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Correlation between the microscopy and qPCR methods (SYBR Green) to detect and quantify *Rhizophagus irregularis* in grapevine roots

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Summary

For an easier identification and quantification of *R. irregularis* in grapevine, a molecular tool was developed so that each DNA concentration calculated would relate to the degree of root system colonization. To correlate the results obtained by qPCR and microscopy, a different approach for the visualization technique was chosen. It combined the detailed standard method of evaluation on microscopic slides with the global magnifying glass evaluation method in the grids. The global assessment sampling was closer to the qPCR sampling that was made on a representative fraction of the whole root system. This fact became the base of successful correlation between microscopy and qPCR. The result of these measures were the attribution of an average qPCR value to each level of colonization defined as five different classes. Around 0.66 ng·µL^{-1}, the DNA concentration corresponded to the first contacts between the fungus and the grapevine roots (class M1), while around 42 ng·µL^{-1} it accounted for the beginning of the mycorrhizal symbiosis (class M2). A satisfactory mycorrhization level could be concluded from a 258 ng·µL^{-1} DNA concentration (class M3), while all values above 563 ng·µL^{-1} (class M4) showed a full mycorrhization level. The development of this qPCR tool allowed the fast and accurate evaluation of the mycorrhization level in the root system without having to realize any microscopic observation.

K e y w o r d s : nursery; *Vitis vinifera* L. (grapevine); mycorrhiza; *R. irregularis*; microscopy; qPCR; SYBRGreen.

Introduction

Arbuscular Mycorrhizal Fungi (AMF) are mostly symbiotic organisms. They belong to the group of *Glomeromycota* and are probably involved since the middle Paleozoic in facilitation of plant nutrition (Tisserant *et al.* 2013, Finlay 2008). According to Öpik and Davison (2016), only 10-20 % of all AMF have been described, colonizing about 72 % of all plants families (Brundrett and Tedersoo 2018). They perform vital functions by absorbing phosphate, ammonium, and nitrate from the soil (Eckhardt 2005, Bucher 2007, Veresoglou *et al.* 2012) making it easier for plants to adapt to their environment and are fundamental to sustainable plant productivity (Ceballos *et al.* 2013, Smith and Read 1997). Moreover, AMF increase protection against environmental stresses (Lamabam *et al.* 2011) and are involved in the water cycle in terms of improving soil structure and its water holding capacity (Miller *et al.* 2000). Therefore, their importance in agriculture and especially in the nurseries, is undeniable. One of the most common AMF species is *Rhizophagus irregularis* which has been isolated worldwide in agricultural and natural soils. Its isolate DAOM-197198 has been utilized as a commercial inoculant for two decades (Badri *et al.* 2016).

Different staining techniques are available to visualize and to quantify AMF structures (Vierheilig *et al.* 2005). These techniques are primarily used to evaluate the quantity as well as the quality of the symbiosis. They evaluate different mycorrhizal structures such as arbuscules, vesicles, intra-radical spores, as well as the extra-radical structures including hyphae and spores. The most used method of visualization still remains the standard method of Phillips and Hayman (Phillips and Hayman 1970, Brundrett *et al.* 1984, Trouvelot *et al.* 1986) based on the non-vital staining of mycorrhizal structures, followed with coloration methods (Vierheilig *et al.* 1998, Dickson *et al.* 2003, Dickson 2004). There is also the global visualization technique, represented by the grid-line intersection method (Brundrett *et al.* 1996) as well as more complex microscopy methods, which allow the inclusion of arbuscules (Giovannetti and Mosse 1980, McGonigle *et al.* 1990, Smith and Dickson 1991).

All these methods, however, remain rather subjective, with a low repeatability rate and with an interpretation that can vary from one technician to another (Sun and Tang 2012). The visualization techniques encounter other limits, related to fungus identification. It is difficult to make the distinction between different mycorrhizal fungi observed, especially in absence of spores which are essential for such recognition. Another drawback of identification by spores is that their presence may not reflect a symbiotically active organism community (Krüger *et al.* 2009). With an increasing interest for natural products, laboratories are led to evaluate mycorrhizal colonization, focused on a particular strain. However, especially in the case of an evaluation on open-field plants, it is difficult to distinguish the carefully selected commercial strain from another naturally present strain. To overcome the limitations of detection by microscopy, protein

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based methods (Hepper *et al.* 1988, Friese and Allen 1991, Perotto *et al*. 1992, Tisserant *et al.* 1998, Treseder and Allen 2002) and DNA based molecular methods (Simon *et al.* 1992, Clapp *et al.* 1995, Di Bonito 1995, van Tuinen 1998, Redecker 2000, Jansa *et al.* 2002b, 2008, Lee *et al.* 2008) were developed. Even though conventional PCR has been shown to effectively detect and discriminate AMF as genera, species or isolates (van Tuinen *et al.* 1998, Jansa *et al.* 2002b), it does not allow the accurate quantification of DNA.

