* volatiles (in monoculture and interaction with *S. plymuthica*) displayed a lower Fe content at the root level compared to the ones exposed to *S. plymuthica* alone and in control (Table 1). Zinc content decreased in the plants treated with *F. culmorum* compared to the control only in shoot tissues. By contrast, Cu content decreased in plants exposed to *F. culmorum*-emitted volatiles only at the root level. Additionally, microbial volatiles differentially affected Mo content mainly at the shoot level (Table 1). Among the nutrients considered, only Fe content was affected both in root and shoot, suggesting that Fe homeostasis might be one of the main targets of the VOCs

produced by these microbes. Iron deficiency is a nutritional disorder which impairs plant growth. Although, the shoot Fe content decreased about 40%, 35% and 27% in maize plants under S, F and SF treatments respectively, the plant biomass was not affected. Shi et al. (2018) observed that the shoot biomass significantly decreased in B73 and Mo17 maize lines (differing in chlorosis susceptibility) with a decrease of Fe concentration by about 62% and 30% respectively, while small (B73) and any (Mo17) difference in root biomass was observed. Such findings highlight the concept that the critical Fe concentration in plants depends on the genotype. However, such low content of Fe might negatively impact on maize plants later, in a more advanced developmental stage.

Furthermore, plants exposed to volatiles emitted by *F. culmorum* lead to a content decrease of other nutrients like Zn and Mo in the shoot. Molybdenum variation might be associated with the sulfur status of the plants. Transporters mediating Mo and S uptake in plants belong to the same protein family of SULTR transporters (Bittner 2014). Variation of Mo content in plants is associated with the variation of S availability as well as of Fe availability (Courbet et al. 2019 and references therein). At the root level, volatiles-emitted by *F. culmorum* strongly affected also Cu content, which is known to interact with Fe homeostasis in plants (Bernal et al. 2012).

The mineral content profile (ionome) of plants is a complex network of nutrients, and an impaired availability of a given element has an impact on the whole ionome profile (Pii et al. 2015). Therefore, the changes observed in the nutrient status of plants under different microbial volatiles exposure might result from the nutrient interaction that occurs in plants. It is not possible to identify the direct nutrient target of volatiles blend at this stage.

3.2. Analysis of volatiles in soil

Volatiles were collected from the soil for each treatment using Tenax traps and were analyzed by high-resolution GC/MS. A separation of the volatile metabolites trapped in the different soil treatments was observed in the PLS-DA plot (Figure 3).

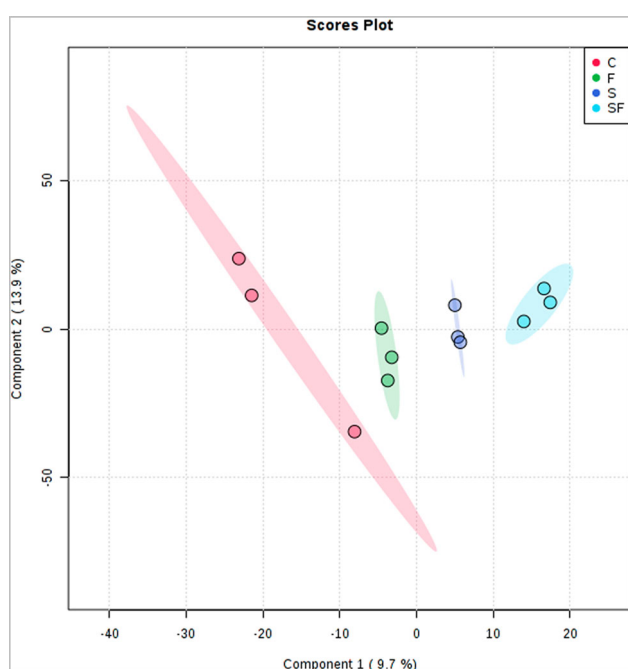


Figure 3. PLS-DA 2D plot of the volatiles collected from the different treatments. C, control; F, *F. culmorum* in monoculture; S, *S. plymuthica* in monoculture; SF, both *S. plymuthica* and *F. culmorum* in interaction. The explained variance is shown in brackets.

