REGIONAL-SCALE ANALYSIS OF ARBUSCULAR MYCORRHIZAL FUNGI: THE CASE OF BURGUNDY VINEYARDS

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Abstract

Aim: To improve knowledge of arbuscular fungal communities for a sustainable management in vineyards.

Methods and results: In 16 plots across Burgundy under contrasted soil properties and agricultural practices, we assessed arbuscular mycorrhizal fungal (AMF) diversity in vine roots, using pyrosequencing of ribosomal Internal Transcribed Spacers (ITS). AMF sequences could be retrieved from all plots across Burgundy, both in organic and in conventional vineyards with high chemical inputs. Sequences from the survey were almost exclusively affiliated to molecular taxa in the Glomerales, including six "core species" found in all plots, corresponding to 77% of all sequences, suggesting a relatively low species diversity in vine roots. A large part of the molecular taxa had no close similarity to previously-reported sequences.

Conclusion: AMF diversity observed in vine roots was relatively low and a significant proportion of molecular taxa shared between the sites. Nevertheless, some differences in the AMF community composition were observed between the plots.

Significance and impact of the study: This is the first large-scale study of AMF diversity in French vineyards using high-throughput sequencing, which will contribute to a better understanding of ecology of these fungi in vine roots, thus providing essential knowledge for future applications in sustainable agriculture in vineyards

Key words: arbuscular mycorrhiza, high-throughput sequencing, agroecology, Burgundy, internal transcribed spacers (ITS)

Objectif : Améliorer les connaissances sur la communauté des champignons mycorhiziens à arbuscules pour une gestion durable des vignes.

Résumé

Méthodes et résultats : Nous avons évalué la diversité des champignons mycorhiziens à arbuscules (CMA) dans les racines de vignes, en utilisant un pyroséquençage basé sur le marqueur ITS (espaceurs internes transcrits), dans 16 parcelles à travers la Bourgogne ayant des propriétés du sol et des pratiques culturales contrastées. Les résultats montrent que les CMA peuvent être trouvés dans toutes les parcelles de Bourgogne, aussi bien dans les parcelles biologiques que dans les vignes conventionnelles avec de hauts niveaux d'intrants chimiques. Les séquences de l'étude ont été presque exclusivement affiliées à des taxons moléculaires au sein des Glomerales, dont 6 espèces structurantes, c'est à dire des espèces trouvées dans toutes les parcelles, correspondant à 77% des séquences totales, suggérant ainsi une diversité relativement faible d'espèces dans les racines de vigne. Une grande partie des taxons moléculaires n'avaient pas de similitude avec des séquences précédemment décrites.

Conclusion : La diversité observée était relativement faible et une proportion significative de taxons moléculaires étaient communs entre les sites. Néanmoins, quelques différences dans la composition de la communauté des CMA ont été observées entre les parcelles.

Signification et impact de l'étude : Il s'agit de la première analyse à grande échelle de la diversité des CMA dans les vignobles français en utilisant un séquençage à haut débit, et cela contribue à mieux comprendre l'écologie de ces champignons dans les racines de vigne, fournissant ainsi une connaissance essentielle pour de futures applications pour une gestion durable des vignobles.

Mots clés : mycorhizes à arbuscules, séquençage haut débit, agroécologie, Bourgogne, espaceurs internes transcrits (ITS)

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INTRODUCTION

Vitis vinifera (L) is an economically important crop, in particular in some regions where vine production is a major economic resource. Grape production and quality is highly dependent on soil condition and composition (Renouf *et al*. 2010). However, soil quality and structure is affected by erosion caused by environmental factors like water-induced erosion (Quiquerez *et al.* 2008), but also by anthropogenic factors (Chevigny *et al.* 2014). The costs of erosion can counterbalance, to a non-negligible part, the income generated by the sale of wine, in particular because of the loss of N and P provided by chemical fertilization. In Burgundy, which is one of the major regions of vine production and exportation worldwide, soil erosion is a threat for this high-quality vine production. It is therefore essential to better understand the relationship between vines and their soil, in terms of physical, chemical and biological properties.

