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Linking soil's volatilome to microbes and plant roots highlights the importance of microbes as emitters of belowground volatile signals

Denis Schenkel,^{1,2} Aurélie Deveau,³ Jun Niimi,¹ Pierre Mariotte,^{4,5} Amarante Vitra,^{4,5} Marco Meisser,⁶ Alexandre Buttler^{4,5,7} and Richard Splivallo^{1,2,4*}

¹Institute for Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue Str. 9, 60438, Frankfurt am Main, Germany.

²Integrative Fungal Research Cluster, 60325, Frankfurt, Germany.

³Institut national de la recherche agronomique (INRA), Unité Mixte de Recherche 1136 INRA-Université de Lorraine, Interactions Arbres/Microorganismes, Centre INRA-Grand Est-Nancy, 54280, Champenoux, France.

⁴Laboratory of Ecological Systems (ECOS), Station 2, École Polytechnique Fédérale de Lausanne (EPFL), School of Architecture, Civil and Environmental Engineering (ENAC), 1015, Lausanne, Switzerland.

⁵Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Site Lausanne, Case postale 96, 1015, Lausanne, Switzerland.

⁶Agroscope, Route de Duillier 50, Case Postale 1012, 1260, Nyon, Switzerland.

⁷Laboratoire de Chrono-Environnement, UMR CNRS 6249, UFR des Sciences et Techniques, 16 route de Gray, Université de Franche-Comté, F-25030, Besançon, France.

Summary

Plants and microbes release a plethora of volatiles that act as signals in plant–microbe interactions. Characterizing soil's volatilome and microbiome might shed light on the nature of relevant volatile signals and on their emitters. This hypothesis was tested by characterizing plant cover, soil's volatilome, nutrient content and microbiomes in three grasslands of the Swiss Jura Mountains. The fingerprints of soil's volatiles were generated by solid-phase micro-extraction gas chromatography/mass spectrometry, whereas high-throughput sequencing

was used to create a snapshot of soil's microbial communities. A high similarity was observed in plant communities of two out of three sites, which was mirrored by the soil's volatilome. Multiple factor analysis evidenced a strong association among soil's volatilome, plant and microbial communities. The proportion of volatiles correlated to single bacterial and fungal taxa was higher than for plants. This suggests that those organisms might be major contributors to the volatilome of grassland soils. These findings illustrate that key volatiles in grassland soils might be emitted by a handful of organisms that include specific plants and microbes. Further work will be needed to unravel the structure of belowground volatiles and understand their implications for plant health and development.

Introduction

Vascular plants, with more than 300 000 species (Christenhusz and Byng, 2016), occupy a wide range of habitats spanning from deserts to rainforests. Adaptation of plants to varying environmental conditions is oftentimes facilitated through belowground interactions with microbes, which might improve plant fitness and resistance to stress, as well as shape the coexistence pattern between species (Hacquard *et al.* 2015; 2017).

Microbes surrounding plant roots are predominantly composed of bacteria and fungi (Mendes *et al.* 2013). At the plant root level, 80%–90% of terrestrial plants are associated with mycorrhizal fungi (Smith and Read, 2008; Bonfante and Genre, 2010). These beneficial microbes simultaneously colonize roots and soil, thereby increasing plants nutrient uptake (Farzaneh *et al.*, 2011) and resistance to drought (Mariotte *et al.*, 2013; 2017) and pests (El Komy *et al.*, 2015; Frac *et al.*, 2018). Root colonization by these symbionts is highly dynamic and competitive as more than 100 symbiotic fungal species might simultaneously co-exist on the roots of a single plant (Bahram *et al.*, 2011; Deveau, 2016). Besides mycorrhizal fungi, plant roots also contain non-mycorrhizal fungal endophytes, endosymbiotic microbes living within plant tissues (Rodriguez *et al.*, 2009; Kia *et al.*, 2017). Depending on plant-fungal species combination, these endophytes can

Received 6 February, 2019; revised 13 March, 2019; accepted 19 March, 2019. *For correspondence. *E-mail richard.splivallo@a3.epfl.ch; Tel. (+49) 69 798 42193; Fax (+49) 69 798 29527.

influence plant development in different ways, ranging from growth inhibition to growth promotion (Kia *et al.*, 2017). For instance, endophytic *Fusarium* species have been shown to inhibit plant development and *Helotiales* strains to enhance it (Almario *et al.*, 2017; Kia *et al.*, 2017). Finally, roots are also prone to attacks by pathogenic fungi (i.e. *Fusarium* and *Verticillium*) and oomycetes (i.e. *Pythium* and *Phytophthora*) dwelling in soil, which might cause wilts, blights and rots in host plants (Parry *et al.*, 1995; McCormick *et al.*, 2011). Overall, these examples highlight the importance of root colonizing fungi in plant health and development.

Bacteria are another important component of the plant root microbiome that plays a key role in plant nutrition, root development and plant health. Some bacterial strains enhance nutrient acquisition in plants (Nissinen *et al.*, 2012; Bulgarelli *et al.*, 2013; Vandenkoornhuysen *et al.*, 2015; Kielak *et al.*, 2016), support stress adaptation (Vandenkoornhuysen *et al.*, 2015) or, like *Bacillus amyloliquefaciens*, suppress plant pathogens (Santhanam *et al.*, 2015; Gómez Expósito *et al.*, 2017; Saechow *et al.*, 2018). Others can have deleterious or no visible effects on plants (Mansfield *et al.*, 2012). Bacterial diversity has been shown to decrease from the bulk soil to the rhizosphere, the thin layer of soil in direct contact with roots, and from the rhizosphere to the endosphere that makes up the inner root space (Turner *et al.*, 2013; Mendes *et al.*, 2014; Edwards *et al.*, 2015; Zgadzaj *et al.*, 2016). Members of the *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* bacterial phyla are mostly represented in the rhizosphere and endosphere of numerous plant species (Ofek-Lalzar *et al.*, 2014; Schlaeppi *et al.*, 2014). Some studies performed under controlled greenhouse conditions have demonstrated a relative stability in root associated bacterial communities; however, dynamic bacterial communities that react to changing environmental factors are surely more common under natural conditions (Edwards *et al.*, 2015; Marupakula *et al.*, 2016).