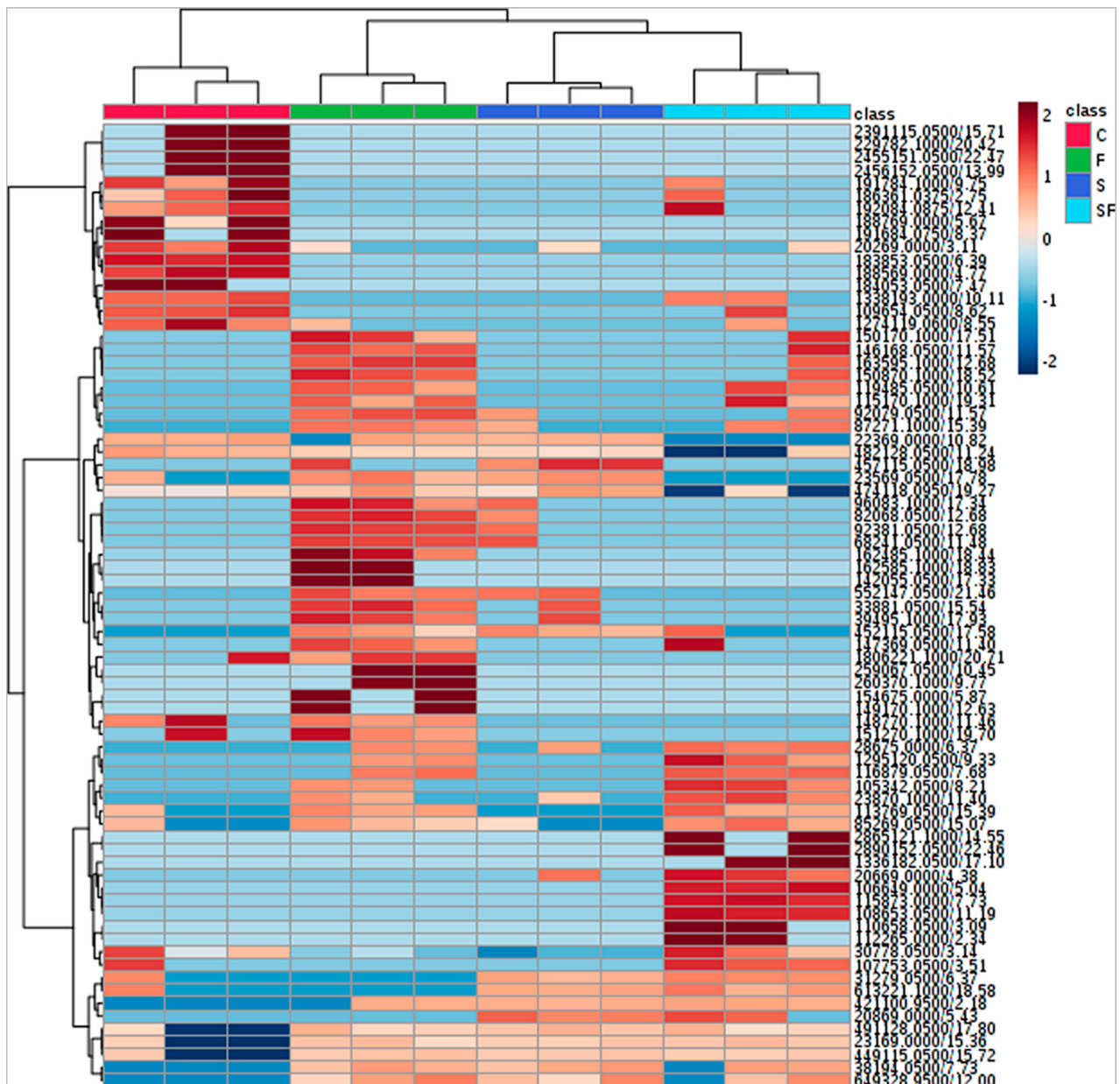


Figure 4. Heatmap showing the hierarchical clustering of the samples based on Euclidean distance. The top 75 mass features for the different treatments ranked by t-tests are shown. Columns represent the samples, in three replicates. Rows represent mass features. Blue, low abundance; Red, high abundance. C, control; F, *F. culmorum* in monoculture; S, *S. plymuthica* in monoculture; SF, both *S. plymuthica* and *F. culmorum* in interaction.

Several mass features in the heatmap were only present in some treatments (Figure 4). Nevertheless, there are no significant differences in the volatile compounds trapped in the treatments and control. It is known that different blends of VOCs are produced by diverse soil microbes in monocultures and interactions (Tyc et al. 2015; Schulz-Bohm et al. 2017). In soils, the production levels of volatile compounds emitted by microbes are presumed to be low (Insam and Seewald 2010) and variations in soil physico-chemical characteristics (for instance, the change of humidity levels when the nutrient solution was added in the pots) can lead to rapid evaporation of volatile compounds resulting in inconsistent outcomes (Ryu 2015).

Next to the volatile organic compounds bacteria also emit inorganic volatiles such as nitric oxide (NO), hydrogen sulfide (H₂S), ammonia or hydrogen cyanide (HCN) (Audrain et al. 2015) which cannot be detected with our trapping system, and that might be responsible for the differences in nutrient content observed in the present study. For

example, it is known that NO regulates several processes in plant cells (Moreau et al. 2010) as well as Fe homeostasis (Chen et al. 2010). Furthermore, it has been reported that inorganic volatile compounds affect plant and microbe growth. For instance, the volatile compound HCN, produced by *Pseudomonas* spp., *Rhizobium* spp., *Chromobacterium violaceum* and *S. plymuthica* IC14, has a direct inhibitory effect on plants, fungi and bacteria (Castric et al. 1981; Antoun et al. 1998; Blumer and Haas 2000; Blom et al. 2011b).

4. Conclusion

The interactions among soil organisms contribute to the proper functioning of an ecosystem. However, the role of belowground VOCs on plant nutritional status is largely underexplored.

An evident variation in the nutrient content of the plants was observed in response to microbial VOCs. The content of nutrients like, K, Fe, Zn and Mo was altered in all treatments

compared to the control plants, indicating that the different volatile blends have an impact on the nutrient status of the plants. Particularly, volatiles emitted by *S. plymuthica* and *F. culmorum* affected the content of micronutrients, such as Fe, Zn, Cu and Mo.

Overall, such findings highlight that microbial volatiles emitted belowground can affect the nutritional status of plants from the distance.

Further efforts should be directed to investigate the effect of microbial volatiles emitted belowground on plants in order to understand better the communication mechanisms occurring in the soil.

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PG, LMS and GV conceived and designed the study. LMS, CA and GV performed the experiments. GV wrote the manuscript. LMS and CA revised the manuscript. PG revised critically the manuscript. All the authors approved the final version of the article.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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