Arbuscular mycorrhiza is a widespread symbiosis involving 80 % of land plant species and arbuscular mycorrhizal fungi (AMF), which all belong to the monophyletic phylum Glomeromycota. AMF are well known for their potential in soil stabilization, which can be useful to limit soil erosion (Rillig and Mummey 2006). These fungi have beneficial effects on plants, enhancing nutrient uptake, in particular phosphate, or resistance against pathogens, including pathogenic fungi and nematodes well known to destroy a large amount of crops (Gianinazzi *et al.* 2010). They can potentially reduce the input of chemical fertilizers, but often are not taken into account in agriculture because of the still relatively poor understanding of their ecology in agricultural ecosystems.

In vineyards, AMF were shown to enhance plant performance, including nutrient uptake (Schreiner 2007), plant growth (Linderman and Davis 2001) or water use efficiency (Valentine *et al.* 2006). Previous studies have shown that plant age or genotype may influence AMF community composition, as well as soil properties or cultural practices (Gianinazzi *et al.* 2010; Peyret-Guzzon *et al*. 2016). Conversely, different AMF species may have a different impact on plant growth or health (Linderman and Davis 2001). However, a better understanding of the impact of biotic (plant diversity) and abiotic (soil management) factors on AMF composition in vineyards may contribute to improving wine production or quality through the ecosystem services they can provide (for review see Trouvelot *et al*. 2015).

In this project, in order to better understand AMF diversity in vineyards, the objectives were to (i) identify parameters influencing AMF communities in Burgundy vineyards and (ii) determine a possible core set of species commonly present in these vineyards. In this study, 16 vineyard plots were selected from those monitored by the Bureau

Table S1 - Summary of the 16 vineyard locations in Burgundy.

n	City	GPS position	Cultural practices	Rootstock	Grape variety
Y7b	Saint-Bris-le-Vineux	47°45'14"N, 3°38'38"E	Conventional	41B	Pinot noir
Y5	Chitry	47°45'16"N, 3°41'18"E	Conventional	41B	Chardonnay
Y6b	Saint-Cyr-les-Colons	47°45'3"N, 3°44'41"E	Conventional	41B	Pinot noir
Y8	Irancy	47°42'58"N, 3°40'9"E	Conversion	41B	Pinot noir
SL9	Igé	46°23'37"N, 4°44'49"E	Conventional	SO4	Chardonnay
SL12	Chaintré	46°15'50"N, 4°45'56"E	Conversion	SO4	Chardonnay
SL10	Vergisson	46°18'44"N, 4°43'22"E	Conventional	SO4	Chardonnay
SL8	Bissy-la-Mâconnaise	46°29'27"N, 4°47'20"E	Conventional	SO4	Chardonnay
CD3	Vosne-Romanée	47°9'10"N, 4°58'5"E	Organic	SO4	Pinot noir
CD4	Vosne-Romanée	47°9'39"N, 4°56'54"E	Organic	SO4	Pinot noir
CD13	Nuits-Saint-Georges	47°10'18"N, 4°56'15"E	Conventional	SO4	Pinot noir
CD16-1	Aloxe-Corton	47°4'9"N, 4°51'20"E	Conventional	161-49C	Pinot noir
CD16-2	Aloxe-Corton	47°4'15"N, 4°51'45"E	Conventional	161-49C	Pinot noir
CD10	Chassagne-Montrachet	46°55'32"N, 4°42'44"E	Organic	SO4	Chardonnay
CD17-1	Santenay	46°55'2"N, 4°41'57"E	Organic	161-49C	Chardonnay
CD17-2	Santenay	46°54'58"N, 4°41'55"E	Organic	161-49C	Chardonnay

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Interprofessionnel des Vins de Bourgogne (BIVB, Beaune, France), which are representative of soil properties and agricultural practices in Burgundy vineyards. AMF colonization levels were determined and diversity was analyzed in these plots using 454 pyrosequencing based on the ITS marker gene, designated as the universal barcode marker for Fungi (Schoch *et al.* 2012).