Belowground plant–microbe interactions are in part regulated by chemical signals secreted by one organism that induces changes in the other. These signals might be water-soluble proteins or small molecules (Martin and Kamoun, 2011). For instance, soil microbes release phytohormones (i.e. auxins and gibberellins) that might modulate plant growth and immunity (reviewed in the study by Persello-Cartiaux *et al.*, 2003). Plants are also able to shape their root microbiome through root exudates, a broad range of water-soluble metabolites [i.e. amino and organic acids, sugars, peptides; reviewed in the study by van Dam and Bouwmeester (2016)]. Exudates are, however, not the only signals encountered in plant–microbe interactions. Volatile organic compounds, small molecules with a low boiling point and high vapour pressure, are indeed another important group of chemical signals exchanged between plants and microbes. Because of their volatile nature, volatile organic compounds can diffuse in the soil and convey messages

many centimetres away from their emitters (Rasman *et al.*, 2005; Wenke *et al.*, 2010; Peñuelas *et al.*, 2014; Massalha *et al.*, 2017; Schulz-Bohm *et al.*, 2018; Sharifi and Ryu, 2018). To date, 841 volatiles that might act as signals to plants have been documented from soil associated microbes, even though a much higher number might exist (Schenkel *et al.*, 2015). For instance, 2,3-butanediol released by Gram-positive *Bacillus* bacteria promote the growth of *Arabidopsis* plants (Ryu *et al.*, 2003). Likewise, a mixture of 2-methyl-propanol, 3-methyl-butanol, methacrylic acid and isobutyl acetate produced by the fungal genus *Phoma* induce growth promotion in tobacco (Naznin *et al.*, 2013). Plants are similarly able to attract bacteria from bulk soil by the release of volatiles through their roots as recently illustrated for the grass species *Carex arenia* (Schulz-Bohm *et al.*, 2018). The latter examples highlight, however, that studies unravelling the role of specific signals generally focused on simplified laboratory setups, which are far from representing natural communities. Those communities are much more complex in terms of plant assemblages, root microbiomes and exchanged signals. Yet, studies of signals in complex communities have to date been hindered by technical challenges, as reflected by the scarce information available on biogenic volatiles in soil [reviewed in the study by Peñuelas *et al.* (2014)].

The aim of this study was to address the latter gap in knowledge. Specifically, the first goal was to investigate to which extent belowground microbial communities, soil's chemical properties and plant communities correlated to the soil's volatilome. The second aim was to identify specific emitters of relevant volatile signals. Three semi-natural grasslands, diverse plant communities mostly composed of grasses and forbs, were used here to address these questions. A comprehensive approach of metabolomics by gas chromatography/mass spectrometry (GC/MS) and high-throughput sequencing was employed to extensively characterize soil's volatilome as well as belowground fungal and bacterial communities. Multivariate statistics were then used to highlight any possible associations among soil's volatilome, soil nutrients, plants, bacteria and fungi.

Results

Vegetation cover

Vegetation of all three sites was dominated by grass species, ranging in abundance from roughly 75% in site 1 to slightly more than 50% in site 3 (Fig. 1A). Sites 1 and 2 were the most similar in terms of grass species, with the three dominant species *Lolium perenne*, *Poa trivialis* and *Dactylis glomerata* making up more than 75% of all grasses in both sites (Fig. 1B). By contrast, these two species were mostly absent in site 3, where *Agrostis capillaris* and *Festuca rubra* made up more than

85% of all grass species. Forbs and legumes ranged second in abundance after grasses in all three sites. As for the pattern observed for grasses, sites 1 and 2 were the most similar in terms of forbs and legumes plant community composition (Fabaceae and Asteraceae), and differed the most from site 3. A comparable pattern can be seen in terms of the number of plant species that are common or specific to the three sites (Table S1). Forage yields were $7.3 (\pm 0.6)$ tons ha^{-1} (site 1), $7.0 (\pm 0.8)$ tons ha^{-1} (site 2) and $4.6 (\pm 0.6)$ tons ha^{-1} (site 3) dry weight. Based on the Mann–Whitney statistical test, only the yield of site 3 was significantly lower than the one of the two other sites.

Soil parameters

Soil was characterized in the three sites in terms of nutritional properties. Microbial carbon, total nitrogen and microbial nitrogen were the highest in sites 2 and 3 (Fig. S1A, S1B, Mann–Whitney test, $p < 0.05$). Nitrate, soil inorganic nitrogen, soil phosphorus and microbial phosphorus were the highest in site 2. By contrast, ammonium was the highest in site 3, which had the biggest proportion of forbs and legumes. Soil carbon was the only parameter that significantly increased from site 1 to 3, according to the increasing altitude. Significant differences were also observed in nutrient ratios (Fig. S1C). For instance, microbial and soil C:P ratios were highest in site 3, microbial N:P ratios were lowest in site 1 and soil N:P ratios lowest in site 2. Differences were furthermore observed in soil pH and mean annual temperatures (Table S2).

Soil's volatilome

Soil samples were further characterized in terms of volatilome for all three sites. Chromatograms were highly complex as illustrated in Fig. S2, hindering the structure identification of most volatiles. Raw GC/MS data are provided for a representative sample of each site (along with an alkane series) as supplemental material. Nevertheless, the goal here was not to definitely identify compounds but rather to compare the volatilome of the three sites, which can be done without full structure identification. To this end, fingerprints of volatile organic compounds were generated with the Tagfinder software that creates a matrix of TAGs, corresponding to specific mass fragments (m/z) within a retention time window (Luedemann *et al.*, 2008). Processing the data of all sites and further filtering resulted in 298 TAGs. A heatmap illustrates those 298 TAGs (Fig. 2). Considering a TAG to be present in one site if present in at least one sampling plot (Fig. S6), 62% of all TAGs were common to all three sites, while the proportion of TAGs shared between two sites ranged from six to 10%, with site 1 and 2 having the highest proportion in common (Table S1). By contrast zero to 9% of the TAGs were site specific, and site 3 had the highest proportion of site-specific TAGs (Table S1). Overall, similarly to what has been observed in plant cover, these results highlight more similarities in the volatile profiles of sites 1 and 2 and marked differences with site 3.

Soil's bacterial and fungal communities

Microbial communities (fungi and bacteria) were further characterized from the soil of each plot. High throughput sequencing yielded on average (\pm standard deviation)

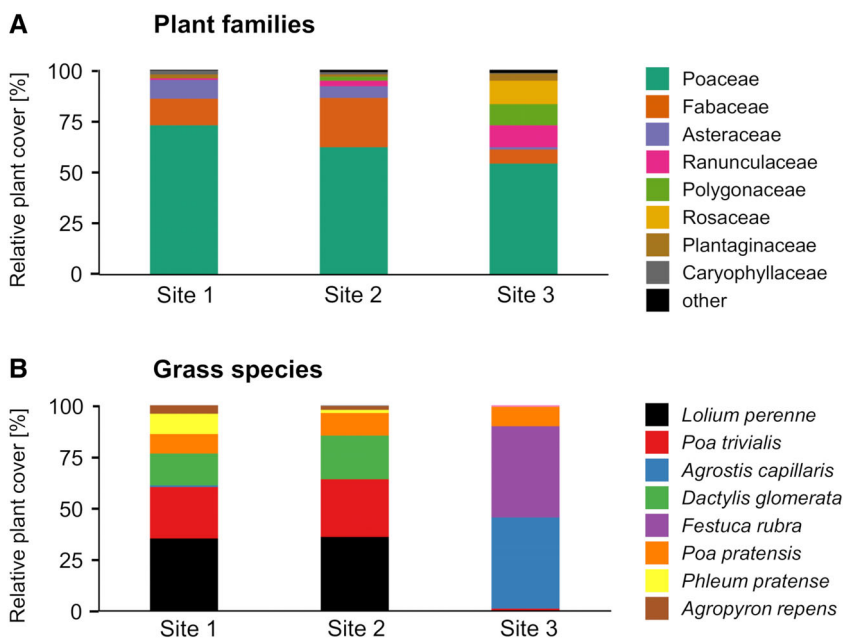


Fig. 1. Plant community composition in the three grassland sites. A. Relative proportion of botanical families of the eight most dominant plant families in the three study sites. B. Species distribution within the Poaceae family. Sites 1 and 2 were the most similar in terms of plant families and grass species.

