Agriculture, Ecosystems and Environment 313 (2021) 107369

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

The effects of field inoculation of arbuscular mycorrhizal fungi through rye donor plants on grapevine performance and soil properties

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ARTICLE INFO

Keywords:
Vitis vinifera L.
Secale cereale L.
Funneliformis mosseae
Mycorrhizal communities
Cover crops
Sustainable soil management

ABSTRACT

Grapevines are highly dependent on arbuscular mycorrhizal fungi (AMF) for normal growth and development. However, vineyard soils may have low AMF abundance and diversity due to conventional soil management practices that are detrimental for these fungi. In this context, the establishment of AMF-inoculated cover crops can be a highly convenient strategy to reestablish soil mycorrhizal potential, as it combines the advantages of a vigorous inoculum source coming from mycorrhizal donor plants with the overall benefits of green covers for grape quality, microbial diversity and soil health. In this work, the potential benefits of *Funneliformis mosseae*-inoculated under-vine cover crops on grapevine growth, physiology and production were compared to those derived from 1) the establishment of non-inoculated under-vine cover crops, and 2) conventional herbicide-based weed control in the under-vine space. In addition, grapevine root AMF community composition was analyzed to assess if the introduction of a non-native AMF species induced changes on resident mycorrhizal community assemblies and to unveil potential variations in AMF diversity associated to herbicide replacement by green covers. Results indicated that under-vine cover crops, inoculated or not, led to a general vigor decrease in grapevines, probably due to competition between the two species. However, after a heat wave that occurred at harvest time in the second year of the experiment, grapevines growing in plots with inoculated cover crops had the highest photochemical reflectance indices and net photosynthesis rates, and partially compensated production losses due to berry sunburn. Root mycorrhizal community analysis by the end of the experiment revealed that the inoculated *F. mosseae* isolate colonized grapevine roots from inoculated plots, while it was absent in the other ones. Moreover, inoculation of this AMF did not lead to a replacement of native root AMF communities, but allowed further colonization by other resident Glomeraceae and non-Glomeraceae AMF taxa. Overall, the work herein demonstrates that the introduction of *F. mosseae* through donor plants is a suitable field inoculation method for grapevines and can help them to better withstand heat waves.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are a natural and integral component of healthy soil ecosystems. They form mutualistic symbioses with most crop species, including grapevines, that are in turn dependent on them for their normal growth and development (Trouvelot et al., 2015). Mycorrhizal symbiosis offers multiple ecological services to agricultural systems, i.e. by promoting plant/crop growth, nutrient uptake and stress tolerance, and thus, reducing fertilizer and phytochemical requirements; by enhancing soil aggregation, water retention, nutrient cycling and microbial diversity, thereby improving soil quality; and by phyto-stabilizing certain contaminants (Gianinazzi et al., 2010).

However, current agricultural practices (e.g. high nutrient and biocide inputs, soil tillage, monoculture, cultivation of non-mycotrophic crops) are detrimental to the abundance and diversity of AMF communities and their associated beneficial microorganisms (Jansa et al., 2006). This results in agrosystems that are deprived of the full range of

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https://doi.org/10.1016/j.agee.2021.107369
Received 3 December 2020; Received in revised form 11 February 2021; Accepted 13 February 2021
Available online 1 March 2021

benefits that AMF can provide to the crops (Gosling et al., 2006). In order to offset this negative impact and to reestablish mycorrhizal potential in agricultural soils, two possible strategies can be used: 1) adoption of field practices that enhance indigenous populations of AMF (Brígido et al., 2017; Brito et al., 2008; Lehman et al., 2012), and/or 2) plant/soil inoculation with selected AMF species (Belew et al., 2010; Camprubí et al., 2008; Juntahum et al., 2020; Nogales et al., 2009, 2008).

The establishment of cover crops is a sustainable soil management practice that promotes the proliferation of natural mycorrhizal communities (Brígido et al., 2017; Brito et al., 2013; Kabir and Koide, 2000; Soti et al., 2016). Moreover, cover crops are an alternative to herbicides and to soil tillage for weed control, and provide a variety of benefits to the soil, by preventing erosion, improving water holding capacity and infiltration rates (Basche and DeLonge, 2019), and by increasing soil organic matter as well as overall biodiversity (Costello and Daane, 1998; Kim et al., 2020; Steenwerth and Belina, 2008; Vukicevich et al., 2016). Furthermore, mycorrhizal hyphae originating from cover crops can colonize cash crop roots and form mycorrhizal links between the two species, favoring the transfer of nutrients (Cheng and Baumgartner, 2005).

Nevertheless, native AMF are usually subjected to intense selection pressures in monocultures, especially in conventionally managed soils (Oehl and Koch, 2018). The resulting AMF communities are typically dominated by few taxa, that are presumably well adapted to those agricultural conditions but that are less mutualistic i.e. provide little or no benefit to the plant (Verbruggen and Kiers, 2010). In such agrosystems, inoculation with selected AMF may be a good strategy to improve crop performance as well as to increase productivity.

Several AMF species have been domesticated and many commercial mycorrhiza-based inoculants are available worldwide (Gianinazzi, 2014). However, whereas direct field inoculation in annual crops is feasible when appropriate machinery is available, direct inoculation of individual plants in woody perennial crops such as grapevines, is overly laborious, expensive, and technically challenging due to the deep root system of this species. Hence, the development of new cost-effective methods for AMF inoculation in vineyards are needed. In this context, the establishment of mycorrhizal cover crops beneath grapevines as neighboring donor plants (under-vine cover crops) represents a simple and efficient method to increase mycorrhizal potential in the soil as well as to inoculate grapevines with well-selected and effective AMF. Strategically, this methodology combines the advantages of a vigorous inoculum source [the extraradical mycelium coming from a donor plant is much more infective than spores and mycorrhizal root fragments (Brito et al., 2009)] with the significant benefits of cover crops on AMF diversity, soil health, and grape quality (Lopes et al., 2008; Schipanski et al., 2014; Soti et al., 2016).

The objective of this work was therefore to test the hypothesis that the establishment of mycorrhizal cover crops as AMF donor plants is a suitable strategy for inoculating grapevines under field conditions, and that it provides an overall improvement in plant performance as well as on soil properties. Furthermore, this work aimed to assess if the introduction of a non-native AMF species induced changes on resident mycorrhizal communities and to disclose potential variations in AMF diversity due to cover crop establishment.

2. Material and methods

2.1. Experimental system

2.1.1. Experimental design

A field experiment was set at the experimental vineyard of the Instituto Superior de Agronomia - Universidade de Lisboa campus (Lisbon, Portugal, 38°42′27.5′'N; Lng: 9°10′56.3′'W), which was planted in 2006. Grapevines (*Vitis vinifera* L.) of the white 'Viosinho' variety grafted onto 1103 Paulsen rootstock were used in this study. Plants were spaced 1.0 m within and 2.5 m between rows, and grapevines were trained on a vertical shoot positioned with two pairs of movable wires. Spur-pruning on a unilateral Royat Cordon system was used to trim plants approximately 20 cm above the upper wires. Standard vineyard floor management practices consisted of mowing spontaneous vegetation in the inter-row space, while vegetation in the under-vine space was controlled by herbicide spraying (2–3 applications per year).

The experimental design consisted of a randomized complete block design (Fig. S1). The field was divided into three complete blocks consisting of nine adjacent rows ~25 m wide and 100 m in length. Each block was further sub-divided into three elemental plots with the following three experimental under-vine soil treatments: herbicide-based weed control (H), non-inoculated rye cover crops (RC) and inoculated rye cover crops (IRC) (Fig. S1).

Each elemental plot within the blocks included 33 plants in three adjacent rows of 11 grapevines each- two buffer rows and a central one, which was used for data collection. The first and the last three plants of the central row were also considered as buffer plants, and thus, all data were collected from the central three or five plants in each elemental plot (Fig. S1).

2.1.2. Experimental set up

Starting in December 2016, the under-vine soil of the vineyard was manually prepared. A superficial ditch (10 cm wide ×100 cm long × 10 cm deep) was dug beneath each grapevine (Fig. 1).

For the IRC treatment, 10 g of *Funneliformis mosseae* inoculum (isolate BEG95, Symbiom®, Czech Republic) was placed at the bottom of the ditches. The inoculum was covered with a 5 cm soil layer and rye seeds collected from Mesquitela (Portugal) were sown (5.5 g of seeds beneath each vine). The seeds were then covered with the remaining soil (Fig. 1).

The same procedure was followed in RC plots, but instead of the inoculum only the inoculum-carrier material, consisting of expanded clay devoid of mycorrhizal propagules, was placed at the bottom of the ditch. After the carrier material was covered with soil, rye seeds were sown as described above. In RC and IRC plots, the spontaneous vegetation growing next to rye plants was manually controlled.

