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Dynamics and resilience of soil mycobiome under multiple organic and inorganic pulse disturbances

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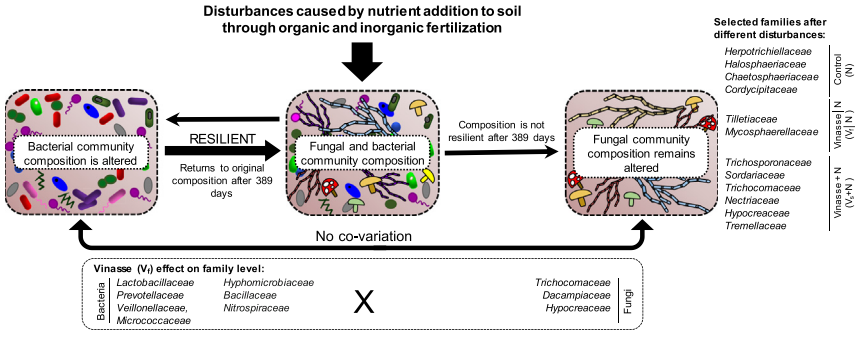
HIGHLIGHTS

- Fungal community responded differently to the organic and inorganic amendments.
- Disturbances change soil fungal community without cyclical variation.
- Vinasse increased fungi with copiotrophic and oligotrophic lifestyles.
- The changes in the fungal and bacterial communities were not correlated.
- Soil fungal community was neither resistant nor resilient to disturbances.

GRAPHICAL ABSTRACT



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ABSTRACT

Disturbances in soil can cause short-term soil changes, consequently changes in microbial community what may result in long-lasting ecological effects. Here, we evaluate how multiple pulse disturbances effect the dynamics and resilience of fungal community, and the co-occurrence of fungal and bacterial communities in a 389 days field experiment. We used soil under sugarcane cultivation as soil ecosystem model, and organic residue (vinasse - by-product of sugarcane ethanol production) combined or not with inorganic (organic residue applied 30 days before or together with mineral N fertilizer) amendments as disturbances. Application of organic residue alone as a single disturbance or 30 days prior to a second disturbance with mineral N resulted in similar changes in the fungal community. The simultaneous application of organic and mineral N as a single pulse disturbance had the greatest impact on the fungal community. Organic amendment increased the abundance of saprotrophs, fungal species capable of denitrification, and fungi described to have copiotrophic and oligotrophic lifestyles. Furthermore, the changes in the fungal community were not correlated with the changes in the bacterial community. The fungal community was neither resistant nor resilient to organic and inorganic disturbances over the one-year sampling period. Our findings provide insights on the immediate and delayed responses of the fungal community over one year to disturbance by organic and inorganic amendments.

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1. Introduction

Microorganisms play a key role in the functioning of soil ecosystems, including soil nutrient cycling and plant growth (Fuhrman, 2009; Graham et al., 2016). However, identifying the drivers of microbial community composition in soil is challenging due to the multitude of processes and shifts in environmental conditions. Disturbances caused by nutrient addition to soil through fertilization cause short-term soil changes and may result in long-lasting ecological effects that create temporal and spatial heterogeneity in soil ecosystem performance (Bissett et al., 2013). Soil microbial communities are sensitive to these effects of fertilization, and their responses to organic and/or mineral fertilizers in soils have been studied extensively in recent years (Cassman et al., 2016; Fu et al., 2017; Hartmann et al., 2015; Lazcano et al., 2013; Lupatini et al., 2019; Pan et al., 2014). Organic amendment can affect microbial diversity and shift the relative abundances of copiotrophic and oligotrophic bacteria in the community (Leite et al., 2017; Lourenço et al., 2018b; Lupatini et al., 2017; Suleiman et al., 2018; Trivedi et al., 2013; Trivedi et al., 2015) and soil microbiome may be resilient, resistant or sensitive (its change the composition) to nutrients addition (Allison and Martiny, 2008; Griffiths and Philippot, 2013; Shade et al., 2012). However, most studies have focused on the responses of bacteria rather than fungi to different fertilization strategies.

Although often grouped together under the broad term 'microbial community', fungal and bacterial taxa may not respond similarly to organic and inorganic fertilizer addition in soil because of their physiological and ecological differences. Fungi play key roles in the soil by acting as decomposers, pathogens and mutualists (Gao et al., 2015; Kuramae et al., 2013a,b). Saprotrophic fungi are key regulators of nutrient recycling and the central agents in the decomposition of organic matter (Boddy et al., 2008), but this group also includes a number of fast-growing molds and yeasts with limited abilities to break down and utilize complex organic substrates (Frankland, 1998; Hudson, 1968). Plant symbiont mycorrhizal fungi acquire carbohydrates from their hosts and in return facilitate soil nutrient uptake by plants (Smith and Read, 2008). Ectomycorrhizal fungi (ECM) harbor sets of genes for carbon degradation and have partial decomposition capabilities as well (Kohler et al., 2015) while arbuscular mycorrhizal fungi (AMF) influence degradation of organic matter, acquire and transfer a portion of released nutrients to their associated host plants (Bunn et al., 2019). Fungi may also be present as plant pathogens living in soil (soil-borne) or in organic debris. In addition to affecting the rate of decomposition, nutrient cycling and plant health, soil fungal communities affect the resiliency of ecosystem functioning (Gessner et al., 2010; Valentín et al., 2014). So, due to the different niches of the members of the fungal community, the response after disturbances can be variable. For instance, terrestrial litter fungi display a wide range of adaptations to degrading specific chemical compounds, which can reflect in successional patterns of the community members (Gessner et al., 2010). On the opposite, Valentín et al. (2014) showed that the fungal community inhabiting the advanced stages of wood decay substrate have slight impact on decomposition rate suggesting a stronger resilience of the diverse fungal community.

Soil fungal communities are susceptible to perturbations caused by nutrient amendment (Cassman et al., 2016; Hartmann et al., 2015), which decreases fungal biomass and diversity and alters fungal community composition (Edwards et al., 2011; Paungfoo-Lonhienne et al., 2015; Wallenstein et al., 2006). The addition of mineral fertilizer and the combination of organic and mineral fertilization reduce the proportion of dominant saprotrophs by 40% that consequently affect cellulose decomposition and decrease the bacterial and fungal interactions (Wang et al., 2017). For mycorrhizal fungi, the biological activity, abundance and diversity are higher in soils treated with organic fertilizer than in soils treated exclusively with mineral fertilizer (Maeder et al., 2002; Song et al., 2015; Verbruggen et al., 2010). Song et al. (2015) have shown that AMF propagules are down-regulated by nutrient-rich fertilization but induced by N, P or K-deficiency. These alterations of the soil-borne fungal community may be linked to soil nutrient fluctuations and carbon inputs from plants or residues (Allison et al., 2007; Song et al., 2015). The increased nutrient loads may also select for reduced mutualist effectiveness, as demonstrated for mycorrhizal fungi (Kiers et al., 2010; Lau et al., 2012).

