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ORIGINAL PAPER

Bacterial and fungal communities vary with the type of organic substrate: implications for biocontrol of soilless crops

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Abstract Biocontrol strategies using organic substrates such as wood fibers and biocontrol agents such as *Trichoderma* are currently developed to control soil pathogens such as *Fusarium oxysporum*. Nonetheless, such biocontrol methods give discording results, notably because microbial communities of organic substrates actually are not taken into account. Therefore, there is a lack of information concerning the variability of microbial composition related to the organic substrate type. Here we studied peat, wood and coir fibers, that are substrates known for their different biocontrol efficiency against Fusarium wilt of cucumber. We analyzed in microcosms the microbial composition of wood fibers, coir fibers and peat, incubated up to 60 days, by using an amplicon-sequencing approach based on 16S rRNA gene for bacteria and the internal transcribed spacer (ITS) for fungi. Diversity was assessed by sequencing the 16S rRNA for bacteria and ITS2 region for fungi. Results showed that bacterial richness was threefold higher for coir fiber and peat than for wood fiber. Fungal richness was three times higher for wood and coir fibers compared to peat. Bacterial and fungal patterns showed a dominance of α- and γ- *Proteobacteria* and *Sordariomycetes* for coir fiber; β- and γ-*Proteobacteria* and *Eurotiomycetes* for wood fibers; *Flavobacteria*, *Leotiomycetes* and *Sordariomycetes* for peat. In conclusion, results show that substrates have different microbial composition. Finally, for a proper use of a biocontrol strategy is important to take into account the type of substrate.

Keywords Biocontrol · Microbial communities · Bacterial 16S rRNA gene · Fungal internal transcribed spacer · Fusarium

Introduction

Producing healthy fruits and vegetables by reducing pesticides uses as well as the maintenance of high crop yields is central in agroecological practices. Biological strategies with integrative approach to protect crop against bio-aggressors were then suggested (Mercier and Manker 2005). Despite promising results with the use of biological control agents belonging to e.g., genus *Trichoderma*, *Pseudomonas* or *Bacillus* to fight against *Fusarium oxysporum* (Lecomte et al. 2016), the effectiveness of this strategy varies greatly from one production condition to another. Biocontrol indeed requires the optimization of (1) the inoculum density, (2) its formulation and (3) the method for the inoculation of the substrates used for crops. Because these strategies are based on the control much more than on the suppression of the pathogens, the producers must have acquired an in-depth knowledge of (1) the natural resistance of the considered fruits or vegetables and their susceptibility to the pathogens, according to the plant growth stage and culture conditions prevailing in greenhouse (Chaparro et al. 2012), (2) the favorable conditions so that the pathogen is able to attack

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plants, (3) the biocontrol agents potentially useful to control pathogens. Microorganisms used as biocontrol agents are added to the culture substrates—one of the application of bio augmentation—with the risk that they do not survive (Lebeau 2011). Another strategy relies on the selection of substrates used for crops (Koohakan et al. 2004). The protection against crop diseases varies according to the growing media (Bonanomi et al. 2010). In particularly, wood and coir fibers were recently tested with success for the protection of cucumber against *Fusarium oxysporum* (Montagne et al. 2016). The specific physicochemical characteristics of these substrates were already demonstrated (Domeño et al. 2011), but little is known regarding the microorganism that colonize these growing media. In view of the impact of the diet on the intestinal microbiota (Turnbaugh et al. 2009), it is interesting to study the indigenous microorganisms of these different organic substrates.

So far the microbial composition of organic substrates (wood fibers, coir fibers and peat) used for cucumber crop is unknown, our work aimed to study and compare their bacterial and fungal compositions. For this, we studied substrates, with different times of incubation, by using an amplicon-sequencing approach based on 16S rRNA gene for bacteria and the internal transcribed spacer (ITS) for fungi.

Experimental

Organic substrates

Three types of substrates were studied: wood fiber (PiF for pine and PoF for poplar), coir fiber (Co) and sphagnum peat (SpP). These substrates induce different plants protection against Fusarium (Montagne et al. 2016). Two distinct incubation processes (A and B) were performed, to test different batches of each substrate (Table 1).

A first incubation "A" was performed over a 3-month period with substrates PiF1s, PiF2d, PoFs, CoP, CoF and SpP. A second incubation "B" (over a 2-month period) was performed on new batches of the same substrates, and two additional substrates were studied: PiF2s and SpPn.

