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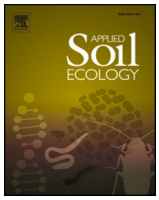

Changes in the root fungal microbiome of strawberry following application of residues of the biofumigant oilseed radish

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ABSTRACT

Biofumigation has been proposed as an environmentally friendly method of plant protection against soil-borne pathogens, but its effects on microbial communities are still incompletely understood. Using high throughput DNA sequencing, we investigated the effects of oilseed radish residues on the root fungal microbiome of strawberry in the presence of a soil-borne fungal pathogen, *Verticillium dahliae*. Results of our greenhouse study show that early flowering occurred in response to residue addition, suggesting a plant stress-response and there was a significant decrease in berry yield. The fungal microbiome of roots was significantly restructured by both biofumigation and inoculation with *Verticillium*. In particular, the abundance of root endophyte- and arbuscular mycorrhizal functional guilds was reduced significantly as a result of biofumigant and *V. dahliae* addition, whereas the abundance of saprotrophs increased significantly when both treatments were applied together. Alpha diversity analyses of fungi associated with roots indicated a significant increase in species richness following *Verticillium* inoculation, whereas the biofumigant alone or in the presence of *V. dahliae* resulted in no significant effect, suggesting that apparently some rare taxa may have been enriched/stimulated in the presence of the pathogen. Further investigations should reveal whether negative effects of biofumigation on potentially beneficial root associated endophytes and arbuscular mycorrhizal fungi are host genotype- or soil-dependent.

1. Introduction

Microbial interactions in the rhizosphere play fundamental roles in influencing the growth, stress tolerance and health of plants. Nearly one third of the total losses of potentially attainable worldwide yields of agriculturally important crops has been attributed to pathogens (Oerke and Dehne, 2004). Current strategies for plant protection based on cultivation practices, including use of resistant cultivars and application of pesticides, are not always sufficient. Pathogen resistance to pesticides, their hazardous effects on our environment and the European Directive 2009/128/EC (2009) on sustainable use of pesticides necessitate the development of alternative solutions that are environmentally sound and sustainable. In this context, use of biocontrol agents is being explored to control pathogens, particularly soil-borne pathogens that are difficult to control with current practices. However, the effects of biocontrol agents have been shown to differ with respect to host genotype response, indicating a need to find better alternatives (King and Parke, 1993; Smith et al., 1997; Smith and Goodman, 1999).

Biofumigation has been investigated as an alternative method of plant protection, especially for soil-borne pathogens, but also weeds (Mazzola et al., 2001; Yohalem and Passey, 2011; Wei et al., 2015). Use of synthetic soil fumigants such as methyl bromide and metam sodium is associated with negative effects on soil fauna and flora (Davis et al., 1996; Toyota et al., 1999). Discontinuation of conventional chemical fumigation is not recommended as it leads to substantial yield losses, e.g. in strawberries (Wilhelm and Paulus, 1980), therefore alternative methods need to be developed.

Incorporation of residues of biofumigant crops (typically *Brassica* species) results in a number of possible effects, including physical mixing of the soil, addition of plant available nutrients and stimulation of saprotrophs through addition of the organic residues, direct effects of biofumigant chemicals and introduction of microbial inoculum originating from the phyllosphere and rhizosphere communities of the biofumigant crop. These effects, individually or collectively, in turn have

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direct or indirect effects on plant growth and nutrient status of the main crop, target or non-target pathogens, soil fauna and other fungal and bacterial communities, including plant-beneficial symbionts, such as arbuscular mycorrhizal fungi. Biofumigation involves the effects of isothiocyanates (ITCs) that are released through hydrolysis of glucosinolates (GLS) of *Brassica* species (Matthiessen and Kirkegaard, 2006) and has been found to suppress soil-borne pathogens. Other antimicrobial compounds such as thiocyanates, nitriles and oxazolidine have also been found to be released after incorporation of biofumigant crop plant material (Matthiessen and Kirkegaard, 2006; Gimsing and Kirkegaard, 2009). Concentrations of some volatile ITCs have been shown to decrease by 90% within 24 h of incorporation of *Brassica* residues whereas non-volatile compounds such as ionic thiocyanates persist longer than ITCs (Brown et al., 1991), however persistence of ITCs up to 45 days has also been demonstrated (Poulsen et al., 2008; Gimsing and Kirkegaard, 2009). Possible differences in residence time of biologically active compounds in different soils, may explain the variable outcomes in different reports. The effects of biofumigation on soil microbial communities and potential plant pathogens have been studied using brassicas (Friberg et al., 2009; Omirou et al., 2011) but effects on the microbial community structure in the roots/rhizosphere of the main crop are less well studied.

Most research on biofumigation has focused on *Brassica* spp. as the biofumigant crop (Rumberger and Marschner, 2003; Cohen and Mazzola, 2006; Friberg et al., 2009; Omirou et al., 2011). Several attempts have been made to study the impact on the structure of rhizosphere microbial communities (Mazzola et al., 2001; Cohen and Mazzola, 2006; Bressan et al., 2009; Omirou et al., 2011). These studies have shown effects on microbial communities such as an increase in the abundance of *Bacillus* and *Streptomyces* spp., as well as Ascomycetous communities, but no effect on ammonia-oxidizing bacteria. In a microcosm study, the effects of a non-brassicaceous and non-glucosinolate oilseed meal (*Linum usitatissimum*) and glucosinolate containing brassicaceous oilseed meal (*Brassica juncea*) amendments on bacterial and microbial communities were also compared, suggesting that mustard in particular, has the potential to alter soil microbial community structure (Hollister et al., 2013). Not only the incorporation of brassicaceous plant material, but also the effect of addition of synthesized ITCs on soil bacterial and fungal communities have been studied (Hu et al., 2015; Hanschen et al., 2015). Elfstrand et al. (2007) used non GLS-producing plant materials such as green-manure and reported an increase in soil microbial biomass and enzyme activity and increased yield of maize. Incorporation of residues from different plant species has also been shown to affect the structure of soil microbial communities, including the relative abundance of pathogenic *Pythium* spp. and antagonists of soil-borne pathogens such as *Trichoderma* spp., fluorescent pseudomonads, *Streptomyces* spp. and actinomycetes (Cohen and Mazzola, 2006; Perez et al., 2008; Mazzola and Zhao, 2010; Mazzola et al., 2001, 2012).