Although fungi and bacteria co-inhabit in soil environment, we expect that the effects of organic fertilization on the function of these communities will differ given the many differences in phenotype, phylogeny, and life history. Furthermore, compared to bacteria, we presume a longer interval of recovery of the fungal composition from disturbance. For example, soil fungal growth rates appear to be 10-fold slower, and fungi tend to be mediators of slower carbon cycling than soil bacterial community (Rinnan and Bååth, 2009). The rapid responses of bacteria to alterations of environmental conditions and their high turnover rates might provide an early indication of return to the original state after disturbance making them less stable overall, as they are more sensitive to disturbance (Suleiman et al., 2016). The patterns and mechanisms of soil microbiota recovery after disturbance are less studied for fungi, and it has been suggested that the trajectories and dynamics of fungi during recovery are distinct from those of bacteria (Ho et al., 2017). The response of the microbial community to multiple disturbances can reshuffle important soil processes and generate alternative microbial states that provide opportunities to disentangle fungal complexities and dynamics.

The main purpose of this study was to determine the dynamics of the fungal community over 389 days under multiple pulse disturbances due to the addition of organic fertilizer with or without inorganic fertilizer in a tropical soil cultivated with sugarcane (Fig. 1). Furthermore, there is a lack of studies tracking the changes in the fungal community after fertilization disturbances through a year of sugarcane plantation. In this sense, we make use of the organic fertilizer vinasse, in our case a sugarcane residue produced during ethanol production. This residue has high organic and salt contents and its large production volume mainly from sugarcane plantation, represents a great challenge to environment due to negative impacts in the water bodies, soil and atmosphere (greenhouse gases emission) (Hoarau et al., 2018; Lourenço et al., 2019). Hence, understanding the impact of this residue in the soil mycobiome is vital. Therefore, the objectives were to (1) determine the patterns of fungal community composition and diversity in response to single and consecutive pulse disturbances with the liquid organic fertilizer; (2) determine the key fungal families that adapted in the soil over time; (3) determine the fungal community interactions and co-occurrence with bacteria; and (4) unravel how various above- and belowground abiotic factors influenced the observed patterns of fungal community. We hypothesized that soil fungal community turnover would be higher in the first days after organic addition and gradually decrease toward stability due to nutrient depletion over the one-year experiment.

2. Materials and methods

2.1. Experimental design and soil collection

The field experiment was set up in an area planted with sugarcane variety RB86-7515 located at Paulista Agency for Agribusiness Technology (APTA), Piracicaba, Brazil. In detail, three replicate blocks and a total of 12 plots (4 treatments × 3 blocks) with sugarcane corresponding to the fourth ratoon cycle were established. In each plot, sugarcane was planted in four 8-m-long rows with a spacing of 1.5 m between rows. A randomized complete block design was adopted. The treatments in this experiment included the application of organic residue vinasse 30 days before (day 0) or at the same time as mineral nitrogen fertilization (Fig. 1). Accordingly, the experiment was established with the following treatments: (V_f) vinasse applied at day 0 (without N); (N) inorganic fertilizer in ammonium nitrate form, applied at day 30;

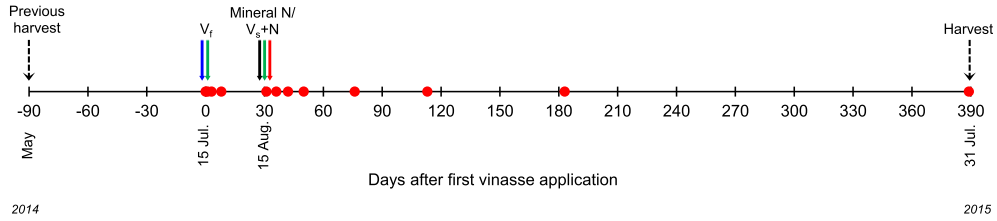


Fig. 1. Time of application of mineral fertilizer N (ammonium nitrate) and vinasse to sugarcane and sugarcane harvest time. The treatments were as follows: V_f, vinasse applied at day 0; N, inorganic fertilizer ammonium nitrate applied at day 30; $V_f|N$, vinasse applied at day 0 and ammonium nitrate applied at day 30; and $V_s + N$, vinasse plus ammonium nitrate applied only at day 30. The red points represent the different sampling time points, the dashed arrows represent the period of sugarcane harvest, and the colors of the bold arrows represent the different treatments: N, black; Vf, blue; Vf|N, green; and Vs + N, red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

($V_f|N$) vinasse applied at day 0 and ammonium nitrate applied at day 30; and ($V_s + N$) vinasse plus ammonium nitrate applied only at day 30. The treatments were chosen based on prior results for sugarcane management practices (Pitombo et al., 2016) (the experimental timeline is described in detail in Lourenço et al. (2018b)). In plots with mineral nitrogen, ammonium nitrate was applied at a rate of 100 kg ha^{-1}, and in the vinasse treatments, a volume of 100 m^3 ha^{-1} (V_f and V_s) was broadcast over the whole plot using a sprayer based on ranges of recommended rates in sugarcane plantations. The N fertilizer was band-applied on the soil surface 10 cm from the sugarcane plants. The treatments with vinasse received higher N input, as vinasse residue is a source of mineral and organic N. The chemical data of the vinasses used are presented in Table 1. As a large volume of vinasse is necessary for this type of study, it was not possible to store vinasse for use on both application dates; therefore, vinasses with slightly different compositions from the same sugarcane mill were used. In all treatments, straw (16 Mg ha^{-1}) from the previous sugarcane harvest was left on the soil.

The soil is classified as an Oxisol soil (soil taxonomy) (USDA, 2014). The physicochemical properties of the soil are presented in Table 2. Soil sampling started on 16 July 2014 by collecting samples at six positions per plot (two samples from the three central sugarcane rows of each plot) from the top 10-cm layer of each of the treatment replicates at nine different time points: 1, 31, 36, 42, 50, 76, 113, 183, and 389 days after the first vinasse (V_f) application. One section of this study focused on the effect of single vinasse on the soil microbial community. For this purpose, two extra days (3 and 8) were added to the sampling days listed above. Furthermore, to test microbial stability, i.e., the resistance and resilience of the microbial community (fungal and bacterial) after the first vinasse application, soil samples without vinasse or mineral N were collected. These samples were collected at the plots from the N treatment on day 1 (the mineral N fertilizer was applied at day 30), for the purpose of analyses, the samples were named as day 0. One portion of the collected soil was stored at −80 °C for DNA extraction, and the remaining soil was stored at −20 °C until analysis of soil chemical properties such as pH, soil moisture and NO_3^--N and NH_4^+-N concentrations. Soil moisture content was measured gravimetrically using 10 g of field-moist soil sample in an oven at 105 °C for 24 h. The pH of the soil sample was measured in a 1:2.5 soil/water suspension. Soil inorganic N was extracted with 1 M KCl, and the filtrates were analyzed for NO_3^--N and NH_4^+-N by a colorimetric method using a continuous flow analytical system (FIAlab-2500 System).

2.2. DNA extraction

Total community DNA was extracted from 0.25 g of each soil sample in triplicate using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's instructions. All DNA samples were stored at −20 °C until use in downstream analyses. DNA concentration and quality were determined spectrophotometrically (NanoDrop 1000, Thermo Scientific, Waltham, MA, USA), fluorometrically (Qubit 2.0 Fluorometer, Life Technologies, Carlsbad, CA, USA), and by agarose gel electrophoresis.

