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Additional Information

High-throughput amplicon sequencing-based analysis of active fungal communities inhabiting grapevine after hot-water treatments reveals unexpectedly high fungal diversity

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ABSTRACT

The ecology of total fungal communities in grapevine is so far largely derived from the studies on culture-dependent methods or cultivation-independent rDNA approaches. Sequencing the ribosomal RNA transcripts (rRNA) would rather reveal the functionally and metabolically active important taxa of the fungal community and provide insights into its activity in the wood. The present study investigated changes in the potentially active fungal communities of internal grapevine wood after Hot-Water Treatment (HWT) in planting material from Czech Republic and Spain at two different moments of the propagation process and from two plant zones. We examined fungal communities using both traditional microbiological approach and high-throughput amplicon sequencing (HTAS) of internal transcribed spacer 2 (ITS2) region in extracted total RNA. HTAS from metatranscriptomic RNA increased the resolution of the fungal community analysis and revealed a highly diverse mycoflora of grapevine wood compared to the traditional method. Fungal diversity differed between grapevine genotypes and showed a temporal variation over the vegetative period. Grapevine planting materials exhibited high fungal diversity after HWT, which demonstrates that the HWT process does not sterilize the internal wood of grapevine. HWT reduced the infection caused by fungal trunk disease pathogens but was not completely effective in eliminating their growth. This study provides important and practically useful insights into dynamics of active fungal communities in hot-water treated plants and represents the first approach to study active fungal communities on grapevine grafted plants by comparing traditional and next-generation sequencing methods.

Keywords:

Endophytic fungi, grapevine trunk diseases, ITS2, metatranscriptomics, mycobiota, NGS, plant microbiome

1. Introduction

Hot water treatment (HWT) is an efficient, environmentally safe and commercially viable method of suppressing a wide range of pests and pathogens in planting material of grapevine and other crops (Waite and May, 2005). For grapevine, it comprises the submersion in water of dormant cuttings, rootlings or grafted rootlings for a given temperature and time (Waite and Morton, 2007; Gramaje et al., 2009). The HWT mechanism is the application of heat to the material in order to denature the pathogens and kill arthropods and nematodes. It is especially remarkable the effectiveness of this method in controlling crown gall (Ophel et al., 1990; Burr et al., 1996), phylloxera (Buchanan and Whiting, 1991), phytoplasma diseases (Caudwell et al., 1997; EPPO, 2012) and the European quarantine agent *Xylella fastidiosa* (Goheen et al., 1973; Purcell et al., 2013; EFSA PLH Panel, 2015), or reducing the incidence of fungal trunk pathogens (Fourie and Halleen, 2006; Gramaje et al., 2009; Halleen and Fourie, 2016).

Grapevine trunk diseases are caused by a wide range of taxonomically unrelated fungal species that colonize the wood of spurs, cordons, cuttings and trunk, compromising the translocation of nutrients and water throughout the vine, which eventually leads to death of the woody tissues. Nurseries are particularly vulnerable to trunk disease infections since propagation of grapevines creates wounds for pathogen invasion as well as the means of spreading inoculum in asymptomatic planting material (Gramaje and Armengol, 2011). Chemical control of these endogenous fungal pathogens inhabiting the vascular tissue of grapevines is difficult. Standard treatments applied to the surface of cuttings to manage other fungal diseases in nurseries do not penetrate the cutting tissue sufficiently to be effective (Gramaje et al., 2018). HWT is therefore the only currently recognized means of controlling internal infections of fungal trunk pathogens in propagating material. However, there is a perception in the vine nursery industry that the HWT process sterilizes completely both the surface and the internal wood of cuttings, making the plants more vulnerable to any kind of

new fungal infection. The effects of the changes on the population of internal microorganisms (endophytes and/or fungal pathogens) on cuttings that result from HWT protocols are still unknown.

Fungal communities in plants can be examined by different approaches. Cultivation-based techniques have been frequently used before, however these methods tend to misrepresent fungal activity and underestimate species richness, because fungi may be hidden, highly selective and slow growing. Molecular-based approaches have progressively replaced morphological approaches to characterize microbial communities in nature. They allow the detection and identification of more microbial organisms, including species that cannot be obtained in culture (Amann et al., 1995). The new advances in high-throughput sequencing technology have increased both the resolution and scope of fungal community analyses and have revealed a highly diverse and complex mycobiota of plant vascular systems (Studholme et al., 2011). To date, most studies have investigated the ecology of total fungal communities by sequencing the ribosomal RNA genes (rDNA) (Lindahl et al., 2013), which provide a description of all members of the community, regardless of activity level. For example, DNA based methods are unable to distinguish between viable or dead organisms with intact genetic material (England et al., 1997; Demanèche et al., 2001).

Sequencing the ribosomal RNA transcripts (rRNA), an elegant approach without PCR bias for amplicon generation, instead reveal the metabolically active fungal taxa of the community and provide insights into their activity in environmental samples (Urich et al., 2008). The study of the potentially active fungal communities by using rRNA sequences has been carried out in environments such as soil (Baldrian et al., 2012; Kuramae et al., 2013; Barnard et al., 2013), on decaying plant material (Rajala et al., 2011) and in the atmosphere (Womack et al., 2015), but has not been applied to elucidate changes of fungal communities in the wood of economically important crops, such as grapevine.

In grapevine, the ecology of total fungal communities is so far largely derived from the studies on culture-dependent methods (Casieri et al., 2009; Martini et al., 2009; Gónzalez and Tello, 2010; Hofstetter et al., 2012; Pancher et al., 2012; Bruez et al., 2014, 2016, 2017) or cultivation-independent rDNA approaches (Pancher et al., 2012; Bruez et al., 2014, 2016), and information is lacking on the relationships between the diversity of the total community and community of active microbes estimated. The present study investigated changes in the potentially active fungal communities of internal grapevine wood after HWT in Czech Republic and Spain. For this purpose, we examined fungal communities in grapevines using both, traditional microbiological approach and HTAS of internal transcribed spacer 2 (ITS2) region in extracted total RNA. Anderson and Parkin (2007) indicated that the ITS provides a more active part of the fungal community than 18S rRNA as it is continually transcribed but quickly removed in the processing of mature rRNA.

We tested the following hypotheses: (1) the ITS2 region of rRNA is a suitable marker for revealing active fungal species in a community, (2) only part of the fungal community colonizing the wood of grapevine is metabolically active at a particular HWT temperature/time combination, (3) the HTAS procedure significantly enhances the characterization of fungal diversity compared to traditional methods, (4) metabolic activity of fungal species is related to the origin of grapevine planting material, which changes after one season in the vineyard, (5) HWT process does not sterilize completely the internal wood of cuttings but reduces the infection caused by fungal trunk disease pathogens.

2. Materials and methods

2.1. Planting material and treatments

Two experiments were simultaneously carried out in Spain and the Czech Republic in 2015 to examine the effect of Hot-Water Treatments (HWT) on the total fungal mycoflora of dormant grapevine grafted cuttings. In each country, a stock of 450 dormant grafted plants ready to be sold to producers cv. Garnacha Tintorera grafted onto rootstock 110 R grafted and Sauvignon Blanc grafted on SO4 were obtained from commercial nurseries in Spain and the Czech Republic, respectively. Grafted plants were obtained following the propagation process in nurseries described in Gramaje and Armengol (2011). No chemicals and biocontrol agents were applied during the different stages of the propagation.

In April 2015, this planting material was allocated at random to 3 bundles of 150 grafted plants. One bundle was assigned to no-HWT (control). The remaining two bundles were assigned to either HWT at 50ºC 30 min or HWT at 53ºC during 30 min. For HWT, planting materials were placed in a hydrating bath for 1 h in order to pre-soak material before treatment. Following hydration, plants were placed in mesh polyethylene bags and immersed in a temperature controlled bath at the experimental temperatures and times for the treatment. On removal from the HWT bath, vines were immediately plunged into a cool bath of clean water at ambient temperature for 30 min in order to stop the heating process. Vines were then removed from the bath and allowed to drain until there was no free moisture on the surface of the cuttings. Then, two groups of 20 grafted plants were randomly collected from each bundle. For each treatment, one of the groups of 20 grafted plants were subjected to fungal isolation and the other to isolation of total RNA followed by HTAS. All HWTs were carried out at the Polytechnic University of Valencia.

The remaining grafted plants (110 per treatment) were planted immediately in a commercial vineyard, following standard cultural practices in each country during the grapevine growing season. At the end of the growing season (October 2015), two groups of 20 grafted plants were

randomly collected from each treatment and, again, one of these groups of 20 grafted plants was subjected to fungal isolation and the other to HTAS.

2.2 Fungal isolation and molecular identification

Isolations from Czech and Spanish planting material were performed at the Polytechnic University of Valencia. In each plant, isolations were performed from sections (2 cm long), which were cut from two different areas: the basal end of the rootstock cuttings (crown area) and the grafting area. These sections were then washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution, and washed twice with sterile distilled water. Fifteen internal wood fragments per section were placed on malt extract agar (MEA) supplemented with 0.5 g L^{-1} of streptomycin sulphate (MEAS) (five fragments per three Petri dishes). Plates were incubated for 10-15 days at 25ºC in the dark. All emerging colonies were transferred to PDA for molecular identification.

For DNA extraction, 300 mg of fungal mycelium and conidia from single spore isolates grown on PDA for 2 to 3 weeks at 25°C in the dark were scraped and homogenised twice in a Fastprep®-24 tissue homogenizer (MP Biomedicals, USA). Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer's instructions. DNA was visualized on 1% agarose gels stained with RedSafe (iNtRON Biotechnology, Lynnwood, WA, USA). DNA was stored at −20°C.

The identification of all isolates was performed by analysis of the ITS region of DNA amplified using the fungal universal primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). Then, further molecular identification was conducted for specific groups of grapevine trunk pathogens. *Phaeoacremonium* species were identified by sequence analysis of the β-tubulin gene using primer sets T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and

Donaldson, 1995). Identification of Botryosphaeriaceae species was confirmed by analysis of elongation factor 1-α gene amplified using EF1-728F and EF1-986R primers (Carbone and Kohn, 1999). Identification of *Cylindrocarpon*-like anamorphs was confirmed by sequencing part of the histone H3 gene (HIS) with primers CYLH3F and CYLH3R (Crous et al., 2004). The fungal identification reads for four plant samples taken randomly were pooled resulting in a total of five replicates for every batch of 20 plants.

2.3. RNA extraction

RNA extraction from Czech and Spanish planting material were performed at the Mendel University in Brno. RNAs were extracted from the same plant parts from which the fungal isolations were carried out: the grafting area and the root crown. Each analysed plant part was debarked and the wooden plant tissue was scrapped by sterile scalpel under sterile conditions in flow box. For this purpose 100 mg of wood plant tissue was grinded precisely in the mortar cooled to a –80°C, and 50 mg of this homogenate was used for RNA isolation using Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, USA) according to manufacturer's instructions. Total RNA yield and quality was measured using Bioanalyzer 2100 (Agilent Technologies, Palo Alto, USA) using Agilent RNA 6000 Nano Kit, Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, USA) using Quant-iT™ RNA Assay Kit (Thermo Fisher Scientific, Waltham, USA). Samples with RNA Integrity Number (RIN) lower than 7 were excluded from further analysis. Only RNA concentrations higher than 5 ng μl^{-1} were used, higher concentrations were adjusted to 5 ng μl^{-1} too. The adjustment was carried out based on fluorimetry as the most reliable method. After RNA quantification, four samples taken randomly were pooled resulting in a total of five replicates every batch of 20 plants. Subsequently, RNA was transcribed to cDNA as described by Eichmeier et al. (2010).