Precisely, pH, organic matter and dry bulk density were initially measured based on different standard methods, NF EN13037 (2000), NF EN13039 (2011), NF EN13041 (2000), respectively.

In details, the incubation protocol was adapted from the XP U44-163 standard (Montagne et al. 2015): 500 ml of substrate were placed at the bottom of a 2–l air-tight jar; 69.02 ml of a KNO_3 solution at 4.185 mg/ml were added to each jar to avoid nitrogen being a limiting factor for microbial development. A predetermined volume of distilled water was added to each substrate, and its hydric potential was adjusted to ≈ -30 kPa (pF 1.7). A beaker of water was placed in each jar, and then the jars were closed tightly and placed in an incubator at 28 °C for two or three months depending on the incubation A or B. To be close to horticultural conditions (producers use nutrient solution with pH near to 6–7), the pH of all substrates except SpP was set at 6 with a phosphate solution (0.1 M K_2HPO_4, 0.01 M KH_2PO_4).

All the substrates were sampled at 30, 60 and 90 days and at 10 and 60 days for the incubations A and B, respectively, and some of them were targeted to apply a community profiling approach (Table 1).

DNA extraction

In total, 0.250 g of dry matter were ground (MM301, Retsch, Germany) in a 25 ml stainless steel bowl with a 1.5 cm diameter stainless steel bead (2 × 1 min, 20 Hz). Then, DNA was extracted using a FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA). Extracted DNA was quantified with SPECTROstar Nano, (BMG LABTECH LVi Plate, Germany).

Amplicon library construction and sequencing

The V3-V4 region of the 16S rRNA gene and the ITS2 region of the fungal internal transcribed spacer were amplified with the primer sets 341f (5′TACCAGGGTATC TAATCCT-3′; Muyzer et al. 1993)/784r (5′-ACGGRAG GCAGCAG-3′; Gamalero et al. 2012) and ITS2_PlaGe (5′-GCTGCGTTCTTCATCGATGC-3′/IT5_PlaGe (5′-GGAA GTAAAAGTCGTAACAAGG-3′; White et al. 1990). PCR were conducted in a final reaction volume of 50 µl containing 0.6 ng/µl of DNA, 0.6 µM of primer 341f/784r or 0.2 µM of primer ITS2_PlaGe/IT5_PlaGe, 200 µM of dNTPs, 0.6 unit/µl of Taq polymerase (DyNAzyme EXT DNA Polymearse, Fisher Scientific), 1 × DyNAzyme EXT Buffer (Fisher Scientific), 2.5 mM of $MgCl_2$ and 500 ng/µl of bovine serum albumin. The following cycling conditions were employed for V3-V4 region: 1 cycle at 94 °C for 60 s, followed by 30 cycles of 94 °C for 60 s, 65 °C for 60 s, 72 °C for 60 s, and a final extension cycle at 72 °C for 10 min (CFX96, C1000 TouchTM, Thermal Cycler, Bio-Rad, United States). For ITS2 amplification, the conditions were: 1 cycle at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s, and a final extension cycle at 72 °C for 7 min. All amplicons were purified with Clean PCR beads (Mokascience) and quantified with a nanodrop (ND-8000). The second amplification was performed with primers containing the Illumina adapters and index using protocol described in Lluch et al. (2015). All amplicons were purified and

Table 1 Physicochemical characteristics of the substrates according to the manufacturer and detail of samples selected for sequencing from the two incubations

Substrate and type	Origin and process	C:N	Organic matter (g dw/kg)	Samples selected for sequencing	Incubation
PiF1s	Pine 1	612	996	PiF1s-60d-1-A*	A
–	–			PiF1s-60d-2-A	A
Wood fiber	Twin-screw grinding			PiF1s-10d-1-B	B
				PiF1s-10d-2-B	B
				PiF1s-60d-1-B	B
				PiF1s-60d-2-B	B
PiF2d	Pine 2	812	996	PiF2d-60d-1-B	B
–	–				
Wood fiber	Disk grinding				
PoFs	Poplar	567	990	PoFs-60d-1-B	B
–	–				
Wood fiber	Twin-screw grinding				
CoF	Coconut	130	962	CoF-60d-1-A	A
–	–			CoF-60d-2-A	A
Coir fiber	Fiber grinding			CoF-10d-1-B	B
				CoF-10d-2-B	B
				CoF-60d-1-B	B
				CoF-60d-2-B	B
SpP	Sphagnum	50	900	SpP-60d-1-A	A
–	–			SpPn-10d-1-B	B
Peat	Bog sampling			SpPn-10d-2-B	B
				SpPn-60d-1-B	B