2.3. Illumina sequencing of fungal intergenic markers

PCR amplification of fungal ribosomal internal transcribed spacers (region ITS2) was performed using ITS9F (Ihrmark et al., 2012) and ITS4R (White et al., 1990) primers (5′-GAACGCAGCRAAIIGYGA-3′ and 5′-TCCTCCGCTTATTGATATGC-3′) with barcodes, respectively. To do this, 8-base barcodes were added to the 5-end of the reverse primers using the self-correcting barcode method of Hamady et al. (2008). The reaction was conducted in 25 μl containing 15.6 μl of H_2O, 2.5 μl of 10× PCR-buffer + magnesium, 2.5 μl of dNTPs (2 mM), 0.15 μl of fast startExp-Polymerase (5 U/μL), 1 μl of $MgCl_2$ (25 mM), 1.25 μl of BSA, 0.5 μl of each primer (forward and reverse) and 1 μl of DNA template in a thermocycler (Bio-Rad, CA, USA) with the following conditions: initial denaturation for 5 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 60 s at 54 °C and 90 s at 72 °C and a final extension for 10 min at 72 °C. Each sample was purified with Agencourt AMPure XP beads (Beckman Colter, Berea, CA, USA), and the library quality was assessed on a Qubit 2.0 Fluorometer (Thermo Scientific) and an Agilent Fragment Analyzer system. The samples were pooled based on the concentration of 20 ng/ul of DNA for each sample. Finally, the library was sequenced on an Illumina MiSeq using the V3 chemistry platform. All MiSeq data were uploaded to the ENA and are publicly accessible under project number PRJEB30929.

Table 1
Chemical characteristics of the different vinasse batches used for the first (V_f) and second (V_s) vinasse applications to the soil.

Vinasse[a]	Application time	pH	C org[b]	N tot[c]	NH_4^+-N[d]	NO_3^-N[e]	P	K	C/N
			g L^{-1}	g L^{-1}	mg L^{-1}	mg L^{-1}	g kg^{-1}	g kg^{-1}	
V_f	Jul. 15, 2014	4.8	28.8	0.51	45.7	8.8	0.11	3.5	57/1
V_s	Aug. 15, 2014	3.9	31.4	0.89	41.6	4.1	0.23	4.7	35/1

Abbreviations are as follows:
[a] V_f: Vinasse applied at day zero (15 July 2014) and V_s: Vinasse applied at day 30 (Aug. 15, 2014).
[b] C org: Total organic carbon.
[c] N tot: Total organic nitrogen.
[d] NH_4^+-N: Ammonium.
[e] NO_3^--N: Nitrate.

Table 2
Physicochemical properties of soil (0 to 20 cm) (mean ± standard deviation).

pH[a]	OM[b]	P[c]	K	Ca	Mg	H + Al[d]	CEC[e]	Soil texture[f]		
								Clay	Silt	Sand
	g dm^{-3}	mg dm^{-3}	$mmol_c dm^{-3}$					g kg^{-1}		
5.0 ± 0.1	21.1 ± 1.3	14.6 ± 1.1	0.7 ± 0.1	17.4 ± 3.2	11.9 ± 2.7	34.9 ± 3.0	65.1 ± 5.5	631 ± 11	151 ± 8	218 ± 2

Abbreviations are as follows:
[a] ($CaCl_2$; 0.0125 mol L^{-1}).
[b] Organic matter.
[c] Available phosphorus, K, Ca, and Mg were extracted with ion exchange resin.
[d] Buffer solution (pH 7.0).
[e] CEC (Cation exchange capacity).
[f] Soil texture determined by the densimeter method.

2.4. Illumina sequencing of bacterial markers

In this study, a subset of the samples from Lourenço et al. (2018b) was used to evaluated the impact of solely organic vinasse in the bacterial community and its interaction with fungal community. Single DNA template from different samples was used for bacterial and fungal community analysis. PCR amplification and sequencing of the 16S rRNA were performed from the soil DNA using 515F and 806R primers (Caporaso et al., 2011), targeting the variable V4 regions (5′-GTGCCA GCMGCCGCGGTAA-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′) resulted in amplicons of ~300–350 bp. The bacterial community amplification and data processing are described in detail in Lourenço et al. (2018b).

2.5. Data processing and analysis

Sequenced paired-end reads were joined using VSEARCH (Rognes et al., 2016) and subjected to quality and length filtering, adapter sequence trimming and PhiX contaminant removal with BBDuk2 from the BBMap tool suite (Bushnell, 2016). Chimeras were identified and removed in the de novo mode of the UCHIME algorithm (Edgar et al., 2011). Singletons were removed, and the resulting sequences were clustered into operational taxonomic units (OTUs) with the usearch_global method implemented in VSEARCH at 97% sequence identity (Rognes et al., 2016). The chimera removal processes were then performed using de novo mode in UCHIME (Edgar et al., 2011). Representative sequences for each OTU were taxonomically assigned by RDP Classifier with a bootstrap threshold of 0.8 using the Unite database 7.2 as a reference (Kõljalg et al., 2013).

Rarefaction curves from non-rarefied data using the sequence sample size and number of different OTUs was used to indicate that the measurement has met the requirements depth (Fig. A.1). Rarefaction curve were calculated in RStudio version 1.0.136 running R version 3.3.1. using the phyloseq (McMurdie and Holmes, 2013) and ranacapa (Kandlikar et al., 2018) packages. Estimates of alpha diversity were calculated in QIIME 1.8 (Caporaso et al., 2012). These estimates included the observed Chao1, Simpson and Shannon diversity indices (Chao, 1984) As alpha diversity measures are sensitive to differences in sampling effort, estimates were calculated based on rarefied data sets that were randomly subsampled to 2847, 1584, and 127 sequences for the different comparisons in this study: (I) the effects of vinasse on the soil fungal community, (II) the differences between treatments, and (III) the microbiomes in the vinasse treatments, respectively.

Multivariate dispersion analysis was performed to test the differences in variances among the treatments using the PERMDISP2 procedure in PRIMER v7 software. Permutational Multivariate Analysis of Variance (PERMANOVA) using the Bray-Curtis distance method from "adonis" command and analysis of similarity (ANOSIM) using the "anosim" commands in the vegan package at 999 permutations and $\alpha = 0.05$ were performed to test factors including treatment, time (days during experiment), and their interactions. We generated discriminant analysis of principal components (DAPC) plots to visualize and determine the effect of vinasse on fungal and bacterial community composition using the function 'adegenet' in the R library (Jombart et al., 2010). We also performed multivariate regression tree (MTR) analyses based on Bray–Curtis dissimilarities of the fungal community using the function "mvpart" to explore fungal dynamics over time for each treatment (De'ath, 2002). For the analysis, the data were log-transformed, and the resulted tree was plotted after 500 cross-validations (Breiman et al., 1984) to prevent overfitting. Next, the PCoA of the MTR was plotted with the function "rpart.pca" from the "mvpart" package (De'ath, 2007; Therneau and Atkinson, 1997).