This limiting factor has promoted the development of quantitative real-time PCR (Heid *et al.* 1996) relying on measuring the intensity of a fluorescent signal that is proportional to the amount of DNA generated during the PCR amplification (Heid *et al.* 1996 , Wittwer *et al.* 1997a). The qPCR is currently well-used to quantify concentration of DNA in a simplified model system (Redecker 2000, Filion *et al.* 2003, Alkan *et al.* 2004, 2006, Gamper *et al.* 2010). However, little is still known about what information this method provides as compared to the traditional measurements of AMF abundance in roots and soil. Currently, no correlation has been made between DNA extracted from roots and mycorrhizal colonization level.

Some studies show that the qPCR method assesses different biological units than the microscopy-based approaches (Gamper *et al.* 2008) and that these approaches were not comparable. Several authors have focused on the problem of the detection of a mycorrhizal fungus by PCR or qPCR (Fillon *et al.* 2003, Alkan *et al.* 2004), as well as the correlation with microscopy (Alkan *et al*. 2006, Gamper *et al.* 2008) without succeeding.

In this study, we hypothesized that the difficulty in correlating the qPCR values to microscopy observation reported by several authors (Alkan *et al.* 2006, Gamper *et al.* 2007) could be solved by using another, more global microscopic approach, which takes into account all the mycorrhizal structures, inside and outside the root and in all types of roots, without prejudicing the quality and quantity assessment of mycorrhiza implantation in the roots.

Material and Methods

Host plants: *Vitis vinifera* variety 'Cabernet Sauvignon' clone 169 grafted on the rootstock *Vitis riparia* x *Vitis rupestris* variety 101-14 'Millardet' and 'de Grasset' clone 1034 was used for the establishment of the correlation curve and validation of the accuracy of the global method in microscopy evaluation. The rootstocks 101-14 759, 3309 144, P1103 768, R110 180, R140 265; SO4 203, Gravesac 264, Fercal 242, RSB 141, 41B 195 and 420A 11 were used to verify the performance of the method via technical triplicates and biological duplicates.

Mycorrhizal inoculum: *Rhizophagus irregularis* strain Pont Rouge (DAOM 181602, DAOM 197198), concentration: 140 spores per liter of substrate.

Substrate composition: 40 % Irish blonde peat, 30 % maritime pine bark, 10 % medium coconut fiber, 20 % fine coconut fiber, 1 kg·m^{-3} starter fertilizer NPK 16-4-17, 0.28 g·m^{-3} mycorrhiza MYC500, 1 g·m^{-3} OSMOCOTE EXACT 8/9, 1.5 g·m^{-3} OSMOCOTE EXACT 5/6, pH 6 Ec 1.0 mS·cm^{-1}.

Sample preparation for microscopy and qPCR assay: The plants were grown in peat substrate in greenhouse under a sprinkling system from April 2017 to November 2017. For the correlation experiment, the roots were collected plant by plant, monthly, from June to October 2017, washed and dried in the open air for 4 h. The roots were segmented into small fragments and mixed together to create a homogeneous and representative sample. 1 g of each sample was used for the qPCR assay and 1 g was used for the microscopy analysis. To assess the performance of the qPCR method via technical triplicates and biological duplicates, the roots were collected in November 2017. 1 g per sample and per analysis was prepared, which corresponds to 2 x 1 g for DNA extraction for each plant. Each DNA extraction was analyzed three times by qPCR, corresponding to R1, R2 and R3.

Root staining for microscopy observation: A modified Philips and Hayman (1970) non-vital CBE method was used as a stain process. Root fragments were cleared in 10 % KOH at 90 °C for 90 min and stained with a trypan blue lacto glycerol 1:1:1 solution at 70 °C for 20 min. Stained samples were stocked in water at 4 °C until analysis.