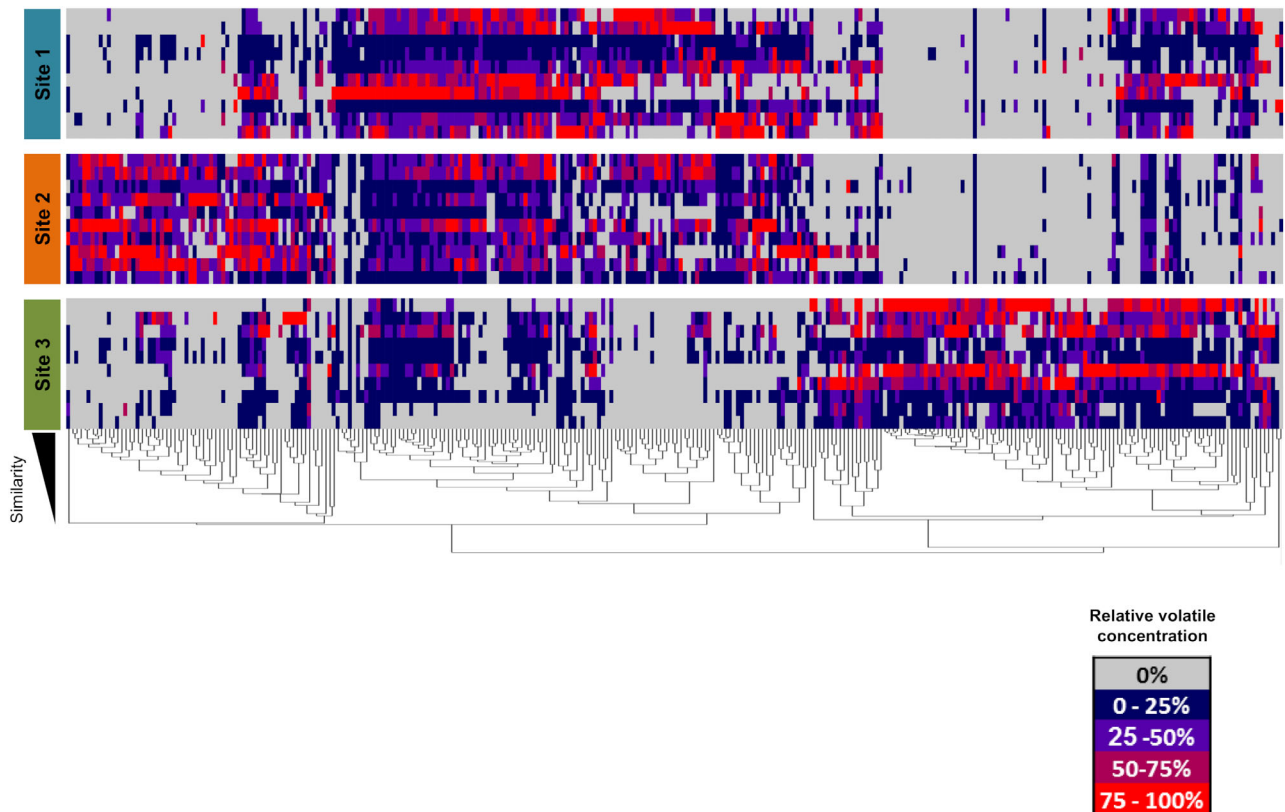


Fig. 2. Heatmap of volatile fingerprints at the three sites. Heatmap illustrating the relative proportion of the 298 TAGs (volatile signals) detected in the soils of each plots for sites 1, 2, and 3. The cluster tree, based on correlations, highlights three major clusters: one made of TAGs present almost exclusively in site 2, one made of TAGs common to sites 1 and 2, and one made of TAGs almost only appearing in site 3.

6727 (\pm 2183) fungal reads and 30360 (\pm 2510) bacterial reads per sample. Once rarefaction was completed, reads were assigned to 1063 fungal and 3950 bacterial Operational Taxonomic Units (OTUs) during affiliation. These observed OTUs were distributed among plots of all three sites with an average of 267 (\pm 41) fungal OTUs and 2065 (\pm 150) bacterial OTUs in site 1, 261 (\pm 18) fungal and 2002 (\pm 177) bacterial OTUs in site 2 and 249 (\pm 17) and 1686 (\pm 204) bacterial OTUs in site 3. Microbial richness was displayed as observed taxa and diversity was estimated based on Shannon index (H'). Observed richness and estimated diversity among the three sites did not significantly differ for fungi but did so for bacteria (Fig. S3, $p < 0.05$).

Fungal and bacterial community composition was explored at different taxonomic levels. The Ascomycota phylum dominated fungal community composition in each site (more than 62% of the total diversity), followed by members of the Basidiomycota and Glomeromycota phyla (Fig. S4). At the class level, the Dothideomycetes (phylum: Ascomycota) were the least abundant in site 3, which had the highest proportion of Leotiomyces (Ascomycota). Differences were less apparent for classes within the Basidiomycota and Glomeromycota due to

the important variability in relative abundance observed among plots of the same sites (Fig. S4). Seven bacterial phyla represented more than 95% of the total diversity, with each phylum making up at most 23% of the overall diversity (Fig. S5). Differences in abundance could also be observed at the class level for bacteria and highlighted once again that site 3 differed the most from the other two sites (Fig. S5).

The standout of site 3 compared with the other sites can similarly be seen at the family level and focusing on the microbial families that represented more than 2% of the reads (Table 1). These included ten fungal families out of 505 and five bacterial families out of 1768 (refer to Tables S4 and S5 for the data at different taxonomic level). Overall, 60% of fungal and 86% bacterial OTUs differed in relative abundance among all sites (Kruskal-Wallis test, $p < 0.05$). Specifically, site 3 stood out in terms of the Davidiellaceae, Clavariaceae, Geoglossaceae and Glomeraceae fungal families and the Planctomycetaceae, Acidobacteriaceae and Xanthobacteraceae bacterial families (Table 1). The peculiarity of site 3 could also be seen at the OTU level. Indeed, site 3 shared from 5% to 11% of microbial OTUs with the other two sites while this number was markedly higher for sites 1 and 2 (19% to 21%). In comparison, the proportion of

OTUs that were common to all sites were 22% for fungi and 54% for bacteria, and all sites had a comparable proportion of site-specific OTUs (2%–4% for bacteria, 14%–16% for fungi) (Table S1).

Overall the data illustrate a slightly higher location specificity for fungi compared with bacteria and shows that site 3 differed the most from the other sites in terms of microbial community composition.

Soil volatiles have the strongest associations with plants and microbes

Multiple factor analysis (MFA) was performed with the aim to assess the correlation strength among the soil volatiles and the other factors considered here (plants, microbes, soil properties). The MFA of Fig. 3 highlights that samples from the three sites were distinguishable from each other, and that the MFA model overall explained 39.2% of total variance (axis 1: 22.1%, axis 2: 17.1%). The loadings visible in Fig. 3, indicate that some plants, microbes, nutrients and volatiles/TAGs were driving differences among sites. Three plant species and six TAGs that were the most characteristic of each site have been colour coded in red (panel Vegetation, Fig. 3) and blue (panel Volatiles, Fig. 3). Table S3 furthermore provides the structural identification of those TAGs/volatiles in a handful of cases. Similarly, specific microbial OTUs drove the differences among the sites (refer to Table S4 and S5 for a full list of microbial OTUs, including their occurrence in each site and corresponding statistics). Colour coding microbial OTUs at the phylum level (panels Fungal OTUs and Bacterial OTUs, Fig. 3) did not reveal any obvious site-specific differences in microbial community composition. The shape of the data cloud (triangle shaped

for fungi and more donut shaped for bacteria) nevertheless confirms the higher level of site specificity for fungi compared with bacteria already observed previously.