Verticillium dahliae is one of several soil-borne plant pathogens causing damage to various crops, including strawberries (Heale and Karapapa, 1999; Fradin and Thomma, 2006; Klosterman et al., 2009). It is estimated that as few as three microsclerotia per gram of soil can cause approximately 50% loss in strawberries (Harris and Yang, 1996). Crop rotation using *Brassica* species has been shown to decrease numbers of microsclerotia of *V. dahliae* but not the total colony counts of *Pythium* spp. in a strawberry field studied by Subbarao et al. (2007). Incorporation of *B. juncea* seed meal and *Ricinus communis* has been found to increase the yield of tomato and strawberry (Seigies and Pritts, 2006; Handiseni et al., 2012). However, studies of incorporation of *Raphanus sativus* var. *oleiferus*, *B. juncea*, and *Sinapis alba* have demonstrated negative effects on seed germination and establishment of cucurbit crops (Ackroyd and Ngouajio, 2011). Other studies of wheat emergence (Mazzola et al., 2012) have demonstrated negative effects of *B. napus* and *S. alba*, but not of *B. juncea*. In Europe and some parts of USA, oilseed radish has been shown to be effective and is used for controlling nematodes in sugar beet (Brown et al., 2008). Use of oilseed radish as a green manure, has also been shown to promote marketable yield of cucurbit crops more than the use of other crucifers (Ackroyd and Ngouajio, 2011). Knowledge of oilseed radish effects on economically important soil-borne pathogens, particularly *V. dahliae*, *Rhizoctonia solani* and microbial communities sharing the same niche is still limited.

Most of the studies of soil microbial communities mentioned above have been conducted using cultivation-dependent approaches and/or molecular methods with relatively low taxonomic resolution, such as terminal restriction fragment length polymorphism (T-RFLP) or denaturant gradient gel electrophoresis (DGGE). The use of these methods is known to underestimate the total microbial diversity (Torsvik and Øvreås, 2002; Weinert et al., 2011). Sanger sequencing provides somewhat better resolution but recent studies using high throughput techniques, such as phylochips and 454-pyrosequencing have revealed taxa that were previously missed (Weinert et al., 2011; Xuan et al., 2012). Mazzola et al. (2015) used 454 sequencing to evaluate the long-term effect of *Brassica* seed meal amendment on composition of the apple rhizosphere microbiome. The seed meal treatment rhizosphere was modified, containing specific fungal and bacterial elements previously associated with pathogen suppression and the amended soils were resistant to reinfestation by *Pratylenchus penetrans* and *Pythium* spp. and associated with enhanced tree performance. Studies using T-RFLP and DGGE have shown reductions in soil microbial diversity following incorporation of *B. juncea* or *B. oleracea* or ITC derivatives (Rumberger and Marschner, 2003; Friberg et al., 2009; Omirou et al., 2011; Hanschen et al., 2015).

The aim of the present study was to investigate the effects of oilseed radish as a biofumigant on the composition of the strawberry root fungal microbiome in the presence and absence of a soil-borne fungal pathogen, *Verticillium dahliae*. Oilseed radish was chosen for its good ability to promote early establishment in the field and its resistance to cold stress (Pegg and Amarowicz, 2009). We hypothesized that biofumigation and *Verticillium* addition will (i) change composition of the root fungal microbiome, (ii) have greater impact on fungi that are in intimate association (e.g. endophytic or symbiotic) with strawberry roots than other fungi.

2. Materials and methods

2.1. Plant material and growth conditions

Certified frigo plants of strawberry (*Fragaria* × *ananassa* Duch., cultivar 'Honeoye') were obtained from E. Dahlén AB, Vara, Sweden. This cultivar is known to be susceptible to *V. dahliae* (Simpson et al., 2002) and is grown commercially in Sweden. Oilseed radish (*Raphanus sativus* var. *Oleifera* L.) was chosen as the biofumigant plant species and the seeds were obtained from Olssons Frö, Helsingborg, Sweden.

An agricultural field soil with no known history of strawberry cultivation was used in this study. The soil was collected from a field north of the Swedish National Veterinary Institute, Ultuna, Uppsala and analyzed for its physical and chemical traits (Agri Lab, Uppsala, Sweden). To analyze plant-available nutrients, soils were extracted with ammonium acetate lactate solution (0.1 M ammonium lactate and 0.4 M acetic acid) (Egnér et al., 1960) and total elements were extracted with hydrochloric acid – this approach is used routinely in Scandinavia for assessment of nutritional status of agricultural soils. Concentrations of different extractable elements (Ca, K, Mg, P) were measured by inductively coupled plasma (ICP) emission spectroscopy. According to FAO (2006) soil texture and classification, the soil type was 'loamy sand' and cambisol respectively. Details of the chemical characteristics and soil texture are given in Table S1.

2.2. Pathogen inoculum preparation

Verticillium dahliae, a soil-borne pathogen is known to occur in strawberry field soils in Sweden (personal communication, C. Winter,

SJV, Norrköping) but documented information about its prevalence is not yet available. The pathogen isolate, *V. dahliae* 12086 used in this study originated from strawberry and was obtained from C. Dixelius, SLU, Uppsala. It was grown as stationary cultures in potato dextrose broth (PDB 12 g/l, Accumedia, Michigan, USA) for 10 days at 24 °C. Fungal mycelium (fresh weight 4 g) was mixed into successively larger amounts of field soil up to a final weight of 300 g. This soil-inoculum mixture was applied as a layer equally and uniformly in the field soil in pots (size 27 × 17 × 14 cm) before sowing the oilseed radish seeds.

2.3. Experimental design

The oilseed radish seeds were sown at a depth of 1.0–2.0 cm at the seeding rate recommended by the United States Department of Agriculture (see Pest Management (595) – Biofumigation, 2008), which corresponded to 9 seeds per pot. Pots with no seeds were prepared as controls. All pots were arranged in a randomised design in a greenhouse with a 14:10 h, 15:13 °C day:night cycle with a daytime photon flux density of 250 μmol m^{-2} s^{-1} and maintained at 80% of total water holding capacity of soil by regularly weighing on a balance and replenishing with water to a constant weight. Incorporation of the oilseed radish plants was done when they reached the flowering stage, after approximately 9 weeks. The plants were lifted carefully and their roots were gently washed under running tap water before chopping them into small pieces <1 cm. The chopped plant material was crushed further before incorporating into soil, covered with approximately 5 cm of soil and watered on the surface. This approach was followed to minimise loss of volatile compounds released during hydrolysis of glucosinolates and decomposition of organic residues. Soon after incorporation (i.e., 0 h and 24 h), six soil samples per pot were taken for fungal community analyses using a soil borer (diameter 1 cm) at a depth of 7 cm diagonally across the pot (soil samples were also collected in the same manner for the treatments where no strawberry was planted in the later stages of the experiment).