To identify fungal families significantly associated with the treatments at each time point, we used linear discriminant analysis effect size (LEfSe, LDA Effect Size) implemented in the web-based tool Microbiome Analyst (Dhariwal et al., 2017). The LEfSe results included three LDA value distributions that estimated the effect size of each feature and a biomarker abundance comparison chart for different families. A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study. To display co-trends between the fungal and bacterial communities, data were analyzed using co-inertia analysis computed with the 'ade4' package in R (Dray and Dufour, 2007). Co-inertia is a dimensional reduction procedure designed to measure the similarity of two sets of measurements associated with a single set of cases. Relationships of belowground variables such as fungal or bacterial data with physiochemical variables and aboveground data such as greenhouse gas fluxes were determined by redundancy analysis (RDA) with the Hellinger transform OTU table using CANOCO 5 (Biometris, Wageningen, The Netherlands).

3. Results

3.1. Soil fungal community composition and diversity in response to single and consecutive pulse disturbances

Of a total of 126 samples, 124 samples were annotated and recovered from each of the eleven sampling time points, with three replicates per time point. After quality filtering, a total of 912,961 high-quality ITS sequences with an average depth of 7363 reads per sample clustering into 980 OTUs remained for community analysis. The alpha diversity of the fungal communities measured by the Chao1, Shannon and Simpson indices were significantly ($p = 0.00$) lower in the $V_s + N$ treatment, compared to all other treatments (average of all timepoints) (Table 3). From day 36 to the end of the experiment (389 days), the fungal diversity was lowest in the treatment with vinasse simultaneous with mineral N ($V_s + N$).

To assess the effects of the vinasses amendment and time on fungal community structure, taxonomic profiles were compared at different time points with a dissimilarity test. PERMANOVA analysis revealed that treatment (Pseudo-F value = 3.97; P = 0.00), time (days) (Pseudo-F value = 2.57; P = 0.00) and an interaction between treatment and time point had a significant effect on the fungal community

Table 3
Soil fungal microbial alpha-diversity measured in nine time points. The treatments are: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate, applied at day 30; $V_f|N$: vinasse applied at day 0 and ammonium nitrate applied at day 30; and $V_s + N$: vinasse plus ammonium nitrate applied only at day 30.

ANOVA test[a]					Chao1			Simpson		Shannon
Treatment					***			***		***
Day					ns			ns		ns
Treatment × day					ns			ns		***
Tukey's test[b]	Days after vinasse application									Average
	1	31	36	42	50	76	113	183	389	
Chao1										
N	107.5	92.4	99.6	104.8	106.0	112.7	100.5	96.2	116.3	104.0 b
V_f	86.8	100.0	100.0	102.8	118.5	107.1	97.6	97.5	108.9	101.2 ab
$V_f\|N$	103.5	120.3	103.1	114.8	109.0	97.9	100.1	98.7	111.2	106.1 b
$V_s + N$	104.7	106.5	93.5	84.8	92.0	85.4	86.0	85.8	87.8	91.8 a
Simpson										
N	0.92	0.91	0.92	0.92	0.92	0.91	0.92	0.92	0.93	0.92 b
V_f	0.92	0.91	0.91	0.91	0.91	0.92	0.91	0.92	0.91	0.91 b
$V_f\|N$	0.91	0.91	0.91	0.93	0.93	0.92	0.90	0.91	0.92	0.91 b
$V_s + N$	0.88	0.93	0.80	0.82	0.85	0.89	0.84	0.87	0.84	0.86 a
Shannon										
N	4.59 a	4.43 a	4.52 b	4.60 b	4.50 ab	4.43 a	4.53 b	4.57 b	4.67 b	4.54 b
V_f	4.58 a	4.49 a	4.53 b	4.53 b	4.45 ab	4.51 a	4.40 b	4.61 b	4.50 b	4.51 b
$V_f\|N$	4.51 a	4.50 a	4.43 b	4.65 b	4.69 b	4.57 a	4.29 ab	4.45 ab	4.51 b	4.51 b
$V_s + N$	4.25 a	4.68 a	3.36 a	3.55 a	3.92 a	4.12 a	3.79 a	4.00 a	3.61 a	3.92 a

[a] Symbols in the caption refer to overall ANOVA results for the given experiment. Significant difference: * $p \le 0.05$; ** $p \le 0.01$ and ns: Non-Significant.
[b] Means followed by the same capital letter in the column at each treatment and lowercase letter at each day of sampling do not differ significantly by the Tukey's test ($p < 0.05$).

structure (Pseudo-F value = 1.44; P = 0.00, Table A.1). Discriminant analysis of principal components (DAPC) at a family taxonomic level revealed that the fungal community structure differed among the treatments (Fig. 2) and changed during the experiment. However, for the mineral N treatment the different days clustered closely, so the distribution among different days was small (Fig. 2) compared with the treatments with vinasse. The fungal communities in the different treatments were similar on day 1. However, after 183 days, the fungal communities in the mineral N, V_f and $V_f|N$ treatments exhibited similar patterns, whereas the fungal community of the $V_s + N$ treatment showed a very distinct pattern (Fig. 2).

Considering that the factors treatment and time (days) had a significant effect on the fungal community structure (Table A.1), further analyses were performed using time as a factor. We used a multivariate regression tree (MRT) approach to follow the fungal community dynamics in each treatment. The PCoA ordination given by MRT analysis and DAPC showed that the fungal community dynamics was not cyclical (Figs. 2 and 3) and differed among the treatments. However, the treatment with vinasse (V_f) seems to have cyclical changes in the composition, as day 389 is converging on day 1, but it is still in a different branch of the regression tree. Mineral N application alone caused small changes in fungal community structure (Fig. 2 and 3a), and the treatments with vinasse (V_f, $V_f|N$, $V_s + N$) had higher sample dispersion among days than the mineral N treatment. In the $V_s + N$ treatment, the fungal community structure changed at day 36 and remained different from those of the other treatments until the end of the experiment

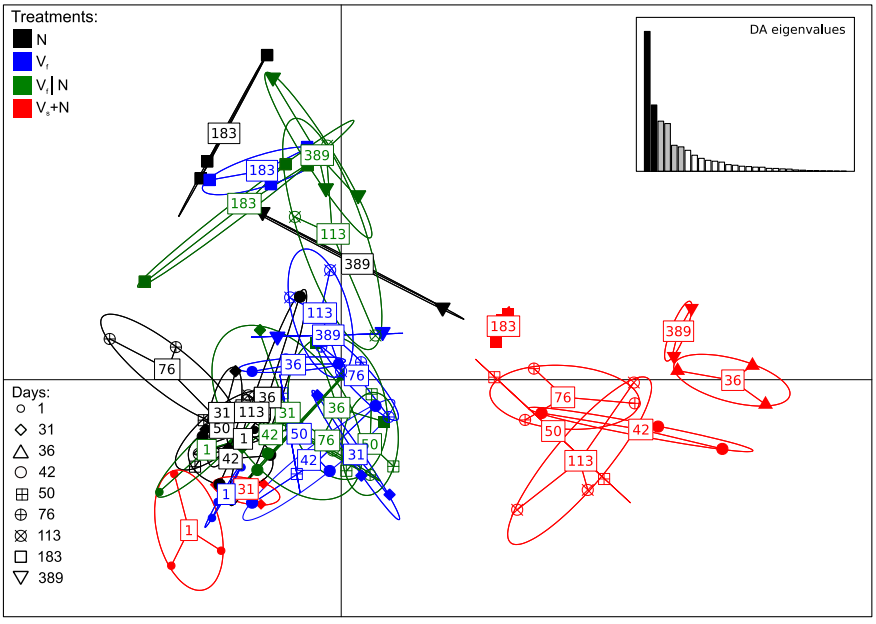


Fig. 2. Discriminant analysis of principal components (DAPC) plot of the effect of vinasse and mineral N on soil microbial taxonomy (Hellinger transformation, rarefied OTU table, family level).