2.4. Library preparation and sequencing

cDNA was used for library preparation according to Illumina (San Diego, USA) protocol, 16S Metagenomic Sequencing Library Preparation. For the 1st amplification cycle the primers gITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) were used. For the 2nd amplification primers constructed by adding the forward and reverse overhanging Illumina adaptors, exactly ITSNGSf (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGARTCTTTG) and ITSNGSr (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC) were used. Then, Nextera XT indexes were added according to the same Illumina (San Diego, USA) protocol. For all amplification steps Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, UK) was used. Final PCR products were separated on 1.3% agarose (Serva, Heidelberg, Germany). PCR products in size approximately 510 bp were cut of and purified with NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. Final PCR products were measured with the Bioanalyzer 2100 using Agilent DNA 12000 Kit, Modulus™ Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, USA) using Quant-iT™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, USA) and finally with MCNext™ SYBR® Fast qPCR Library Quantification Kit (MCLAB, San Francisco, USA) used with Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Sequencing was carried out with MiSeq Reagent Kit v3, 2 × 300 pair-end reads with the 600 cycles (Illumina, San Diego, USA) on MiSeq instrument (Illumina, San Diego, USA).

2.5. Bioinformatic analysis of sequencing reads

The sequence quality was checked by fastQC-0.10.1 (Andrews, 2010), for the trimming and merging of paired reads was used CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark). The distance of evaluated reads in trimming and merging step was set from 180 to 400 nts. Primer and Illumina adapter sequences were also trimmed out. Subsequently, the reads were mapped to the references of grapevine ITS (KF544886, KF454243, KT344630) and the un-mapped reads were used for downstream processes. The un-mapped reads were exported to fasta format by CLC Genomics Workbench 6.5.1 (CLC Bio, Aarhus, Denmark).

The data were clustered into Operational Taxonomic Units (OTU) using blastclust software (Altschul et al., 1997) with length coverage threshold = 0.9 and score coverage threshold = 99. OTUs from data sets were extracted using a custom script and then, MUSCLE v3.8.31 (Edgar 2004) was used for multiple OTU sequence alignment. Singletons were not considered and were removed from the analysis. Consensus sequences for individual OTUs were prepared with MEGA 7.0.18 (Kumar et al., 2016). These consensus sequences from each of the OTUs were identified using blastN in GenBank/NCBI.

2.6 Statistical analyses of the fungal community diversity and composition

The fungal community composition was evaluated using non-metric multidimensional scaling (NMDS) plots of Bray-Curtis distances using the metaMDS function of the vegan package in R (Oksanen et al., 2013; R Core Team 2016). Analysis of variance (ANOVA) was performed to investigate which OTUs significantly differed in abundance among experimental factors after Bonferroni correction. Fungal richness (N), the Shannon diversity (H’) and Pielou’seveness (J’) were calculated using the vegan package and compared using a 2-way ANOVA with treatment and sampling moment as factors.

3. Results

3.1. High-throughput amplicon sequencing-based procedure is superior to traditional isolation in detection and identification of active fungal communities

The diversity estimators showed that a more diverse fungal community was revealed by HTAS approach than the traditional isolation approach (Fig. 1). Datasets of 40,480,800 quality sequences were produced from Czech and Spanish planting material by HTAS approach (n=120). The total OTU number was 10,585 defined by 97% sequence similarity. The large majority of these OTUs were Ascomycota (26 orders, 50 families and 129 genera) followed by Basidiomycota (17 orders, 33 families and 55 genera), Mucoromycota (2 orders, 2 families and 2 genera) and Zygomycota (one order, one family and one genus). OTUs were assigned to 45 orders, 82 families and 189 genera (Table S1). Four fungal orders including Agaricales, Capnodiales, Hypocreales and Pleosporales were predominant (relative abundance >5%), and accounted for 40% of the total sequences. At family level, Erysiphaceae, Lasiosphaeriaceae, Mycosphaerellaceae, Nectriaceae and Pleosporaceae were the predominant groups, accounting for 21.7% of the total sequences in the wood samples.

By traditional isolation approach, a total of 93 fungal taxa were identified (Tables 1-4), representing 22 orders, 35 families and 67 genera (Table S2). Ascomycota, representing 15 orders, 24 families and 55 genera was the predominant fungal division followed by Basidiomycota (5 orders, 6 families and 7 genera), Mucoromycota (2 orders, 2 families and 2 genera) and Zygomycota (one order, one family and 3 genera). Three fungal orders including Eurotiales, Hypocreales and Pleosporales were predominant (relative abundance >5%), and accounted for 46.1% of the total fungi. At family level, Bionectriaceae, Nectriaceae,

Microascaceae, and Trichocomaceae were the predominant groups, accounting for 36% of the total fungi isolated from the wood samples.

Rarefaction curves for the data separated by origin of planting material and technique (HTAS or ISO) did not reach saturation, suggesting that deeper sequencing and more sampling efforts would uncover more taxa (Fig. S1).

3.2. Fungal communities differ between grapevine genotypes

Both HTAS and traditional isolation approaches showed that fungal diversity significantly differed in their relative abundances and composition in comparisons of grapevine genotype after Bonferroni correction ($P < 0.01$) (Fig. 2A, B). By HTAS approach, OTUs were assigned to 40 orders, 67 families and 126 genera in Sauvignon Blanc/SO4, while 34 orders, 49 families and 97 genera were associated with Garnacha Tintorera/110R planting material. Samples of Sauvignon Blanc/SO4 were dominated by the orders Agaricales, Hypocreales and Pleosporales (35.8% of the total sequences). In Garnacha Tintorera/110R, Capnodiales, Hypocreales and Pleosporales were the dominant orders (35.6% of the total sequences). Taxa responsible for the differences among Czech and Spanish planting material are shown in Table 5. Only 66 out of 189 genera were shared among grapevine genotypes (Table S3).

By isolation approach, OTUs were assigned to 15 orders, 24 families and 45 genera in Sauvignon Blanc/SO4 planting material, while 18 orders, 24 families and 45 genera were associated with Garnacha Tintorera/110R planting material. Samples of Sauvignon Blanc/SO4 were dominated by the orders Hypocreales and Pleosporales (50% of the total sequences). In Tintorera/110R samples, Eurotiales and Hypocreales were the dominant orders (34.9% of the total sequences). Taxa responsible for the differences among Czech and Spanish planting material are shown in Table 5. Only 17 out of 45 genera were shared among grapevine

genotypes (Table S3). Four fungal genera including *Bionectria*, *Dactylonectria*, *Geomyces* and *Mucor* were isolated from planting material but not detected by HTAS approach.

3.3. Fungal diversity exhibits a temporal variation over the vegetative period

Fungal community composition differed strongly between sampling moments in all treatments for both types of planting material (Fig. 3). In general, species richness was higher when plants were sampled after one growing season than immediately after HWT (Fig.1). However, fungal community diversity, as determined by Shannon diversity and Pielou's evenness indexes was not significantly different between sampling moments ($P > 0.05$) (Fig. 1).

3.4. Grapevine planting materials exhibit high fungal diversity after hot-water treatment

In general, species richness decreased with increasing temperature of treatments (Fig. 1A); but this difference was not significant ($P > 0.05$), with the exception of HWT at 50ºC in Czech planting material by HTAS approach after one growing season, HWT at 50ºC in Czech planting material by isolation approach after HWT, and HWT at 50 and 53ºC in Czech planting material by isolation approach after one growing season. Species diversity was also statistically indistinguishable, with the exception of HWT at 50ºC in Czech planting material by isolation approach after HWT (Fig. 1B). Fungal communities were more evenly distributed when sampling after HWT than sampling after one growing season (Fig. 1C). Species evenness was not significantly different among treatments within a sampling moment ($P > 0.05$).

3.5. Hot-water treatment affects fungal diversity composition at order level

The relative abundances of the top 10 orders (each relative abundance, >1%) for the Czech Republic and Spanish planting material by HTAS approach are shown in Figs. 4a and 4b, respectively. In Sauvignon Blanc/SO4, the order Hypocreales was detected in all treatments, plant parts and sampling moments (Fig. 4A). In the non-treated control, Agaricales (27.7%), Pleosporales (24.6%) and Hypocreales (13.5%) were the predominant fungal orders of all samples. Hypocreales (27.6%), Pleosporales (18.6%) and Sordariales (18.1%) were the predominant fungal orders of all samples after HWT at 50ºC. In samples hot-water treated at 53ºC, Sordariales (38.0%), Hypocreales (17.4%) and Pleosporales (9.8%) were the predominant fungal orders. In Garnacha Tintorera/110R, the orders Hypocreales, Helotiales and Pleosporales were detected in all treatments, plant parts and sampling moments (Fig. 4B). Helotiales (27.1%), Hypocreales (22.4%) and Togniniales (18.5%) were the predominant fungal orders of all samples in the control treatment. Hypocreales (31.4%), Pleosporales (18.6%) and Togniniales (21.0%) were the predominant fungal orders of hot-water treated samples at 50ºC. In samples treated at 53ºC, Togniniales (30.7%), Hypocreales (24.5%) and Pleosporales (22.4%) were the predominant fungal orders.

The relative abundances of the top 10 orders (each relative abundance, >1%) for the Czech Republic and Spanish planting material by isolation approach are shown in Figs. 4c and 4d, respectively. In Sauvignon Blanc/SO4, the orders Hypocreales and Eurotiales were detected in all treatments, plant parts and sampling moments (Fig. 4C). Hypocreales was the predominant order in the control, 50 and 53ºC treatments, accounting for 51.5%, 63.7% and 54.2% of all samples, respectively. The second predominant order was Eurotiales (20.6%, 26% and 40.1%). In Tintorera/110R, the orders Hypocreales and Eurotiales were detected in all treatments, plant parts and sampling moments (Fig. 4D). Hypocreales was the predominant order in the control, 50 and 53ºC treatments, accounting for 50.9%, 62.2% and 69.2% of all samples, respectively. Other predominant orders in the control treatment were Togniniales (10.4%) and

Botryosphaeriales (7.6%). In samples hot-water treated at 50ºC and 53ºC, other predominant orders were Togniniales (9.9% and 4.25%, respectively) and Eurotiales (9.6% and 11.4%, respectively).

3.6. Hot-water treatment affects fungal diversity composition at genus level

HTAS revealed the diversity of fungal communities in different samples at the genus level (Fig. S2A, B). In Sauvignon Blanc/SO4, only the genus *Alternaria* was detected in all treatments, plant parts and sampling moments (Fig. S2A). In the control treatment, *Psathyrella* (18.9%), *Neofusicoccum* (9.5%) and *Alternaria* (9.2%) were the predominant fungal genera of all samples. *Coniochaeta* (17%), *Clonostachys* (10.2%), *Sterigmatosporidium* (9.4%), *Phaeoacremonium* (8.9%) and *Fusarium* (8.6%) were the predominant fungal genera of all samples after HWT at 50ºC. In samples hot-water treated at 53ºC, *Coniochaeta* (38.3%), *Cladosporium* (10.4%) and *Phaeoacremonium* (9.8%) were the predominant fungal genera. In Garnacha Tintorera/110R, only the genus *Cadophora* was detected in all treatments, plant parts and sampling moments (Fig. S2B). *Cadophora* (27.3%) and *Phaeoacremonium* (20.1%) were the predominant fungal genera of all samples in the control treatment. *Phaeoacremonium* (22.2%), *Fusarium* (15.3%), *Alternaria* (11.8%) and *Acremonium* (9.8%) were the predominant fungal genera of hot-water treated samples at 50ºC. In samples treated at 53ºC, *Phaeoacremonium* (32.8%) and *Fusarium* (18.6%) were the predominant fungal genera.