In H plots, 10 g of the inoculum-carrier material devoid of mycorrhizal propagules was placed at the bottom of the ditch, and the soil was deposited back. In March, a pre-emergence herbicide treatment (based on glyphosate: 8 L/ha) and in May a post-emergence herbicide treatment (based on diquat 1.5 L/ha) were applied. All herbicide applications were done according to standard practices and following manufacturer's labeling instructions.

At the end of 2017 after the first growing year, the dry rye plants were removed, superficial soil was ploughed, and new rye seeds were sown at 5 cm depth in IRC and RC plots. No AMF re-inoculations were performed. All other vineyard management practices were carried out as described for the first season, except irrigation, as detailed below.

2.1.3. Soil water content and temperature monitoring

Vineyard soil water content was accessed through the entire the experimental period with capacitance probes at 10, 20, 30, 40, 50 and 60 cm depth. The probes were located in a contiguous grapevine line outside the experimental plots.

From May to mid-August, the vineyard was drip-irrigated according to the data collected from the capacitance probes. In 2017, from May to July, watering was applied twice a week. From July onwards, irrigation amount was reduced to control excessive grapevine vegetative growth, and soil water content was frequently beyond the refill point, as commonly done in commercial vineyards during berry maturation stages (Fig. S2a). However, in 2018, to avoid potential excessive water competition between cover crops and grapevines, from July to the beginning of August, irrigation amounts were increased and soil water content was kept above the refill point but not exceeding 50 % of field capacity (Fig. S2b).

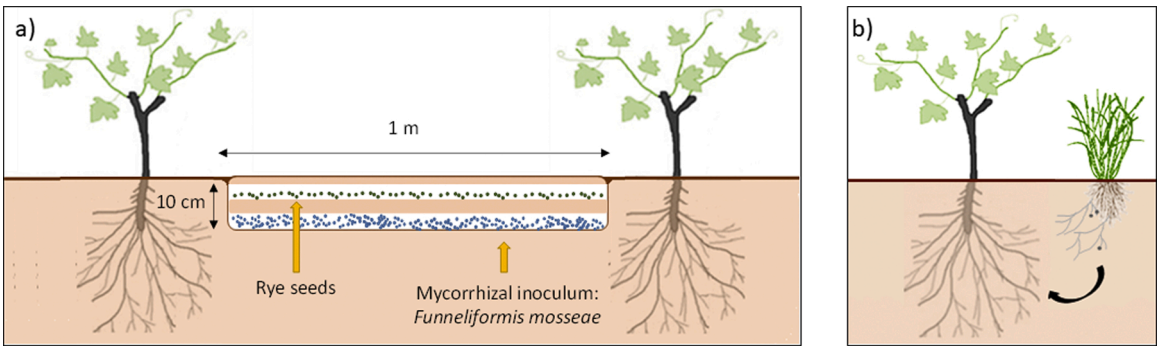

Fig. 1. Schematic representation of arbuscular mycorrhizal fungal inoculation through cover crops. Inoculum is placed at 10 cm depth in the under-vine ditch and rye seeds are sown above (a). Several months later, inoculated rye plants are expected to create extraradical mycelium capable of infecting grapevine roots (b).

During 2017 and 2018, the maximum, minimum and average air temperatures were accessed at the meteorological station located at the Instituto Superior de Agronomia (Lisbon). On August 3rd–5th of 2018, there was a heat wave in Portugal, with daily temperatures above 40 °C in Lisbon (Fig. S2d). After the heat wave, irrigation water amount was increased up to field capacity to promote a faster grapevine recovery after acute heat stress and potential moderate water deficiency stress (Fig. S2b).

2.2. Data collection

2.2.1. Soil characteristics

Composite soil samples were collected from each elemental plot to analyze soil physio-chemical and microbiological characteristics. Sample collections were made during the grapevine dormant period in November 2017 and December 2018. Each sample was divided into two portions. The first portion was air-dried, homogenized, and sieved to collect the soil fraction < 2 mm. This was then used to characterize pH and electric conductivity in water suspension (1:2.5 m/V), organic carbon (Tinsley method), extractable phosphorous, potassium (Egner–Riehm method), as well as other macro and micronutrients, as described in (Nogales et al., 2019).

The remaining portion was used to analyze soil dehydrogenase activity by the triphenyl tetrazolium chloride method (Tabatabai, 1994), which is an indicator of the overall microbial activity and soil quality, and to set the most probable number (MPN) assay to estimate the number of mycorrhizal infective propagules in the soil (Porter, 1979; Powell, 1980). *Allium porrum* L. plants (leek) were used as trap plants, which grew in greenhouse conditions for six months. Afterwards, they were harvested and stained with 0.05 % Trypan blue in lactic acid (Koske and Gemma, 1989; Phillips and Hayman, 1970) to assess mycorrhizal colonization (positive/negative). The number of infective propagules in the soil was evaluated through the mathematical model of Jarvis et al. (2010).

2.2.2. Grapevine vegetative growth, physiology, yield, and berry composition

Along the two growing seasons (2017 and 2018), at berry-touch and harvest, normalized difference vegetation index (NDVI) and photochemical reflectance index (PRI) were measured using PlantPen NDVI 300 (PSI, Czech Republic) and PlantPen PRI 200 (PSI, Czech Republic) portable devices, respectively. The first parameter is commonly used as an indirect estimation of plant vigor, chlorophyll content, and N and P uptake (Sembiring et al., 1998), while PRI is used as indicator of photosynthetic efficiency and stress status (Garbulsky et al., 2011). Two leaves from the upper 1/3 of the canopy were selected from each of the five central vines of each elemental plot. The average NDVI and PRI values per elemental plot were considered for the statistical analysis.

At veraison and at harvest time in 2017 and 2018, individual leaf gas exchange parameters were recorded using an Infrared Gas Analyzer (Licor 6400, LICOR Bio Sciences, USA), equipped with a 2×3 cm^2 transparent leaf chamber: net photosynthesis rate (P_n), stomatal conductance to water vapour (g_s), transpiration rate (E) and intercellular CO_2 concentration (C_i). For this purpose, two fully developed leaves, located on the upper 1/3 of the canopy and directly exposed to sunlight were selected from three central vines per elemental plot. Air flow rate was maintained at 500 mol s^{-1}. The average values of the three plants per elemental plot were considered for the statistical analysis.

At harvest time in August 2017 and 2018, shoot numbers were assessed and leaf area per shoot was estimated in a sample of two representative fertile shoots per vine from three central vines per elemental plot. Leaf area per grapevine was estimated by multiplying the average leaf area per shoot by the number of shoots per vine following the method of Lopes and Pinto (2005). The average plant leaf area value per elemental plot was considered for the statistical analysis.

In both years, at harvest time in mid-August, bunch number and grape yield per plant were assessed on the five central grapevines of each elemental plot. For berry composition analysis, a sample of 200 berries per plot was harvested from both sides of the canopy, from four different parts of the bunches (top, front, back and bottom) and from bunches located in different positions across the entire fruiting zone. Total soluble solids, pH, and titratable acidity were all analyzed according to the procedures defined by the International Organization of Vine and Wine (OIV, 1990). In addition, berry sunburn damage was evaluated after the heat wave that occurred in Portugal at the beginning of August 2018 by visually assessing the fraction of dry berries per bunch in each plant.

In winter of 2017 and 2018, grapevine vigor was assessed in the five central plants of each elemental plot by measuring one-year-old pruning mass per grapevine. The average value obtained for each plot was considered for the statistical analysis.

2.2.3. Mycorrhizal colonization

At the end of rye's growing cycle in July 2017 and 2018, roots were collected from each elemental plot and a composite sample was kept. Similarly, at the end of the trial in December 2018, composite grapevine root samples were collected from five central plants of each experimental plot.

One portion of the composite rye and grapevine root samples was stained as described in Section 2.2.1 and root colonization rates were determined by the gridline intersect method (Giovannetti and Mosse, 1980) using an optical microscope Olympus with an amplification of 40×. The other portion was used for further molecular analyses, as described below.

2.2.4. Mycorrhizal community characterization

2.2.4.1. DNA extraction and sequencing. Grapevine and rye root samples collected from the elemental plots in 2018 were washed in water,

ground to powder in liquid nitrogen, and stored at −80 °C until use. DNA extractions were performed using MO BIO's PowerSoil DNA Isolation Kit (Qiagen, California, USA), following manufacturer's instructions. DNA concentration and quality were measured in a Synergy HT (Biotek, Germany) equipment and using the software Gen5™. DNA integrity was further verified by agarose gel electrophoresis.