An overall interpretation of the MFA is furthermore achievable through the RV scores which reflects how strongly different loadings (parameters) are associated among each other. RV scores were the highest among volatiles and fungi or plants or bacteria ($RV_{VOLATILES/FUNGI} = 0.77$, $RV_{VOLATILES/PLANT} = 0.76$, $RV_{VOLATILES/BACTERIA} = 0.74$), and were considerably lower between volatiles and nutrients ($RV_{VOLATILES/NUTRIENTS} = 0.52$).

In summary, this highlights a strong association between the soil's volatiles/bacteria, volatiles/fungi and volatiles/plants, and exemplifies a comparable association strength of those three factors.

Volatiles concentrations correlate to the abundance of specific plants and microbes

To get a better understanding of specific interactions among volatiles and the three most relevant factors of the MFA (plants, bacteria and fungi), a correlation analysis was applied treating each site independently (*i.e.*, correlation between relative volatiles/TAGs concentrations, plant species and microbial OTUs). The correlation matrices were further filtered by statistics, taking in account only positive or negative significant correlations ($p < 0.05$, t-test) and setting non-significant correlations to zero (Tables S6 (fungi), Table S7 (bacteria), Table S8 (plants)). We first determined which TAGs consistently correlated (positively or negatively) to the same plants or microbial OTUs in at least two sites. This revealed for positive correlations 36% bacterial OTUs, 26% fungal OTUs, and 27% plant species. By contrast,

Table 1. Dominant microbial families with more than 2% average occurrence in the three sites.

Rank	Families (class, phylum)	Site 1 (%)	Site 2 (%)	Site 3 (%)	Average among sites	STE
<i>Fungi</i>						
1	Davidiellaceae (Dothideomycetes, Ascomycota)	17.1	12.2	4.1	11.1	3.8
2	Clavariaceae (Agaricomycetes, Basidiomycota)	0.7	0.2	9.8	3.5	3.1
3	Geoglossaceae (Geoglossomycetes, Ascomycota)	0.1	0.1	10.2	3.5	3.4
4	Glomeraceae (Glomeromycetes, Glomeromycota)	4.3	3.7	1.2	3.1	0.9
5	Strophariaceae (Agaricomycetes, Basidiomycota)	5.3	1.6	1.1	2.7	1.3
6	Sporormiaceae (Dothideomycetes, Ascomycota)	1.7	4.5	1.3	2.5	1.0
7	Phaeosphaeriaceae (Dothideomycetes, Ascomycota)	1.7	3.7	1.4	2.3	0.7
8	unknown family 421 (Agaricomycetes, Basidiomycota)	0.2	0.1	6.5	2.3	2.1
9	Bolbitiaceae (Agaricomycetes, Basidiomycota)	2.4	3.7	0.4	2.2	1.0
10	Pyronemataceae (Pezizomycetes, Ascomycota)	2.1	3.0	1.1	2.1	0.6
<i>Bacteria</i>						
1	DA101 soil group (Spartobacteria, Verrucomicrobia)	10.7	3.1	13.9	9.2	5.3
2	Planctomycetaceae (Planctomycetacia, Planctomycetes)	5.4	5.2	11.9	7.5	4.3
3	Chitinophagaceae (Sphingobacteriia, Bacteroidetes)	3.9	2.2	1.9	2.7	1.5
4	Acidobacteriaceae (Subgroup 1) (Acidobacteria, Acidobacteria)	1.0	0.3	5.6	2.3	1.3
5	Xanthobacteraceae (alpha-proteobacteria, Proteobacteria)	1.9	1.4	3.4	2.2	1.3

STE = standard error.

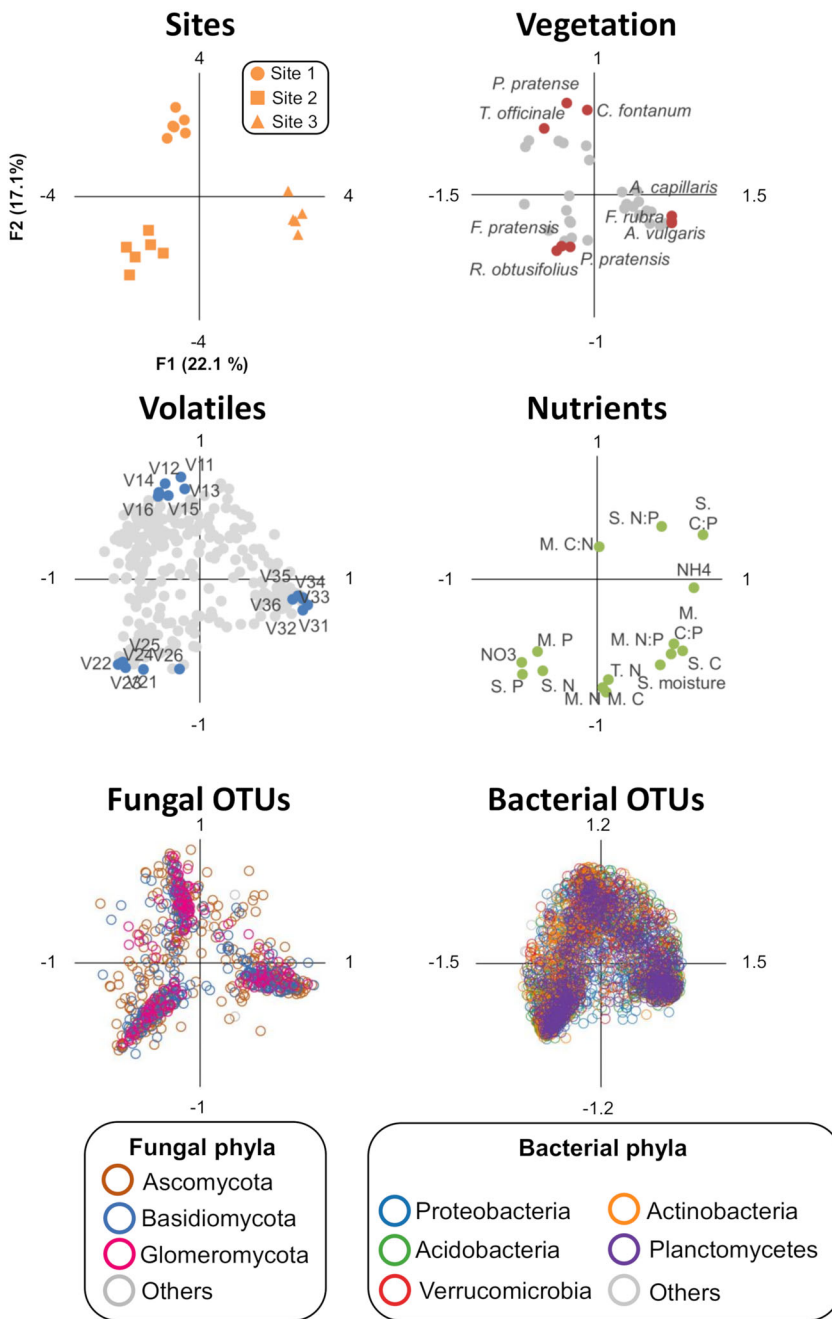


Fig. 3. The MFA was constructed based on vegetation (28 plant species), soil properties (14 parameters), soil volatiles (298 TAGs) and microbial OTUs (1063 fungi and 3950 bacteria). Scores and loadings are shown in six separate plots for the ease of visualization and highlight the relative specificity of some fungal/bacterial OTUs, soil nutrients or TAGs in each site.

negative correlations with TAGs were observed for only 3% bacterial OTUs and 1% fungal OTUs (none were observed with plants).