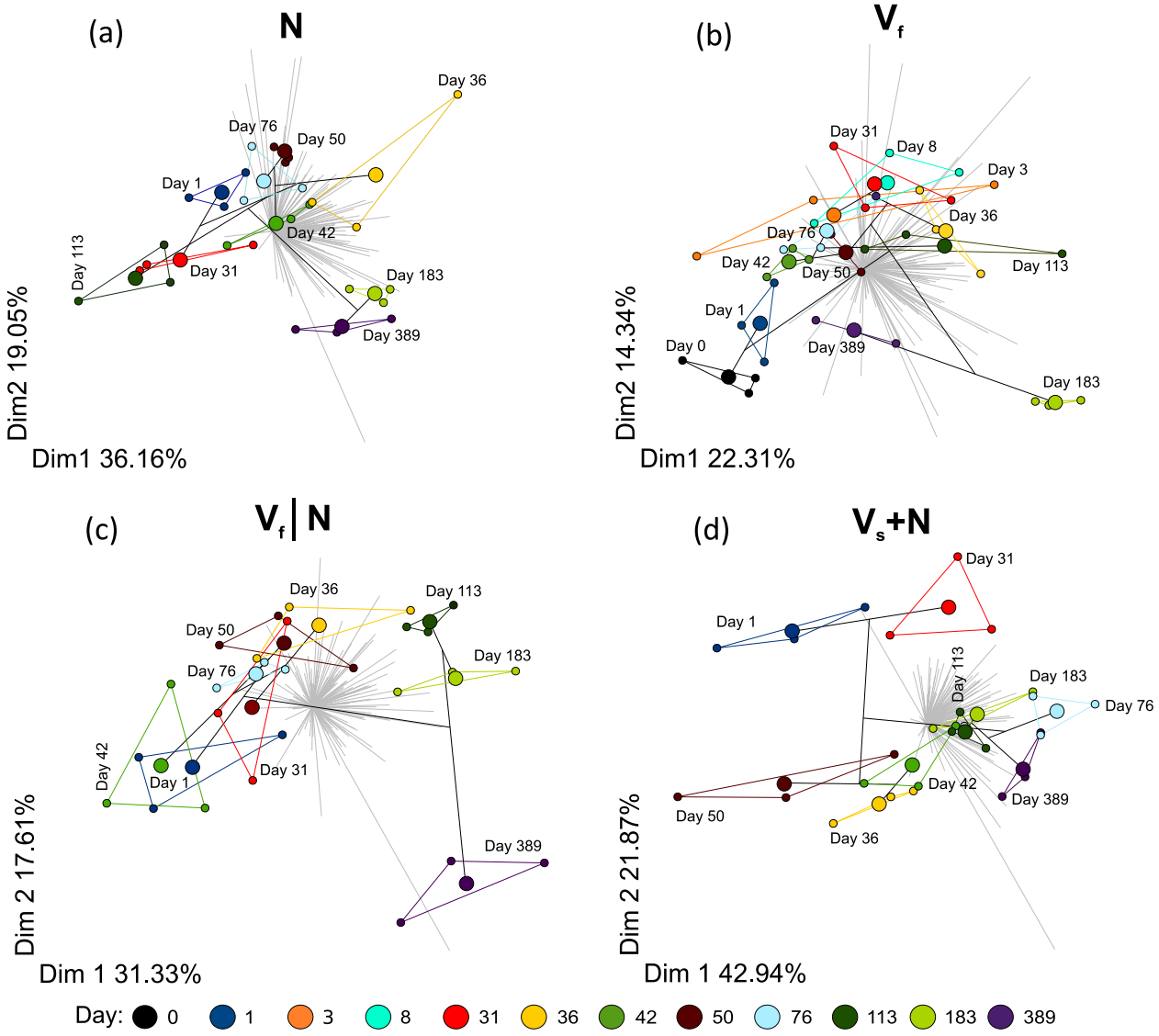


Fig. 3. Cyclical community composition dynamics after vinasse and N fertilizer application. Multivariate regression tree (MRT) analysis was used to estimate the impact of time on the fungal community structure independently for each treatment. Nine (A, C, D) and twelve (B) different leaves (large colored circles) were defined based on fungal abundance and composition. The community composition within leaves is represented in a principal coordinate analysis (PCoA) plot, where small points represent individual samples and large points represent the group mean (within the leaf). The gray barplot in the background indicates families whose differential abundance explains the variation in the PCoA plot. The treatments were as follows: (a) N, inorganic fertilizer ammonium nitrate applied at day 30; (b) V_f, vinasse applied at day 0; (c) $V_f \mid N$, vinasse applied at day 0 and ammonium nitrate applied at day 30; and (d) $V_s + N$, vinasse plus ammonium nitrate applied only at day 30 (log transformation, rarefied OTU table, family level).

(day 389) (Fig. 3d). The combined application of vinasse plus N ($V_s + N$) appeared to be a greater determinant of changes in the fungal community than the separate application of each fertilizer (N; V_f; $V_f \mid N$) (Fig. 2).

3.2. Fungal taxa associated with the treatments

The fungal community was composed of ten phyla dominated by Ascomycota (67.9%), followed by Basidiomycota (9.6%) and Mortierellomycota (0.9%). Unclassified phyla represented 20.9%, while other phyla represented 0.7% of the fungal community (Figs. A.2, A.3, A.4).

The specific fungal groups in each treatment were determined by linear discriminant analysis effect size (LEfSe). The treatments with vinasse (V_f, $V_f \mid N$ and $V_s + N$) had the most enriched fungal classes (Figs. 4 and A.4), namely Tremellomycetes (Order Trichosporonales and Tremellales), Sordariomycetes (Order Sordariales, Hypocreales, Trichosphaeriales, Microascales, Chaetosphaeriaceae, and Magnaporthales), Eurotiomycetes (Order Eurotiales and Chaetothyriales), Exobasidiomycetes (Order Tilletiales), Mucoromycetes (Order Mucorales), Microbotryomycetes (Order Sporidiobolales), Saccharomycetes (Order Saccharomycetales), and Dothideomycetes (Order Capnodiales and Pleosporales). The relative abundances of the families *Trichosporonaceae*, *Sordariaceae*, *Trichocomaceae*, *Nectriaceae*, *Hypocreaceae*, *Tremellaceae,* and Sporidiobolales Incertae sedis increased significantly in the $V_s + N$ treatment, whereas *Tilletiaceae* and *Mycosphaerellaceae* increased in the $V_f \mid N$ treatment (Figs. 4 and A.4). Chaetothyriales family Incertae sedis and Trichosphaeriales family Incertae sedis were overrepresented in the V_f treatment, whereas *Herpotrichiellaceae,* Hypocreales family Incertae sedis, *Halosphaeriaceae*, *Chaetosphaeriaceae*, and *Cordycipitaceae* had higher abundance in the control than in the vinasse treatments (V_f, $V_f \mid N$ and $V_s + N$) (Fig. 4). Similar results were found using the random forest (RF) algorithm (Fig. A.5). According to both the LEfSe and RF results, the most important variable for predicting