* 10d and 60d mean 10 days and 60 days of incubation of substrates. -1- and -2- is the number of the replicate. Disk grinding leads to finer fibers than twin-screw grinding. SpPn corresponds to SpP but its pH is set at 7

quantified as previously described. The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by qPCR using the KAPA Library Quantification Kit. Amplicons libraries were mixed with 15% PhiX control according to Illumina's protocols. One sequencing run was performed at GeT-PlaGe sequencing facility with a MiSeq reagent kit v2 (500 cycles).

Clustering Miseq reads into operational taxonomic units (OTUs)

The sequencing data were analyzed with Mothur (Schloss et al. 2009) with standard operating procedures described in Kozich et al. (2013). Briefly, 16S rRNA gene sequences were aligned against the 16rRNA gene SILVA database. All sequences that did not align correctly were removed from the data sets. Chimeric sequences were detected with Uchime and removed. Taxonomic affiliation of 16S rRNA gene was performed with a Bayesian classifier against the 16S rRNA gene training set (v9) of the Ribosomal Database Project. Sequences were divided into groups according to their taxonomic units (OTUs) at a 97% identity threshold.

ITS read pairs were combined with Mothur, and the variable ITS2 regions of ITS sequences were extracted with the Perl-based software ITSx. Then sequences were clustered at a 97% identity cutoff using Uclust, and taxonomic affiliation was performed with a Bayesian classifier (80% bootstrap confidence score) against the UNITE database.

Microbial community analyses

In order to enhance the reproducibility of community profiles, only abundant OTUs representing at least 0.1% of the library size were used for microbial community analyses (Barret et al. 2015). Normalization between samples was performed through rarefaction of 8000 sequences per sample. Both α diversity indexes (Simpson inverse) and β diversity were calculated with Mothur (Schloss et al. 2009). Analysis of Similarity (ANOSIM) was performed to

assess the effects of different factors on the microbial community structure ($P < 0.05$). Beta diversity was assessed using Bray–Curtis dissimilarity index. Ordination of the similarity between microbial communities was performed with nonmetric multidimensional scaling (NMDS) plots.

Results and discussion

Diversity and composition of bacteria and fungi were compared between the three types of organic substrates. In detail, we compared richness (number of OUTs), Simpson diversity index, taxonomic composition (microbial classes) and microbial structure (with beta diversity and nonmetric multidimensional scaling).

Bacterial and fungal diversity in substrates and factors involved in the change of the microbial structure

After applying recommended filters, a median of 29,128 16S rRNA gene sequences and 53,390 ITS2 sequences were obtained per sample. According to Good's coverage estimator, the median coverage was 92% for 16S rRNA gene sequences and closed to saturation (99%) for ITS2 sequences (data not shown). Mean bacterial and fungal richness observed was 80 and 30 (number of OTU), for 16S rRNA gene and ITS2 sequences, respectively, after normalization of abundant OTUs. The distribution and the mean values of richness and diversity are shown for each type of substrate (Table 2) irrespective of the number and the time of incubation. For 16S rRNA gene, coir fibers show the highest number of OTU (124) than peat and wood fibers (108 and 38, respectively). Regarding ITS, wood fibers show the highest number (35), as compared to coir fibers and peat (30 and 10, respectively).

These differences are supported by Simpson diversity indices, bacterial diversity is higher for coir fiber and peat (with an index around 21) than wood fiber (with an index of 7.5) and fungal diversity is around 2.7 for wood and coir fibers and 2.4 for peat. Analysis of similarity of microbial composition (ANOSIM) highlights significant differences in the microbial community structure when you consider the three types of substrates ($P < 0.001$ for bacterial and fungal community structure). The communities for both incubations (A and B) are significantly different for bacteria ($P < 0.001$) but not for fungi ($P = 0.068$). Conversely, the microbial community structure was not impacted by the incubation time of 10 and 60 days ($P = 0.585$ and $P = 0.914$, for bacterial and fungal community structure, respectively). The structure of the microbial communities was stabilized in the early stage of incubation (from 10 days). The characteristics of the substrate (type of organic matter, physical structure resulting from the process, etc.) strongly act on the microbial composition. Thus, the manufacturing process has an important role in the defining of microbial structure. Microbial communities depend strongly of the type of substrate. This result can be extrapolated to the effects of diet on the microbiota, observed in the gut or the rumen (Turnbaugh et al. 2009; Russell and Rychlik 2001).