Considering the predominant numbers of positive correlations among TAGs and microbes or plants, we further questioned which organisms were behind those correlations. Further processing the data of Tables S6, S7 and S8 revealed that single bacterial and fungal OTUs were significantly and positively correlated to a maximum of 15%–18% (average among sites) of all 298 TAGs. This proportion was markedly lower for plants where single species correlated with a

maximum 9% of all TAGs. Table 2 lists the top 15 plant species or fungal and bacterial OTUs correlating to the highest proportion of TAGs (average among sites) along with their relevant taxonomic data. These top 15 organisms represented seven plant families, where the Fabaceae and Poaceae were each comprised of three species (Fabaceae: *Lotus comiculatus*, *Trifolium repens* and *Trifolium pratense*; Poaceae: *Poa pratensis*, *Poa trivialis* and *Dactylis glomerata*). These families/species were also important contributors to plant cover (Fig. 1). At the family level, a little less than half of the top 15 microbial taxa could not be assigned/identified (Table 2).

Table 2. Top 15 bacterial and fungal OTUs or plant species correlating to the most volatiles.

Rank	OTU number	Phylum	Class	Order	Family	Genus	Species	Site 1 (%)	Site 2 (%)	Site 3 (%)	Average among sites (%)	STE (%)
<i>Fungi</i>												
1	174	Basidiomycota	Agaricomycetes	Unknown	Unknown	Unknown	Unknown	13	21	19	18	2
2	479	Glomeromycota	Unknown	Unknown	Unknown	Unknown	Unknown	14	12	19	15	2
3	42	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	<i>Psilocybe</i>	<i>inquinata</i>	13	10	19	14	3
4	105	Glomeromycota	Unknown	Unknown	Unknown	Unknown	Unknown	12	10	19	14	3
5	680	Ascomycota	Dothideomycetes	Pleosporales	Unknown	Unknown	Unknown	13	4	19	12	4
6	828	Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	<i>Cistella</i>	Unknown	13	4	19	12	5
7	121	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	<i>Conocybe</i>	<i>gigasperma</i>	12	5	19	12	4
8	29	Ascomycota	Eurotiomycetes	Unknown	Unknown	Unknown	Unknown	13	3	19	12	5
9	142	Basidiomycota	Agaricomycetes	Agaricales	Unknown	Unknown	Unknown	8	8	19	12	4
10	110	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	<i>Coprinopsis</i>	<i>babosiae</i>	11	3	19	11	5
11	8	Ascomycota	Unknown	Unknown	Unknown	Unknown	Unknown	9	5	19	11	4
12	281	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	<i>Gibberella</i>	<i>avenacea</i>	0	2	30	11	10
13	413	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Unknown	Unknown	13	17	2	11	5
14	720	Ascomycota	Sordariomycetes	Chaetosphaeriales	Chaetosphaeriaceae	<i>Cylindrotrichum</i>	Unknown	18	0	14	11	6
15	307	Basidiomycota	Agaricomycetes	Auriculariales	Unknown	Unknown	Unknown	12	2	18	11	5
<i>Bacteria</i>												
1	56	Actinobacteria	Thermoleophiia	Gaiellales	Unknown	Unknown	---	9	18	19	15	3
2	3297	Proteobacteria	Deltaproteobacteria	Myxococcales	Sandaracinaceae	Unknown	---	21	7	17	15	4
3	635	Planctomycetes	Physisphaerae	WD2101 soil group	Unknown	Unknown	---	22	11	9	14	4
4	3783	SHA-109	Unknown	Unknown	Unknown	Unknown	---	23	7	11	14	5
5	2292	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	<i>Gemmatimonas</i>	---	20	11	9	14	3
6	1450	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	<i>Mesorhizobium</i>	---	12	4	22	13	5
7	367	Planctomycetes	Physisphaerae	WD2101 soil group	Unknown	Unknown	---	16	1	21	12	5
8	1001	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Unknown	---	4	13	20	12	6
9	3700	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	<i>Planctomyces</i>	---	16	4	16	12	4
10	1850	Actinobacteria	Acidimicrobia	Acidimicrobiales	Unknown	Unknown	---	10	7	19	12	3
11	1366	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Unknown	---	15	10	11	12	2
12	2066	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Unknown	Unknown	---	17	2	17	12	5
13	1436	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	<i>Sphingobacterium</i>	---	13	1	21	12	6
14	1647	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	Unknown	---	3	9	23	12	6
15	362	Actinobacteria	Actinobacteria	Corynebacteriales	Mycobacteriaceae	<i>Mycobacterium</i>	---	4	11	20	12	5
<i>Plants</i>												
1	---	Vascular plants	Magnoliopsida	Asterales	Asteraceae	<i>Taraxacum</i>	<i>officinale</i>	10	11	6	9	2
2	---	Vascular plants	Magnoliopsida	Fabales	Fabaceae	<i>Lotus</i>	<i>corniculatus</i>	0	3	19	7	6
3	---	Vascular plants	Magnoliopsida	Brassicales	Brassicaceae	<i>Capsella</i>	<i>bursa-pastoris</i>	17	3	0	7	5
4	---	Vascular plants	Magnoliopsida	Lamiales	Plantaginaceae	<i>Veronica</i>	<i>arvensis</i>	0	5	12	6	4
5	---	Vascular plants	Magnoliopsida	Poales	Poaceae	<i>Poa</i>	<i>pratensis</i>	3	13	0	5	4
6	---	Vascular plants	Magnoliopsida	Lamiales	Orbanchaceae	<i>Rhinanthus</i>	<i>alektorolophus</i>	0	0	14	5	5
7	---	Vascular plants	Magnoliopsida	Lamiales	Plantaginaceae	<i>Plantago</i>	<i>major</i>	12	0	0	4	4
8	---	Vascular plants	Magnoliopsida	Poales	Poaceae	<i>Dactylis</i>	<i>glomerata</i>	1	1	9	4	2
9	---	Vascular plants	Magnoliopsida	Geraniales	Geraniaceae	<i>Geranium</i>	<i>rotundifolium</i>	0	11	0	4	4

(Continues)

Table 2. Continued

Rank	OTU number	Phylum	Class	Order	Family	Genus	Species	Site 1 (%)	Site 2 (%)	Site 3 (%)	Average among sites (%)	STE (%)
10	---	Vascular plants	Magnoliopsida	Ranunculales	Ranunculaceae	<i>Ranunculus</i>	<i>repens</i>	4	6	0	3	2
11	---	Vascular plants	Magnoliopsida	Fabales	Fabaceae	<i>Trifolium</i>	<i>repens</i>	3	0	7	3	2
12	---	Vascular plants	Magnoliopsida	Poales	Poaceae	<i>Poa</i>	<i>trivialis</i>	5	0	4	3	1
13	---	Vascular plants	Magnoliopsida	Ranunculales	Ranunculaceae	<i>Ranunculus</i>	<i>acris</i>	6	1	2	3	2
14	---	Vascular plants	Magnoliopsida	Caryophyllales	Polygonaceae	<i>Rumex</i>	<i>obtusifolius</i>	4	5	0	3	2
15	---	Vascular plants	Magnoliopsida	Fabales	Fabaceae	<i>Trifolium</i>	<i>pratense</i>	7	1	1	3	2

STE = Standard error.