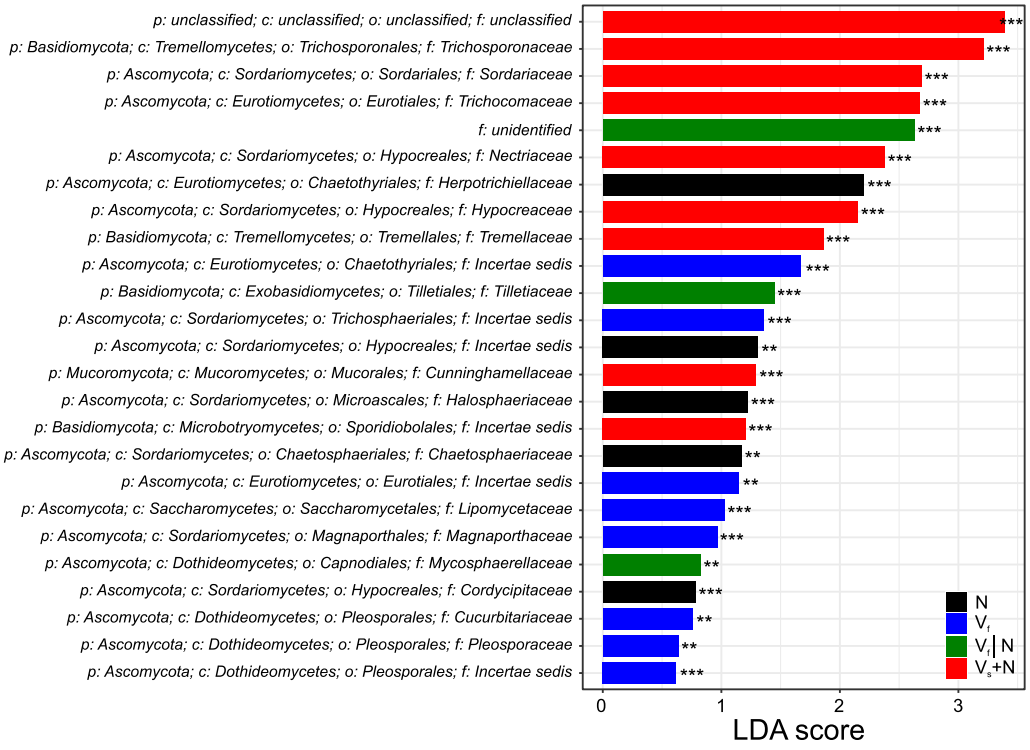


Fig. 4. Linear discriminant analysis effect size (LEfSe) of statistically different abundances of families between treatments (average of days). The treatments were as follows: V_f: vinasse applied at day 0; N: inorganic fertilizer ammonium nitrate applied at day 30; V_f | N: vinasse applied at day 0 and ammonium nitrate applied at day 30; and V_s + N: vinasse plus ammonium nitrate applied only at day 30. The bars show the treatments with the highest mean and the logarithmic LDA score. Significant differences: $^{**}p \leq 0.05$ and $^{***}p \leq 0.01$. p:, c:, o: and f indicates Phylum, Class, Order and Family level, respectively.

changes in the soil fungal community was the family *Trichosporonaceae* (Order Trichosporonales), which was most abundant in the V_s + N treatment.

3.3. Weather conditions, soil analysis, and CO_2-C emissions

The weather conditions, soil analysis, and CO_2 emissions were described in Lourenço et al. (2018b); additional information on pH, mineral N concentration and moisture is provided in Supplementary Fig. A.6, and CO_2 emissions data are presented in Fig. A.7. and additional Supplementary results. According to RDA, abiotic factors (ammonium and nitrate content, pH, moisture, soil and air temperature) explained ~9.2% of the fungal community variation (axis 1, 3.07%; axis 2, 1.96%), suggesting that unmeasured biotic or abiotic factors explained the remaining ~94.97% of the variation (Fig. A.8; Pseudo-F = 1.6, P = 0.001).

3.4. Effect of single vinasse application on fungal and bacterial communities and on their co-occurrence

To assess the dynamics and resilience of the soil fungal and bacterial communities after single vinasse application (V_f), samples were obtained at 12 time points, including soil samples without vinasse and mineral N. The application of vinasse alone to the soil altered the resident fungal community over time (Fig. 5a) (PERMANOVA: Pseudo-F = 1.25, P = 0.044; ANOSIM: $R = 0.16$, p = 0.03) but did not alter the Chao1, Simpson and Shannon diversity indices. The fungal community was not resilient, however; although the community remained different at day 389, it appeared to begin returning to the previous state (day 0) (Figs. 3b, 5a). LEfSe analyses showed that the relative abundances of *Trichocomaceae* (Order Eurotiales), *Dacampiaceae* (Pleosporales), *Hypocreaceae* (Order Hypocreales), and Sporidiobolales family Incertae sedis changed significantly after vinasse application in the soil (Table 4). By contrast, the resident bacterial community was resilient in the V_f treatment and recovered to the original state after 31 days (Fig. 5b; Lourenço et al., 2018b).

For the single vinasse treatment (V_f), RDA analysis showed that abiotic factors (ammonium and nitrate content, pH, moisture, soil and air temperature) explained ~27.1% of the fungal community variation (axis 1, 6.77%; axis 2, 5.96%) (Fig. A.9a; Pseudo-F = 1.4, P = 0.001). RDA analysis showed that the best explanatory variables for soil fungal community changes were precipitation (5.1%; p = 0.011), soil moisture (4.5%; p = 0.041) and pH (5.0%; p = 0.008). For the soil bacterial community, these factors explained ~20.8% of the whole bacterial community variation (axis 1: 8.26%; axis 2: 4.09%); however the test on all axes was non-significant (Fig. A.9b; Pseudo-F = 1.00, P = 0.35), suggesting that unmeasured biotic or abiotic factors explained the remaining variation.

To assess the degree of concordance between the fungal and bacterial community compositions, we performed co-inertia analysis. A non-significant concordance between ordinations was found (RV test = 0.356; P = 0.753, based on 999 replicates), suggesting that the structures of the bacterial and fungal communities were not associated with each other. Furthermore, no correlations were found between the bacterial and fungal families responsible for the changes in the soil microbial community after vinasse application (Fig. A.10).