To identify AMF communities in grapevine and rye roots, a targeted metagenomic approach based on the amplification of LSU-D2 region of rDNA genes was followed, as in Campos et al. (2018). Amplicons were sequenced in an Illumina MiSeq platform by a 2 × 250 bp paired end protocol at GenoScreen company (Lille, France). Preparatory procedures prior to Illumina sequencing, such as equimolar pooling of PCR reactions, addition of adaptors, and PCR product purification were performed by GenoScreen.

2.2.4.2. Sequence analysis and OTU assignation. Raw sequence data were processed using MOTHUR (Schloss et al., 2009). Low-quality and short sequence reads were filtered, and chimeric sequences removed via the *Chimera Vsearch* tool (Rognes et al. 2016). A *pre-clustering* step was applied to correct for sequencing related errors as well as to constrain overestimation of operational taxonomic units (OTUs). A distance matrix was constructed with *dist.matrix* tool and sequences were clustered into OTUs at ≤ 97 % similarity using the *cluster* tool. Singletons were removed from the resulting table containing the number of sequence reads per OTU, and representative sequences were obtained for each OTU through the *get.oturep* command.

Then, the OTU abundance table was exported to R environment and the number of sequences per sample was normalized using the function *rrarefy* from the R package 'vegan' (Oksanen et al., 2019) to remove sampling depth effects. To assess sampling effort, a rarefaction analysis was done using the *rarecurve* function also from 'vegan' package (Oksanen et al., 2016). The normalized OTU abundance table was used for further biodiversity analyses.

Affiliation of each representative sequence was assessed at the genus level by performing a BLAST analysis against the NCBI and MARJAAM databases. For determining which OTUs corresponded to the inoculated AMF, a new clustering analysis was performed using the representative sequences of the OTUs belonging to *Funneliformis* genus, four sequences of *F. mosseae* isolate BEG 95 obtained in Nogales et al. (2020) and four sequences of the same isolate retrieved from NCBI database. For this, previously all sequences were aligned in MEGA 7 (Kumar et al., 2016) and exported to MOTHUR. Pairwise distances were obtained using *dist.seq* command and sequences were clustered at ≤ 97 % similarity using the *cluster* command.

The study of α and β-diversity was performed in two steps. First, grapevine root samples of H, RC and IRC experimental treatments were considered to assess potential differences in grapevine root AMF communities due to the under-vine treatments. Then, only rye and grapevine root samples of RC and IRC treatments were considered to assess potential differences in AMF community composition and structure due to the host species (rye or grapevine) or due to the cover crop type (inoculated or not). In both cases, α-diversity was analyzed by Shannon diversity index (H′) and the study of β diversity was conducted based on Bray-Curtis dissimilarity index (Bray and Curtis, 1957) as a measure of distance between pairs of AMF communities. Both indexes were calculated from the normalized OTU abundance table using the R package 'vegan' (Oksanen et al., 2019).

2.3. Statistical analysis

For the analysis of soil physico-chemical and biological parameters, rye root colonization rates as well as for plant growth/vigor, yield and must quality parameters, a linear model considering *Under-vine treatment, Year* and *Block* as fixed effects factors was fitted. *Block* factor was considered a fixed effects factor because the three blocks were established to control specific conditions of the field site. Due to the sequential nature of the data on each plot over the two years (repeated measures), in the fitted model, random errors associated to different experimental units were assumed to be independent, whereas random errors associated with observations made in different years in the same plot were not.

Plant physiology data were also analyzed fitting a linear model, but in this case *Under-vine treatment, Phenology stage* and *Block* were considered as fixed effects factors.

As the experimental design was a randomized complete block design, interactions between *Under-vine treatment* and *Block* were not included in the models. Statistical differences among group means were assessed by Duncan's test at $p \leq 0.05$. Analyses were conducted in SPSS Statistics vs. 23 (IBM) software.

The effects of different under-vine treatments on grapevine root colonization and on H' index calculated for grapevine root AMF were analyzed by one-way ANOVA tests. Then, Bray–Curtis distances calculated for each pair of experimental treatments (H, RC and IRC) were analyzed by permutational multivariate analysis of variance (PERMANOVA) (Anderson and Walsh, 2013) using *Adonis* function of "vegan' package" in R (Oksanen et al., 2019). The significance of the *Under-vine treatment* factor was assessed through comparison with 999 randomized data sets. Non-metric multidimensional scaling (NMDS) was additionally used to represent AMF community variation according to the under-vine treatment.

A two-way ANOVA was conducted to assess the effect of host species (grapevine or rye) and cover crop type (RC or IRC) on H' index. Bray–Curtis distances calculated for the different pairs of experimental treatments were analyzed by a PERMANOVA (Anderson and Walsh, 2013) using *Adonis* function of "vegan' package" in R (Oksanen et al., 2019). The significance of the main effects (plant host species or cover crop type) as well as of the interaction between them was assessed through comparison with 999 randomized data sets. Non-metric multidimensional scaling was also used to represent AMF community variation among plant host species and cover crop type in a two-dimensional space.

Venn diagrams created in Venny 2.1 online tool (Oliveros, 2015) were used to study the number of unique and shared OTUs in 1) grapevine roots from different under-vine treatments and 2) grapevine and rye roots from plots with inoculated and non-inoculated cover crops. Significant differences in the frequencies of shared OTUs grouped by genera among the under-vine treatments were determined by one-way ANOVA or by the Kruskall Wallis test, and significant differences in the frequencies of shared OTUs related to the host species and cover crop types were determined by a two-way ANOVA using SPSS Statistics vs. 23 (IBM) software.

3. Results

3.1. Soil characteristics variation throughout experimental years

The soil had a Clay texture, and had a pH between 6.5 and 6.6, average organic matter content (approximately 3 %), and high P concentration (93.1–101.8 mg/kg). Soil Ca, Mg, K and Cu concentrations were also high, as shown in Table S1. *Year* factor had a significant effect on all parameters except on pH, EC, K, and B (Table S1), but no significant main effects were detected for the *Under-vine treatment* factor. However, when data were analyzed separately by year, in 2017 organic matter content was significantly higher in IRC plots than in H plots, while RC plots presented intermediate values. In 2018 the tendency was the same, but it was not statistically significant.

Regarding biological parameters, no significant differences were observed in soil dehydrogenase activity and in the number of infective mycorrhizal propagules due to *Year* or *Under-vine treatment* factors (Fig. S3a and b). However, albeit not statistically significant due to the high variability of the data, the number of mycorrhizal propagules in the

soil tended to be higher in IRC plots, as this number increased 3.1 and 4.6 times respect H plots in 2017 and 2018 respectively, and 2.6 and 2.1 times respect the RC plots in 2017 and 2018, respectively (Fig. S3b).

3.2. Mycorrhizal colonization of rye and grapevine plant roots

The *Under-vine treatment* factor had a significant effect on rye colonization rate and no interactions were detected with the other factors (*Year* and *Block*). Inoculation with *F. mosseae* promoted a significant increase in rye colonization rate, especially in 2017, with 15.6 % higher rates in IRC than in RC plots. In 2018 the increase was less obvious, colonization rates being 9.4 % higher in IRC than in RC plots (Fig. 2).

Concerning grapevine root colonization rate, by the end of the experimental period in 2018, the different under-vine management practices had no significant effects. This was evident as rates of 0.64 ± 0.008, 0.63 ± 0.022 and 0.68 ± 0.003 (average value ± standard error) were found in grapevines growing in H, RC and IRC plots, respectively.

3.3. Under-vine treatment effects on grapevine growth and physiological parameters

The significance of the effects of *Year*, *Under-vine treatment* and *Block* factors on plant growth parameters, as well as the probability associated to their interactions are shown in Fig. 3. *Year* factor had a significant effect on plant shoot number and total leaf area but not on pruning weight (Fig. 3a–c). Moreover, there was a significant effect of the *Under-vine treatment* factor on total plant leaf area (Fig. 3b). This was especially evident in 2018, when grapevines from RC and IRC plots showed a respective decrease of 43.8 % and 38.6 % on this parameter in comparison to that from H plots (Fig. 3b). Pruning weight showed a similar tendency, albeit it was not statistically significant (Fig. 3c).