Eighteen days after incorporation, but before planting of strawberries, soils were sampled again, as described above. The soils from six replicate pots of each treatment were pooled, mixed thoroughly and filled into new pots (size 9 × 9 × 20 cm) containing burnt clay beads (size, 8–12 mm, ECONOVA Garden AB, Sweden) at a ratio of 2 vol soil:1 vol beads. The beads were used to facilitate aeration of the roots. Honeoye seedlings were then planted (one per pot) in this mixture, in accordance with treatments summarized below. In total 24 pots were filled with biofumigated soil (+B) and 24 pots with non-biofumigated soil (−B). Half of each of these treatments were inoculated with *Verticillium* (+V) and half uninoculated (−V). This resulted in 12 pots for each treatment – half were then planted with strawberry seedlings and the remaining half served as no plant controls (bulk soil). The biofumigation and *V. dahliae* treatments were applied factorially, resulting in four main treatments, no biofumigant, no *Verticillium* (−B−V); biofumigant, no *Verticillium* (+B−V); no biofumigant, plus *Verticillium* (−B+V); biofumigant, plus *Verticillium* (+B+V). Three replicates per treatment, were harvested at 90 days and 120 days after strawberry planting. All the pots were placed in a greenhouse at 23 °C. The pots were watered and rotated regularly throughout the experimental period to minimise border effects. The cumulative numbers of flowers and fresh berry weight per plant were recorded. The effects of these four treatments were assessed in three physical compartments, bulk soil, rhizosphere soil of strawberry and the strawberry roots themselves. The bulk soil samples were collected from pots containing soil only. Rhizosphere soil was collected by carefully removing the plant roots, together with firmly adhering soil and suspending this material in phosphate buffer saline solution (PBS, 0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^{3-}, pH 7.4; Medicago AB, Sweden) for 30 min, centrifuged at 9000*g* for 10 min at 4 °C (Biofuge PrimoR, Heraeus Sorvall) and the soil pellet was stored at −20 °C for further analysis. Roots were carefully washed with ice cold MilliQ water to remove the soil particles attached to the roots and soil removal was confirmed microscopically. They were gently washed three times with 0.1% Triton X-100 for 2 min and thereafter washed with sterile MilliQ water. All collected roots were subsequently freeze-dried for 48 h (CoolSafe, ScanLaf A/S, Denmark), homogenized by milling in 2 ml tubes containing 2.38 mm diameter stainless steel beads (MOBIO laboratories, California, USA) in a bead-beater for 30 s twice at 5000 RPM. Milled root material was stored at −20 °C until used for DNA extraction.

2.4. Analysis of fungal communities

The DNA extraction procedures for rhizosphere and root associated samples, and PCR products for pyrosequencing were as specified in a previous study (Nallanchakravarthula et al., 2014), but bioinformatic and statistical analyses were performed differently, as described below.

2.4.1. DNA extraction and selection of samples for pyrosequencing

To analyze fungal communities that were intimately associated with roots (including both fungi adhering firmly to roots and residing inside roots), DNA from roots that had been washed thoroughly with Triton X-100 (as described above) was extracted using DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's recommendations. 50 milligrams of the homogenized root material was used for DNA extraction. The concentration of extracted DNA was determined using NanoDrop (ND-1000 Nanodrop technologies, USA) and used as a template for generating PCR amplicons for pyrosequencing.

To analyze soil fungal communities, we used two different nucleic acid extraction methods and the resultant DNA extracts were checked individually for quality and reproducibility on a DGGE gel following PCR amplifications. In an earlier pilot study, there was a clear effect of the nucleic acid extraction method on the soil fungal communities. The first method was based on 'CTAB' (hexadecyltrimethylammonium bromide) (Griffiths et al., 2000) which involved extraction of nucleic acids from 0.5 g of soil. The soil samples were weighed in 2 ml 'Lysing matrix B' tubes (Fisher Scientific, USA) containing 0.5 ml CTAB buffer (120 mM and pH 8.0) and 0.5 ml phenol:chloroform:isoamylalcohol (25:24:1 V/V). The cells were lysed at 5000 RPM for 30 s in a bead beater (Precellys 24; Bertin Technologies, France). The nucleic acids were extracted and precipitated using chloroform and isoamyl alcohol (24:1) followed by further precipitation using polyethylene glycol (30%). The precipitated DNA was washed with ice-cold ethanol (70%), air dried and re-suspended in 30 μl of MilliQ water and stored at −20 °C for further analysis. The second nucleic acid extraction method was based on MOBIO's RNA PowerSoil® total RNA isolation and DNA elution accessory kits (MOBIO laboratories, California, USA) according to manufacturer's recommendations. The concentration of extracted DNA was determined (as described above). The soil DNA from the above two extraction methods was pooled to give better coverage of the resulting fungal communities and to minimise extraction-method related bias, as described by Nallanchakravarthula et al. (2014) and used as a template for generating PCR amplicons for pyrosequencing.

Prior to pyrosequencing, samples from 0 h, 24 h and 18 d after biofumigant incorporation, and 90 d and 120 d after strawberry planting were subjected to DGGE-based analysis following PCR amplification. Based on the finding that the maximum treatment effect on fungal community composition occurred at the 90-day harvest, samples (0 h, 18 d and 90 d) were chosen for further analysis using pyrosequencing.

2.4.2. Pyrosequencing

Pyrosequencing analysis of the fungal communities was carried out as described by (Ihrmark et al., 2012) using primers fITS7 (5′-GTGARTCATCGAATCTTTG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). In brief, each 50 μl reaction mixture consisted of 200 μM of dNTPs, 2.75 mM $MgCl_2$, primers at 500 nM and 300 nM, 0.025 U polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in buffer. The thermocycling conditions were: 5 min at

94 °C; 35 cycles (30 s at 94 °C; 30 s at 57 °C; 30 s at 72 °C); 7 min at 72 °C. Three technical replicates were run for each sample. The PCR amplification products were analyzed on 1% (w/v) agarose gels pre-stained with Nancy red-520 (Sigma Aldrich, USA). The triplicate PCR products of each sample were pooled, purified using Agencourt® AMPure®, PCR purification kit (Beckman Coulter, Massachusetts, USA) and quantified using a Qubit Fluorometer (Invitrogen, USA). Equal concentrations of samples were pooled and the resultant mixture was freeze-dried (CoolSafe™, ScanLaf A/S, Denmark) overnight. Pyrosequencing of root and soil amplicons was carried out separately on two 1/8th of a GS FLX Titanium Pico Titer Plate (LGC Genomics, Germany and MACROGEN, South Korea) according to the manufacturer's instructions (Roche, Branford, CT).