4. Discussion

Different types of mineral and organic fertilizers are added to soils to increase nutrient and carbon content with the aim of improving soil fertility and agricultural production. The impact of fertilization on soil microbiota is of growing concern due to the importance of microbes in soil ecosystems. Our findings provide insights on the fungal community responses in soil over one year under multiple pulse disturbances by mineral fertilizer and organic vinasse residue. Important to highlight that this is one of the first study evaluating the impact of organic and

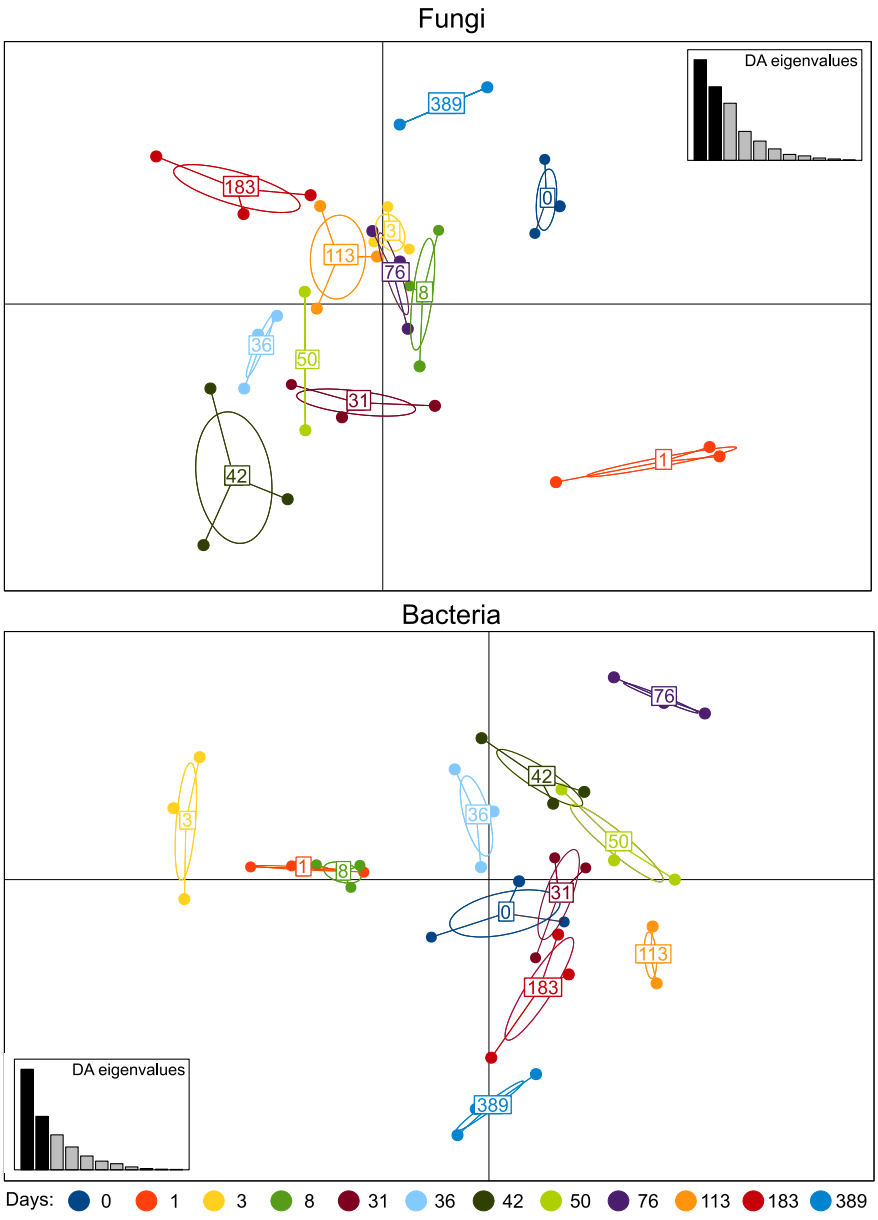


Fig. 5. Discriminant analysis of principal components (DAPC) plot of the effect of vinasse only (V_f) on soil fungal and bacterial taxonomy (Hellinger transformation, rarefied OTU table, family level).

inorganic fertilizer right after application on soil fungal community through an entire year. Most of the studies have analyzed only in a single sampling date, consequently indicating either short-term or long-term effects on microbial community and not the dynamics of the microbial community.

The soil fungal community responded differently to the organic and inorganic amendments. The addition of organic vinasse together with inorganic N fertilizer to soil covered with sugarcane straw had the highest impact on the fungal community. It is worth noting that the amount of straw present in the soil was lower at the last soil sampling (389 days) than before fertilization due to straw decomposition. At the first soil sampling, the amount of sugarcane straw on the soil was approximately 16 t ha^{-1} of dry matter. The annual decomposition rate of straw typically ranges from 60% to 98% throughout the crop season (Carvalho et al., 2017; Fortes et al., 2012), and the amount of sugarcane straw at different time points is expected to vary (Carvalho et al., 2017; Fortes et al., 2012; Oliveira et al., 1999; Varanda et al., 2019), as are the different fungal functional groups, especially those related to organic matter (lignin, cellulose and hemicellulose) decomposition (Fortes et al., 2012; Rachid et al., 2016). Consequently, fungal community resilience in a short period under specific soil conditions is unexpected (Ågren et al., 2001; Boer et al., 2005). In addition, vinasse may act as a primer upon addition to soil by decreasing the C:N ratio (Silva et al., 2013) and thus accelerating the changes in fungal community structure. Moreover, Rachid et al. (2016) suggested that different levels of sugarcane straw (0%, 50%, and 100% of the original straw deposition) and, consequently, different amounts of organic C have different effects on the fungal community.

Vinasse increased different fungal families after one day of application in soil (day 1; Table 4), particularly families from the phyla Ascomycota (*Dacampiaceae, Hypocreaceae, Trichocomaceae*) and Basidiomycota (*Sporidiobolales Incertae sedis*). Although the multiple pulse disturbances with vinasse and/or with mineral N induced changes in the fungal community, the combined application of vinasse and mineral N (V_s + N) as a single pulse disturbance on the same day resulted in the largest changes in the fungal community (Fig. 2). The V_s + N treatment decreased the alpha diversity and increased the abundance of *Trichosporonaceae* compared to the other treatments. The relative

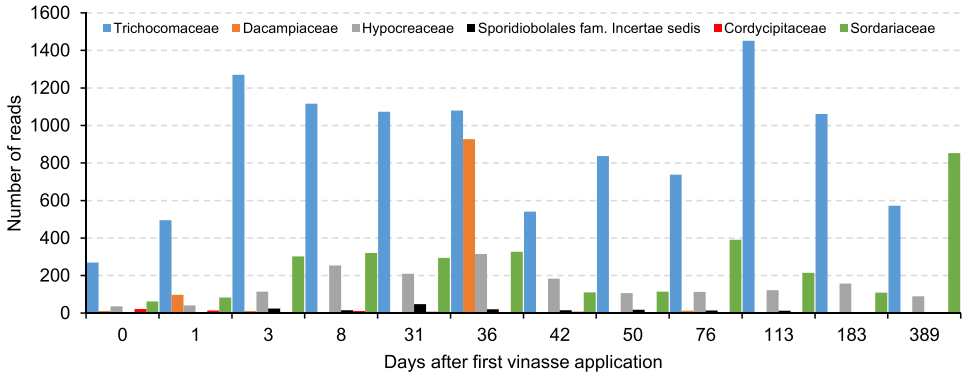
Table 4
(a) Fungal microbial community members at the family level whose abundances differed statistically according to linear discriminant analysis effect size between days after first vinasse (V_f) application in the soil and (b) their relative abundance.

(a)

Significant difference between days – Vinasse Effect [a]	"P values"	LDAscore
p: Ascomycota; c: Eurotiomycetes; o: Eurotiales; f: Trichocomaceae	0.010	2.77
p: Ascomycota; c: Dothideomycetes; o: Pleosporales; f: Dacampiaceae	0.038	2.67
p: Ascomycota; c: Sordariomycetes; o: Hypocreales; f: Hypocreaceae	0.047	2.15
p: Basidiomycota; c: Microbotryomycetes; o: Sporidiobolales; f: Incertae sedis	0.048	1.38
p: Ascomycota; c: Sordariomycetes; o: Hypocreales; f: Cordycipitaceae	0.059	1.07
p: Ascomycota; c: Sordariomycetes; o: Sordariales; f: Sordariaceae	0.072	2.6

[a] p:, c:, o: and f: means Phylum, Class, Order and Family level, respectively.