Estimation of mycorrhizal colonization by microscopy evaluation. The global approach: 1 g of colored roots by sample was spread in a glycerol solution on a Petri dish with a numbered 1x1 cm grid on the bottom. The extent and density of mycorrhizal structures (Fig. 3) were evaluated under magnifying glass in each of the 30 grids analyzed. A class from 0 to 5 was attributed to the total of 30 grids depending on the total volume colonized by hyphae in one grid. Class M0 means that no mycorrhization was observed, M1 corresponds to less than 10 % of the roots volume colonized, M2 indicates a mycorrhization level below 30 %, M3 indicates a mycorrhization rate from 30 to 70 %, M4 indicates a mycorrhization rate up to 90 % and M5 when the mycorrhization is superior to 90 % (Figs 1 and 4). The M mean was calculated for each sample according to the formula:

$$M = \frac{(\sum M0 * 0) + (\sum M1 * 1) + (\sum M2 * 2) + (\sum M3 * 3) + (\sum M4 * 4) + (\sum M5 * 5)}{\sum Grids}$$

M – Mycorrhization rate; M0 – total number of grid classed M0, M1 – total number of grid classed M1 to M5 – total number of grid classed M5.

The arbuscular, vesicular and spore extent and density were assessed by a rating of each structure (St) from 0 (St0, absence of mycorrhizal structures, to 3 for St3, important presence of mycorrhizal structures (Fig. 2). The mean was calculated according to formula:

$$St = \frac{(\sum St0 * 0) + (\sum St1 * 1) + (\sum St2 * 2) + (\sum St3 * 3)}{\sum Grids}$$

St – Mycorrhizal structure (Arbuscule, Vesicle or Spore); St0 – total number of grid classed St0, St1 – total number of grid classed St1 to St3 – total number of grid classed St3. Maximal score – the maximum rating that can be assigned to indicate the estimated amount.

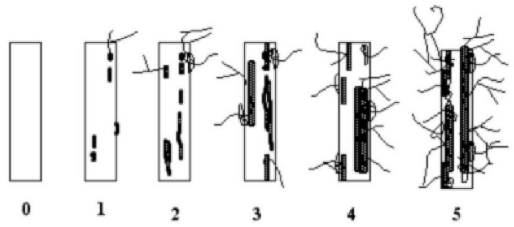

Fig. 1: Scoring mycorrhizal colonization. Illustration of the extension of the hyphae in the root segments according to the class assigned. This method is transposable to the evaluation in the grids where the sample represents the segment. M0 no mycorrhization observed, M1 ≤ 10 % of the root's volume colonized, M2 ≤ 30 %, M3 ≤ 70 %, M4 ≤ 90 % and M5 ≥ 90 %.

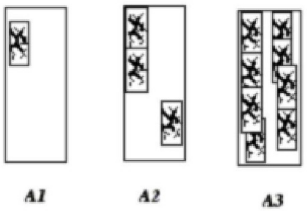

Fig. 2: Scoring arbuscule abundance. Illustration of the structures density in the mycorrhizal part of root segments under the class assigned (ex: arbuscules). Method is transposable to the evaluation in the grids. A0 no arbuscule observed, A1 low density, A2 intermediate density, A3 abundant arbuscules.

Fig. 3: Structures evaluated. From left to right, from top to bottom: hyphae; arbuscules; vesicles; intra-radical spores and vesicles; hyphae and extra-radical spores; extra-radical structures.

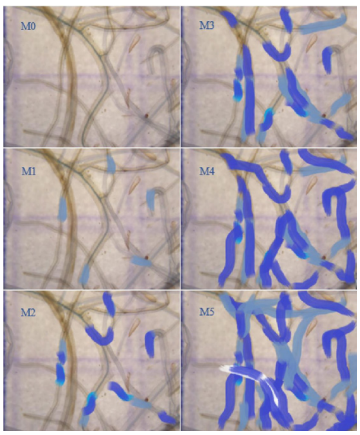

Fig. 4: Grid evaluation method illustration. Classification in classes from M0 to M5 according to the mycorrhization rate observed in one grid.

Only the global mycorrhization rate M was paired to DNA concentrations. The classes designating the arbuscules, the vesicles and the spores were taken into account for a correction of the correlation curve.