The diversity of fungal communities in different samples at the genus level by isolation approach is shown in Figs. S2c and S2d. In Sauvignon Blanc/SO4, the genera *Fusarium, Penicillium* and *Trichoderma* were detected in all treatments, plant parts and sampling moments (Fig. S2C). *Trichoderma* was the predominant genus in the control, 50 and 53ºC treatments, accounting for 40.4%, 51.1% and 41.5% of all samples, respectively. Other predominant genera

were *Fusarium* (5.9%, 12.7% and 10.6%) and *Penicillium* (16.1%, 25.4% and 40.4%). In Tintorera/110R, the genera *Fusarium* and *Penicillium* were detected in all treatments, plant parts and sampling moments (Fig. S2D). *Fusarium* was the predominant genus in the control, 50 and 53ºC treatments, accounting for 36.9%, 57.6% and 57.4% of all samples, respectively. Other predominant genera were *Acremonium* (3.5%, 12% and 9.8%), *Penicillium* (5.8%, 6% and 7.5%) and *Phaeoacremonium* (9.6%, 9.1% and 4.3%).

3.7. The fungal communities associated with grapevine trunk diseases differ in their temporal distribution after hot-water treatment

Among the identified taxa, 10 genera are generally regarded as being associated with grapevine trunk diseases (GTDs): *Botryosphaeria*, *Cadophora*, *Dactylonectria*, *Diaporthe*, *Diplodia*, *Eutypa*, *Ilyonectria*, *Neofusicoccum*, *Phaeoacremonium* and *Phaeomoniella*.

In the HTAS approach, HWT reduced significantly the fungal trunk pathogens communities in Sauvignon Blanc/SO4 at both sampling moments, with the exception of the genera *Diaporthe* and *Phaeoacremonium* that exhibited a significant increase in the number of OTUs at 53ºC for 30 min (Fig. 5A). In Garnacha Tintorera/110R, all fungal communities associated with GTDs decreased the number of OTUs after HWT, with the exception of *Phaeoacremonium* spp. immediately after HWTs at 50ºC and 53ºC (Fig. 5B). In the isolation approach, HWT reduced significantly the fungal trunk pathogens communities in Sauvignon Blanc/SO4 at both sampling moments, with the exception of the genera *Cadophora* that exhibited a significant increase in the number of isolates immediately after HWT 53ºC for 30 min (Fig. 6A). In Garnacha Tintorera/110R, fungal communities associated with GTDs decreased the number of OTUs after HWT, with the exception of *Cadophora* immediately after HWTs at 53ºC, and *Phaeoacremonium* and *Phaeomoniella* that exhibited no significant differences in hot-water

treated samples with respect to the control after one growing season (Fig. 6B). *Diplodia* spp. and *Botryosphaeria* spp. isolation increased at 50ºC and 53ºC after one growing season, respectively.

4. Discussion

This study provides important and useful insights into the dynamics of fungal communities in hot-water treated plants and represents the first approach to study active fungal communities on grapevine grafted plants by using HTAS of metatranscriptomic RNA combined with traditional isolation approach.

The HTAS procedure was found to be superior to the isolation method in detection and identification of active fungal communities. This result indicates that isolation on culture media misses a functionally and significant part of fungal communities and our current knowledge largely based on this approach is incomplete. In both procedures, Ascomycota were the most abundant fungal division, being consistent with other studies on the fungal endophytic communities of woody tissues of grapevine (Casieri et al., 2009; Hofstetter et al., 2012; Pancher et al., 2012; Bruez et al., 2014, 2016, 2017), and were followed by Basidiomycota, Mucoromycota and Zygomycota.

Out of the 93 fungal species identified by traditional isolation, 55 were isolated from Sauvignon Blanc/SO4 planting material and 59 species from Garnacha Tintorera/110R planting material. In previous research conducted with nursery planting material in Switzerland, Casieri et al. (2009) and Hofstetter et al. (2012) isolated 66 and 85 fungal species from healthy one-year-old vines, respectively. Species accumulation curves in both grapevine genotypes analysed by two methods suggest that the fungal community associated with grafted plants is still far

from totally sampled. Therefore, the fungal endophyte diversity that can be associated with grapevine remains possibly largely unknown.

Different genera and species of fungi were associated with both grapevine genotypes. This agrees with the results obtained by Casieri et al. (2009) when examining the fungal communities associated with five one-year-old *V. vinifera* grafted plants in Switzerland. This result is not surprising since the plants examined in our study came from different nurseries, and fungal communities in different soils and in the air of different areas are likely to be different. In previous research, the climate (Arnold and Lutzoni 2007) and the sampling locality (Arnold et al., 2001; Higgins et al., 2007) were suggested to have an influence on the composition of the endophytic mycoflora. Further studies with grapevine genotypes collected from the same environmental conditions and nursery facilities are also needed to elucidate if grapevine genotypes can shape the fungal communities living in the wood.

Our results demonstrate that shifts existed in the fungal communities colonising the grapevine wood tissues over a period of seven months. Bruez et al. (2016) hypothesized that abiotic factors, such as the changes in the presence of nutrients moving in the xylem vessels during the growing season and the climate, could explain the shifts observed in the mycoflora of adult vines in France. In the present study, colonization of fungi living in soil could have influenced the fungal community structure at the base of the rootstock in nursery plants. For instance, the incidence of some fungal genera such as *Dactylonectria* and *Phaeoacremonium* increased in vines collected at the end of the growing season, compared to those analysed prior to planting in nursery fields. *Dactylonectria* spp. are soilborne pathogens and their presence in nursery fields and their capacity to infect grafted plants is well known (Agustí-Brisach et al., 2013b, 2014; Berlanas et al., 2017; Carlucci et al., 2017). *Phaeoacremonium* spp. have been detected in nursery soils and isolated from xylem vessels of grapevine seedlings used as bait plants (Agustí-Brisach et al., 2013a). Pathogenicity studies using artificially infested soils also

showed that *Phaeoacremonium* spp. can infect and colonize grapevine roots (Gramaje et al., 2015). Further research is needed to determine how the endophytic fungi inhabiting grapevines can evolve over time in each step of the nursery propagation process. Investigation of endophyte interactions in grapevine tissues is essential to select and apply biocontrol agents, and to counter grapevine trunk diseases more efficiently.

In the present experiment, the fungal diversity identified by both traditional isolation and HTAS approaches was unexpectedly high after HWT. Crous et al. (2001) noticed drastic reductions of endophytes and common fungal pathogens occurring in woody tissues of the hot-water treated cuttings in isolations made inmediately after treatment. By contrast, Rooney and Gubler (2001), in studies conducted with the grapevine trunk disease pathogens *Phaeomoniella chlamydospora* and *Phaeoacremonium inflatipes*, indicated that despite the initial shock to these pathogens by HWT at 51ºC for 30 min they could recover from this treatment and survive. In a recent study carried out in France, Bruez et al. (2017) did not find differences in fungal communities between either HWT or non-HWT grapevine plants 14 or 15 years after treatment.

In our study, several fungal species associated with grapevine trunk diseases as well as some fungal species reported as being suitable biocontrol agents against grapevine pathogens were able to tolerate temperatures of up to 53ºC. Both *in vitro* and *in vivo* HWT trials already demonstrated that pathogenic fungal species belonging to the genera *Cadophora* and *Phaeoacremonium* can survive at temperature above 53ºC (Gramaje et al., 2008, 2009, 2010). In addition, the potentially plant-protective fungi *Trichoderma* spp. was abundant in the wood of Sauvignon Blanc/SO4 after HWT. *Trichoderma* spp. are considered opportunistic and avirulent plant symbionts (Harman et al., 2004), and the antagonistic activity of some strains against pathogenic fungi has been revealed (Perveen and Bokhari, 2012; Schwarze et al., 2012). Further research, aimed at studying the interactions between potential biocontrol agents and

fungal pathogens in grapevine wood, needs to be undertaken in order to elucidate the inhibition of the development of some fungi.

In conclusion, our investigation showed that the HWT process does not sterilize the internal wood of cuttings, reduces the infection caused by fungal trunk disease pathogens, but is not completely effective in eliminating their growth. Hot-water treated plants could get re-infected in the field once planted out. This does, however, provide interesting possibilities of combining the HWT with biological control agents within an integrated management program (Gramaje et al., 2018), which could ensure that plants remain almost pathogen free for a longer period. Finally, fungal endophytes isolated in this study will also provide future resources for identification of potential biological control agents for the management of trunk diseases in grapevine nurseries and young vineyards.

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Table 1

Czech plants analyzed immediately after hot-water treatment. Number of plants and percentage of wood fragments from which the different fungi were isolated for the different treatments (control, 50 ºC for 30 min. and 53 ºC for 30 min.) and plant zones (grafting area or base of the rootstock).

Order	Family	Genus	Species	Control				50ºC 30 min				53ºC 30 min			
				Grafting area		Rootstock		Grafting area		Rootstock		Grafting area		Rootstock	
				No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%
Hypocreales	?	*Acremonium*	sp.	-	-	-	-	-	-	-	-	1	0.3	-	-
Pleosporales	Pleosporaceae	*Alternaria*	sp.	3	3.3	2	3.7	1	0.7	-	-	-	-	-	-
-	-	*Ascomycota*	sp.	-	-	-	-	-	-	1	0.3	-	-	-	-
Eurotiales	Trichocomaceae	*Aspergillus*	sp.	-	-	-	-	-	-	-	-	-	-	1	0.3
Hypocreales	Bionectriaceae	*Bionectria*	sp.	3	5.7	-	-	-	-	-	-	-	-	-	-
Helotiales	?	*Cadophora*	*luteo-olivacea*	-	-	-	-	-	-	-	-	1	0.7	-	-
Sordariales	Coniochaetaceae	*Coniochaeta*	*velutina*	-	-	-	-	-	-	1	0.3	2	0.7	2	1.0
Sordariales	Coniochaetaceae	*Coniochaeta*	sp.	-	-	-	-	-	-	1	0.7	-	-	1	0.3
Hypocreales	Nectriaceae	*Cylindrocladium*	sp.	2	1.7	1	0.3	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Dactylonectria*	*pauciseptata*	-	-	1	1.0	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Dactylonectria*	*torresensis*	-	-	4	4.3	-	-	-	-	-	-	-	-
Diaporthales	Diaporthaceae	*Diaporthe*	*ampelina*	-	-	1	1.7	-	-	-	-	-	-	-	-
Diaporthales	Diaporthaceae	*Diaporthe*	sp.	-	-	2	3.0	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Fusarium*	sp.	5	7.3	3	1.7	4	3.7	1	0.3	8	9.3	1	0.3
?	Myxotrichaceae	*Geomyces*	sp.	-	-	-	-	-	-	-	-	-	-	1	0.3
Sordariales	Chaetomiaceae	*Humicola*	*grisea*	-	-	-	-	-	-	-	-	-	-	1	0.3
Pleosporales	Didymosphaeriaceae	*Kalmusia*	*variispora*	-	-	-	-	-	-	1	1.3	-	-	-	-
Sordariales	Coniochaetaceae	*Lecythophora*	*hoffmannii*	-	-	-	-	-	-	-	-	1	0.7	-	-
Sordariales	Coniochaetaceae	*Lecythophora*	sp.	-	-	-	-	-	-	-	-	1	1.0	1	0.3
Hypocreales	Nectriaceae	*Mariannaea*	*superimposita*	-	-	1	0.3	-	-	-	-	-	-	-	-
Mucorales	Mucoraceae	*Mucor*	sp.	4	3.0	3	2.0	11	13.0	3	2.0	-	-	1	0.3
Ophiostomales	Ophiostomataceae	*Ophiostoma*	*piceae*	-	-	1	0.3	-	-	-	-	-	-	-	-
Pleosporales	Montagnulaceae	*Paraconiothyrium*	*sporulosum*	-	-	-	-	-	-	3	2.3	-	-	1	0.3
Pleosporales	Montagnulaceae	*Paraphaeosphaeria*	sp.	-	-	1	0.3	-	-	-	-	-	-	-	-
Eurotiales	Trichocomaceae	*Penicillium*	sp.	6	3.3	10	9.7	12	15.7	16	29.0	15	40.7	9	15.3

Microascales	Microascaceae	*Petriella*	*guttulata*	-	-	3	2.0	-	-	2	0.7	-	-	-	-
Hypocreales	Bionectriaceae	*Stromatonectria*	sp.	-	-	1	0.7	-	-	-	-	-	-	-	-
Eurotiales	Trichocomaceae	*Talaromyces*	*assiutensis*	-	-	2	1.3	-	-	-	-	1	0.3	-	-
Hypocreales	Hypocreaceae	*Trichoderma*	sp.	17	70.0	17	25.3	20	64.7	18	36.7	15	34.0	8	22.7
Xylariales	Amphisphaeriaceae	*Truncatella*	*angustata*	-	-	1	1.0	-	-	-	-	-	-	-	-
		Unknown		2	3.7	2	1.7	-	-	-	-	2	0.6	-	-

[a]Number of plants from which each fungi was isolated. In each treatment and zone of the plants 20 plants were analyzed.