As shown in Table 1, the vegetative index NDVI was only affected by the *Phenology stage* factor. However, when NDVI data were analyzed separately at each phenology stage, at veraison 2018, grapevines growing in H plots showed significantly higher values than plants growing in RC and IRC plots (Fig. 4). In the other vegetative index, PRI, both, the *Phenology stage* and the *Under-vine treatment* factors had a significant effect in 2017. That year, at harvest time, plants growing in RC plots had lower PRI values than plants growing in H and IRC plots. In veraison 2018, PRI was still lower in RC plots than in H plots, but in that case, grapevines from IRC plots had intermediate values (Fig. 4). This trend changed later at harvest, when PRI values were measured right after the heat wave (Fig. S2d), as the highest values were found in grapevines growing in IRC plots. In turn, plants growing in RC showed again the lowest values, and plants of H plots had intermediate values (Fig. 4).

All individual leaf gas exchange parameters, except Ci, were also influenced by the phenology stage in 2017, but not in 2018 (Table 1). The overall effect of the *Under-vine treatment* factor was only significant for Ci in 2017. When data were analyzed separately at each phenology stage and year, at harvest time in 2017 plants from RC plots showed the highest Ci values. However, in 2018, only P_n showed significant differences related to the under-vine treatment, which were detected after the heat wave at harvest time: grapevines growing in IRC plots showed significantly higher values than the ones from RC plots, and plants of H plots had intermediate values (Fig. 4), following the same trend as PRI.

3.4. Under-vine treatment effects on berry yield and must composition

As shown in Table 2a, the *Under-vine treatment* factor was not significant for berry yield or for the number of berry bunches. However, when data were analyzed separately by year, after the heat wave in 2018, a significant yield decrease was observed in grapevines growing in plots with cover crops compared to the ones growing in H plots, but the decrease was more pronounced in plants from RC plots (Table 2b). This was supported by the analysis of the percentage of berry sunburn, which was significantly higher in plants growing in RC plots, intermediate in IRC grown grapevines, and lowest in grapevines from H plots (Fig. S4).

Regarding berry must composition, the effect of the *Year* was significant in all the measured parameters, but the effect of the *Under-vine treatment* factor was only detected on must pH (Table 2a). However, when data were analyzed individually per year, no differences were observed among the three under-vine treatments (Table 2b).

3.5. Mycorrhizal community characterization

A total of 72,995 clean sequences were obtained from the nine grapevine and six rye composite root samples, which were assigned to a maximum number of 187 OTUs after singletons were removed. Rarefaction curve (Fig. S5) indicated that the number of sequences obtained provided adequate coverage of the AMF OTU richness in grapevine and rye roots.

The distribution of the different OTUs identified in grapevine and rye root samples grouped by genus, and their relative frequency, are shown in Fig. 5. Altogether, 13 distinct OTUs belonging to *Funneliformis* genus were identified. Among these, six clustered together with already available sequences of *F. mosseae* BEG isolate 95 and were thus considered to be the same AMF isolate (Table S2).

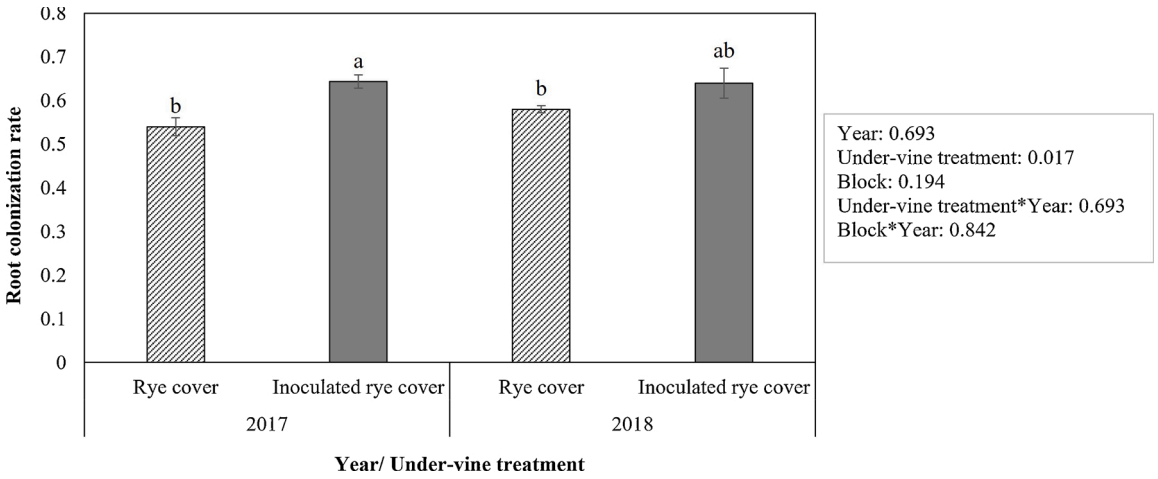

Fig. 2. Mycorrhizal colonization rate in rye plants in July 2017 and July 2018. Different letters indicate significant differences according to Duncan's *a posteriori* test. The box next to the graphic indicates the *p*-values of the test for the effects of the different factors and their interactions.

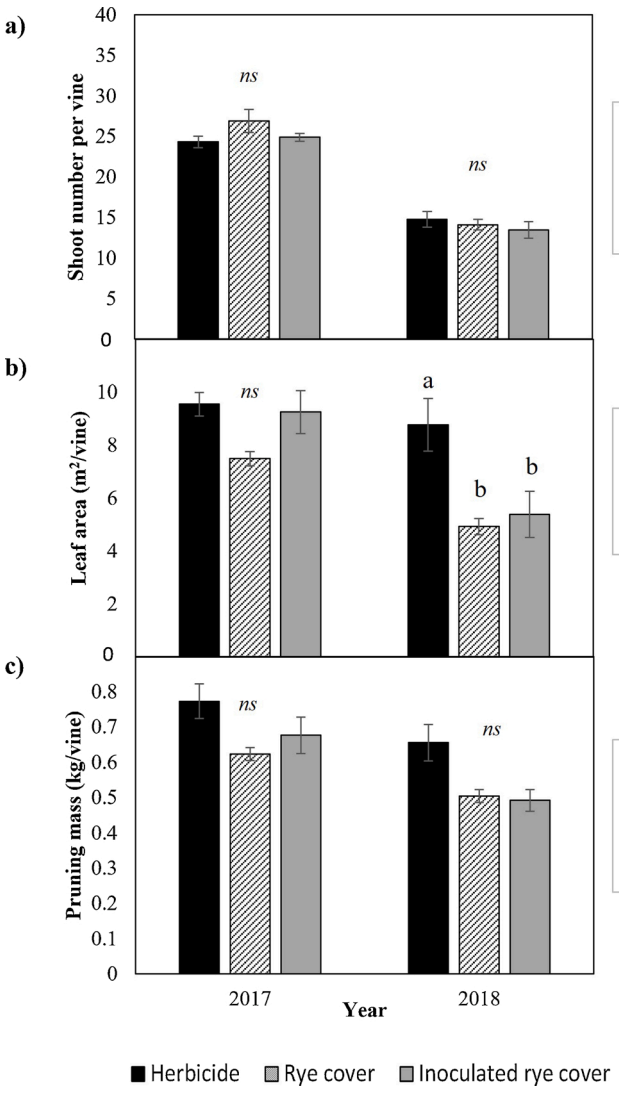

Fig. 3. Shoot number (a), total plant leaf area (b), and pruning mass (c) of 'Viosinho' variety grapevines growing in plots treated with herbicide or in plots with inoculated or non-inoculated rye cover crops. Vertical bars indicate the average value of three experimental plots (5 plants per plot) ± standard error, and different letters on top of the bars indicate statistically significant differences according to Duncan *a posteriori* test while "*ns*" indicates no significant differences ($p \leq 0.05$). The box next to each graphic indicates the *p*-values of the tests for the effects of the different factors and their interactions.

Table 1
P-values of the test for the effects of *Phenology stage*, *Block* and *Under-vine treatment* and their interactions on normalized difference vegetation index (NDVI), photochemical reflectance index (PRI), photosynthesis rate, stomatal conductance, internal CO_2 concentration and transpiration rate. Significance: $p \leq 0.05$.

Effects	NDVI		PRI		Photosynthesis rate		Stomatal conductance		Internal CO_2 concentration		Transpiration rate	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Phenology stage	<0.001	<0.001	<0.001	<0.001	0.011	0.133	0.032	0.082	0.055	0.763	0.035	0.819
Block	0.664	0.252	0.189	0.682	0.130	0.686	0.176	0.856	0.048	0.877	0.226	0.717
Under-vine treatment	0.373	0.583	0.040	0.051	0.408	0.411	0.412	0.929	0.040	0.970	0.291	0.921
Phenology stage * Block	0.521	0.270	0.195	0.020	0.892	0.142	0.801	0.302	0.923	0.508	0.857	0.234
Phenology stage * Under-vine treatment	0.979	0.843	0.053	0.037	0.907	0.475	0.570	0.917	0.330	0.247	0.545	0.899

3.5.1. Effects of under-vine treatments on grapevine root mycorrhizal communities

When grapevine root AMF communities from H, RC and IRC plots were studied, no differences were detected in Shannon diversity index, being this index 1.82 ± 0.278 in H plots, 1.95 ± 0.197 in RC plots and 1.62 ± 0.688 in IRC plots (average value of three plots ± standard error).