MATERIALS AND METHODS

1. Sites and sampling

Across Burgundy (France), 16 vineyard plots (planted between 1976 and 1987) were selected in 2013 among those monitored by the BIVB, in Yonne, Côte-d'Or and Saone-et-Loire (Figure 1, Tables S1 and S2). They were selected based on their agricultural practices (conventional, conversion or organic) and rootstock. At four sampling points in each plot, young thin roots of nine consecutive vines were collected. Root samples were washed with water to remove the soil. A part of each sample was set aside for mycorrhizal colonization estimation using the method described in Trouvelot *et al.* (1986). For each sample, mycorrhizal frequency (F%), intensity of mycorrhizal root colonization (M%), and relative arbuscular richness (A%) were calculated. The remaining root samples were stored at -80° until DNA extraction. Soil was sampled in triplicate for the 16 plots, and soil properties were analyzed in the Laboratoire d'Analyse des Sols d'Arras (France).

2. DNA extraction, PCR amplification and pyrosequencing

Root samples were ground in liquid nitrogen using mortar and pestle. Genomic DNA was extracted from 70mg of roots using a DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was eluted in 70 µl and 50 µl AE buffer and kept at -20 °C.

Samples were prepared for 454 pyrosequencing in a two-step PCR procedure in order to amplify the ITS 2 region used as gene marker. A first PCR was performed using 0.4 U of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, Courtaboeuf, France), 1x Phusion HF buffer, 0.5 µM of the primers SSUmCf and LSUmBr (Krüger *et al.* 2009), 0.2 mM of each dNTPs and 1µl of genomic DNA, in a final volume of 20 µl. The PCR conditions used were 5 min at 99 °C, 35 cycles of 10 s at 99 °C, 30s at 63 °C and 1min at 72 °C, followed by 10min at 72 °C, using an Eppendorf Mastercycler epgradient S (Vaudaux-Eppendorf, Schönenbuch, Switzerland). Each PCR product was checked on an agarose gel and diluted at 1/100 to be used as template in the nested PCR. The nested PCR was performed using

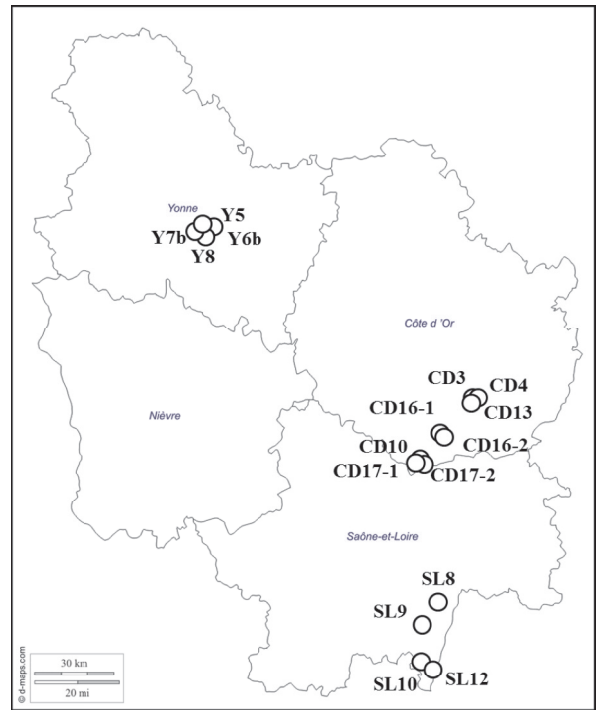
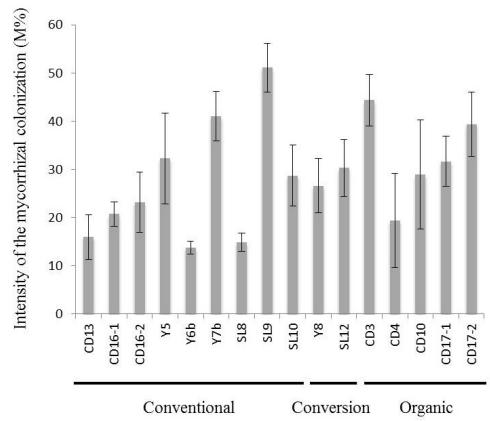


Figure 1 - Map with the location of the 16 vineyards in Burgundy.



Suppl. Figure 1 - Intensity of mycorrhizal colonization (M%) in the 16 plots studied. Error bars correspond to standard deviation.