Comparison of taxonomic composition and microbial structure associated with the type of substrates

The taxonomic composition of each substrate was then investigated in more details, using bacterial and fungal classes as taxonomic unit (Fig. 1a, b). CoF is characterized by a high proportion of α- and *γ-Proteobacteria* and *Actinobacteria*, with 60% and 20%, respectively (Fig. 1a). Pine wood fibers bacterial communities (PiF1s and PiF2d) are mainly composed of α- *β*- and *γ-Proteobacteria* and *Actinobacteria*. The taxonomic composition of PoFs is distinct from PiF1s and PiF2d, with the high prevalence of *γ*-P*roteobacteria* (60 and 20% for PoFs and both PiFs, respectively). The presence of *Flavobacteria* reached 18% in PoFs while showed lower presence in both PiFs. Thus, Pine wood fibers are closer to coir fiber than to Poplar wood

Table 2 Results after read analysis and after Simpson diversity calculations for each type of substrate

Samples	16S rRNA gene sequences		ITS2 sequences	
	OTUs	Simpson index	OTUs	Simpson index
Wood fibers ($n = 8$)	38 ± 19	7.56 ± 7.20	35 ± 15	2.81 ± 1.37
Coir fibers ($n = 6$)	124 ± 24	21.01 ± 15.62	30 ± 12	2.72 ± 0.99
Peat (pH 6) ($n = 3$)	108 ± 47	21.36 ± 16.45	10 ± 2	2.38 ± 0.96

Microbial richness (number of Operational Taxonomic Units—OTUs) and diversity (Simpson inverse) were estimated with abundant Operational Taxonomic Units obtained with 16 rRNA gene and ITS sequences, after homogenization at 8000 reads (n for the number of sample)

Note the variation of the number of bacterial OTUs between the three types of substrate. Wood fibers differed with low bacterial diversity and high fungal diversity, in comparison with others types