Mycorrhizal communities from grapevine roots were dominated by OTUs belonging to *Rhizoglomus* and to uncultured or non-described Glomeraceae species (Fig. 5). In total, 28 OTUs were found to be common to the three under-vine treatments (H, RC and IRC), all belonging to these two genera (Fig. 6a). Furthermore, there were no significant differences in the frequencies of these shared OTUs grouped by genus among the experimental treatments (Fig. 6b).

When non-inoculated cover crops were installed in the under-vine space (RC plots), three new OTUs belonging to *Septoglomus* genus appeared, representing 9.4 % of the sequences on average. Two of these were exclusive to this experimental treatment (representing 1.7 % of the sequences), while the other one was also found in grapevine roots from inoculated plots (Fig. 5).

A considerate number of exclusive OTUs were identified in grapevine roots of IRC plots (Fig. 6b): while 36 unique OTUs were identified in IRC plots, 23 and 17 were identified in RC and H plots, respectively. In inoculated plots, unique OTUs included three OTUs belonging to the

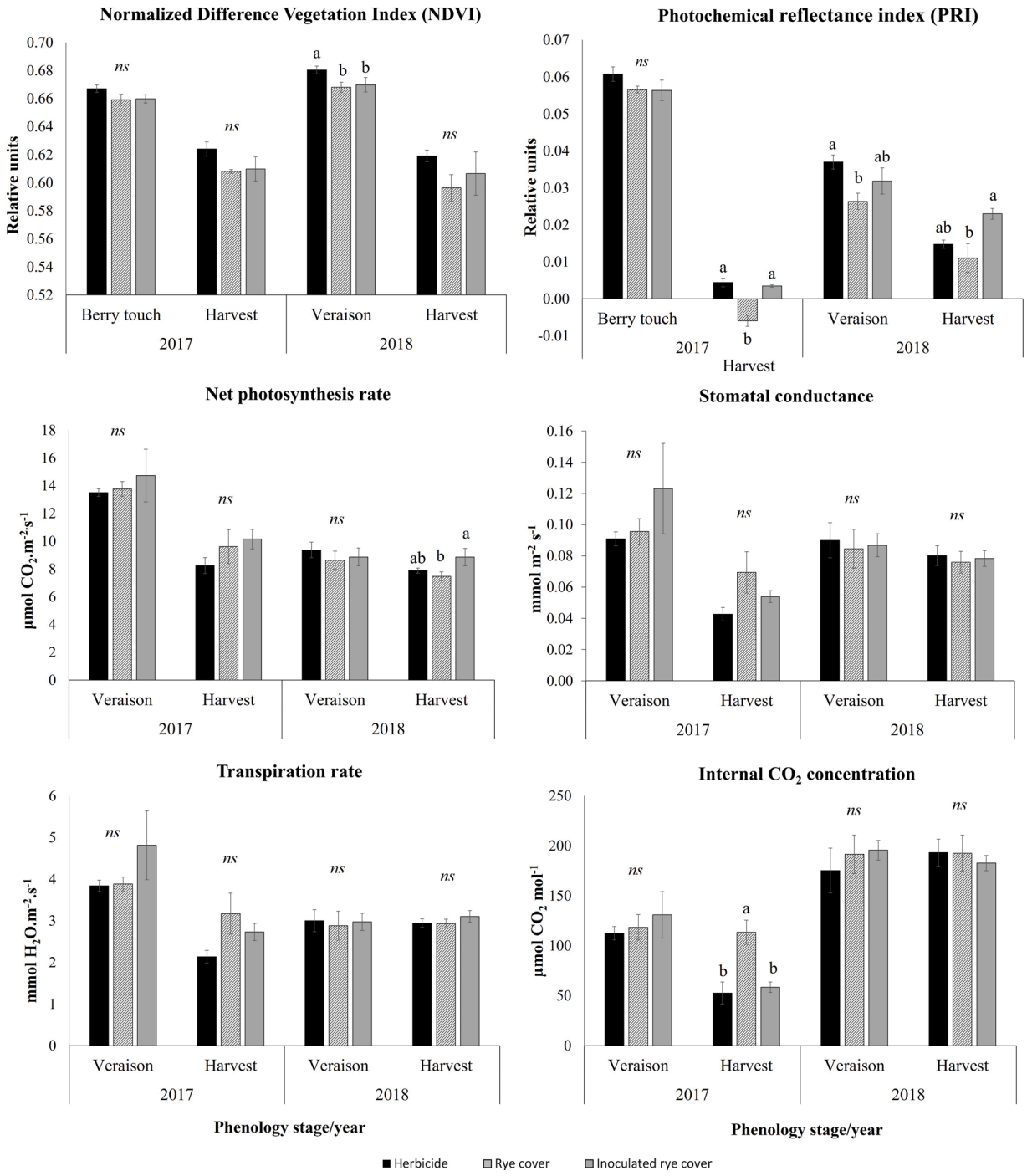

Fig. 4. Vegetation indices and gas exchange parameters recorded in 'Viosinho' variety grapevines growing in plots with herbicide treatments, and from plots with inoculated or non-inoculated rye covers in 2017 and 2018. Different letters indicate significant differences according to Duncan *a posteriori* test, and "*ns*" indicates no significant differences ($p \leq 0.05$). Vertical bars indicate the average value of three experimental plots (3 or 5 plants per plot) ± standard error.

Archaeospora genus (0.45 % of sequences), two to the *Claroideoglomus* genus (0.05 % of sequences), 14 to the *Rhizoglomus* genus, 15 to uncultured species from the Glomeraceae family, and three to the *Funneliformis* genus (0.7 % of the sequences). One of these clustered together with sequences obtained from the inoculated *F. mosseae* isolate BEG95, while the other two were identified as *F. geosporum* (Table S2).

Roots from the RC and IRC treatments shared 22 OTUs that were absent in H plots, which belonged to *Rhizoglomus* (14), *Septoglomus* (1), and to uncultured Glomeraceae species (7) (Fig. 6a).

Regardless of the differences observed in AMF community assemblies at genus level, especially concerning the identity of low abundant and exclusive OTUs, β-diversity analysis did not show significant differences in grapevine AMF community structures between different under-vine treatments (PERMANOVA based on Bray Curtis dissimilarity index, $p = 0.873$). These results were further supported by the lack of significant differences in NMDS ordination (Fig. 6c).

3.5.2. *Effects of host plant species and cover crop inoculation on root mycorrhizal communities*

Similar to the results obtained in grapevine roots, the most frequent OTUs in rye roots also belonged to *Rhizoglomus* and to uncultured Glomeraceae (Fig. 5). The Venn diagram in Fig. 7a, which shows shared

Table 2
P-values of the test for the effects of *Year*, *Block* and *Under-vine treatment* factors, as well as for their respective interactions on grape production and must quality parameters (a), and the average values of production and quality parameters measured in 2017 and 2018 in three plots per experimental treatment (composite grape samples collected from 5 five plants per plot) ± standard error (b). Different letters indicate significant differences according to Duncan *a posteriori* test. Significance: $p \leq 0.05$.

a)

Effects	Yield Number of clusters per vine	Yield	Must composition Total soluble sugars	pH	Titratable acidity
Year	0.196	0.011	0.013	0.006	<0.001
Block	0.466	0.410	0.334	0.452	0.502
Under-vine treatment	0.559	0.021	0.862	0.029	0.117
Year * Block	0.826	0.297	0.363	0.178	0.281
Year * Under-vine treatment	0.826	0.032	0.826	0.416	0.761

b)

Under-vinetreatment	Number of clusters per vine		Yield (kg per vine)		Total soluble sugars (ºBrix)		pH		Titratable acidity (g tartaric acid. l^{-1})	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
Herbicide	17 ± 1.4	18 ± 1.7	2.9 ± 0.30	3.1 ± 0.29 **a**	25.6 ± 0.34	22.7 ± 0.59	3.4 ± 0.03	3.2 ± 0.01	5.0 ± 0.08	6.5 ± 0.07
Rye cover	19 ± 2.8	20 ± 2.7	3.3 ± 0.42	0.9 ± 0.15 **b**	25.8 ± 0.96	23.1 ± 0.14	3.3 ± 0.03	3.2 ± 0.01	5.4 ± 0.15	7.0 ± 0.08
Inoculated rye cover	16 ± 0.8	16 ± 0.3	2.7 ± 0.22	1.7 ± 0.28 **c**	25.3 ± 0.07	23.3 ± 0.78	3.3 ± 0.01	3.2 ± 0.01	5.2 ± 0.08	6.9 ± 0.22