The remaining portion belonged to seven fungal and eight bacterial families, which include for fungi the Hyaloscyphaceae, Psathyrellaceae, Nectriaceae, Entolomataceae, Chaetosphaeriaceae, Strophariaceae and the Bolbitiaceae families. The latter two families belonged to the dominant taxa listed in Table 1. Bacterial families were represented by the Sandaracinaceae, Gemmatimonadaceae, Phyllobacteriaceae, Acetobacteraceae, Nitrosomonadaceae, Sphingobacteriaceae, Mycobacteriaceae and Planctomycetaceae. The latter family was listed as a dominant taxon in Table 1.

Overall these findings highlight that some microbial OTUs are correlated to a higher proportion of volatiles than single plant species. Our data also reveal the taxonomic identity of the organisms that are correlated to the most TAGs.

Discussion

The possibility to gain relevant insight in belowground plant-microbe interactions by profiling soil volatiles in association with plant and microbial community compositions was explored here. Overall, volatile profiles from the soils collected in two sites were highly similar to each other and greatly differed from a third location. Of the four factors considered here, soil nutrients had the lowest association with the soil's volatilome. This is not surprising since soil nutrients have no direct influence on soil volatiles. Yet, they might indirectly influence the physiology of volatile emitters and shape soil's microbiome (Faoro *et al.*, 2010; Wang *et al.*, 2017; Xue *et al.*, 2018), potentially explaining the small effect observed here in the soil volatilome. Indeed the relative proportion of available carbon to nitrogen (C:N) or to phosphorus (C:P), and the ratio of nitrogen to phosphorus (N:P) are known to impact plant biomass and metabolism (Elser *et al.*, 2010; Güsewell, 2005; Huaranca Reyes *et al.*, 2018; Zheng, 2009). This is further supported by the fact that fertilization treatments of soil have been documented to affect mono- and sesquiterpenes emission in plants (Ormeño and Fernandez, 2012). Similarly, the uptake of soil's nutrients by plants was shown to influence the production of volatiles, as illustrated by the finding that phosphorus foliar concentrations negatively correlated to isoprene and monoterpenes emissions (Fernández-Martínez *et al.*, 2018). Additionally, nutrients might similarly affect volatiles synthesized by bacteria (Garbeva *et al.*, 2014).

Microbial community structures and compositions were additional factors considered here for their associations with soil's volatilome. Our data highlight that soil microbes were strongly associated with soil's volatilome. Bacteria and fungi are well known emitters of volatile compounds (Lemfack *et al.*, 2018), and we have speculated earlier based on undescribed (and mostly uncultivable) microbial taxa that soil might contain a huge diversity of undescribed volatiles (Schenkel *et al.*, 2015). This hypothesis is corroborated here

by the fact that most volatiles of the present study could not be identified. This should, however, be interpreted with caution since part of the difficulty in identifying volatiles might be attributed to low signals in the chromatograms. However, it is also likely that many of the volatiles of the present study have not been described/characterized yet as they originate from a highly complex matrix (soil) that has been little studied (Peñuelas *et al.*, 2014). A similar observation about the need to characterize the structure of unknown bacterial volatile has been done earlier (Kai *et al.*, 2009). Future efforts should thus focus on identifying specific soil volatiles and linking those volatiles to distinct microbes and biosynthesis pathways.

In addition to the strong associations evidenced by the MFA, significant positive correlations were observed between volatile signals and specific plants. Current knowledge of volatile emission by plant roots is scarce (Schenkel *et al.*, 2015) and to the best of our knowledge, there is no published data on the root volatilomes of any of the plant (grass) species listed as relevant in Table 2. By contrast some data exists on the volatilomes of some of the relevant microbial taxa listed in Table 2. For instance, the volatile profile of the plant pathogen *Gibberella avenacea*, (teleomorph of *Fusarium avenaceum*) has been investigated during infection of maize ears (Becker *et al.*, 2014). Additionally, the volatile profiles of other *Fusarium* species have been characterized in numerous other studies (Bitas and Kang, 2015; Lemfack *et al.*, 2018; Schenkel *et al.*, 2018; Wang *et al.*, 2018). Two unidentified OTUs belonging to the Glomeromycota phylum known for its ability to form arbuscular mycorrhizae with plants were also put forward as possible emitter of volatiles (Table 2). Studies on the volatile emission in *in vitro* cultures of arbuscular mycorrhizal fungi have been hindered by the microscopic size of those organisms and probably by technological challenges in cultivation (Mukhongo *et al.*, 2016). Nevertheless, arbuscular mycorrhizal fungi are known to alter the above-ground volatile emission of the plants they colonize (Schausberger *et al.*, 2012), thus it is conceivable that a similar modulation might occur at the plant root level. Lastly, microbes do not only have the ability to emit volatiles but possibly also to adsorb or even metabolize them as recently suggested for fungi and soil volatiles (Schenkel *et al.*, 2018) or for bacteria and the volatile cumene (Eaton and Nitterauer, 1994; Habe *et al.*, 1996). Yet, the higher proportion of positive correlations compared with negative ones between volatile signals and plants or microbes observed in our data suggests that overall, soil organisms are emitting more volatiles than they consume.

In conclusion, the soil volatilome should be considered as a signature resulting from the net effect of all organisms and processes acting in the soil. Additionally, the scarce information on the volatilome of organisms listed as relevant here begs for characterizing their volatile profiles.

The data presented here furthermore highlight a higher level of site-specificity for fungi than for bacteria. Certain dominant fungi have closer associations with plant roots

(i.e. forming mycorrhizas) than bacteria, and the higher specificity observed for fungi might thus be driven by the stark differences in plant communities between sites 1 and 2 compared with site 3. However, our sampling strategy might also have influenced this outcome. Indeed, bacteria might closely associate with plant roots at the level of the rhizosphere, a thin zone of soil directly under the influence of plant roots that is known to contain most microbes relevant for plant health (Berendsen *et al.*, 2012). Considering that our sampling strategy, established to minimize wound-induced root volatiles, included both rhizosphere and bulk soil, our results should be interpreted with a note of caution. Indeed, signals from rhizosphere bacteria might be diluted by signals from bulk soil bacteria. A stricter sampling of rhizosphere microbial communities might thus have revealed more of the plant associated active microbes. However, the detection of some OTUs belonging to the Proteobacteria phylum, and that represented approximately 20% of the bacterial diversity in our data, validates at least partially our sampling strategy. These microbes are indeed known as common rhizosphere inhabitants and endophytes in several grass species (Duc *et al.*, 2009; Hurek *et al.*, 2002; Iniguez *et al.*, 2004; Sevilla *et al.*, 2001). Additional studies focusing on the bacterial communities in the rhizosphere of single plant species will be needed to pinpoint microbial specificity/ubiquity.