The standard method mentioned in this work follows the recommendations of Trouvelot *et al.* (1986). The assigned classes were recalculated in percentage for the mycorrhization rate and frequency.

DNA extraction from spores: Pure genomic DNA was extracted from spores by adapting the DNA Plant II extraction kit protocol (Macherey Nagel, Düren, Germany). Spores were obtained from a commercial product by diluting 10 g of powder in 50 mL of water. After 4 h of soaking, the mix of powder and water was carefully vortexed to resuspend the spores and filtered using three sieves of 500 µm, 250 µm and 50 µm. 100 viable spores of average size were suspended in 1,5 ml of sterile deionized water and concentrated by centrifugation at 10 000 rpm for 15 min. After removing the water, the concentrated spores were crushed with a plastic pestle to release the DNA and then suspended in 2 mL of extraction buffer. The end of the protocol followed the supplier's recommendations.

DNA extraction from roots: Total genomic DNA was extracted from 1 g of root sample prepared as described above using the DNA Plant II extraction kit (Macherey Nagel, Düren, Germany) according to the manufacturer protocol.

Quantitative real-time PCR: The qPCR reactions were carried out with the components supplied in the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA) containing antibody-mediated hot-start Sso7d-fusion polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye enhancers, stabilizers and a blend of passive reference dyes. The 20 µL reaction mixtures contained the following components (final concentrations): reaction buffer (1X), 0,5 µM of forward and reverse primers, 2 µL of DNA template and ultrapure sterile water to make up the final volume.

Each set included a template control containing water to check for contamination in the reaction components. The qPCR was carried out in a C1000 Touch Thermal cycler (Bio-Rad, Hercules, USA), according to the following program: 2 min. at 98 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s and a melting curve going from 65 °C to 95 °C with an increment of 0,5 °C every 5 s, following the supplier recommendations'. The threshold cycle (Ct) was calculated by the Bio-Rad CFX Manager 3.1 software. The primers used were designed by Alkan (Alkan *et al.* 2006) for *Rhizophagus irregularis* and *R. intraradices* detection.

Standard curve preparation using genomic DNA: The standard curve was prepared by using spectrophotometrically quantified genomic DNA isolated from spores as described above. Concentration gradients of DNA ranging from 8,85 pg to 8,85 ng. The concentration of template DNA was plotted against Ct, by means of the CFX Manager 3.1 software. The Ct value was plotted against the logarithmic value of the DNA concentration measured to obtain a standard quantification curve (Fig. 5). The assay was repeated twice, with each dilution reaction set up in triplicates.

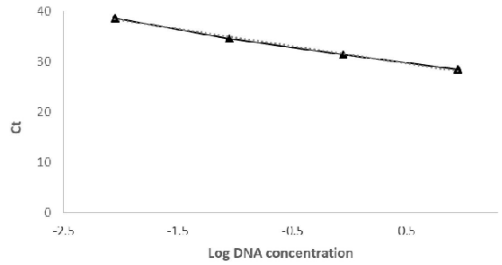

Fig. 5 : Standard curve obtained by plotting threshold cycle (Ct) against genomic DNA concentration (log value). y = -3,383x + 31,385, R^2 = 0, 99.

Statistical analysis: Divergence and correlation between the results were analyzed using Kruskal-Wallis ANOVA test and the AHC (Ascending Hierarchical Classification) and K-means tests for distribution into clusters (Fig. 6). Significance levels of $p < 0{,}05$ were used.

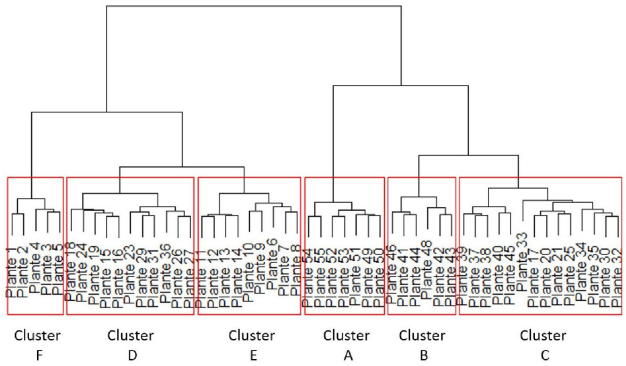

Fig. 6: Dendrogram obtained by ACH statistical analysis. The 59 samples are divided in 6 clusters (A-F) depending on their level of DNA concentration and mycorrhization level.