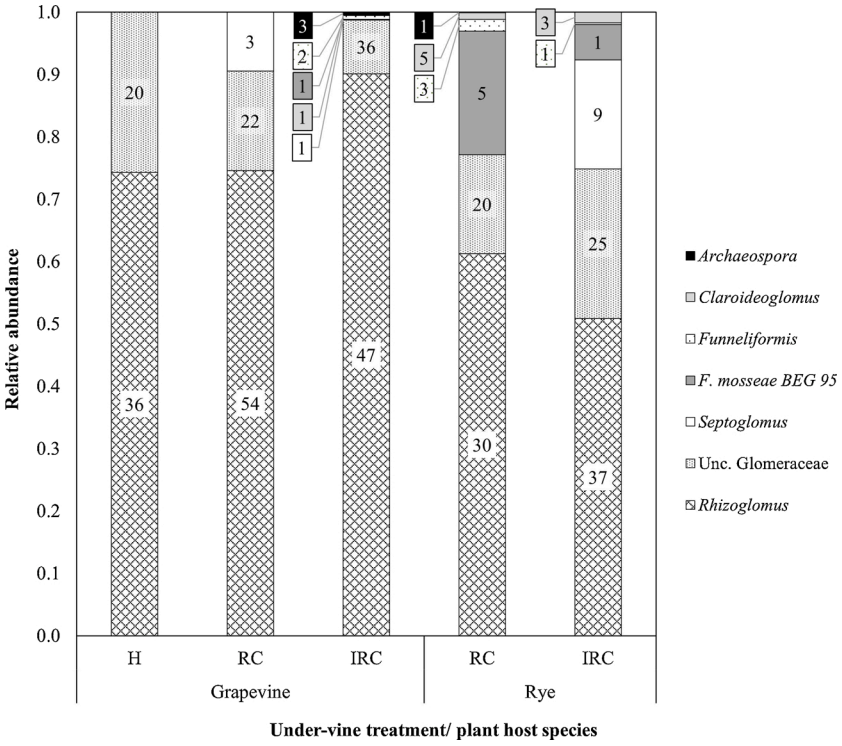

Fig. 5. Relative sequence abundance of different AMF genera in grapevine roots ('Viosinho' variety grapevines grafted onto 1103 Paulsen rootstock) and in rye roots from plots under the three soil management regimes (H-herbicide; RC-rye cover; IRC-inoculated rye cover). Numbers in each bar section indicate the number of OTUs belonging to each mycorrhizal fungal genus.

and exclusive OTUs detected in grapevine and rye roots collected from RC and IRC plots, indicated that altogether, 24 OTUs were present in both species, which belonged these two groups, i.e. to *Rhizoglomus* genus and to undescribed members of the Glomeraceae family. As illustrated in Fig. 7b, there were no significant differences in the relative abundance of these taxa grouped by genus related to the host species ($p = 0.749$ and $p = 0.749$, for *Rhizoglomus* and undescribed Glomeraceae, respectively), or to the cover crop type ($p = 0.892$ and $p = 0.735$, for *Rhizoglomus* and uncultured Glomeraceae, respectively).

Besides these two taxa, in rye plants of inoculated and non-inoculated plots, OTUs corresponding to the inoculated *F. mosseae* BEG 95 were identified (five in RC and one in IRC plots), which indicates a transference of the inoculated AMF from IRC to RC plots (Fig. 5). However, the high frequency of *F. mosseae* BEG 95 revealed in rye roots of RC plots was due to a large presence in only one root sample.

In rye plants from IRC plots, one additional OTU belonging to *F. geosporum* species was also identified (0.6 % of the sequences), and in RC plots, three additional OTUs of *Funneliformis* genus were identified, accounting for 1.9 % of the sequences. These belonged to a different *F. mosseae* isolate, to *F. geosporum* and to an uncultured *Funneliformis* isolate, respectively, as indicated by BLAST analysis performed against NCBI and MARJAAM databases (Table S2).

Operational taxonomic units belonging to *Claroideoglomus* genus were also present in rye roots from both, RC and IRC plots (1 % and 1.7 % of sequences, respectively) (Fig. 5). Three of these OTUs were shared among the two cover crop types, while the other two were exclusively

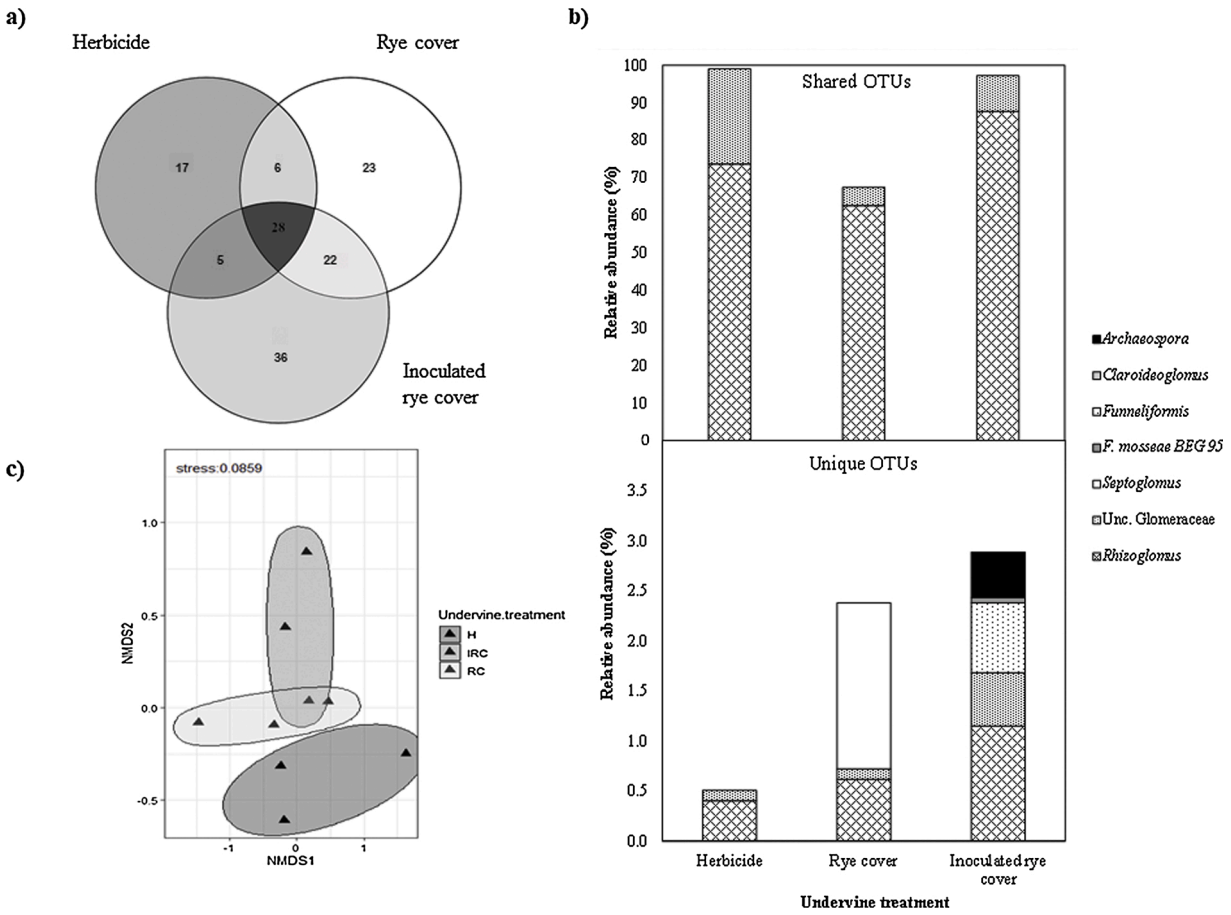

Fig. 6. Venn diagram illustrating the number of unique and shared OTUs in grapevine roots ('Viosinho' variety grapevines grafted onto 1103 Paulsen rootstock) collected from plots under different soil management regimes (H-herbicide; RC-rye cover; IRC-inoculated rye cover) (a); Relative abundance of shared (top) and unique (bottom) OTUs in grapevine roots grouped by genera (b); and non-metric multidimensional scaling (NMDS) plot showing relationships between the AMF communities in grapevine root samples from the three experimental treatments (c).

identified in RC plots (0.2 % of the sequences). One OTU of the *Archaeospora* genus was also present exclusively in rye roots of RC plots, while *Septoglomus* genus was only present in IRC plots (nine OTUs representing 17.4 % of the sequences, from which three were also detected in grapevine roots) (Fig. 5).