1U of Phusion High Fidelity polymerase, 1x HF buffer, 0.5µM of the primers ITS3m (Zhong *et al.* 2010) and ITS4 (White *et al.* 1990) with 5-bp barcodes, 0.2 µM of each dNTPs and 2 µl of diluted PCR product, in a total volume of 50 µl. PCR conditions were 30 s at 98 °C, 30 cycles of 10 s at 98 °C, 30 s at 64 °C and 20s at 72 °C, followed by 10min at 72 °C, in an Eppendorf Mastercycler ep gradient S. PCR success was checked on an agarose

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Table S2 - Soil properties in the 16 plots across Burgundy.

	CD16-1	CD16-2	CD17-1	CD17-2	CD3	CD4	CD10	CD13	SL8	SL9	SL10	SL12	Y5	Y6b	Y7b	Y8
Clay ($g.kg^{-1}$)	328	358	302	313	314	338	475	466	418	411	457	263	519	542	451	416
Fine silt ($g.kg^{-1}$)	205	206	203	172	280	229	221	153	258	323	208	201	256	282	288	297
Coarse silt ($g.kg^{-1}$)	218	201	119	112	235	136	132	109	204	100	124	328	80	93	93	87
Fine sand ($g.kg^{-1}$)	141	121	111	100	81	68	74	64	66	44	148	124	37	20	47	49
Coarse sand ($g.kg^{-1}$)	108	114	265	303	90	229	98	208	54	122	63	84	108	63	121	151
Organic carbon ($g.kg^{-1}$)	16.1	13.4	16.1	18.2	16.8	16.6	14.5	47.1	16.5	24.7	16.2	12.6	23.6	27.2	30.6	17.7
Total nitrogen ($g.kg^{-1}$)	1.21	0.981	1.17	1.1	1.24	1.23	1.17	3.74	1.35	1.99	1.28	0.999	2.13	2.57	2.57	1.47
C/N ratio	13.3	13.7	13.7	16.6	13.6	13.5	12.4	12.6	12.2	12.4	12.6	12.6	11.1	10.6	11.9	12
Organic matter ($g.kg^{-1}$)	27.8	23.3	27.8	31.4	29.1	28.7	25.2	81.4	28.6	42.7	28	21.8	40.9	47.1	52.9	30.6
pH	7.99	8.02	8.12	8.06	8.02	8.06	8.11	7.84	8.07	8.09	7.96	7.63	8.06	7.96	7.95	8.09
Total $CaCO_3$ ($g.kg^{-1}$)	118	161	411	351	85	301	106	330	229	402	25	6	257	144	348	417
Active $CaCO_3$ ($g.100g^{-1}$)	2.29	2.66	5.63	3.59	2.35	4.15	2.04	8.97	6.31	18.1	<0.5	<0.5	9.53	5.63	12.3	13.1
P_2O_5 ($g.kg^{-1}$)	0.219	0.168	0.13	0.655	1.24	0.403	0.197	0.456	0.076	0.057	0.175	0.096	0.426	0.541	0.439	0.319
CEC ($cmol.kg^{-1}$)	20.5	18.9	17.5	18.3	21.4	23	25	31.5	23.9	23.4	20.9	15.9	27.4	32.2	28.3	21.6
Ca^{2+} ($cmol.kg^{-1}$)	20.7	21.2	17.7	18.4	21.1	22.9	24.4	30.9	23.9	23.9	21.6	16.1	26.8	32.2	28	21.7
Mg^{2+} ($cmol.kg^{-1}$)	0.991	0.576	0.558	0.647	0.72	0.85	1.14	1	0.807	1.05	0.964	0.892	1.43	1.35	1.43	0.903
Na^{+} ($cmol.kg^{-1}$)	0.0179	0.0168	0.0182	0.0208	0.0212	0.034	0.0314	0.019	0.0243	0.0703	0.0294	0.0285	0.0501	0.0391	0.0375	0.0257
K^{+} ($cmol.kg^{-1}$)	0.595	0.657	0.514	0.82	1.07	0.662	0.754	2.13	0.973	0.776	0.543	0.357	1.35	1.03	1.16	0.9
Fe^{2+} ($cmol.kg^{-1}$)	0.0109	0.0125	0.0108	0.015	0.0099	0.0106	0.0126	0.0059	<0.005	0.0134	0.0094	0.0171	0.0059	0.0116	<0.005	0.0129
Al^{3+} ($cmol.kg^{-1}$)	0.0292	0.0309	0.032	0.0622	0.0367	0.03	0.0489	<0.02	<0.02	0.0664	0.0238	0.0666	0.0205	0.0427	<0.02	0.0715
Mn ($mg.kg^{-1}$)	15.1	15.4	12.4	17.6	13.5	14.3	16.4	23.5	17.9	17.6	26.3	45.3	16.4	16.4	18.6	12.2
Zn ($mg.kg^{-1}$)	8.04	7.49	5.86	11.3	16.7	12.6	6.22	19.3	3.5	8.17	12.2	7.43	7.81	6.23	11.9	8.55
Si ($g.100g^{-1}$)	0.118	0.115	0.086	0.108	0.113	0.115	0.121	0.071	0.091	0.087	0.089	0.083	0.115	0.123	0.085	0.092
Total Cu ($mg.kg^{-1}$)	153	140	92.9	208	229	208	89.3	114	118	193	122	246	99.4	46.1	168	138
Total S ($mg.kg^{-1}$)	298	262	280	386	275	375	232	705	359	474	237	221	516	491	528	431