[b]Percentage of wood fragments from which fungal colonies were isolated. In each treatment and zone of the plants 300 wood fragments were analyzed (20 grafted plants, with 15 fragments per plant).

Table 2

Czech plants analyzed after one growing season. Number of plants and percentage of wood fragments from which the different fungi were isolated for the different treatments (control, 50 ºC for 30 min. and 53 ºC for 30 min.) and plant zones (grafting area or base of the rootstock).

				Control				50ºC 30 min				53ºC 30 min			
				Grafting area		Rootstock		Grafting area		Rootstock		Grafting area		Rootstock	
Order	**Family**	**Genus**	**Species**	**No.[a]**	**%[b]**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Hypocreales	?	*Acremonium*	sp.	-	-	-	-	1	0.3	-	-	-	-	1	0.3
Agaricales	Stophariaceae	*Agrocybe*	*praecox*	1	0.3	-	-	-	-	-	-	-	-	-	-
Pleosporales	Pleosporaceae	*Alternaria*	sp.	13	13.7	3	2.0	1	0.3	4	3.3	-	-	3	1.3
Eurotiales	Trichocomaceae	*Aspergillus*	sp.	-	-	1	1.7	-	-	-	-	1	1.0	1	0.3
Dothideales	Dothioraceae	*Aureobasidium*	sp.	-	-	-	-	-	-	-	-	-	-	1	1.0
Hypocreales	Bionectriaceae	*Bionectria*	sp.	1	3.0	-	-	-	-	-	-	-	-	-	-
Eurotiales	Trichocomaceae	*Byssochlamys*	*spectabilis*	2	1.7	1	0.3	-	-	-	-	-	-	-	-
Helotiales	?	*Cadophora*	*malorum*	2	1.7	1	0.3	-	-	-	-	-	-	-	-
Sordariales	Chaetomiaceae	*Chaetomium*	*globosum*	-	-	1	2.0	-	-	-	-	-	-	-	-
Capnodiales	Cladosporiaceae	*Cladosporium*	sp.	-	-	-	-	-	-	-	-	-	-	2	0.7
Sordariales	Coniochaetaceae	*Coniochaeta*	*hoffmannii*	5	5.0	1	0.3	1	1.7	4	2.3	4	2.0	6	2.7
Sordariales	Coniochaetaceae	*Coniochaeta*	sp.	-	-	-	-	-	-	1	0.3	1	2.0	1	0.3
Hypocreales	Nectriaceae	*Dactylonectria*	*torresensis*	1	0.3	5	3.7	-	-	1	1.0	-	-	4	2.0
Diaporthales	Diaporthaceae	*Diaporthe*	*ampelina*	-	-	1	0.3	-	-	-	-	-	-	-	-
Diaporthales	Diaporthaceae	*Diaporthe*	sp.	5	6.3	2	2.0	1	0.3	6	12.7	-	-	-	-
Diversisporales	Acaulosporaceae	*Entrophospora*	sp.	-	-	-	-	-	-	-	-	-	-	1	0.3
Hypocreales	Nectriaceae	*Fusarium*	sp.	2	2.0	2	4.0	15	24.3	12	11.7	11	17.0	9	4.7
Hypocreales	Hypocreaceae	*Gliocladium*	sp.	3	2.3	-	-	1	0.3	-	-	1	2.0	-	-
Hypocreales	Bionectriaceae	*Gliomastix*	sp.	1	0.3	-	-	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Ilyonectria*	*liriodendri*	-	-	3	5.3	-	-	-	-	-	-	-	-
Sordariales	Coniochaetaceae	*Lecythophora*	sp.	-	-	-	-	-	-	2	1.0	-	-	3	1.0
Pleosporales	Massarinaceae	*Massarina*	sp.	-	-	-	-	-	-	-	-	-	-	1	2.3
Mortierellales	Mortierellaceae	*Mortierella*	sp.	-	-	-	-	-	-	1	0.7	-	-	-	-
Hypocreales	Nectriaceae	*Neonectria*	sp.	-	-	1	0.3	-	-	-	-	-	-	1	1.0
Ophiostomatales	Ophiostomataceae	*Ophiostoma*	sp.	1	1.7	-	-	-	-	-	-	-	-	-	-

Pleosporales	Montagnulaceae	*Paraphaeosphaeria*	*sporulosa*	1	0.3	2	1.0	-	-	-	-	-	-	-	-
Eurotiales	Trichocomaceae	*Penicillium*	sp.	12	13.7	12	13.7	16	23.0	14	12.3	19	34.7	18	28.0
Microascales	Microascaceae	*Petriella*	sp.	-	-	-	-	1	1.0	2	3.0	-	-	-	-
Togniniales	Togniniaceae	*Phaeoacremonium*	*croatiense*	17	70.0	17	25.3	20	64.7	18	36.7	15	34.0	8	22.7
Togniniales	Togniniaceae	*Phaeoacremonium*	*fraxinopennsylvanicum*	-	-	1	1.0	-	-	-	-	-	-	-	-
Togniniales	Togniniaceae	*Phaeoacremonium*	*minimum*	2	3.7	2	1.7	-	-	-	-	2	0.6	-	-
Togniniales	Togniniaceae	*Phaeoacremonium*	*scolyti*	1	0.3	1	0.3	1	0.3	-	-	2	0.7	1	0.7
Pleosporales	Didymellaceae	*Phoma*	sp.	1	0.3	-	-	-	-	-	-	-	-	-	-
Pleosporales	Venturiaceae	*Protoventuria*	*alpina*	7	3.3	6	5.3	-	-	-	-	-	-	-	-
Agaricales	Psathyrellaceae	*Psathyrella*	sp.	1	1.0	1	0.3	-	-	-	-	-	-	-	-
Microascales	Microascaceae	*Scedosporium*	*dehoogii*	-	-	-	-	-	-	1	0.3	-	-	-	-
Eurotiales	Trichocomaceae	*Talaromyces*	sp.	-	-	-	-	-	-	1	0.3	-	-	-	-
Hypocreales	Hypocreaceae	*Thichoderma*	sp.	3	1.0	6	5.0	20	34.0	16	25.7	18	32.0	19	33.3
Xylariales	Amphisphaeriaceae	*Truncatella*	*angustata*	-	-	1	0.3	-	-	-	-	-	-	-	-
		Unknown		4	4.0	4	2.6	1	0.3	-	-	-	-	-	-

[a]Number of plants from which each fungi was isolated. In each treatment and zone of the plants 20 plants were analyzed.

[b]Percentage of wood fragments from which fungal colonies were isolated. In each treatment and zone of the plants 300 wood fragments were analyzed (20 grafted plants, with 15 fragments per plant).

Table 3

Spanish analyzed immediately after hot-water treatment. Number of plants and percentage of wood fragments from which the different fungi were isolated for the different treatments (control, 50 ºC for 30 min. and 53 ºC for 30 min.) and plant zones (grafting area or base of the rootstock).

				Control				**50ºC 30 min**				**53ºC 30 min**			
				Grafting area		**Rootstock**		**Grafting area**		**Rootstock**		**Grafting area**		**Rootstock**	
Order	**Family**	**Genus**	**Species**	**No.**[a]	**%**[b]	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Hypocreales	?	*Acremonium*	sp.	5	4.3	4	2.3	7	12.3	10	6.3	7	9.7	4	1.7
?	?	*Acrocalymna*	sp.	-	-	1	0.3	-	-	1	0.3	-	-	1	0.3
?	?	*Acrocalymna*	*vagum*	-	-	1	0.3	-	-	-	-	-	-	-	-
Pleosporales	Pleosporaceae	*Alternaria*	sp.	1	2.7	-	-	1	0.7	2	0.7	-	-	-	-
-	-	Ascomycota	sp.	-	-	-	-	-	-	1	0.3	-	-	-	-
Eurotiales	Trichocomaceae	*Aspergillus*	*amstelodami*	-	-	1	0.3	-	-	5	2.7	-	-	2	1.0
Eurotiales	Trichocomaceae	*Aspergillus*	*ochraceus*	-	-	1	0.3	2	0.7	-	-	-	-	-	-
Eurotiales	Trichocomaceae	*Aspergillus*	*ruber*	1	0.3	-	-	-	-	1	0.3	-	-	-	-
Eurotiales	Trichocomaceae	*Aspergillus*	sp.	-	-	-	-	-	-	1	0.3	2	1.3	1	0.3
Atheliales	Atheliaceae	*Athelia*	*bombacina*	2	1.7	-	-	-	-	-	-	-	-	1	1.3
Hypocreales	Bionectriaceae	*Bionectria*	*ochroleuca*	2	3.3	-	-	-	-	-	-	-	-	-	-
Hypocreales	Bionectriaceae	*Bionectria*	sp.	-	-	-	-	2	0.7	-	-	-	-	-	-
Helotiales	?	*Cadophora*	*luteo-olivacea*	4	4.7	3	3.0	2	0.7	1	0.3	6	6.3	2	1.0
Microascales	Microascaceae	*Cephalotrichum*	*microsporum*	1	1.0	-	-	1	1.0	-	-	-	-	-	-
Capnodiales	Mycophaerellaceae	*Cercospora*	*sophorae*	-	-	1	0.3	-	-	-	-	-	-	-	-
Sordariales	Chaetomiaceae	*Chaetomium*	sp.	-	-	-	-	-	-	-	-	1	0.3	-	-
Capnodiales	Cladosporiaceae	*Cladosporium*	sp.	-	-	-	-	1	0.3	1	0.7	1	0.3	-	-
Hypocreales	Bionectriaceae	*Clonostachys*	*rosea*	5	8.0	6	5.0	3	2.7	3	1.0	1	0.3	-	-
Glomerellales	Glomerellaceae	*Colletotrichum*	sp.	1	2.0	-	-	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Dactylonectria*	*macrodidyma*	-	-	3	1.0	-	-	-	-	-	-	-	-
Diaporthales	Diaporthaceae	*Diaporthe*	*neotheicola*	2	3.0	-	-	-	-	-	-	-	-	-	-
Botryosphaeriales	Botryosphaeriaceae	*Diplodia*	*seriata*	7	9.3	1	2.7	-	-	-	-	1	0.3	-	-
Eurotiales	Trichocomaceae	*Eurotium*	sp.	-	-	-	-	1	0,3	-	-	-	-	1	0.3
Hypocreales	Nectriaceae	*Fusarium*	sp.	7	16.3	11	10.7	9	13.7	15	13.3	14	27.7	14	14.3
?	Myxotrichaceae	*Geomyces*	sp.	-	-	-	-	-	-	-	-	-	-	1	0,7