2.4.3. Bioinformatics

Root and soil reads from two pyrosequencing runs were merged using the "merge.sffiles" script in MOTHUR (Schloss et al., 2009). For initial analysis of the reads, we used the publicly available SCATA pipeline (scata.mykopat.slu.se/?p=home) that has been developed for sequence clustering and analysis of tagged amplicons and is specifically optimized for sequences of fungal ITS-region. The following default criteria were used for quality filtering of the reads: singletons and low quality sequences were removed; sequences shorter than 200 base pairs (after primer and tag trimming) were removed; any sequences with an average quality score below 20 or with a score below 10 at any position were removed. Sequences were clustered using the USEARCH clustering engine (Edgar, 2010) with the following parameters: minimum alignment length for clustering 0.85, maximum alignment length for clustering 0.015, mismatch penalty 1, gap open penalty 0, gap extension penalty 1 and homopolymer reduction at 3 bp, the proportion of primer match was set at 0.9 for forward primer and 0.75 for reverse primer. Sequences were assembled into clusters by single-linkage clustering with a 98.5% sequence similarity threshold. The most common sequence of the cluster was used to represent the operational taxonomic units (OTUs). Individual sequences were classified taxonomically using the "classify.seqs" command in MOTHUR (Schloss et al., 2009) (confidence threshold 80) using the UNITE fungal ITS reference database (Kõljalg et al., 2013) (release ver. UNITE_public_mothur_full_10.10.2017). The OTU table was constructed using abundance data from the SCATA pipeline and further analyses were performed using the QIIME 1.9.0 pipeline (Caporaso et al., 2010). Non-fungal OTUs were removed from the final OTU table using "filter_otus_from_otu_table.py". The filtered OTU table was rarefied using the command "single_rarefaction.py". To confirm the results further, cumulative sum scaling (CSS) (Paulson et al., 2013) or DESeq2 (Love et al., 2014) normalization of the OTU table was also performed using "normalize_table.py". To visualize the taxonomic composition of sequences at various levels the "summarize_taxa_through_plots.py" command was used.

For ecological/functional guild-based annotation of the OTUs, all OTUs were parsed using FUNGuild tools as described by Nguyen et al. (2015). The resulting data set contained the following functional guilds: saprotroph, pathogen, lichenized, endophyte, arbuscular mycorrhizal, ericoid mycorrhizal. Fungi that were difficult to assign to a single guild but could possibly be assigned to multiple guilds (e.g., saprotroph/pathogen or endophyte/mycorrhizal etc.) were termed as 'ambiguous' and the remaining OTUs that could not be classified to any known guild (because they lacked sufficient ecological and/or taxonomic characterization) were grouped as 'unassigned'. All annotations were checked manually against published information on taxonomy, ecology and habitat of different fungal species.

The fungal pyrosequencing data files have been submitted to the European Nucleotide Archive (ENA) and are available under the study accession number PRJEB21579 at the following link https://www.ebi.ac.uk/ena/browser/view/PRJEB21579

2.5. Statistical analyses

The differences in fresh berry yield and changes in alpha diversity were evaluated by analysis of variance (ANOVA) using JMP Pro 13. Using rarefied abundance data, beta diversity patterns were analyzed by nonmetric multidimensional scaling (NMDS) ordinations with the Bray-Curtis dissimilarity measure. Statistical significance of the community data was estimated using nonparametric analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations. Pair-wise comparisons were performed with Bonferroni-corrections to test for significance of different treatments. Alpha diversity indices (Taxa S, Shannon H, Menhinick, Margalef's richness, Brillouin, Fisher's alpha, Dominance D, Berger-Parker dominance, Simpson 1–D, Buzas-Gibson's evenness e^H/S and Equitability J) were calculated using 9999 runs of bootstrapping (PAST 3 statistical package). Diversity estimates were based on sequence abundance data that had been rarefied to 5274 sequences per sample. Singletons were removed from the data prior to analyses. To identify fungal taxa that differed significantly ($P < 0.05$) in abundance across the treatments, we used the "group_significance.py" script with ANOVA implementation in QIIME.

3. Results

3.1. Plant growth parameters

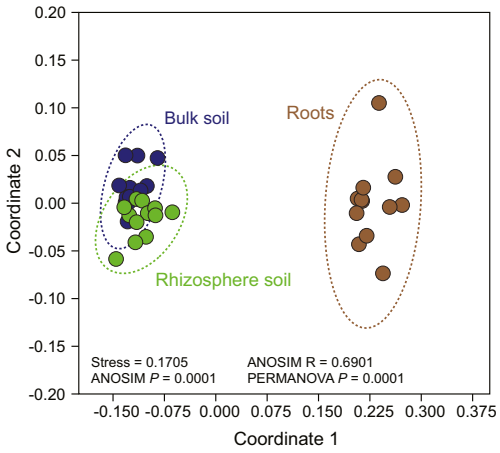
Four weeks after planting, a reduction in strawberry plant growth was observed in the presence of *V. dahliae*, irrespective of the biofumigant addition (data not shown). In the absence of *V. dahliae*, biofumigant residues (+B–V) induced early flowering in over 50% of the plants. At the same time point there were no flowers in the –B–V treatment. A significant decrease in berry yield was observed in response to biofumigant addition in the *V. dahliae* controls (+B–V) (ANOVA $P = 0.024$), but the reduction associated with biofumigant treatment in the presence of *Verticillium* (+B+V) was not statistically significant (Fig. S1). However, a parallel analysis of plant-available nutrients in soil did not show any significant differences across the treatments (data not shown).

3.2. Fungal communities

In total 255,405 reads were obtained from pyrosequencing of 12 root and 36 soil samples and after quality filtering and chimera removal 139,858 sequences (108,882 from roots and 30,976 from soils) remained for downstream analysis. In total, 692 OTUs remained after removal of 10 non-fungal OTUs, 32 OTUs that were classified as 'unknown' at kingdom level and 1575 singletons. However, doubletons (156 OTUs) and tripletons (86 OTUs) were not removed. The pyrosequencing throughput of the root samples was 3.5 fold higher than that of the soil samples, therefore we focus on the root microbiome and present the results from rhizosphere and bulk soils as 'Supplementary data'.