Results

Comparison between standard and global visualization methods by microscopy: In order to develop a more adequate visualization technique, the standard and global methods were compared on 24 random samples (Tab. 1). For the mycorrhization rate and arbuscules density, no significant difference could be found. However, a significant difference was observed in vesicles and spores evaluation. In general, a higher variation was recorded for the standard method showing a larger disparity between the segments. This work showed that by including the spores and vesicles, the global method evaluated all the mycorrhizal structures present in the sample, rendering the correlation to qPCR results possible.

Standard curve preparation: The primers pair were used to validate the detection of the commercial product strain, from isolated spores and an inoculated grapevine sample. Once the detection of the strain was validated, a standard calibration curve was generated (Fig. 5). A linear relationship was obtained between the Ct of the qPCR amplification reaction and DNA concentration, which ranges between 8,85pg and 8,85 ng. The regression equation of this relationship was as follows: y = -3,383x + 31,385 with R^2 = 0,99.

Accuracy of qPCR evaluation: For one plant, two root samples were prepared for DNA extraction. Each sample was analyzed three times by qPCR, corresponding to R1, R2 and R3. The mean and standard error were calculated for the qPCR triplicates (R1 to R3) and for the biological duplicates (A, B). The results obtained for 10 different plants were compared (Tab. 2).

The accuracy of qPCR quantification was showed by low standard error values between technical triplicates. A larger error was observed for the biological repetitions, meaning that the variance between two preparations of the same sample was more important. This can be caused by a non-homogeneous distribution of the mycorrhizal fungus within a specific sample. However, since the sampling was carried out on 10 plants, with all the roots constituting a sample unified, homogenized and segmented, it was possible to conclude to a larger variation in the quality of mycorrhization between the plants of the same production batch.

Correlation between microscopic evaluation and qPCR quantification: To correlate the DNA concentration and the mycorrhization level, a total of 59 plants were analyzed with both methods over five months. The Kruskal-Wallis test showed that there was a correlation between the DNA concentration and the mycorrhization level (M) measured in microscopy (p-value = 9.447e-07). An AHC (Ascending Hierarchical Classification) and K.means analysis allowed the classification of the samples in a dendrogram (Fig. 6) divided into 6 clusters (A to F), explaining 94.8 % of their distribution. The mean, maximum and minimum of each quantitative variable (mycorrhization rate, arbuscular density, vesicular density and DNA concentration) were determined for each cluster (Tab. 3 and Fig. 7). By comparing the results in each cluster between the mycorrhization level and the DNA concentration, a significant correlation was shown and a biological explanation of the results could be made.

Table 1

Comparison between mycorrhizal rate, arbuscules and vesicles values obtained by evaluation with the standard and global method. The means and standard errors of 24 evaluations are presented in this table. Descriptive Statistics and One-Way ANOVA were performed

	Mycorrhizal rate		Arbuscules		Vesicles / spores	
	Global	Standard	Global	Standard	Global	Standard
Means ± *SE*	3.88 ± *0.15*	4.32 ± *0.16*	2.21 ± *0.08*	2.37 ± *0.10*	1.96 ± *0.07*	1.47 ± *0.08*
Variance	0.549	0.649	0.172	0.220	0.129	0.133
P-value	0.0505		0.224		2.794 E-05	

Table 2

qPCR quantification on 10 rootstock samples with biological duplicates and technical triplicates. The DNA concentration ($ng \cdot \mu L^{-1}$) for each technical replicate. the mean for the three technical replicates and for the two biological duplicates are indicated. Standard error was calculated for each mean (italic letters)