In summary, our findings illustrate some of the challenges in studying the soil's volatilome but also highlight its high complexity. Most of all, soil microbes appear as essential emitters of volatiles and seem to contribute slightly more than plant roots to the complexity of the soil's volatilome. Further studies are nevertheless required to verify whether our observations are global or local and how they vary in space and time.

Experimental procedures

Site description and experimental design

Three semi-natural grasslands located in the Jura Mountains (Canton of Vaud, Switzerland) were characterized in terms of plant communities, soil nutrients, soil volatilomes and microbiomes. Details about each site can be seen in the Supporting Information Table S2.

Five rainout shelters were set up at each site. The experiment was conducted during two growing seasons using plots of 3.6 m² and data for this study were collected in the second year of the experiment (2016). The current study included two plots per shelter (considering five shelters per site, this is equivalent to 10 plots per site), which were irrigated for 8 weeks with different watering regimes to assess the effect of drought. Specifically, a 'control plot' was watered based on the averaged precipitations of the last 30 years while the 'drought plot' received 30% less water. Because statistical testing

revealed that the effect of drought on the soil volatilome was negligible (it significantly influenced the concentrations of only 6% of volatiles for site 1, 3% for site 2 and 4% for site 3, $p < 0.05$, Kruskal–Wallis test), the two plots (drought and the respective control plot) were considered here as equivalent.

Vegetation cover determination, soil sampling (for nutrients, microbiomes, volatilomes) and subsequent plant yield were determined at the end of the 8 weeks period. The specific sampling dates (end of May for site 1, mid-June for site 2 and early July for site 3) differed for each site as they were chosen to reflect peak biomass (based on data obtained in the previous years). The exact number of replicates (plots) sampled at each site was for vegetation determination and soil's volatilome – 10 plots per site; soil nutrients – 6 plots per site and for bacterial and fungal microbiomes – 10 plots per site, except for site 3 for which one bacterial microbiome sample was lost resulting in 9 plots.

Determination of vegetation

Plant community was surveyed using a pin-point method (Daget and Poissonet, 1971) with 80 points per plot, evenly distributed every 20 cm on four lines of 400 cm spaced 20 cm apart. At each point of interception, all plant species in contact with the edge of a 1 mm dagger (presence/absence) were recorded. Relative species cover was determined by dividing the number of contacts per species in each plot by the total number of contacts (Iussig *et al.*, 2015). Above ground biomass (plant yield) was determined after harvesting and expressed as dry matter per plot.

Soil nutrients

Soil samples were collected for the top 12–15 cm layer by pooling 15 spatially randomly distributed cores per plot. The water content was determined gravimetrically by drying soil subsamples at 105°C to a constant weight. Total carbon (C) and nitrogen (N) content in the bulk soil were analysed after ball milling. Soil C and N were determined under high-temperature oxidation using an elemental analyser (CE Instruments model NA2500 Nitrogen Carbon Analyser) and expressed as $\text{mg}\cdot\text{kg}^{-1}$ dry soil. For the determination of microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}), pairs of about 5 g of fresh soil were weighed for each replicate and one sample from each pair was immediately extracted in 25 ml solution of 0.5 M K_2SO_4 , whereas the other sample was put in a vacuum desiccator and subjected to chloroform vapours. After 1 day of fumigation, the fumigated soil sample was extracted with the same solution. Total C and N concentrations in fumigated and non-fumigated samples were

analysed by a TOC/TN analyser (Shimadzu TOC-V). To determine the soil available phosphorus (P) and the microbial phosphorus (P_{mic}), 3 g of fumigated and non-fumigated fresh soil was extracted with 40 ml of 0.5 M NaHCO_3 (Olsen method). Phosphorus concentrations (i.e. orthophosphates) were analysed by colorimetry using a spectrophotometer at 890 nm. Microbial biomass C, N and P were estimated as the differences between the amounts of C, N and P after and before fumigation using an extractability factor of 0.45 for C (Vance *et al.*, 1987), 0.54 for N (Brookes *et al.*, 1985) and 0.4 for P (Brookes *et al.*, 1982). Microbial biomass C, N and P and soil available P are expressed as $\text{mg}\cdot\text{kg}^{-1}$ dry soil. Ammonium (N-NH_4) and nitrate (N-NO_3) concentrations were determined by continuous flow analyses using an automated analyser (SEAL AA3 HR Auto-analyser) after extraction of 5 g of fresh soil with 30 ml of 1 M KCl, and the results expressed as milligram per kilogram dry soil.

Sampling of soil for volatilome and microbiome analysis

Soil was sampled from each plot according to the scheme described in the Supporting Information Fig. S6. In short, six soil cores (12–15 cm depth, 3.2 cm diameter) were taken from each plot. Soil cores contained highly intermingled roots (a few milligram of roots per grams of soil – dry weight each). Separating rhizosphere from bulk soil was not feasible without extensively damaging root systems and thus inducing the release of wound-induced volatiles. Hence, root samples were immediately separated at the sampling site from soil through sieving (3 mm sieve). This resulted in a mixture of bulk and rhizosphere soil that was subsequently used to determine soil's volatilome and microbiome as described hereafter. To minimize volatile loss or drift in microbial population following sampling, the soil samples were cooled to 4°C until volatile profiling was performed (within 24 h from collection) and immediately frozen thereafter.

Soil volatile profiling

A total of 1.00 (± 0.02) g fresh soil of each sample was transferred to 20 ml solid-phase micro extraction (SPME) vials. Volatile fingerprints were generated by SPME-GC/MS and analysed as described previously (Sherif *et al.*, 2016; Schenkel *et al.*, 2018) with the exception of the threshold of 600 set in the Tagfinder software (version 4.1) used to process chromatograms (Luedemann *et al.*, 2008). This analysis resulted in a matrix of TAGs, signals equivalent to mass fragments within specific time ranges [m/z , RT range], and which were normalized to the total ion current (TIC). To filter noise out of the data, only TAGs present in six plots (out of 10) at a single site were considered. TAGs were further

filtered by retention time and pairwise correlations were calculated with adjacent TAGs. TAGs with a correlation of more than 70% with the adjacent TAG and falling in a comparable retention window were assumed to belong to the same compound. The data were subsequently reduced to one representative TAG for each TAG having a comparable RT range, resulting in a matrix of 298 TAGs.

Volatiles comprised TAGs of interest were tentatively identified via NIST Mass Spectral Search Program 2.0 (National Institute of Standards and Technology, Gaithersburg, USA) by comparison of mass spectra and Kovats retention indices (n-alkane). Complete identification was achieved for 2-methylbutan-1-ol and 3-methylbutan-1-ol by injecting authentic standards purchased from Merck (Darmstadt, Germany). Raw chromatograms of a representative soil sample per site and respective alkane series are provided as Supporting Information in the CDF format.