Nonmetric multidimensional scaling (NMDS) analysis of the composition of the fungal community data revealed communities that were statistically significantly distinct from each other in the roots, rhizosphere soil and bulk soil (ANOSIM $P = 0.0001$, PERMANOVA $P = 0.0001$) (Fig. 1), with a particularly clear separation between root and soil communities. Bonferroni-corrected pairwise comparisons based on ANOSIM and PERMANOVA suggest that fungal community composition was statistically different in bulk soil, rhizosphere soil and roots of strawberry plants (Fig. 1).

Rarefaction curves based on individual root samples (Fig. S2a) revealed that sampling depth was adequate but did not reach an asymptote. Between 5274 and 12,889 sequences (median 8839) were obtained per sample that resulted in 314 fungal OTUs in roots. Rarefaction curves based on rarefied sequences (5274 per sample) (Fig. 2a) showed that treatment with no biofumigation, but with *V. dahliae* addition (–B+V), resulted in a statistically significant increase in species



ANOSIM *P*	Bulk soil	Rhizosphere soil	Roots
Bulk soil	–	0.0048	0.0003
Rhizosphere soil	0.0048	–	0.0003
Roots	0.0003	0.0003	–
PERMANOVA *P*	**Bulk soil**	**Rhizosphere soil**	**Roots**
Bulk soil	–	0.0018	0.0003
Rhizosphere soil	0.0018	–	0.0003
Roots	0.0003	0.0003	–

Fig. 1. Nonmetric multidimensional scaling (NMDS) ordination showing differences in fungal community composition in the bulk soil, rhizosphere soil and roots of strawberry plants 90 days after planting. Ellipses represent 95% confidence intervals. The table depicts Bonferroni-corrected *P* values of pairwise comparisons using analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) of fungal community composition in bulk soil, rhizosphere soil and roots of strawberry plants.

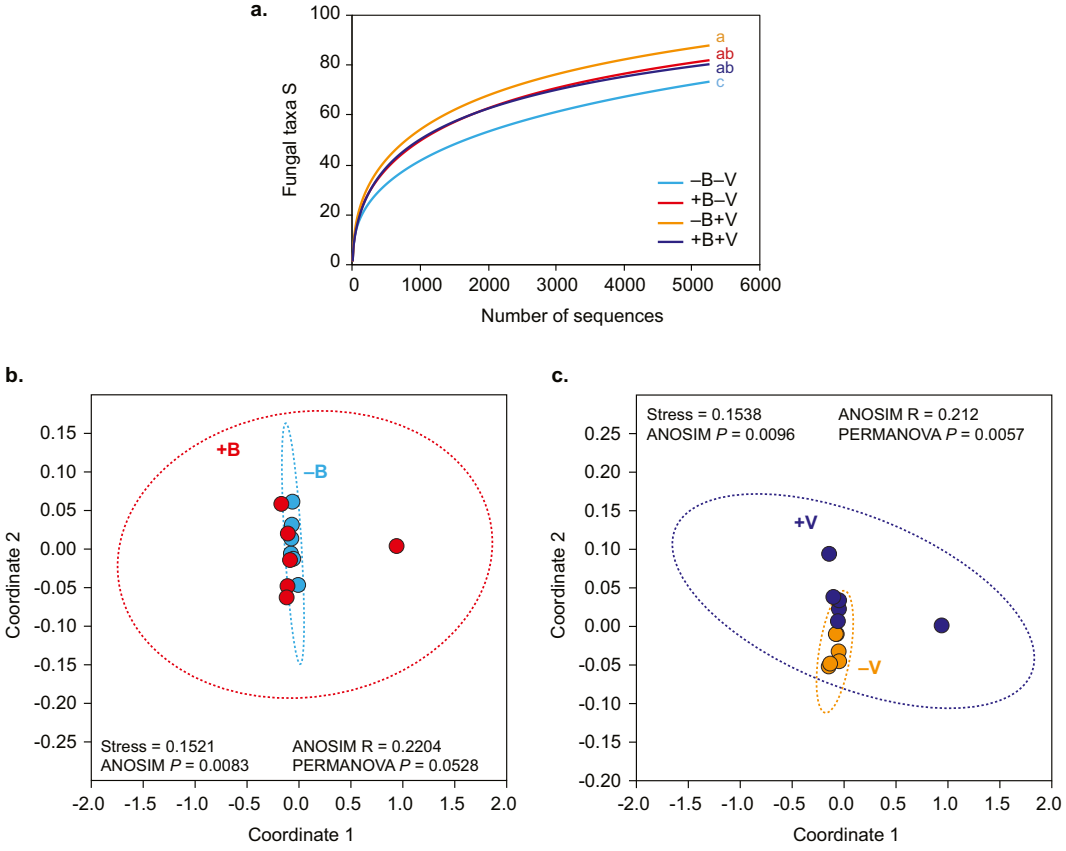


Fig. 2. Rarefaction curves illustrating the numbers of fungal taxa associated with the roots of strawberry plants treated with factorial combinations of oilseed radish (*Raphanus sativus oleifera*) residues as biofumigant, (+/− B) and inoculation with the fungal pathogen *Verticillium dahliae* (+/− V). The fungal community data was rarefied to 5274 sequences per sample. Different letters denote differences among means ($P < 0.05$; ANOVA) (a). Nonmetric multidimensional scaling (NMDS) ordination showing differences in fungal community composition due to biofumigant residues (blue circles, blue ellipse = no biofumigant; red circles, red ellipse = with biofumigant) (b) and to inoculation with *Verticillium dahliae* (orange circles, orange ellipse = no *V. dahliae*; dark blue circles, dark blue ellipse = with *V. dahliae*) (c) in the roots of strawberry plants 90 days after planting. Ellipses represent 95% confidence intervals for +B versus −B or for +V versus −V treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

richness (Taxa S); index values (Table 1) of Shannon H, Menhinick, Margalef's richness, Brillouin and Fisher's alpha also followed a similar trend, while Dominance D and Berger-Parker dominance were highest in –B–V controls. Index values of Simpson 1–D, Buzas-Gibson's evenness e^H/S and Equitability J did not show any significant change across the treatments (Table 1).