gel. Each DNA extract was amplified three times. The three PCR products, with a size around 400 bp, were pooled and purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Meylan, France) following the manufacturer's instructions. After quantification using the Quant-iT™ Picogreen® dsDNA Assay kit (Invitrogen, Life Technologies, Saint-Aubin, France), the purified PCR products were mixed equimolarly to prepare sequencing libraries. The libraries were sent to Genoscreen (Lille, France) for sequencing, using a 454 GS FLX Roche instrument. Raw data of 454 pyrosequencing were submitted to Sequence Read Archive within the Bioproject SRP070752.

3. Sequence and data analysis

Raw data were split in different fasta files after removal of primer and barcode sequences. The filtered sequences were trimmed and denoised using Mothur v.1.32.0 (Schloss *et al.* 2009). The resulting sequences were clustered using UCLUST algorithm of USEARCH (Edgar 2010) at 97 % identity threshold to create Operational Taxonomic Units (OTUs), and singletons were excluded from further analysis. Hence OTUs were assigned to taxonomy: firstly, a Blast search against UNITE database was performed (e-value=1.10^{-5}) and non-Glomeromycota sequences were excluded; Secondly, the EPA algorithm of RAxML (Berger *et al.* 2011) was used to correct and improve the taxonomic assignment of the OTUs to Molecular Taxa (MTs) at the species-level wherever possible. In each sample, 1200 sequences were subsampled from the total number of sequences obtained to avoid an overestimation of the diversity in some of the samples.

Rarefaction curves, diversity indices (Shannon) and richness indices (Chao1) of species-level MTs were obtained using EstimateS software v.9.0.0 (Colwell 2013). Statistic Shannon diversity differences among plots were tested using Kruskal-Wallis test in the stats package implemented in R software (version 2.15.1, R Core Team, 2013). Bray-Curtis distances were calculated from AMF abundance tables at the OTUs level and two MT-levels (genus and species). Variations of species-level MT abundances among plots were tested using a pairwise Wilcoxon test with Bonferroni correction from the stats package implemented in R software. Soil physical properties were log-transformed to normalize data, and differences among plots were statistically tested by ANOVA using stats package implemented in R software.

RESULTS AND DISCUSSION

1. Arbuscular mycorrhiza colonization rate

AMF colonization rates were calculated to analyze whether agricultural practices influence the proportion of mycorrhiza in vine roots. Intensity of mycorrhizal colonization, as well as the abundance of