Overall, 50 OTUs were detected exclusively in rye roots but not in grapevines (Fig. 7a). These belonged to *Claroideoglomus* (4), *Septoglomus* (6), *Funneliformis* (8) and *Rhizoglomus* (18) genera and to uncultured Glomeraceae species (14). On the other hand, 64 OTUs were present in grapevine roots but not in rye: two belonging to *Archaeospora*, two to *Funneliformis* and 37 to *Rhizoglomus* genera, and 23 to uncultured members of the Glomeraceae family.

When H' index was studied in root AMF communities related to the host species or to the cover crop type, no significant main effects or interactions were detected, with *p*-value for the *Host plant species* factor being 0.334 and for the *Cover crop type* factor 0.653.

Regarding β-diversity, neither the *Host plant species* factor ($p = 0.732$) nor the *Cover crop type* factor ($p = 0.710$) had significant main effects, and no interaction was detected among them. These results were supported by the NMDS ordination plot based on Bray Curtis index (Fig. 7c).

4. Discussion

This work aimed to analyze the effectivity of a new field inoculation method through mycorrhizal cover crops as AMF donor plants for grapevines. To this end, the potential benefits derived from mycorrhizal cover crop establishment in soil properties as well as in grapevine performance were studied in comparison to the ones derived from the establishment of non-inoculated under-vine cover crops and from conventional herbicide applications in the under-vine space.

As expected, inoculation with *F. mosseae* led to an overall increase in rye root colonization rates and to a small raise in the number of infective mycorrhizal propagules in the soil. Moreover, although there is a potential risk that inoculated AMF persistence in plant roots might be temporary for some non-native AMF species (Berruti et al., 2017; Bouffaud et al., 2016; Martignoni et al., 2020; Pellegrino et al., 2012; Sýkorová et al., 2012), in our study, two years after the experiment was established, *F. mosseae* BEG 95 was detected in grapevine roots from IRC plots, while it was absent in roots from H and RC plots, indicating that the inoculation of this AMF through rye donor plants was successful.

Even though this inoculation system has already been tested both in *in vitro* and in greenhouse experiments (Cheng and Baumgartner, 2004; Johansen and Jensen, 1996; Lalaymia and Declerck, 2020; Meng et al., 2015; Muneer et al., 2020), and despite the fact that the occurrence of common mycorrhizal networks between grapevine roots and cover crop roots has already been demonstrated in vineyards (Cheng and Baumgartner, 2005), to our knowledge this is the first time that field-grown grapevines have been inoculated through green cover donor plants.

4.1. Effects of grapevine field inoculation through rye donor plants on root mycorrhizal community compositions

In this study, a relatively low AMF diversity was observed at the phylogenetic level in grapevine roots, being AMF communities of the three under-vine treatments dominated by *Rhizoglomus* and by

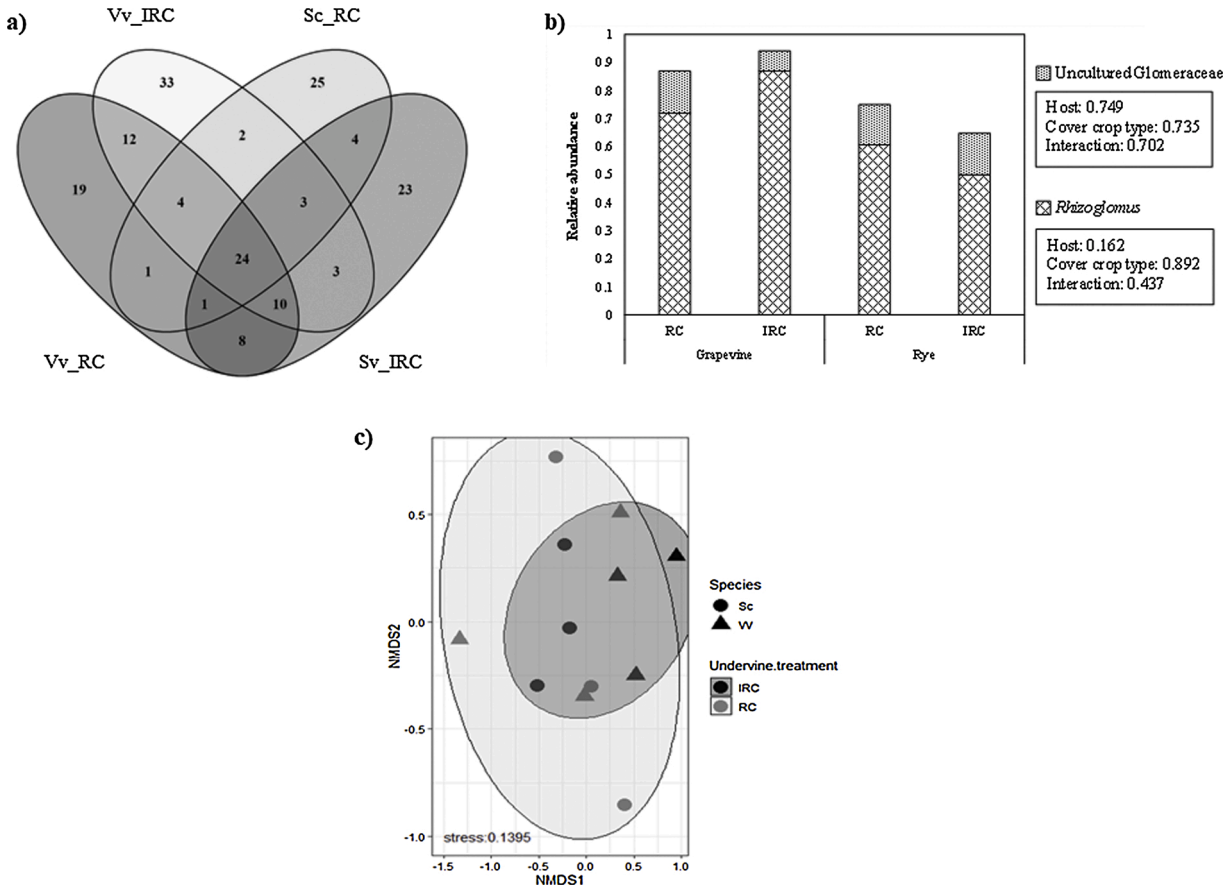

Fig. 7. Venn diagram showing the number of unique and shared OTUs in grapevine and rye roots from inoculated (IRC) or non-inoculated (RC) plots (a); Relative abundance of shared OTUs in grapevine and rye roots from IRC and RC plots (b); and non-metric multidimensional scaling (NMDS) plot showing relationships between the AMF communities in grapevine and rye root samples in plots with inoculated and non- inoculated under-vine cover crops. Colored ellipses represent different cover crop types. Circles and pyramid points represent rye (Sc) and grapevine (Vv) (c).

uncultured *Glomeraceae* species (Fig. 5 and 6b). Other studies have also shown that agricultural soils often have low AMF diversity (Oehl et al., 2003; Schnoor et al., 2011), and in particular, in vineyard soils and in field-grown grapevine roots, fungi from Glomeraceae family are generally dominant, with a large presence of *Rhizoglomus* genus (Balestrini et al., 2010; Bouffaud et al., 2016; Likar et al., 2013; Oehl et al., 2010; Schreiner and Mihara, 2009; Van Geel et al., 2017). However, fungi of the genus *Funneliformis* were not naturally colonizing grapevine roots of 'Viosinho' variety plants grafted onto 1103 Paulsen, as demonstrated by the fact that this AMF genus was only detected in grapevine roots growing in plots with inoculated cover crops. This agrees with other previous works, that also did not find *F. mosseae* or other species of the same genus in grapevine roots from different vineyards under distinct management regimes (Bouffaud et al., 2016; Likar et al., 2013). Contrastingly, OTUs belonging to *Funneliformis* genus were detected in rye roots of both, inoculated and non-inoculated plots (Fig. 5), which suggests that in vineyards, such species may remain associated to host plants other than grapevines, such as cover crops or weeds, as also observed by Schreiner and Mihara (2009). In fact, several studies have shown that roots of vineyard vegetation harbors different AMF communities than the intermingled grapevine roots (Holland et al., 2014; Radić et al., 2012; Schreiner, 2020).