Separate NMDS analyses of the effects of biofumigant addition and *V. dahliae* treatments (Fig. 2b, c) revealed that both treatments significantly (ANOSIM $P = 0.0083$, $P = 0.0096$ respectively) changed fungal community composition in roots. Based on a PERMANOVA test however, the biofumigation effect was not significant ($P = 0.0528$) whereas the *V. dahliae* effect was still strongly significant ($P = 0.0057$) in roots. A further NMDS evaluation based on DESeq2 normalized data (Fig. S2b, c) provided confirmation that both biofumigation and *V. dahliae* –

Table 1
Alpha diversity indices of fungal communities in roots of strawberry plants treated with factorial combinations of oilseed radish (*Raphanus sativus oleifera*) residues as biofumigant, (+/− B) and inoculation with the fungal pathogen *Verticillium dahliae* (+/− V). Diversity estimates are based on sequence abundance data that have been rarefied to 5274 sequences per sample. Singletons were removed from the data prior to diversity analysis. ± represent standard error (SE). Different letters denote differences among means ($P < 0.05$; ANOVA, pair-wise comparisons using Student's t).

Indices	–B–V	+B–V	–B+V	+B+V
Taxa S	74.67 ± 2.19 b	82.33 ± 2.60 ab	88.33 ± 1.33 a	80.67 ± 6.89 ab
Menhinick	1.01 ± 0.03 b	1.13 ± 0.04 ab	1.22 ± 0.02 a	1.11 ± 0.10 ab
Margalef	8.48 ± 0.26 b	9.49 ± 0.30 ab	10.19 ± 0.16 a	9.30 ± 0.80 ab
Dominance D	0.20 ± 0.04	0.16 ± 0.01	0.12 ± 0.01	0.17 ± 0.03
Berger-Parker	0.39 ± 0.05 a	0.30 ± 0.00 ab	0.27 ± 0.02 b	0.30 ± 0.04 ab
Evenness e^H/S	0.14 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.14 ± 0.02
Equitability J	0.54 ± 0.03	0.56 ± 0.02	0.61 ± 0.01	0.55 ± 0.03
Simpson index	0.81 ± 0.04	0.84 ± 0.01	0.88 ± 0.01	0.83 ± 0.03
Brillouin	2.29 ± 0.12 b	2.43 ± 0.08 ab	2.68 ± 0.06 a	2.37 ± 0.16 ab
Shannon H	2.32 ± 0.13 b	2.47 ± 0.08 ab	2.71 ± 0.06 a	2.39 ± 0.16 ab
Fisher's alpha	12.12 ± 0.43 b	13.85 ± 0.53 ab	15.07 ± 0.27 a	13.54 ± 1.37 ab

according to ANOSIM tests ($P = 0.00207$, $P = 0.00411$ respectively) and PERMANOVA ($P = 0.0192$, $P = 0.0154$ respectively) had significantly altered fungal communities in roots.

The relative abundance of different fungal phyla and classes in the strawberry roots is shown in Fig. 3a. Dominant fungal phyla were Ascomycota, Basidiomycota and Glomeromycota that covered 87%, 9% and 4% respectively of the sequences obtained from roots. Among Ascomycota, *Dothideomycetes* was the only class that significantly increased in abundance in the –B+V treatment. The Basidiomycotan class *Agaricomycetes* showed a trend of marked increase in abundance in treatments with biofumigant (+B) but the differences were not significant statistically. *Glomeromycetes* was the dominant class among Glomeromycota that responded strongly to the treatments and decreased in abundance significantly in both +B and +V treatments. Fig. 3b depicts the top twenty-five fungal taxa (based on proportional abundance of ITS sequences) that contributed to around 95% of the total fungal community in roots, whereas the NMDS ordination plot (Fig. 3b [inset]) is based on all the taxa. The relative abundance of *Cadophora orchidicola*, unclassified *Pleosporales*, *Setophoma terrestris*, and unclassified *Helotiales* appeared to be reduced in response to biofumigant residues and/or *V. dahliae* addition. In particular, an unidentified arbuscular mycorrhizal taxon belonging to the *Glomeraceae* was significantly reduced in relative abundance by biofumigation and especially by *V. dahliae* inoculation. In the absence of biofumigation, *V. dahliae* inoculation significantly increased the relative abundance of *Pleosporales*, *Periconia macrospinosa* and *Microdochium bolleyi*.

The above results were explored further by analysis of variation in the abundance of fungi belonging to different functional guilds (Fig. 4a). In the roots the main variation was due to dynamic changes in the abundance of arbuscular mycorrhizal fungi, endophyte and saprotroph guilds in response to biofumigant residues and an even greater effect of inoculation with *Verticillium*. Both root endophyte and arbuscular mycorrhizal guilds were significantly ($P < 0.05$) reduced in abundance by biofumigation and *V. dahliae* addition, however, abundance of saprotroph guild increased significantly ($P < 0.05$). A bivariate plot of the proportional abundance of sequences representing endophyte versus saprotroph guilds (Fig. 4b) revealed a statistically significant ($P = 0.0097$) negative relationship between the two guilds. This relationship remained statistically significant ($P = 0.007$) and negative even when the arbuscular mycorrhizal guild was combined with the endophytes. However, this relationship was not significant when the arbuscular mycorrhizal guild alone was tested against saprotrophs (data not shown). Endophyte and arbuscular mycorrhizal guilds were significantly abundant in the –B–V treatment and the saprotroph guild was significantly abundant in the +B+V treatment.

4. Discussion

To our knowledge this is the first study describing the effect of oilseed radish residues on the composition of the root fungal microbiome in strawberry plants. However, the effects of biofumigation using *Brassica juncea* and *Raphanus sativus* on apple rhizosphere bacterial and fungal communities have been evaluated (Yim et al., 2016). In the present study, strawberry yield was shown to be negatively affected by the application of oilseed radish 18 days before planting. In small-scale field trials (Mazzola et al., 2017) significant phytotoxicity including plant death has been observed in strawberries planted 46 days after *Brassica juncea* seed meal application.