Sodariales	Chaetomiaceae	*Humicola*	sp.	-	-	-	-	-	-	1	0.3	-	-	1	0.3
Hypocreales	Nectriaceae	*Ilyonectria*	*liriodendri*	-	-	3	1.7	-	-	-	-	-	-	-	-
Pleosporales	Lophiostomataceae	*Lophiostoma*	sp.	2	2.3	1	0.3	-	-	5	3.0	1	2.0	-	-
Mucorales	Mucoraceae	*Mucor*	*racemosus*	1	0.3	-	-	-	-	-	-	-	-	-	-
Mucorales	Mucoraceae	*Mucor*	sp.	-	-	2	1.0	6	4.7	3	1.0	3	1.7	2	0.7
Botryosphaeriales	Botryosphaeriaceae	*Neofusicoccum*	*parvum*	-	-	-	-	2	1.7	-	-	-	-	-	-
Hypocreales	Ophiocordycipitaceae	*Ophiocordyceps*	*sinensis*	-	-	1	0.3	-	-	-	-	-	-	-	-
Hypocreales	Nectriaceae	*Paracremonium*	*inflatum*	-	-	-	-	-	-	-	-	-	-	1	0.3
Eurotiales	Trichocomaceae	*Penicillium*	*cinnamopurpureum*	-	-	-	-	-	-	1	0.3	-	-	2	1.0
Eurotiales	Trichocomaceae	*Penicillium*	sp.	3	5.0	9	5.0	7	3.0	8	5.0	8	3.3	9	5.0
Togniniales	Togniniaceae	*Phaeoacremonium*	*fraxinopennsylvanicum*	2	3.3	-	-	1	0,7	-	-	1	4,3	-	-
Togniniales	Togniniaceae	*Phaeoacremonium*	*minimum*	3	5.3	9	10.0	4	11.7	2	4.3	1	1.3	1	0.7
Phaeomoniellales	Phaeomoniellaceae	*Phaeomoniella*	*chlamydospora*	-	-	3	4.0	-	-	-	-	-	-	-	-
Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	sp.	-	-	-	-	1	0.3	-	-	-	-	-	-
Hypocreales	Hypocreaceae	*Trichoderma*	sp.	-	-	1	1.0	-	-	-	-	-	-	-	-
		Unknown		1	0.3	7	4.0	2	0.7	1	1.0	-	-	-	-
Mucorales	Mucoraceae	*Umbelopsis*	*isabellina*	-	-	1	0.3	-	-	-	-	-	-	4	1.7

[a]Number of plants from which each fungi was isolated. In each treatment and zone of the plants 20 plants were analyzed.

[b]Percentage of wood fragments from which fungal colonies were isolated. In each treatment and zone of the plants 300 wood fragments were analyzed (20 grafted plants, with 15 fragments per plant).

Table 4

Spanish plants analyzed after one growing season. Number of plants and percentage of wood fragments from which the different fungi were isolated for the different treatments (control, 50 ºC for 30 min. and 53 ºC for 30 min.) and plant zones (grafting area or base of the rootstock).

				Control				50ºC 30 min				53ºC 30 min			
				Grafting area		Rootstock		Grafting area		Rootstock		Grafting area		Rootstock	
Order	Family	Genus	Species	No.[a]	%[b]	No.	%	No.	%	No.	%	No.	%	No.	%
Hypocreales	?	*Acremonium*	*sclerotigenum*	1	1.7	-	-	10	7.7	1	1.0	10	10.0	3	2.7
Pleosporales	Pleosporaceae	*Alternaria*	sp.	6	7.3	2	1.3	3	2.0	4	2.7	-	-	-	-
Eurotiales	Trichocomaceae	*Aspergillus*	sp.	1	4.7	1	0.3	1	0.3	1	0.3	1	0.3	-	-
Botryosphaeriales	Botryosphaeriaceae	*Botryosphaeria*	*dothidea*	-	-	-	-	-	-	-	-	2	4.3	-	-
Helotiales	?	*Cadophora*	*luteo-olivacea*	1	0.3	-	-	-	-	-	-	1	0.3	1	0.3
Helotiales	?	*Cadophora*	*malorum*	1	1.3	-	-	1	0.3	-	-	-	-	-	-
Capnodiales	Cladosporiaceae	*Cladosporium*	sp.		-		-	2	0.7	3	1.3	-	-	2	2.0
Hypocreales	Bionectriaceae	*Clonostachys*	sp.	8	13.0	6	5.3	1	3.0	-	-	1	1.0	-	-
Agaricales	Psathyrellaceae	*Coprinellus*	*disseminetus*	-	-	1	0.3	-	-	4	2.0	-	-	-	-
Hypocreales	Nectriaceae	*Dactylonectria*	*alcacerensis*	-	-	1	0.3	-	-	1	0.3	1	0.3	1	0.3
Hypocreales	Nectriaceae	*Dactylonectria*	*torresensis*	-	-	7	8.0	-	-	-	-	-	-	-	-
Diaporthales	Diaporthaceae	*Diaporthe*	*ampelina*	2	2.0	-	-	1	0.3	-	-	1	0.7	-	-
Botryosphaeriales	Botryosphaeriaceae	*Diplodia*	*seriata*	-	-	-	-	5	6.7	1	0.3	-	-	-	-
Hypocreales	Nectriaceae	*Fusarium*	sp.	15	27.7	15	35.3	18	43.7	18	37.3	17	42.3	16	56.3
Helotiales	Helotiaceae	*Idriella*	*lunata*	-	-	-	-	-	-	1	0.3	-	-	-	-
Pleosporales	Lophiostomataceae	*Lophiostoma*	sp.	-	-	1	1.3	-	-	-	-	-	-	-	-
Mortierellales	Mortierellaceae	*Mortierella*	*alpina*	3	2.,7	7	7.3	-	-	1	2.3	1	0.3	1	1.0
Mucorales	Mucoraceae	*Mucor*	sp.	1	0.3	3	3.7	-	-	2	0.7	-	-	2	1..7
Botryosphaeriales	Botryosphaeriaceae	*Neofusicoccum*	*parvum*	12	14.3	1	1.3	-	-	-	-	-	-	-	-
Togniniales	Togniniaceae	*Phaeoacremonium*	*minimum*	5	3.3	2	1.3	-	-	7	4.7	3	3.0	3	1.3
Phaeomoniellales	Phaeomoniellaceae	*Phaeomoniella*	*chlamydospora*	2	1.0	1	2.3	-	-	1	2.0	1	2.0	2	1.0
Eurotiales	Trichocomaceae	*Penicillium*	sp.	2	1.7	5	2.7	4	3.3	4	2.0	7	6.3	5	2.7
Calosphaeriales	Calosphaeriaceae	*Pleurostoma*	*richardsiae*	-	-	-	-	1	0.3	-	-	-	-	-	-
Agaricales	Psathyrellaceae	*Psathyrella*	sp.	-	-	-	-	-	-	2	1.7	-	-	2	1.0
Microascales	Microascaceae	*Pseudallescheria*	*ellipsoidea*	-	-	-	-	-	-	1	2.7	-	-	-	-

Cantharellales	Ceratobasidiaceae	*Rhizoctonia*	sp.	-	-	-	-	-	-	-	-	2	0.7	1	0.3
Mucorales	Mucoraceae	*Rhizopus*	*kiliense*	-	-	-	-	-	-	-	-	1	4.7	-	-
Hypocreales	?	*Sarocladium*	sp.	-	-	-	-	-	-	-	-	1	1.0	-	-
Helotiales	?	*Scytalidium*	sp.	-	-	3	5.3	2	4.7	4	1.3	1	2.7	1	4.7
Hypocreales	Hypocreaceae	*Trichoderma*	sp.	1	0.7	1	0.3	1	0.7	4	4.3	-	-	2	4.0
		Unknown		-	-	1	0.3	-	-	-	-	-	-	4	1.7

[a]Number of plants from which each fungi was isolated. In each treatment and zone of the plants 20 plants were analyzed.

[b]Percentage of wood fragments from which fungal colonies were isolated. In each treatment and zone of the plants 300 wood fragments were analyzed (20 grafted plants, with 15 fragments per plant).

Table 5
Taxa responsible for the differences among grapevine genotypes at the genus level (Bonferroni-corrected single ANOVA, *P*<0.01).

	Sauvignon Blanc/SO4	Tintorera/110R
NGS approach		
Aspergillus	3.1%	0.01%
Cadophora	1.2%	14.6%
Cladosporium	3.6%	0.1%
Coniochaeta	19.8%	0.1%
Diaporthe	3.6%	0.4%
Fusarium	5.4%	13.9%
Phaeoacremonium	7.1%	24.9%
Psathyrella	5.8%	1.4%
Sterigmatosporium	3.7%	0%
Trichoderma	2.9%	0.8%
ISOLATION approach		
Acremonium	0.2%	8.3%
Cadophora	0.5%	2.6%
Fusarium	13.6%	47.3%
Mortierella	0.1%	1.9%
Mucor	0.1%	2.2%
Neofusicoccum	0%	2.4%
Penicillium	33.5%	6.5%
Phaeoacremonium	1.2%	7.6%
Phaeomoniella	0%	1.7%
Trichoderma	34.9%	2.4%

Fig. 1. Species richness (**a**), Shannon diversity (**b**) and Pielou's eveness (**c**) of the fungal communities of Czech (CR) and Spanish (SP) planting material analysed by Next-Generation Sequencing (NGS) and traditional isolation (ISO) with the confidence limits. Asterisks denote significant differences with respect to the control ($P < 0.05$). Data on ordinate axis synthesis average values of five replicates of four plants per treatment (Control: no HWT; T50: HWT at 50ºC; T53: HWT at 53ºC).

Fig 2. Non-metric multidimensional scaling (NMDS) of the fungal communities colonising the graft union (Graft) or the base of the rootstock (Root) in Czech (Sauvignon Blanc/SO4) and Spanish (Garnacha Tintorera/110R) planting material analysed by next-generation sequencing (**a**) and traditional isolation (**b**).

Fig. 3. Non-metric multidimensional scaling (NMDS) of the fungal communities colonising the Czech planting material analysed by next-generation sequencing (**a**) or traditional isolation (**c**), and the Spanish planting material analysed by next-generation sequencing (**b**) or traditional isolation (**d**) after no hot-water treatment (C), HWT at 50ºC (T50) and HWT at 53ºC (T53). Plants were analysed after HWT (AT) and after one-growing season (AO).

Fig. 4. Relative abundances of the dominant fungal orders of three treatments (Control: C; HWT at 50ºC: T50; HWT at 53ºC: T53) applied to Czech planting material analysed by next-generation sequencing (**a**) or traditional isolation (**c**), and the Spanish planting material analysed by next-generation sequencing (**b**) or traditional isolation (**d**). Plants were analysed after HWT (AT) and after one-growing season (AO).

Fig. 5. Distribution of the number of OTUs representing fungal trunk disease genera obtained by next-generation sequencing in the Czech (**a**) and Spanish (**b**) planting material after no HWT (Control), HWT at 50ºC (T50) and HWT at 53ºC (T53).