Sample	R1	R2	R3	Technical triplicates means	Biological duplicates means
101-14 A	197.38	121.74	198.73	172.61 ± *25.44*	152.34 ± *20.27*
101-14 B	36.74	134.82	224.63	132.07 ± *54.26*	
3309 A	237.20	235.59	209.85	227.54 ± *8.86*	154.02 ± *73.52*
3309 B	74.07	93.36	74.07	80.50 ± *6.43*	
41B A	39.60	65.08	46.31	50.33 ± *7.63*	58.90 ± *8.57*
41B B	73.07	52.70	76.63	67.47 ± *7.45*	
420A A	133.00	74.58	95.28	100.95 ± *17.10*	81.35 ± *19.60*
420A B	49.57	76.11	59.57	61.75 ± *7.74*	
FER A	76.63	188.20	117.67	127.50 ± *32.57*	117.15 ± *10.34*
FER B	67.34	120.09	133.00	127.50 ± *32.57*	
GRAV A	214.18	101.99	151.36	155.84 ± *32.46*	115.84 ± *40.00*
GRAV B	49.24	97.91	80.37	75.84 ± *14.23*	
P1103 A	292.92	333.36	309.31	311.86 ± *11.74*	277.99 ± *33.87*
P1103 B	317.85	217.11	197.38	244.11 ± *37.30*	
R110 A	76.11	86.62	74.58	79.104 ± *3.79*	107.06 ± *27.96*
R110 B	108.44	146.30	150.34	135.02 ± *13.34*	
R140 A	140.44	196.04	224.63	187.04 ± *24.72*	155.83 ± *31.21*
R140 B	136.67	35.76	201.45	124.62 ± *48.21*	
RSB A	211.28	277.39	411.66	300.11 ± *58.95*	248.41 ± *51.71*
RSB B	169.93	200.08	220.09	196.70 ± *14.58*	
SO4 A	82.03	56.03	87.21	75.09 ± *9.65*	105.77 ± *30.68*
SO4 B	80.37	143.34	185.65	136.46 ± *30.59*	
		Standard error mean		*22.71*	*31.61*

Discussion

In the aim of correlating the results obtained by qPCR and microscopy, a performant visualization technique was developed for which the evaluation was closer to the biological reality of the sample because it took into account the whole representative sample and not only segments separated from the plant. This raises a hypothesis that by this separation, many details could be overlooked such as the presence of spores or hyphae outside the root segment surface (Tab. 1). However, all these structures contain DNA that is detectable in qPCR.

Moreover, the high precision for the microscopy evaluation by standard method hinders the establishment of satisfactory correlation between the mycorrhization level observed and the qPCR value obtained. By rounding the mycorrhization levels to convert them into classes from M0 to M5, the correlation has been confirmed as shown in Tab. 3. This study has demonstrated that a more global view of mycorrhizal colonization of the roots in the sample can be rather beneficial to determine a more accurate status *in situ*.

This study also showed that later in the season there was a disparity between the DNA concentration and the microscopic quantification, characterized by a high presence of mycorrhizal structures for a lower DNA concentration. According to the correlation curve based on the values collected throughout the growth period, the DNA quantity extracted correspond to the M2 or M3 level rather than to the M4 or M5 level observed under the magnifying glass. This disparity has been revealed for the first time under a statistical analysis which has assigned to a specific cluster (C) the majority of the values from September 2017. A high presence of mycorrhizal structures can be observed in cluster C, close from the level observed in cluster B "Strong mycorrhization", but the DNA concentration was average. This suggests an over evaluation of the mycorrhizal levels by microscopy, which took into account senescent mycorrhizal structures depending on the sampling period. In contrast, the qPCR only quantified the

Table 3

Results of the correlation between mycorrhization level and DNA concentration. The correlation was based on the mycorrhization level (rate), the arbuscule (arb) and vesicle (ves) densities measured with the global method and the DNA concentration ($ng \cdot \mu L^{-1}$) obtained by qPCR. The clusters were obtained by analysis of the 59 samples by ACH. The mean, maximum and minimum of DNA concentration of the samples for each cluster is indicated. The standard error *SE* was calculated for each group (italic letters)

Cluster	Class	Rate	Arb	Ves	Mean DNA concentration	Max	Min	Interpretation
A	M5	4.9 ± *0.10*	2.4 ± *0.19*	1.8 ± *0.12*	1705.02 ± *89.11*	1956.4	1510.5	Maximum mycorrhization level
B	M5	4.8 ± *0.12*	2.7 ± *0.14*	1.6 ± *0.11*	845.73 ± *61.68*	1182.3	645.1	Strong mycorrhization
C	M4	4.5 ± *0.13*	2.3 ± *0.14*	2.3 ± *0.12*	400.96 ± *34.66*	563.0	220.1	Average mycorrhization
D	M3	3.0 ± *0.08*	2.0 ± *0.06*	1.6 ± *0.14*	258.48 ± *39.59*	511.8	41.5	Development of the mycorrhization
E	M2	1.5 ± *0.22*	1.6 ± *0.15*	1.3 ± *0.17*	42.62 ± *6.80*	59.9	16.2	Low mycorrhization
F	M1	1.0 ± *0.00*	1.3 ± *0.24*	1.0 ± *0.11*	0.66 ± *0.15*	1.2	0.2	First contact