These results are consistent with those of Vera et al. (1987), who studied the effects of incorporation of different types of cruciferous plant material on the stand establishment and yield of barley, flax, oilseed rape and wheat. In that study, the experimental plots were sown on the day of incorporation, resulting in negative effects of the treatments on both stand establishment and yield in most crops. In another experiment Mazzola et al. (2012) observed a significant reduction or no effect on wheat emergence and wheat biomass of *Brassica* (*B. juncea* and *B. napus*) seed meals compared with non-*Brassica* (*Glycine* max and *Sinapis alba*) seed meals, following incubation for two weeks prior to planting depending on the treatment and the soil type. In contrast, Ackroyd and Ngouajio (2011) found an increase in the marketable yield of a transplanted muskmelon crop in oilseed radish treated plots in which they incorporated yellow mustard, oriental mustard and oilseed radish 7–8 days before planting. Biofumigation using incorporation of *B. juncea*, *Eruca sativa* and *Sinapis alba* 12 days before planting has also been reported to increase strawberry yield when compared with non-treated soil (Koron et al., 2014). Incorporation of *B. juncea* seed meal and *Ricinus communis* one week before planting was found to increase the yield of tomato (Kaskavalci et al., 2009). The choice of the incorporated crop, as well as its cultivar and the rate of application of the plant material before planting the main crop should be considered in order to optimize control strategies based on *Brassica* biofumigation in plant production systems (Mazzola et al., 2001; Rumberger and Marschner, 2003).

Biofumigation may have a direct chemical effect on plant pathogens or may induce biological interactions resulting in pathogen control (or promotion) through changes in microbial community structure (Mazzola et al., 2001; Cohen and Mazzola, 2006; Hoagland et al., 2008; Mazzola et al., 2012). Green manuring has been shown to change the composition of the bacterial, total fungal and symbiotic arbuscular mycorrhizal communities, as well as to increase microbial biomass and soil enzyme activity (Elfstrand et al., 2007). In the present study however, if addition of biofumigant residues had any green manuring or nutritional effect then there should have been a significant increase in the abundance of the fungal 'saprotrophic guild' in rhizosphere soil (Supplementary data Fig. S7b) but we did not observe any major

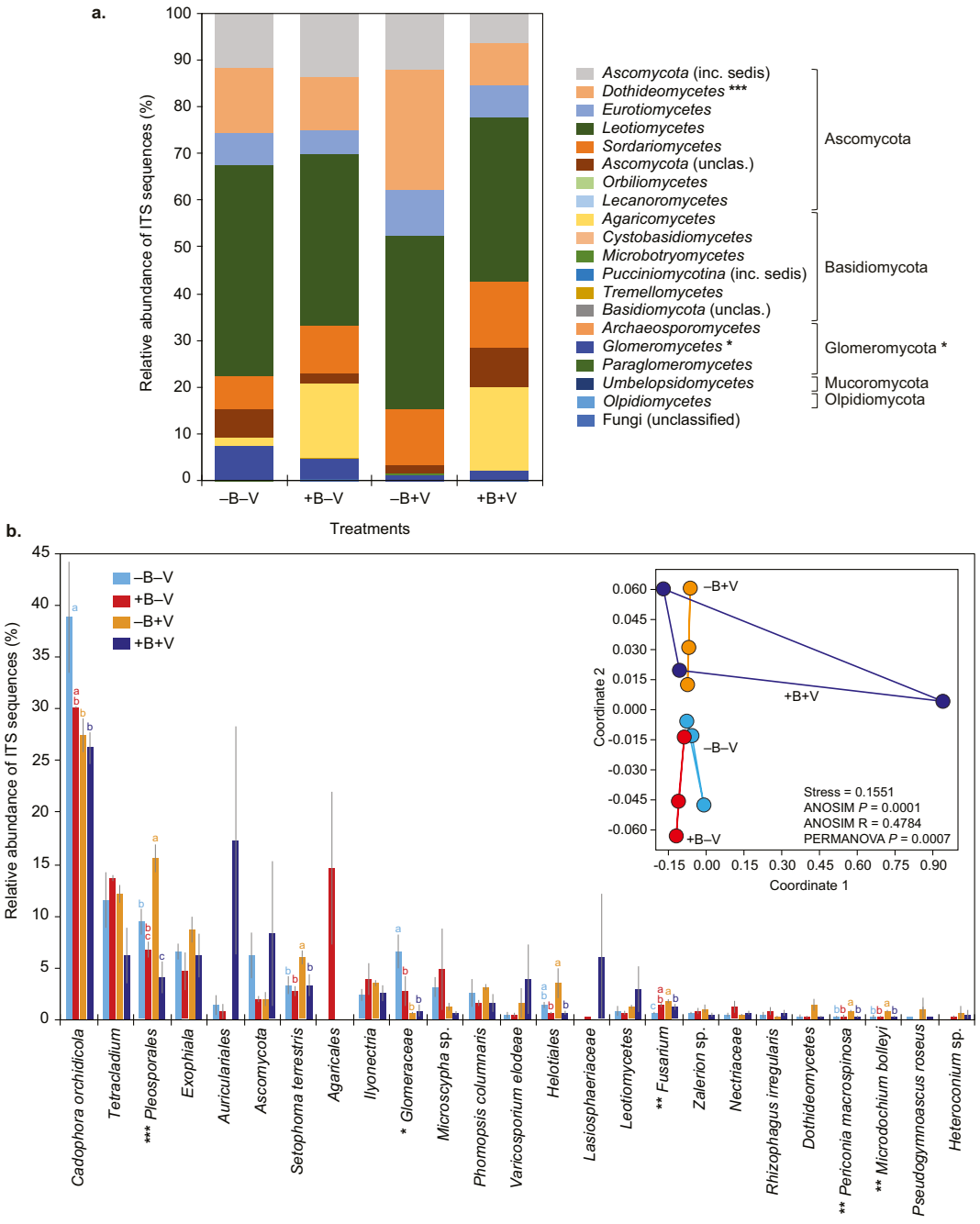


Fig. 3. Relative abundance of ITS sequences of different fungal classes in the roots of strawberry plants treated with factorial combinations of oilseed radish (*Raphanus sativus oleifera*) residues as biofumigant, (+/− B) and inoculation with the fungal pathogen *Verticillium dahliae* (+/− V) (a). Names of fungal phyla are given to the right of brackets grouping classes. The histogram depicts the twenty-five most abundant fungal taxa (that comprise around 95% of the ITS sequences from roots) found in all treatments, whereas the nonmetric multidimensional scaling (NMDS) ordination plot (inset) is based on all taxa (b). Analysis of variance (ANOVA) significance levels: *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

changes in abundance of dominant saprotrophs, suggesting that there was little or no green manuring effect. We therefore believe the observed effects of oilseed radish residues were more likely to be due to the ITCs and phytotoxic chemicals released after mulching of the biofumigant, although these chemicals were not measured directly in this study. In roots, the saprotrophic guild increased significantly in the +B+V treatment, with concurrent decrease in endophyte and arbuscular mycorrhizal guilds, possibly due to chemicals released from the biofumigant.