Fig. 6. Distribution of the number of isolates representing fungal trunk disease genera obtained by traditional isolation in the Czech (**a**) and Spanish (**b**) planting material after no HWT (Control), HWT at 50ºC (T50) and HWT at 53ºC (T53).

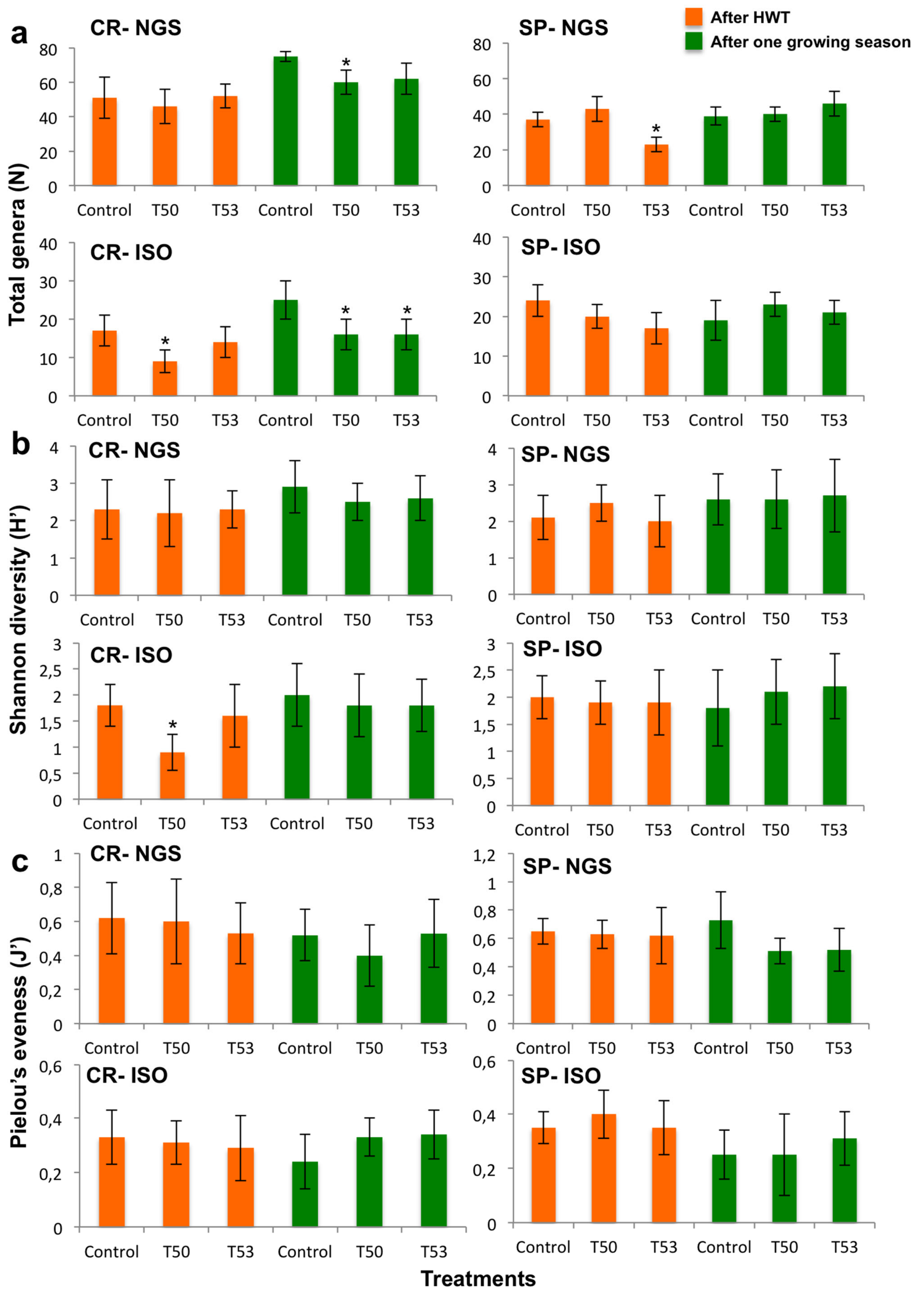

Fig 1

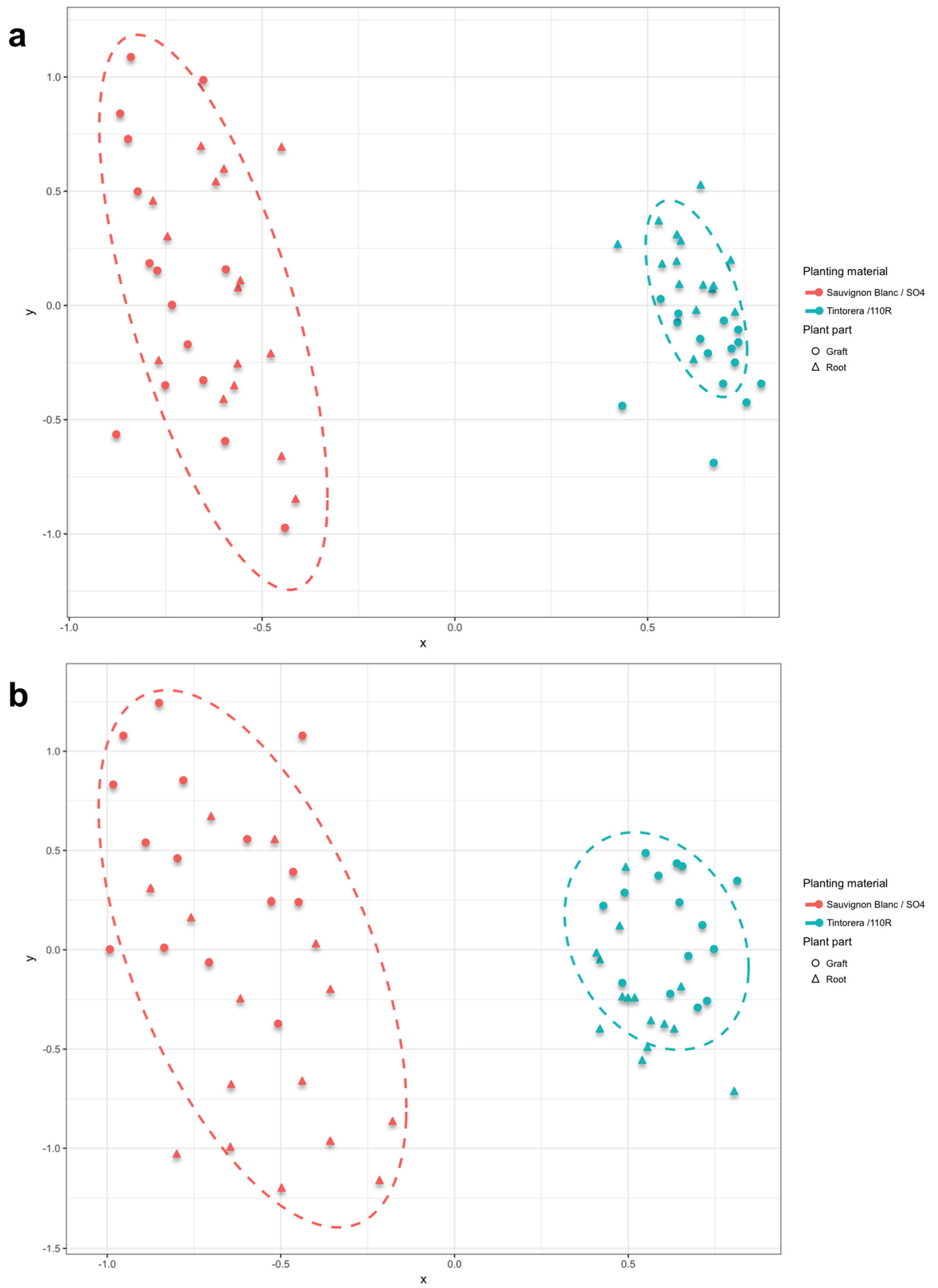

Fig. 2

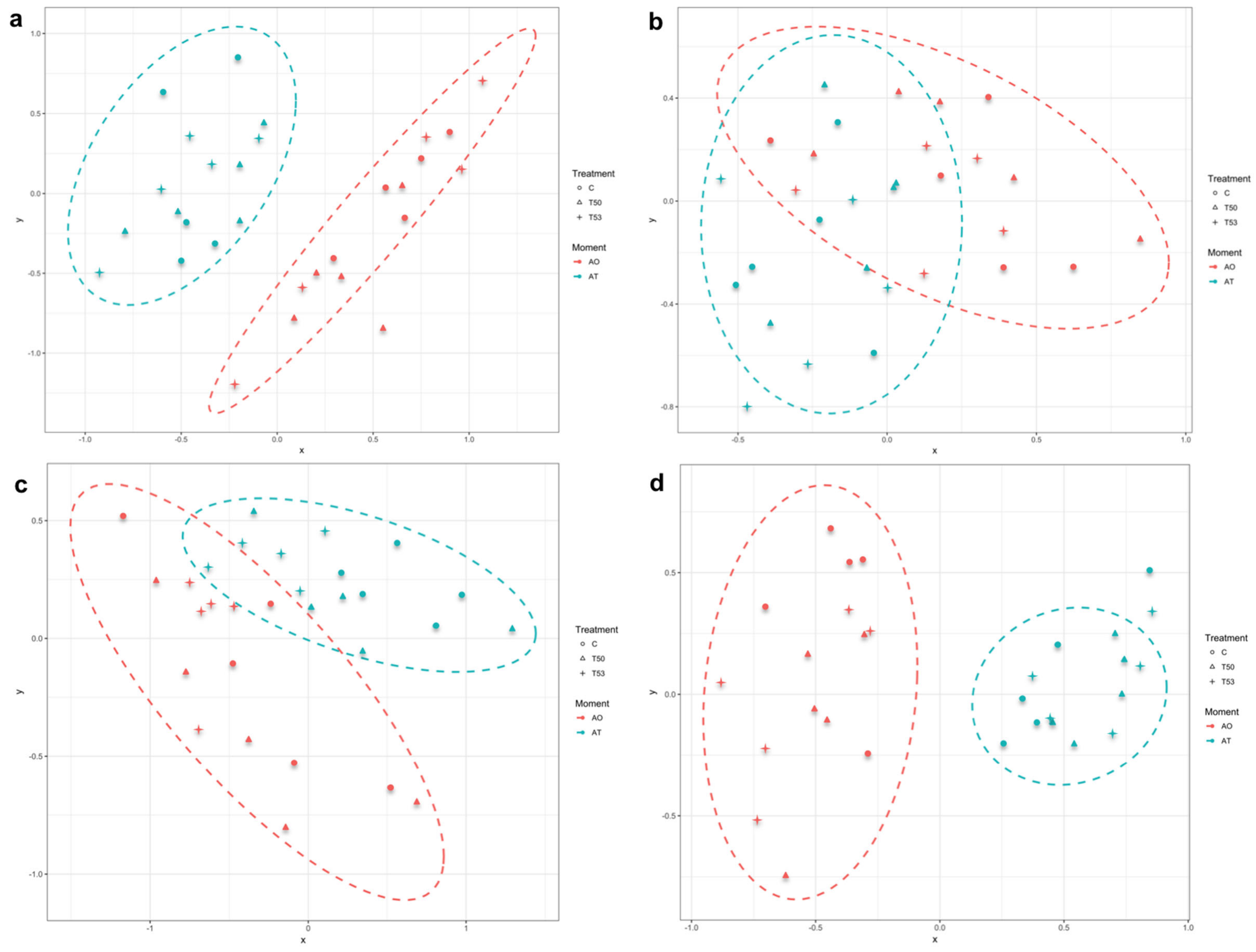

Fig. 3

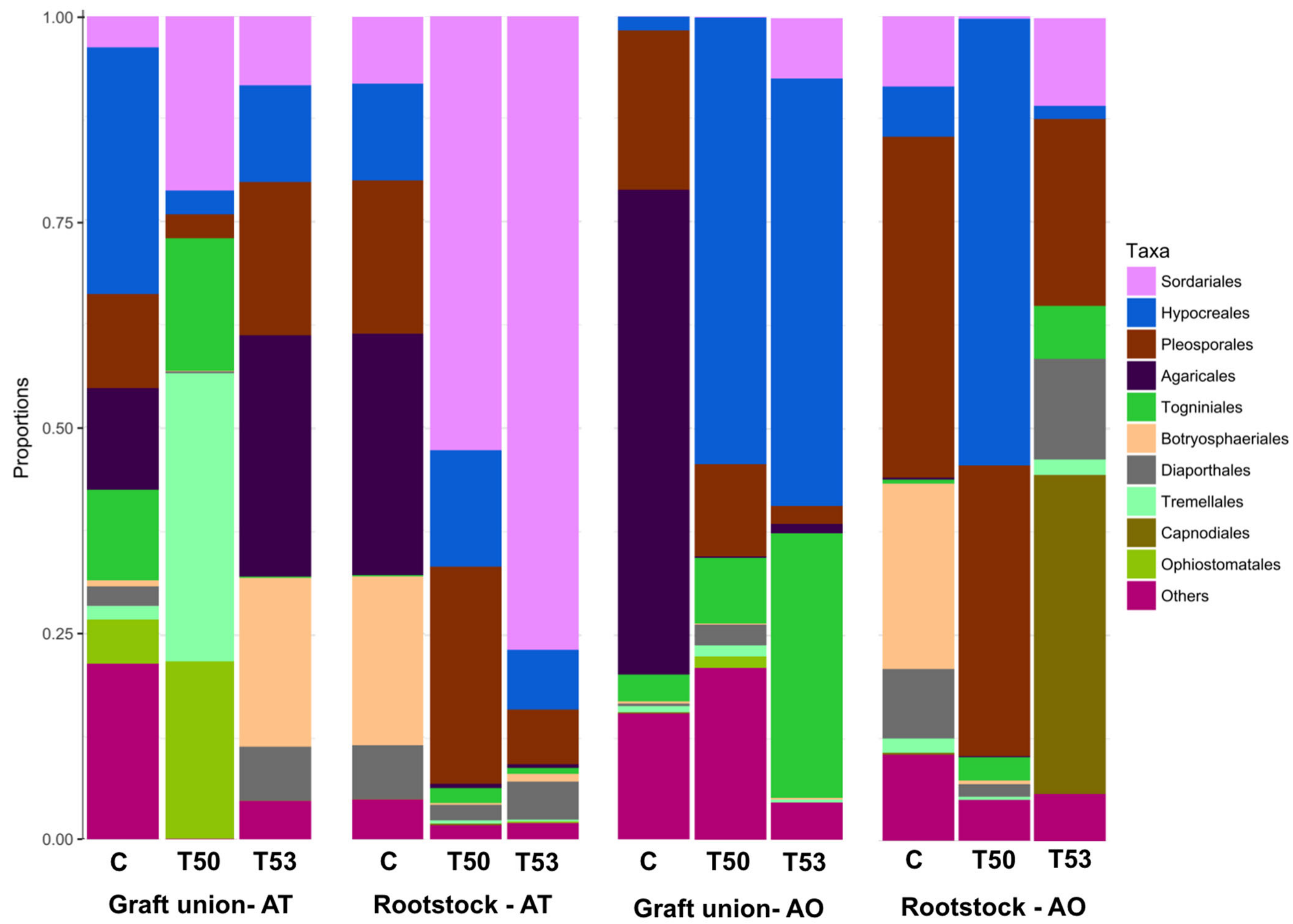

Fig 4a