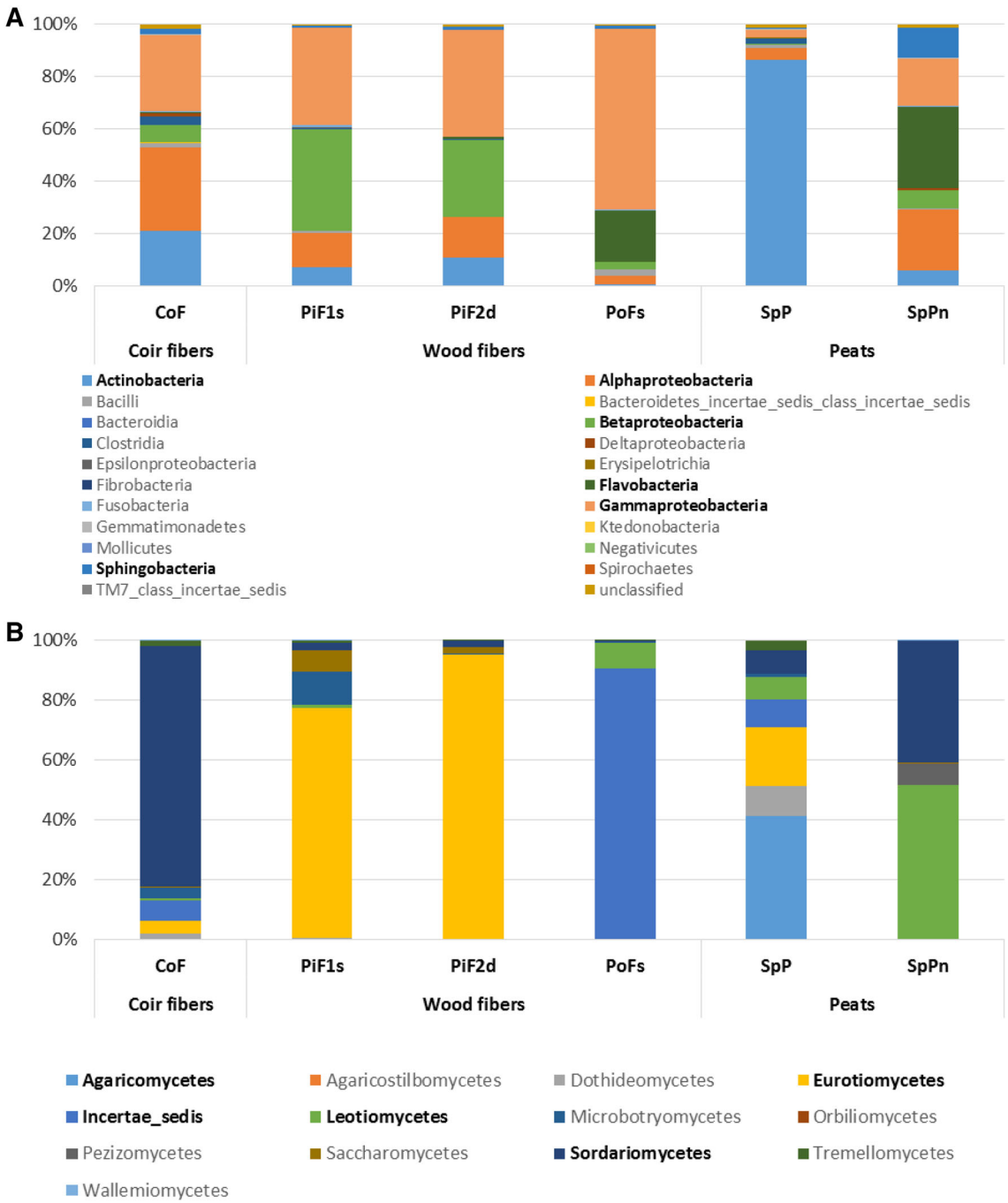

Fig. 1 Relative abundances of phylogenetic microbial classes in substrates, for 16S marker (**a**) and for ITS marker (**b**). Mean % of total reads. Unclassified was removed for fungal classes. Note that the representative classes were different between substrates. The type of substrate (coir, wood fibers and peat) and to a lesser extent the tree species (PiF and PoF) and pH (SpP and SpPn) impact representative classes. Note the similarity of the microbial compositions of two wood fibers, PiF1s and PiF2d, with *Proteobacteria* and *Eurotiomycetes* dominance

fiber (PoFs). PoFs composition is close to SpPn, with *Flavobacteria*, γ- and α-*Proteobacteria* as dominant classes. Finally, SpP bacterial community is mainly composed of *Actinobacteria*. Compared to bacteria, fungal classes (Fig. 1b) are more specific to the substrate. *Sordariomycetes* dominates reaching 80% presence in coir fiber, while *Eurotiomycetes* dominates in PiF1s and PiF2d (75 and 95%, respectively). PoFs is mainly characterized by two classes (*Incertae-sedis* and *Leotiomycete*, with 90 and 8%, respectively). SpPn is represented by *Leotiomycetes*, and by *Sordariomycetes* (50 and 40%, respectively). The class of *Agaricomycetes* is specific of SpP (represented

40%). Some substrates (e.g., SpP or SpPn) host several well-represented fungal classes, while wood fibers consist in one dominant class. The comparison with class level specifies the microbial particularities of each substrate, for example the influence of tree species (Pine or Poplar) or the pH (SpP or SpPn), and it is expected that the three types of substrate (CoF, PiF and SpP) allow the development of specific microorganisms.

To gain more insight into the influence of the type of substrate on the microbial community structure, we performed β-diversity analyses using Bray–Curtis dissimilarity index. Biological replicates for each condition (represented by -1 and -2) are very close (Fig. 2a). The incubation and the time of incubation seem to weakly influence the microbial structure. The bacterial community structure differs according to the type of substrate. The bacterial community structure in wood differs strongly from coir and peat libraries (Fig. 2a). The same differentiation is observed for the fungal communities (Fig. 2b), with differences according to the type of the substrate, and peat community structure was more different than wood or coir community structures.

According to the projection of orders making substrates repartition (Fig. 2a), orders of *Pseudomonadales* and *Burkholderiales* seem to specify wood fibers. These orders belong to γ- and β-*Proteobacteria*. Order of *Sphingobacteriales* is specific to peat (Fig. 1a). Regarding fungal repartition (Fig. 2b), *Eurotiales*, *Diaporthales* and *Pezizales* orders are specific to wood fibers, coir fibers and peat, respectively. These orders belong to the *Ascomycota* division and *Pezizomycotina* subdivision, but the class is different according to the substrate (*Eurotiomycetes*, *Sordariomycetes* and *Pezizomycetes* classes, respectively).