Several studies have also shown that addition of plant biomass including *Brassica* seed meals influences soil microbial community composition, including the abundance of soil-borne plant pathogens (Mattner et al., 2008; Bressan et al., 2009; Friberg et al., 2009; Mazzola et al., 2012). Effects of *Brassica* amendments on fungal community structure in the rhizosphere of apple using 454-sequencing (Mazzola et al., 2015) indicate that fungal diversity was reduced, but studies of apple using Illumina sequencing (Wang and Mazzola, 2019) indicate that the diversity of fungi associated with disease suppression was increased by *Brassica* amendment. In the present study, addition of biofumigant residues did not affect fungal diversity in the roots (Table 1, Fig. 2a) however the presence of the pathogen in the absence of the biofumigant resulted in a significant increase in fungal diversity. Being a

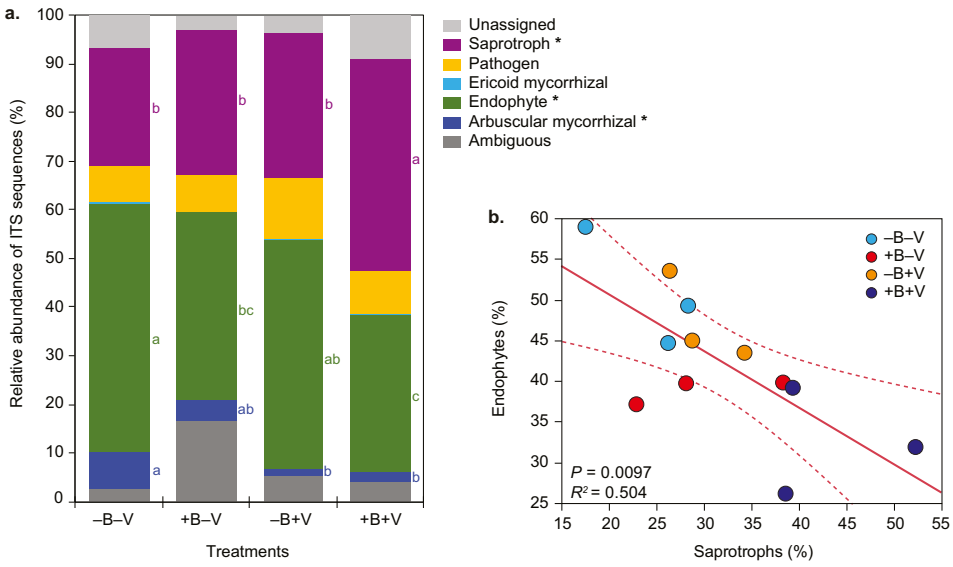


Fig. 4. Relative abundance of ITS sequences representing different functional guilds of fungi in roots of strawberry plants treated with factorial combinations of oilseed radish (*Raphanus sativus oleifera*) residues as biofumigant, (+/− B) and inoculation with the fungal pathogen *Verticillium dahliae* (+/− V) (a). Analysis of variance (ANOVA) significance level * = $P < 0.05$. Regression analysis of the proportion of ITS sequences belonging to endophyte and saprotroph functional guilds in the roots (b) show a statistically significant negative relationship between the two functional guilds. The dotted lines represent 95% confidence intervals.

wilt pathogen, *V. dahliae* colonises roots endophytically, entering the plant vascular tissues (Klosterman et al., 2009) and changing the composition of the fungal communities (Nallanchakravarthula et al., 2014).

In the present study, oilseed radish incorporation was shown to change the composition of the glomeromycotan community, causing a significant decrease in the relative abundance of a taxon in the Glomeraceae. Few studies focus on arbuscular mycorrhizal community responses to biofumigation, but in a strawberry pot culture study using a low resolution method based on TGGE, Koron et al. (2014) found that the numbers and intensities of bands representing mycorrhizal taxa were lower and there was a reduction in root colonisation following biofumigation with *Eruca sativa* in comparison with plants grown in untreated soil. However, Pellerin et al. (2007) showed that there was no effect on root colonisation of maize due to incorporation of *Brassica napus* residues. More recent studies by Wang and Mazzola (2019) using Illumina sequencing have shown reduced microbial diversity and reductions in the abundance of Glomeromycota in the rhizosphere of apple, including *Entrophospora* spp., *Glomus indicum*, *Glomus versiforme*, *Diversispora spuraca*, *Funneliformis mosseae*, *Rhizophagus intraradices* and *Rhizophagus irregularis,* in response to *Brassica* treatments. The results of the present study with strawberry confirm these parallel findings with apple, but additional studies, using other plant species and soils, are needed to determine whether responses of glomeromycotan fungi to biofumigation in other plant species are host genotype- or soil-dependent.

Interestingly, in roots, in the absence of biofumigation but presence of *V. dahliae*, there was a significant increase in relative enrichment of *Periconia macrospinosa* and *Microdochium bolleyi* ('dark septate endophytes'/DSE) and an unclassified taxon belonging to *Pleosporales* (presumably a root endophyte) compared to corresponding control treatment without *V. dahliae* (–B–V). This response of the root endophytes could have been a result of the presence of the pathogen, and this re-structuring of the root microbiome might enable the plant to tolerate deleterious effects of the pathogen. In recent years, growing evidence has accumulated to suggest that root endophytes (both bacterial and fungal, including DSE) may have a potential role in disease suppression (Rodriguez et al., 2009; Busby et al., 2016; Carrión et al., 2019; Dini-Andreote, 2020). However, our mechanistic understanding of this microbiome re-structuring remains poor and more detailed studies are needed to demonstrate whether, in the presence of a root pathogen, plants can selectively allocate more carbon to those endophytic taxa that are able to suppress or antagonize the pathogen. Stable isotope probing based on analysis of ^{13}C-DNA/RNA would be a valuable tool to reveal new insights in pathogen-host-root microbiome interactions.

In conclusion, this study provides new data suggesting that biofumigation of strawberry plants by incorporation of *Raphanus sativus* var. *oleifera* has significant effects on the composition of root fungal communities and may decrease the relative abundance of arbuscular mycorrhizal fungi and some potentially beneficial root endophytic taxa. Further investigations should reveal whether negative effects of biofumigation on strawberry yield and root fungal microbiome are host genotype- or soil-dependent.

Availability of data and material

The authors confirm that all data underlying the findings are fully available without restriction. The fungal pyrosequencing data files have been submitted to the European Nucleotide Archive (ENA) and are available under the study accession number PRJEB21579 at the following link https://www.ebi.ac.uk/ena/browser/view/PRJEB21579.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104116.

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