Microbiome sequencing and data preprocessing

Soil samples (100 g per soil plot) were freeze dried (Christ Alpha 1-4 LD plus, Osterode am Harz, Germany) and subsequently homogenized by mixing/shaking in paper bags. For each sample, the DNA from three subsamples of 250 ± 10 mg soil was isolated with the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction (using lysis buffer SL2 + 150 μ l enhancer). Eventually, the three subsamples of isolated microbial DNA were unified into one sample resulting in 30 samples in total (one for each plot).

Amplicon libraries of bacterial soil inhabitants were generated by amplifying the V4 region of the bacterial 16S rDNA with 515F (Apprill *et al.*, 2015) and 806R (Parada *et al.*, 2016) primers. The internal transcribed spacer 2 (ITS2) region of fungal rDNA was used to characterize fungal soil communities using ITS86F (Vancov and Keen, 2009) and ITS4 (White *et al.*, 1990) primers. In both cases, forward and reverse primers carried the 5-CTTTCCCTACACGACGCTCTTCCGATCT-3 and 5-GGAGTTCAGACGTGTGCTCTTCCGATCT-3 tails. Bacterial amplicon size was approximately 291 base pairs (bp) (Caporaso *et al.*, 2011), fungal amplicon size approximately 400 bp (Vancov and Keen, 2009). Oligo nucleotides were obtained from Eurofins Genomics (Ebersberg, Germany). Polymerase chain reaction (PCR) was performed for three replicates of each sample (2 μ l isolated DNA at 15.5–37.9 ng μ l⁻¹) in 10 μ l 5PRIME 2.5 \times MasterMix including Taq polymerase with 1 μ l of forward and reverse primers (10 μ M) each and 11 μ l DNA free water (Carl Roth, France) in a total reaction volume of 25 μ l per sample. Amplification conditions for bacterial 16S oligonucleotides were 94°C for 3 min, 30 cycles 94°C for 45 s, 50°C for 1 min and 72°C for 90 s. Eventually temperature was hold at 72°C for 10 min and storage at 4°C. Amplification conditions for ITS2 oligonucleotides were 95°C for

2 min, then 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and after the final cycle 72°C for 10 min, followed by 10°C for storage. PCR products without addition of microbial DNA (negative control), mock communities of known fungal or bacterial compositions and unmerged subsamples of two randomly picked samples were added as quality controls. Samples of 50 μ l (30 ng DNA per μ l) were sent for tagging and MiSeq Illumina Next Generation Sequencing (GeT PlaGe INRA sequencing platform, Toulouse, France). The raw data were deposited in the NCBI Sequence Read Archive website (<http://www.ncbi.nlm.nih.gov/sra>) under the SRA study accession number SUB5130080.

Resulting bacterial sequences were further processed with Find Rapidly OTU with Galaxy Solution (FROGS) (Escudé *et al.*, 2017) based on Galaxy metagenomic analysis platform (Afgan *et al.*, 2016). Sequences were demultiplexed, dereplicated, sequence quality was checked, oligonucleotides, linker, pads and barcodes were removed from sequences and sequences were filtered on additional criteria. Sequences were removed from data set, if non-barcoded, if sequences exhibited ambiguous bases or did not match expectations in amplicon size. Remaining sequences were clustered into operational taxonomic units (OTUs) based on iterative Swarm algorithm, and then chimeras and singletons (OTUs containing only one sequence) were removed. Bacterial double affiliation was performed blasting OTUs against SILVA database (Quast *et al.*, 2012) and ribosomal database project (RDP) classifier (Wang *et al.*, 2007). OTUs with affiliation <100% at phylum level (indicated by a RDP bootstrap value <1) were removed from data set. OTUs at lower taxonomic ranks than the phylum level were considered as 'unidentified' when RDP bootstrap value was <0.70. OTUs with high abundances in negative controls were excluded from further analysis, sequencing and affiliation quality was evaluated based on the results obtained for the bacterial mock community.

Fungal sequences were processed as following. After demultiplexing and quality check (quality score = 30, minimal size = 200 bp), bioinformatics analyses were performed using standard procedures as described in the study by Pérez-Izquierdo *et al.* (2017).

For both fungal and bacterial data, per-sample rarefaction curves were calculated to assess sampling completeness, using function *rarecurve()* in package Vegan v3.5–1 (Oksanen *et al.*, 2015) in R (version 3.4.3) (R Core Team, 2017). Based on these, subsequent analyses of diversity and community structure were performed on data sets where samples had been rarefied with the Phyloseq (McMurdie and Holmes 2013) package to achieve equal read numbers according to the minimum number of total reads in any sample (25 143 bacteria, 4127 fungi). Microbial alpha diversity was estimated with the PhyloSeq package, as well. Normal distribution of richness (observed taxa) and diversity (Shannon's H') was tested (Shapiro–Wilk normality

test) for fungi and bacteria individually. Depending on the outcome, richness and diversity of sites 1–3 were compared by ANOVA (normal distribution) or Kruskal–Wallis test (not normal distribution).

Statistical analysis

Tests for statistical significance were computed in Past 3.04 (Hammer *et al.*, 2001) or R [version 3.4.3 (R Core Team, 2017)] and packages within. The exact tests performed are specified in the figure's legends or main text.

Multiple factor analysis. MFAs was performed considering all three sites using XLStat (version 2015 1.01; Addinsoft SARI, Paris, France). MFA requires data matrices (plants, microbes, soil nutrients and volatiles) of the same size (number of scores). Because soil nutrient content had been determined from only six plots per site, only data from the corresponding six plots (five for site 3 due to a missing sample) were considered to generate the relevant matrices for plants, microbes and volatiles. Specifically, the following matrices were used (i) the normalized matrix generated under “Soil volatile profiling” and containing 298 TAGs (volatiles); (ii) for plants, the relative abundance of 38 species were expressed in percentage for each plot; and (iii) for microbes, the relative abundance of 3950 bacterial and 1063 fungal OTUs were expressed as the number of reads detected in each plot. Nutrients and nutrient ratios were expressed as illustrated in the Supporting Information Fig. S1. RV coefficients computed by the MFA range from zero to one and the higher the score, the stronger the association between two data matrices.

Correlation analysis. Correlations among volatiles, plant species and microbial OTU were calculated in Past 3.04 (Hammer *et al.*, 2001) considering each sites separately. Values (Person's R) with non-significant correlations ($p > 0.05$, t-test) were replaced by zero, resulting in three separate correlation matrices that contained only significant positive and negative correlations [Supporting Information Tables S6 (fungi), Table S7 (bacteria), Table S8 (plants)].

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Authors contributions

MM and AB conceived the field trials in Switzerland. AV, MM and DS collected soil samples. AV and MM determined vegetation composition and yield. PM and AB analysed the nutrient content of soil samples. DS and AD characterized the soil's microbiome. DS and RS analysed the soil's volatilome. DS, JN, PM and RS synthesized the data and performed data analysis and statistics. DS, RS and JN wrote the manuscript with input from all other co-authors.

Conflict of interest

The authors declare no conflict of interest of any sort.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Supporting Information Figs S1- S6

Table S1. Supporting Information Tables S1 – S8

Appendix S1. Supplemental Chromatograms (three soil samples and one alkane series)