Bacterial phyla observed in our organic substrates (*Bacteroidetes*, *Actinobacteria*, *Proteobacteria, Acidobacteria* and *Firmicutes)* are frequently observed in soils (Fierer et al. 2007). β- and γ-*Proteobacteria* and *Actinobacteria* are stimulated in presence of fresh organic matter (Bernard et al. 2007). The order of *Burkholderiales* is specific to wood fiber substrates (Fig. 2a), and the class of *Actinobacteria* are more especially observed in pine wood soil, possibly linked to low pH and high C/N ratio (Kuramae et al. 2012). In wood fibers (data not shown), the genus *Pseudomonas* dominates. Numerous *Pseudomonas* sp. belonging to Plant Growth-Promoting Rhizobacteria (PGPR) are known to improve the plant nutrition (Laslo et al. 2012) and to activate the plant defenses system (Faessel et al. 2008). Moreover, all substrates of our study have high rates of cellulose and lignin (about 45 and 30%, respectively, Montagne et al. 2015). Coir fiber (CoF) contains more lignin than wood fibers (which contain more cellulose and hemi-cellulose). This involves a synergistic action of bacteria synthesizers of pectinase and cellulase and fungi decomposers of cellulose and lignin such as *Ascomycota* and *Basidiomycota* (Schellenberger et al. 2010 and de Boer et al. 2005), which dominate in our organic substrates. Moreover, *Actinobacteria* is the only bacterial phylum able to hydrolyze lignin, like fungi of the *Basidiomycota* phylum.

Some microorganisms could outcompete fungal opportunists (Bardin et al. 2004). As pointed out by Vivant et al. (2013), a high fungal diversity (and low bacterial diversity) for wood fibers may constitute a barrier against fungal pathogens. *Penicillium* genus of *Eurotiales* order is also present in wood fibers (data not shown). *Penicillium* genus was studied for its competitiveness against *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Sabuquillo et al. 2010). In a previous study, Montagne et al. (2016) observed a systematic decrease of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* attack on cucumber when cultivated on wood fiber substrate. Moreover, the protection obtained with coir fibers was lower and dependent of climatic conditions and the level of plant's stress. The dominance of *Eurotiomycetes* (in particular the *Eurotiales* order) and with β- and γ-*Proteobacteria* (in particular the *Burkholderiales* and *Pseudomonadales* orders) may explain the wood fibers protection. On the contrary, the variable level of protection obtained with coir fibers could be explained by the variations in the bacterial community structures.

Conclusion

Actinobacteria, *Proteobacteria*, *Bacteroidetes*, *Ascomycota* and *Basidiomycota* dominate in substrates. Microbial composition depends on the type of substrates (wood fibers, coir fibers and peat). Wood fibers show microbial particularities with a low bacterial diversity, high fungal diversity and dominance of *Eurotiomycetes* (85%) and *Proteobacteria* (90%) classes. As the food consumption is defining intestinal microbiota, the choice of growing media for soilless crop is decisive determining the microorganisms present in the substrate. The presence of specific microorganisms when using wood fibers can be a reason explaining the substrate-induced crop's protection. Here, we showed that better understanding the microbial properties of organic substrates allows to achieve major advances in the fields of plant health and biological control. Further studies manipulating microbial communities in such substrates are needed to elucidate the relationship