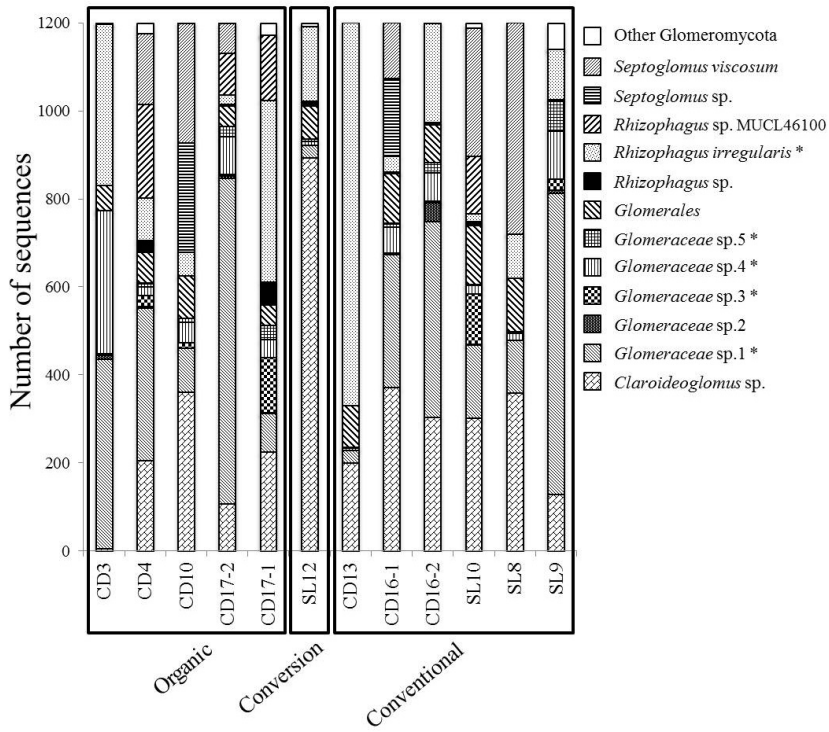


Figure 2 - Number of sequences found for the Glomeromycota taxa identified in each monitored plot. Species with statistically different number of sequences between plots are indicated with *, after Kruskal-Wallis test and P<0.05. Taxa were defined at species level wherever possible, and the box named "other Glomeromycota" corresponds to taxa containing less than 1% of the total number of sequences.

the arbuscules, which are the fungal organs active in plant-fungi exchanges, were calculated using the method described by Trouvelot *et al.* (1986), based on root staining and microscopy. Results showed that in all vineyard plots, arbuscular mycorrhiza was present in vine roots (Suppl. Figure 1), which is the first systematic evidence for this fact in Burgundy vineyards, whatever soil properties or cultural practices. The intensity of mycorrhizal colonization and arbuscule abundance was varying among vineyard plots (Suppl. Figure 1). However, due to the large variability of arbuscule abundance and intensity of mycorrhization in roots in the plots, no correlation with cultural practices could be determined. A significantly lower mycorrhizal colonization was found in the plots in Yonne, with soils with the highest clay content. Lower colonization rate in clay soils has been linked to a lower amount of spores (Aliasgharzadeh *et al.* 2001, Mathimaran *et al.* 2005), probably due to lower oxygen concentration or mechanical barriers affecting hyphal growth. At the same time, these samples were the only ones from rootstock 41B. It is well established that different rootstocks may react differently to AMF (Linderman and Davis 2001), thus in this case it remains unclear whether soil or rootstock are responsible for the low colonization.

Root colonization levels by AMF were previously shown to be influenced by plant species and fertilization level (Zubek *et al*. 2012), or soil biochemical properties (Zaller *et al.* 2011, Chmura and Gucwa-Przepióra 2012, Krishnamoorthy *et al.* 2014). In the present study, soil micro- and macroelements, soil structure and chemical properties were analyzed for all the plots. Each parameter was set into relation to arbuscular mycorrhiza colonization rates. Significant correlations (P<0.05) were observed between mycorrhizal frequency (F%) and C/N ratio (r=0.33), CEC (r=-0.38), magnesium (r=-0.46) and calcium (r=-0.34) soil content. The only significant positive correlation of the intensity of mycorrhizal colonization (M%) with soil parameters was found for copper levels (Cu; r=0.38, P<0.05), whereas arbuscule abundance (A%) was not influenced significantly at all by soil chemical properties. Nutrient uptake is generally known to be enhanced by AMF due to better soil exploration, but the majority of studies has concentrated on P uptake. Copper is essential for plant photosynthesis and Cu

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nutrition in plants has been shown to be improved by the AMF symbiosis (Lehmann and Rillig 2015). Conversely, AMF can reduce Cu toxicity for plants in contaminated soils through several copper transporters (Tamayo *et al.* 2014). Copper is frequently used for disease prevention in vineyards and is the only molecule allowed in organic viticulture, resulting in elevated Cu levels in many vineyard soils. AMF are highly tolerant to this metal and may play a major role in regulating plant copper content in vineyards, especially in organic culture.