(b)



abundance of *Trichosporonaceae* family members increased 6 days after the application of vinasse together with mineral N and remained high until day 389. *Trichosporonaceae* is a family of yeasts reported in soil (Yurkov, 2018) that use nitrite as an inorganic source of N and different organic N sources (ethylamine, L-lysine and cadaverine) and are active in the decomposition of hemicellulose and assimilation of phenolic compounds (Middelhoven, 2005; Middelhoven et al., 2001). The high contents of soluble organic C (10–20 g C L^{-1}) and N (0.4 g N L^{-1}) (Christofoletti et al., 2013; Fuess et al., 2017, 2018; Rodrigues Reis and Hu, 2017) and the presence of organic compounds such as glycerol, ethanol, lactic and acetic acids, and phenolic compounds in vinasse (Freitas et al., 2018; García et al., 1997; Parnaudeau et al., 2008) may facilitate the activities of this fungal family when applied together with mineral N to soil covered with straw. Hence, in soil covered with straw, vinasse functioned as substrate for this fungal family.

Several studies have reported that both mineral and organic long-term fertilization lead to changes in fungal community composition (Ai et al., 2018; Cline et al., 2018; Morrison et al., 2016). In a long-term experiment (113 years), Francioli et al. (2016) showed that the application of organic or organic plus mineral fertilizer strongly increased fungal biomass, whereas long-term application of mineral fertilizer induced only a slight increase in fungal biomass relative to the unfertilized treatments. Both organic and mineral fertilization changed the structure of the fungal community. However, the lack of studies of fungal guilds prevents a clear view of the main families significantly associated with the treatments at each time point. According to the literature, organic vinasse amendment mainly increases the abundance of saprotrophs, species capable of fungal denitrification, and fungi with copiotrophic and oligotrophic lifestyles. *Trichocomaceae* (order Eurotiales) increased after vinasse addition; this order harbors many obligate saprophytic fungi capable of producing extracellular enzymes (Caesar-Tonthat, 2002; Daynes et al., 2012), and some members utilize labile C resulting from organic matter decomposition as a nutrient (Bödeker et al., 2016; Nguyen et al., 2016). In addition to increasing saprophytic fungi, the load of nutrients present in vinasse may contribute to increasing fungi related to N_2O emissions, as evidenced by the detection of the orders Hypocreales and Eurotiales. The detection of these orders indicates that denitrification genes are present in the fungal community, as most species of the orders Hypocreales and Eurotiales have been described to produce N_2O (Mothapo et al., 2015; Wei et al., 2015; Zhou et al., 2016), thereby improving our estimation of the N_2O process (Rohe et al., 2014). As further confirmation, Lourenço et al. (2019) showed that the application of vinasse as organic fertilizer increased N_2O emissions in soils with sugarcane in the same experimental plots used in the present study, and the production of N_2O was correlated with denitrifier fungi (Lourenço et al., 2018a). While others (Carmo et al., 2013; Pitombo et al., 2016) have demonstrated that the combination of straw, vinasse and N fertilizer drives the increase in N_2O emissions, the role of fungi in N_2O emissions from sugarcane has received little attention. *Dacampiaceae* (order Pleosporales) was also highly abundant in the treatments with vinasse, which might be related to the potassium (K) added to the soil by vinasse (2 g L^{-1} K). Pleosporales has been described to be associated with high N fertilization levels and high concentrations of K (Pingel et al., 2019). Furthermore, members of Pleosporales and Sordariales (Ascomycota) are considered potent degraders of lignin and predominantly show oligotrophic features (Entwistle et al., 2013; Ho et al., 2017; Pöggeler, 2011). The class Sordariomycetes and order Sordariales also increased significantly under vinasse fertilization, and most members of this order are considered saprotrophs on dung and wood (Tedersoo et al., 2014). Taken together, our results indicate that the nutrient-rich content of vinasse (organic C, organic N, K) favors mostly copiotrophic but also oligotrophic fungal taxa. The soil environment of sugarcane cultivation is complex having vinasse, mineral fertilizer and sugarcane straw with C:N ratio of 100:1. The vinasse could imply a primer effect increasing copiotrophs microorganisms right after application, however there is also straw that could select microbes related with decomposition of recalcitrant compounds. The findings of the present study thus provide important information on the interactions among organic and mineral amendments, straw and fungi that will aid the understanding of the

implications of the fungal community for environmentally relevant soil processes.

We expected the patterns of the fungal community to differ from those of the bacterial community immediately after organic vinasse amendment (Lourenço et al., 2018b), and fungal community turnover was higher in the first days after organic enrichment but took more time to recover toward previous state (Day 0) compared with bacteria. Lourenço et al. (2018b) showed that the bacterial community was not resistant but highly resilient to vinasse application, with a return to the previous community structure after 31 days, when organic C and mineral N resources were depleted. Vinasse amendment caused a shift in the predominant bacteria from those with copiotrophic lifestyle strategies toward more active bacteria (Lourenço et al., 2018b; Suleiman et al., 2016). By contrast, the fungal community in this study, which was analyzed using the same DNA pool as the bacterial community in Lourenço et al. (2018b), was neither resilient nor resistant to the vinasse and mineral N amendments: the community changed and did not return to the previous state. Some members increased and did not return to their previous abundances in the fungal community, such as the families *Hypocreaceae*, *Sordariaceae*, and *Trichocomaceae* (Table 4 and Figs. A.3, A.4). The changes in the quality and quantity of the sugarcane straw (Fortes et al., 2012) were crucial and probably drove the changes in the fungal community after the first changes due to organic and inorganic disturbances. Due to the different lifestyle strategies of the bacteria and fungi, no interactions were found between the fungal and bacterial communities or among fungal members. The selected fungal families favored by vinasse application in the soil did not return to the relative abundances observed in the beginning of the experiment and remained abundant in the soil for the entire study period.

5. Conclusion

In conclusion, the soil-resident fungal community was neither resistant nor resilient to organic vinasse and mineral amendments over one year of sampling. The combined application of vinasse and mineral N fertilizer was the main driver of the changes in fungal community structure due to the increases in organic C and mineral N and, in turn, decreased C:N ratio. Vinasse application appeared to change trophic guilds related to saprotrophs, fungal denitrification, and copiotrophic and oligotrophic fungi. Although vinasse and mineral N fertilizer altered the soil microbiome, no interaction was found between the fungal and bacterial communities.

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Availability of data and materials

The raw sequences were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB30929 (Fungi) and PRJEB25676 (Bacteria).

Ethics approval and consent to participate

Not applicable.

CRediT authorship contribution statement

Késia Silva Lourenço: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.**Afnan Khalil Ahmad Suleiman:**Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing.**Agata Pijl:**Investigation, Formal analysis, Writing - review & editing.**Heitor Cantarella:**Conceptualization, Writing - review & editing.**Eiko Eurya Kuramae:**Conceptualization, Writing - review & editing.

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

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