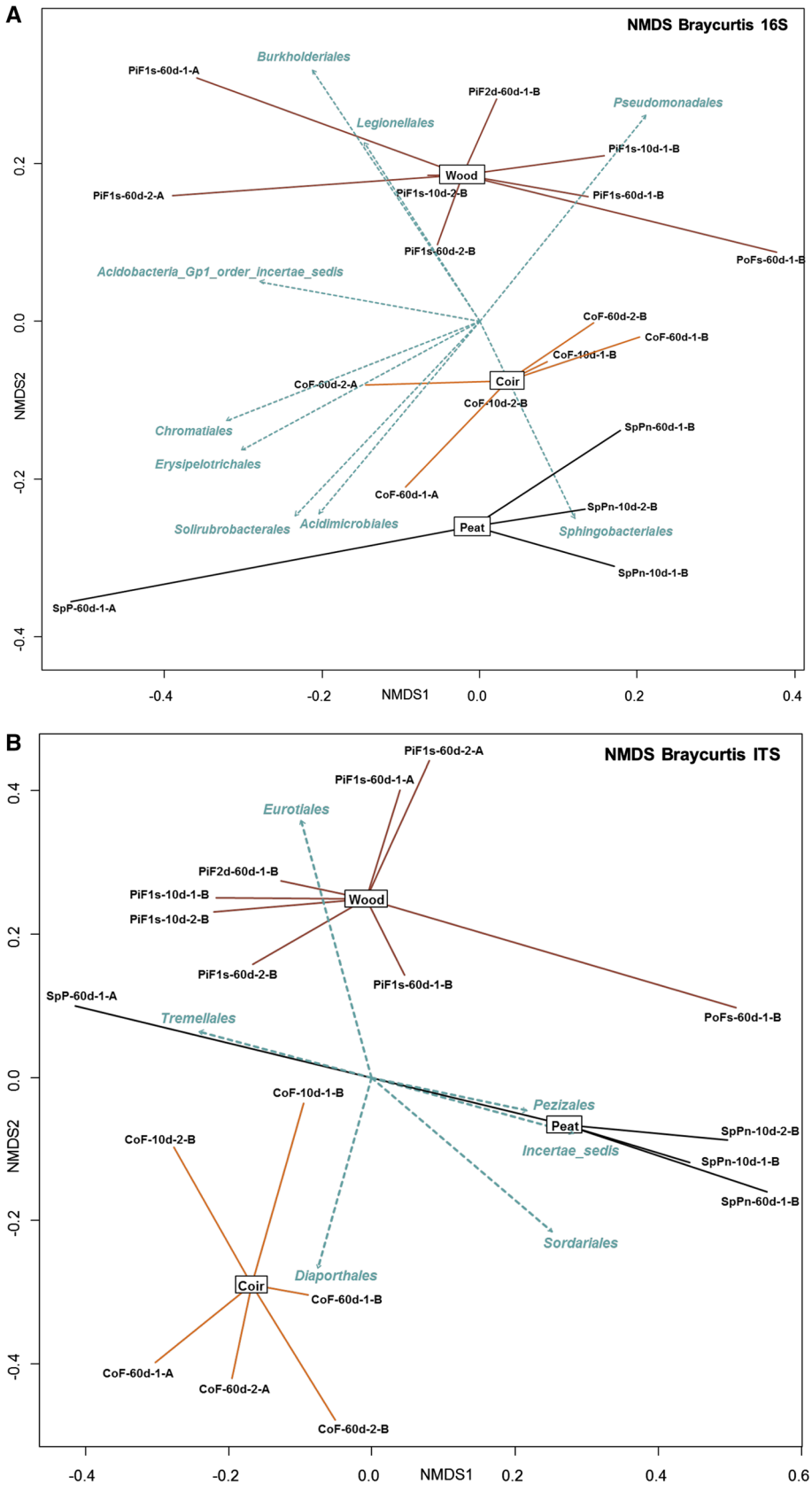

Fig. 2 Influence of substrates on the bacterial (**a**) or fungal (**b**) β-diversity by using Nonmetric MultiDimensional Scaling (NMDS) ordination of Bray–Curtis dissimilarity matrix obtained with 16S or ITS Operational Taxonomic Units (OTUs), respectively. Each dot represents a microbial library observed in one sample. Mothur software and R software were used—stress value is 0.1519532 for bacteria and 0.1518726 for fungi. Projection on the graph of the main bacterial (**a**) or fungal (**b**) orders explains the substrate distribution on the β-diversity Nonmetric MultiDimensional Scaling. Vectors in the bi-plot overlay were constructed from a matrix containing the relative abundances of each bacterial or fungal order. Only correlations ≤ 0.05 were included. The angle and length of the vector indicate the direction and strength of the variable. Note that bacterial and fungal libraries differ according to the type of substrate (Wood fiber, coir fiber and peat). With the projection of orders involved in this representation, we can explain this substrate-dependency with *Pseudomonadales*, *Burkholderiales* and *Eurotiales* specificity of wood fibers, for example

between different bacterial and fungal groups and their potential biocontrol.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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