2. AMF diversity across Burgundy

To analyze AMF diversity, a high-throughput sequencing method was used. After denoising, 252704 sequences classified in 2300 clusters were obtained. Only samples with at least 2100 sequences were kept to analyze AMF species diversity.

Results showed, in agreement with root staining results, that samples from Yonne had a low number of AMF sequences, and therefore they were not included in the following analyses. Concerning the samples analyzed, a relatively low diversity at the phylogenetic level of the order was observed in the vine roots, and 99 % of the sequences belonged to the *Glomerales*, an order known to be highly represented in vine roots (Schreiner and Mihara 2009, Balestrini *et al.* 2010, Lumini *et al.* 2010). Among them, six molecular taxa (*Claroideoglomus* sp., *Glomeraceae* sp. 1, 4 and 5, *Glomerales* sp. and *Rhizophagus irregularis*) correspond to 77% of the total number of sequences. They were found in all monitored plots in a large proportion, so it can be hypothesized that they are generally present in Burgundy vineyards as core set of AMF species. Interestingly, the majority of clusters could not be assigned to known species but only to the genus or family level, as no close matches were available in the sequence databases, suggesting that these may be new to science. No clear trend was observed for a correlation between AMF species composition or diversity index found in the roots on one hand and cultural practices (Figure 2) or rootstock type on the other hand. Therefore, the combination of the variations between plots (soil, local climatic conditions, etc) has a stronger effect on AMF community composition than the cultural practices or rootstock type. The location was also shown to influence AMF diversity but not the cropping system in Croatian vineyards (Radic *et al.* 2014), which suggests the importance of local environment on AMF diversity in vineyards.

The AMF species *Rhizophagus irregularis* was found in all the plots, even if the proportion of this species was affected by organic carbon, organic matter and total nitrogen soil content ($P<0.05$). This species was reported to be ubiquitous with its ecotypes adapted to various environments (Börstler *et al.* 2008). In grapevine, this species was previously found in AMF communities from soil or roots (Balestrini *et al.* 2010, Lumini *et al.* 2010, Holland *et al.* 2014). Therefore, it would be interesting in the future to use the molecular markers developed by Börstler *et al.* (2008) to study intraspecific diversity of this species in relation to cultural practices, in particular in the comparison between grass-covered vineyards and tilled vineyards.

In this project we had the opportunity to study a plot inoculated 17 years ago with *Funneliformis mosseae*. However, this AMF species, which was also never found in the other plots, was not detected in the inoculated plot. In fact, no species from this widespread genus were detected. *Funneliformis mosseae* is known as a disturbance colonizer with an r-type strategy, a quick sporulation and colonization of soil and roots (Sýkorová *et al.* 2007), therefore it is frequently found in arable fields. Our results suggest that even if it may have a role in the first colonization stages, it may not persist on the long term, unlike *Rhizophagus irregularis*. The majority of taxa found in vine roots may indeed be K-strategists, well adapted to competition in a system stable for a long time.

Overall, a large variability in AMF diversity was found among the different plots, but these variations are multifactorial, including soil properties, cultural practices and host genotype. A simplified sampling design taking into account the results presented here, using a limited number of variable factors in a same plot, could increase our understanding of the impact of these factors on AMF communities.

CONCLUSION

Here we report the first large-scale analysis of the diversity of the arbuscular mycorrhiza in French vineyards using high-throughput sequencing. In spite of the relatively low diversity observed and a significant proportion of molecular taxa shared between the sites, some differences in the AMF community composition were observed between the plots. By identifying the spectrum of fungal taxa involved, this study contributes to a better understanding of ecology of these fungi in vine roots, thus providing essential knowledge for future applications in sustainable agriculture in vineyards.

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