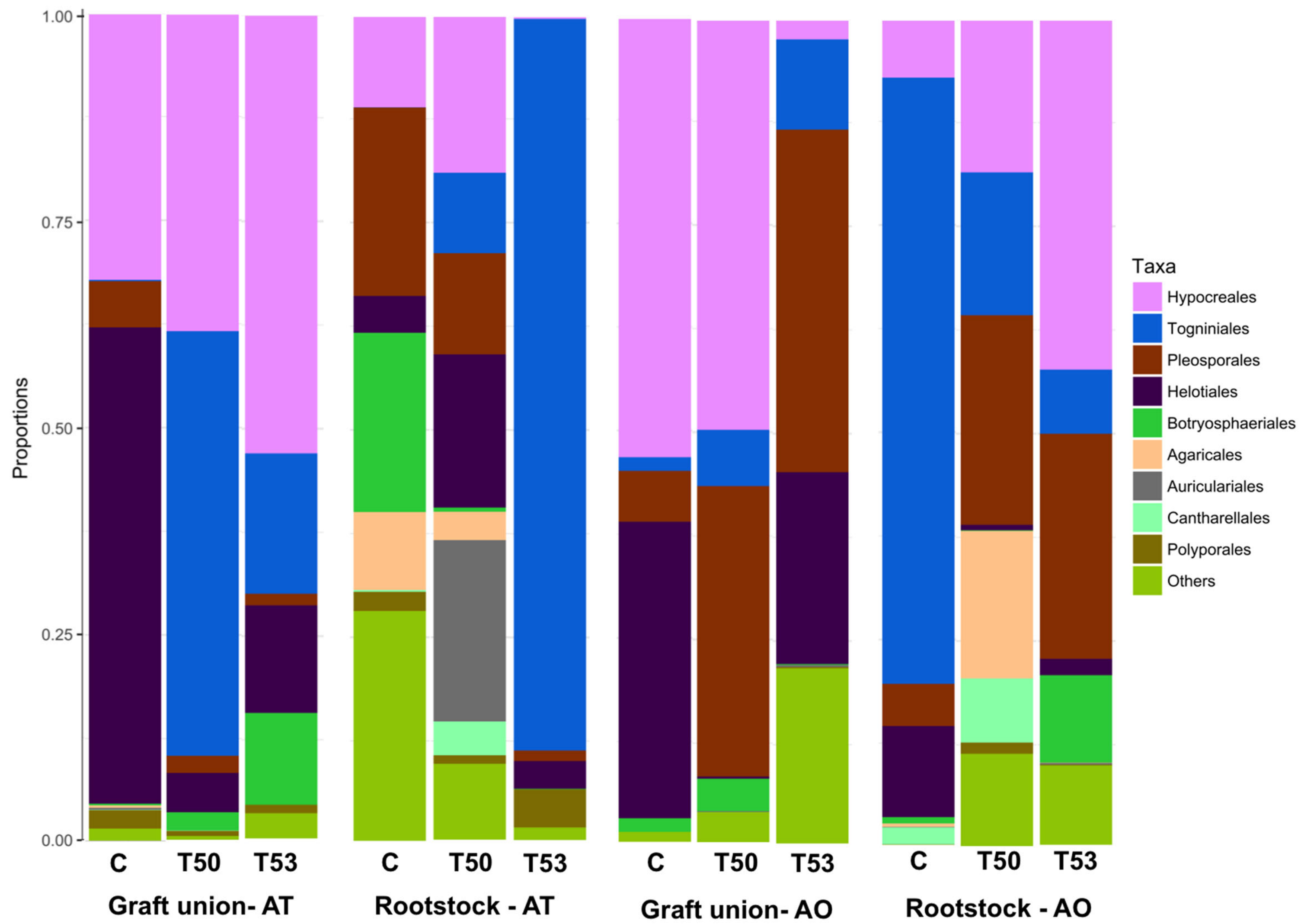

Fig. 4b

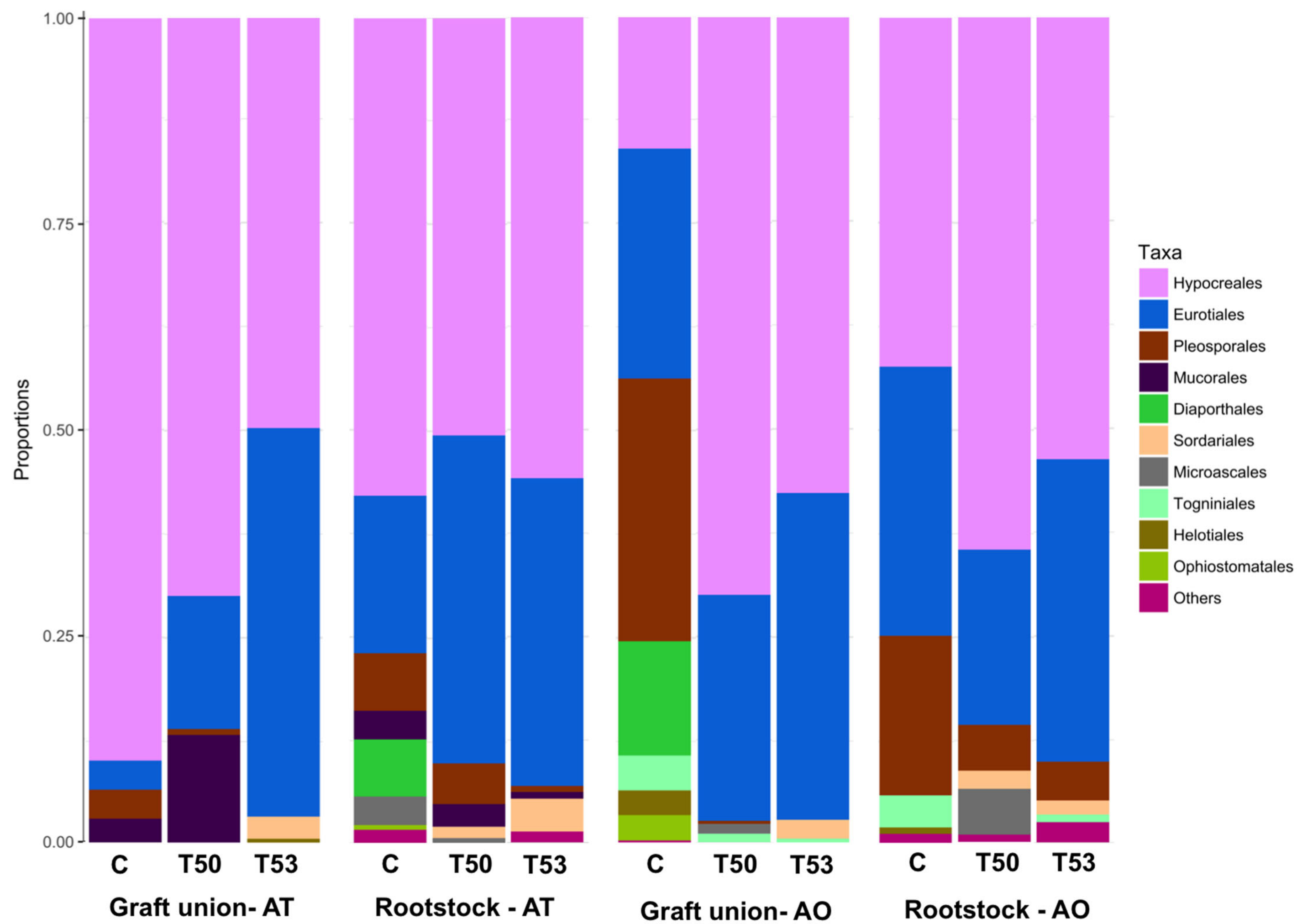

Fig. 4c

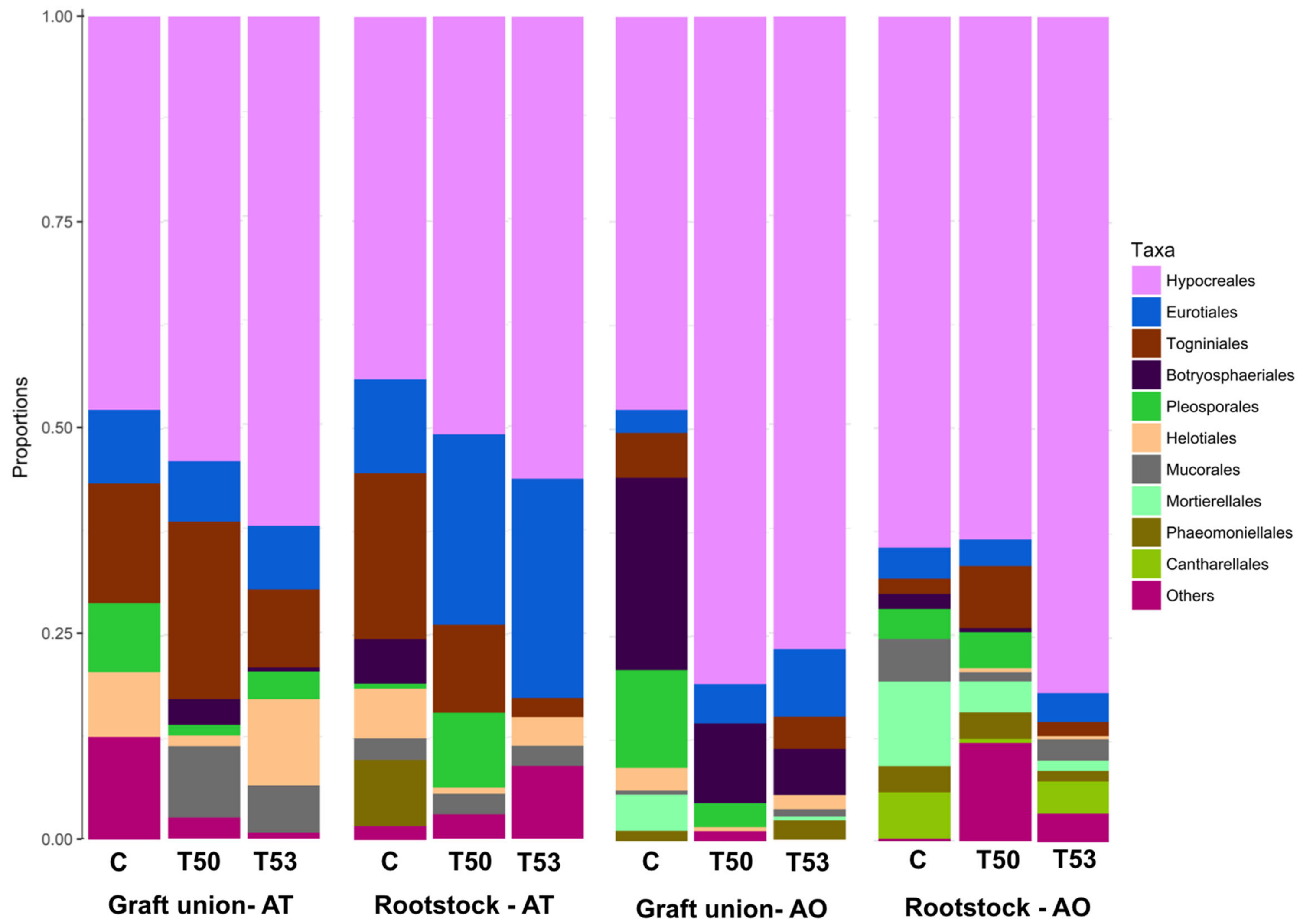

Fig. 4d

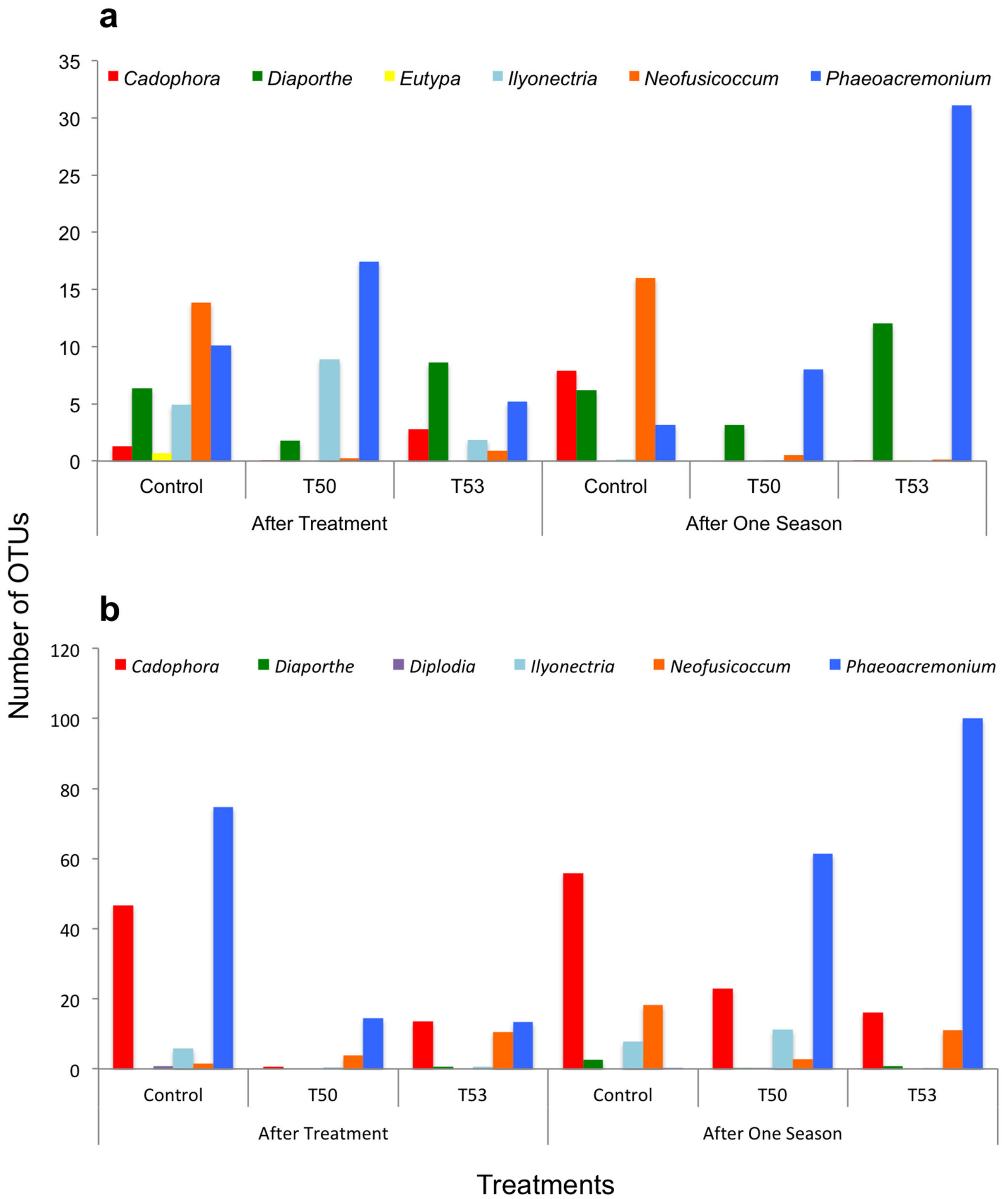

Fig. 5

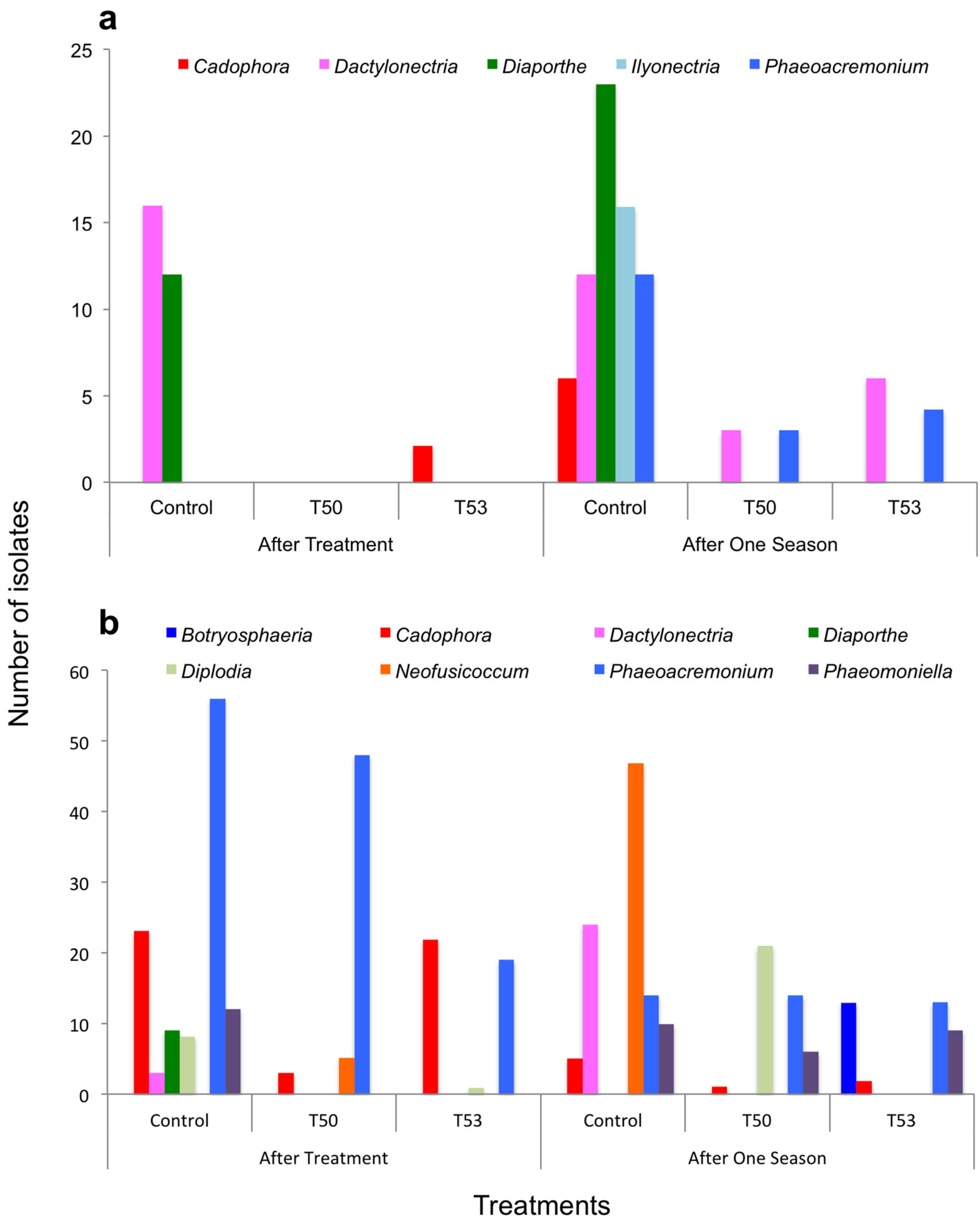

Fig. 6