Furthermore, it is known that the presence of neighboring plant species with different symbiosis preferences can increase species richness and/or induce shifts in the AMF community composition (Brígido et al., 2017; Campos et al., 2018; Meadow and Zabinski, 2012). Although in this experiment mycorrhizal community structures of rye and grapevine roots did not differ significantly (Fig. 7c), the fact that 50 OTUs were present exclusively in rye and 64 in grapevine roots (Fig. 7a) indicates that both species may indeed have different preferences for some particular low abundant AMF taxa. For this reason, the inclusion of vineyard management practices that help to maintain soil AMF diversity, such as the establishment of cover crops or the conservation of resident vegetation during the dormant season, may be crucial to assure an appropriate AMF reservoir for grapevine root colonization. This, in turn, can help cash crops to respond better to environmental changes that may require different functional attributes (Goss et al., 2017; Oehl and Koch, 2018).

On the other hand, in our experimental vineyard, mycorrhizal inoculation through cover crops did not lead to a significant change in α or β-diversity indices in grapevine root AMF communities, as no differences were detected among the three under-vine soil treatments. This agrees with the study of Van Geel et al. (2017), who did not observe differences in AMF diversity between conventionally and organically managed vineyards. These authors suggested that the high soil P concentrations detected in the organic vineyards may have overruled the potential benefits of organic farming on AMF diversity. However, in their study, they found AMF community composition variations according to vineyard management practices (Van Geel et al., 2017). Similarly, in our study, AMF community assemblies differed when herbicide-based weed control was replaced by a rye cover crop establishment, as a new genus (*Septoglomus*) and new unique and/or low-abundant OTUs appeared (Fig. 6a and b). Such increase in new OTUs was even higher in plots with inoculated cover crops, where 36 exclusive OTUs were identified, including the inoculated *F. mosseae* isolate, and novel OTUs from *Funneliformis, Archaeospora* and

Claroideoglomus genera (Fig. 5). Hence, the establishment of cover crops inoculated with *F. mosseae* not only did not lead to a replacement of native AMF communities, but allowed further root colonization by other resident AMF of Glomeraceae and non-Glomeraceae families, as observed also by Berruti et al. (2017). Relevantly, the data herein demonstrate that when management regimes potentially responsible for low AMF diversity are modified, inoculation through cover crops can be a good strategy towards a faster AMF settlement in the unoccupied root habitat.

4.2. Effects of mycorrhizal fungi field inoculation through rye donor plants on grapevine growth and performance

In the experimental vineyard of this study, the establishment of cover crops, inoculated or not, led to a decrease in total grapevine leaf area, especially in 2018. Pruning biomass and NDVI index also showed the same pattern (Figs. 3b, c and 4), indicating that the establishment of rye covers in the under-vine space resulted in a vigor reduction in grapevines. Given that soil nutrient composition did not differ among the experimental plots (Table S1), it seems most likely that both plant species competed for water, despite the fact that irrigation amount was increased in summer 2018 respect to 2017. Hence, the decrease in grapevine vigor may have been caused by a more intense competition among both species in spring, probably due to high water use during rye grain filling phase, which coincides with active vegetative growth in grapevines (Lopes et al., 2004, 2008; Monteiro and Lopes, 2007; Pellegrino et al., 2005). In fact, soil water content in May and June 2018 was lower than in the same period in 2017 (Fig. S2a and b). However, PRI data indicated that although grapevine plants growing in RC plots had lower values of this parameter than plants growing in plots treated with herbicides, the previous inoculation of cover crops with *F. mosseae* was able to compensate, at least in part, the lower performance derived from the competition among the two plant species (Fig. 4).

Later, after the heat wave at harvest time in 2018, there was a general decrease in PRI values. Grapevines are indeed susceptible to extreme weather events such as heat waves (Fraga et al., 2020, 2016), which commonly lead to temperature and UV-B radiation stress (Torres et al., 2018). Nevertheless, grapevines from the IRC plots showed the highest PRI and P_n values (Fig. 4), suggesting that introducing *F. mosseae* through cover crops can help mitigating deleterious effects of heat waves on grapevines by modulating their physiology to allow them to respond more efficiently to those environmental conditions. In fact, this AMF species is known to improve grapevine growth, P uptake, chlorophyll content, P_n and g_s, as well as to promote drought and heat stress tolerance (Kamayestani et al., 2019; Liu et al., 2019; Nogales et al., 2020, 2019; Schreiner, 2007; Wang et al., 2019; Zhang et al., 2018). However, because additional OTUs were also present in grapevine roots from IRC plots, it is not possible to determine whether changes in grapevine performance were directly related to *F. mosseae* colonization or to an indirect effect of the inoculation, through an increase in soil organic matter content (Table S1) or through favoring root colonization by new AMF species/isolates. In fact, there is evidence that AMF communities with different taxon composition lead to distinct growth and nutritional responses in plants (Chen et al., 2017; Moora et al., 2004; Rustioni et al., 2014; van der Heijden et al., 1998; Wang et al., 2008). Moreover, they can differentially affect plant photosynthetic characteristics, as demonstrated by the fact that plants with different root AMF assemblies can present different P_n, g_s, maximum carboxylation rate of Rubisco and maximum RuBP regeneration rates (Chen et al., 2017). Therefore, shifts in mycorrhizal communities can affect plant host's performance, as some AMF might be better suited to enhance certain nutrient's uptake or might promote a higher tolerance to certain environmental stress factors than other fungi (Camprubí et al., 2008; Eftekhari et al., 2012; Klironomos, 2003; Munkvold et al., 2004; Nogales et al., 2019).

Even though AMF inoculation of cover crops led to a relative compensation in water competition between rye covers and grapevines, and although an improvement was observed in plant response to acute heat stress in terms of plant physiology, severe berry sunburn damage was noted after the heat wave of August 2018 in grapevines growing in both, RC and IRC plots (Fig. S4). In H plots, higher leaf area per plant contributed to a higher canopy density and provided shadow protection to berry bunches. In fact, foliage shading has been shown to reduce berry temperatures up to 10 °C (Spayd et al., 2002). On the contrary, the reduced leaf areas in RC and IRC plots likely resulted in excessive sun exposure, which led to significant yield losses. However, these were less pronounced in IRC plots than in RC plots, suggesting that the establishment of *F. mosseae*-inoculated rye cover crops helped to partially compensate yield losses associated with acute heat stress.

Previous studies have shown that berry composition differs between grapes in sun-exposed bunches and those in shaded bunches, although these changes vary according to the grapevine genotype and berry cluster microclimate (Pastore et al., 2013). Similarly, the establishment of green covers can also result in alterations in berry must characteristics, e.g. in titratable acidity (Lopes et al., 2008). However, the data herein for 'Viosinho' grapevines, did not show any must quality variation related to the installation of inoculated or non-inoculated cover crops, although titratable acidity was slightly lower (but not statistically significant) in plants growing in H plots with more shaded bunches.

5. Conclusions

European legislation is becoming more and more restrictive with the use of herbicides due to environmental and health risks that they may cause. For this reason, it is imperative to develop management alternatives that are respectful to human health and that promote sustainability in agriculture and viticulture. In this sense, the establishment of cover crops in the under-vine space may represent a good solution, although special care needs to be taken to avoid excessive competition with grapevines, especially under non irrigated conditions and/or dry climates.

Overall, our results indicate that *F. mosseae* introduction through rye donor cover crops was an efficient method to inoculate grapevines in a 10-year-old vineyard. Moreover, under-vine cover crop inoculation promoted the establishment of grapevine root mycorrhizal communities that were able to increase plant adaptability to extreme weather events, while ensuring grape quality and partially compensating water competition by the surrounding vegetation.

Author contributions

AN, CL, WV and HSP conceived the ideas and designed the methodology; AN, GV, ER and JLC were responsible for field sample and data collection; AN and CC were responsible for data analysis; AN, CL, JMC interpreted data; and AN led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Funding

This study was financially supported by FCT –Fundação para a Ciência e a Tecnologia, I.P. through the project MYCOVITIS (PTDC/AGR-PRO/0676/2014), the project Linking Landscape, Environment, Agriculture and Food Research Centre Ref. UIDB/04129/2020 and the grant SFRH/BPD/108358/2015.

E.R received funding through the Erasmus + Mobility Programme (European Union), MERMOZ (Hauts-de-France regional grant) and AMI (Aide à la mobilité internationale-CROUS-France).

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgements

Authors are thankful to the "Gabinete de Espaços Verdes" of the Instituto Superior de Agronomia for their collaboration and help in vineyard management, to Prof. Elsa F. Gonçalves for her assistance in statistical analyses, to João Graça, Manon Dias and Diana Faria for their help in data and sample collection, and to Prof. Ana Monteiro for providing rye seeds.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2021.107369.

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