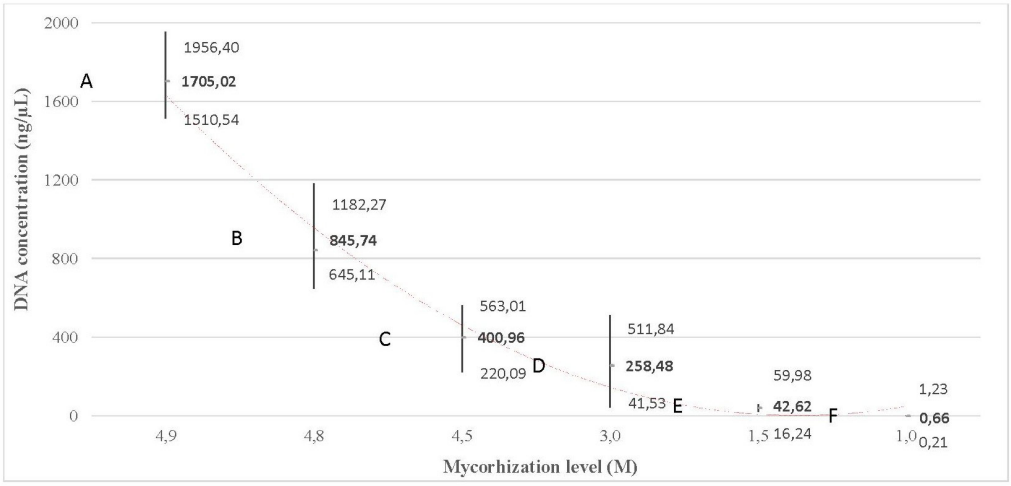

Fig. 7: Correlation curve between mycorrhization level and DNA concentration based on the clusters defined by ACH. The endo-mycorrhizal colonization was quantified with the global microscopy method (class M) and correlated to DNA concentration (ng·µL^{-1}) obtained by qPCR. The mean (in bold letters), the maximum and the minimum of the samples for each cluster is indicated on the polynomial standard curve (red dot). The correlation between the data is validated by the $R^2 = 0.9818$.

active mycorrhizal structures containing DNA. Hence, it is possible to consider a complementarity between microscopy and qPCR approaches. While qPCR is able to discriminate active and non-active mycorrhizal structures and thus to characterize a real mycorrhizal potential in the plant, microscopy can observe other fungal structures in roots and give extra information about root colonization.

In conclusion, this work was able to correlate the extent of colonization by a mycorrhizal fungus, *Rhizophagus irregularis*, in the root system to a range of values obtained by qPCR. The lowest DNA concentration measured (0.66 ng·µL^{-1}) corresponded to the first contacts between the fungus and the roots. This value reflects a few viable hyphae, mostly detectable at the root surface. For an average value of 43 ng·µL^{-1}, the mycorrhizal levels corresponds to the establishment of the mycorrhizal symbiosis. Starting from the value of 258 ng·µL^{-1}, an acceptable mycorrhization level in the root systems can be concluded, corresponding to half of the root system colonized. All the values superior to 563 ng·µL^{-1} correspond to a high mycorrhization level, which signify an established and functional symbiosis. However, as shown statistically (Fig. 6), we could consider simplifying mycorrhizal appreciation in the root system by grouping the 6 classes into 3, such as early mycorrhization, mycorrhizal development, and strong mycorrhization level. It may be also interesting to establish a threshold, all values up to this level indicating early mycorrhization and its development, while all the values above this level indicate a good mycorrhization.

This work was done on grapevine roots in controlled conditions and artificial soil. It will be interesting to use those innovative tools to follow the development of the natural mycorrhization in vineyard conditions, opening new windows of studies to better understand the relation between mycorrhiza and grapevine.

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