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Note

Experimental validation under controlled conditions of real time PCR to quantify arbuscular mycorrhizal colonization in root

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ABSTRACT

Mycorrhizal colonization of roots is traditionally evaluated by empirical methods, such as root microscopy. We compared this method with data from using a real time PCR technique, and determined the correlation between methods, indicating particularities of a promising system for a quick and accurate molecular diagnostic of arbuscular mycorrhization.

Symbiotic associations between plant roots and arbuscular mycorrhizal fungi (AMF) are very common in agro-ecosytems (Machado and Furlani, 2004), representing one of the most important strategies to enhance plant nutrition (Brady and Weil, 2010; Neumann and Martinoia, 2002).

AMF colonization is traditionally evaluated by microscopic observations of particular fungal morphological structures in root fragments after a coloration procedure (Vierheilig et al., 1998). Although this methodology is informative, it is time-consuming and requires a high level of training and experience of the operators (Brundrett and Tedersoo, 2019). Hence, there is a clear need for more standardized methods for AMF quantification.

Molecular techniques analyse the number of nuclei and are promising but these methods are still rarely used to quantify AMF colonization, mainly because the gold standard microscopically-based methodology and molecular techniques are based on different parameters. Many specific primers of the Glomeromycota phylum have been developed (Gollotte et al., 2004; Lee et al., 2008; Krüger et al., 2009), more often targeting the small ribosomal subunit (SSU rDNA) (Öpik et al., 2010). In recent years, the real time polymerase chain reaction (qPCR) has proved to be a valuable tool in the area of AMF field ecology, at the level of AMF species, taxa or community (Alkan et al., 2004; Pivato et al., 2007; Thonar et al., 2012; Bodenhausen et al., 2021). However, the use of this tool compared to the observation approach is complicated by the differing number of ribosomal RNA operons between species. Additionally, the quantification of the ITS region is not a direct quantification of cell numbers. For this reason, some researchers argue against the use of these targets as a reliable reporter of AMF quantification. Here we compared the microscopy and qPCR data, based on a different parameter, in samples along a gradient of root mycorrhization from a controlled experiment, which has never done before, to allow us to validate qPCR as a method to highlight differences in AMF colonization (the experimental design is presented in details in Arruda et al., 2021).

Two crops were used: brachiaria (*Urochloa brizantha* cv. Marandu, Poaceae) and crotalaria (*Crotalaria juncea* L., Fabaceae), inoculated with AMF spores (10 mL of ~18 spores mL^{-1} solution with sterile deionized water to each pot) of *Acaulospora colombiana* (Acaulosporacea); *Rhizophagus clarus* (Glomeracea); and *Dentiscutata heterogama* (Gigasporacea). The couting of spores mL^{-1} was made in subsamples (10 mL, $n = 4$) of the isolated spores solution, using a SMZB magnifying glass (Motic Group Co. Ltd., China), for each AMF species. The amount of spores was standardized for all three AMF species based on the species with the least amount of spores available, by diluting the spore solution for the other

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Table 1
Regression parameter estimates, *p* values and confidence intervals for mycorrhizal colonization of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*C. juncea* L.) cultivated in a soil submitted to distinct manipulated microbiome. Plants were inoculated with: *Acaulospora colombiana; Rhizophagus clarus;* and *Dentiscutata heterogama.*

	f = y0 + ax	Coefficient	*p* value	Standard Error	t	R^2	Adjusted R^2	Standard Error of Estimate	Person's coefficinet (r)
Complete dataset	y0	3.8766	<0.0001	0.0900	43.0945	0.5261	0.514	0.3271	0.7253
	a	0.0181	<0.0001	0.0027	6.5802				
Brachiaria	y0	3.7665	<0.0001	0.1033	36.4577	0.6719	0.6555	0.2938	0.8197
	a	0.0213	<0.0001	0.0033	6.3994				
Crotalaria	y0	4.0487	<0.0001	0.1585	25.5404	0.3368	0.2978	0.3590	0.5803
	a	0.0135	0.0092	0.0046	2.9381				
Acaulospora colombiana	y0	3.8864	<0.0001	0.1108	35.0832	0.5791	0.5528	0.2992	0.7610
	a	0.0164	0.0002	0.0035	4.6915				
Rhizophagus clarus	y0	4.0247	<0.0001	0.1716	23.4556	0.0668	−0.0369	0.3142	0.2585
	a	0.0056	0.4428	0.0070	0.8027				
Dentiscutata heterogama	y0	3.8803	<0.0001	0.2704	14.3491	0.5763	0.5233	0.3667	0.7591
	a	0.0220	0.0109	0.0067	3.2985				

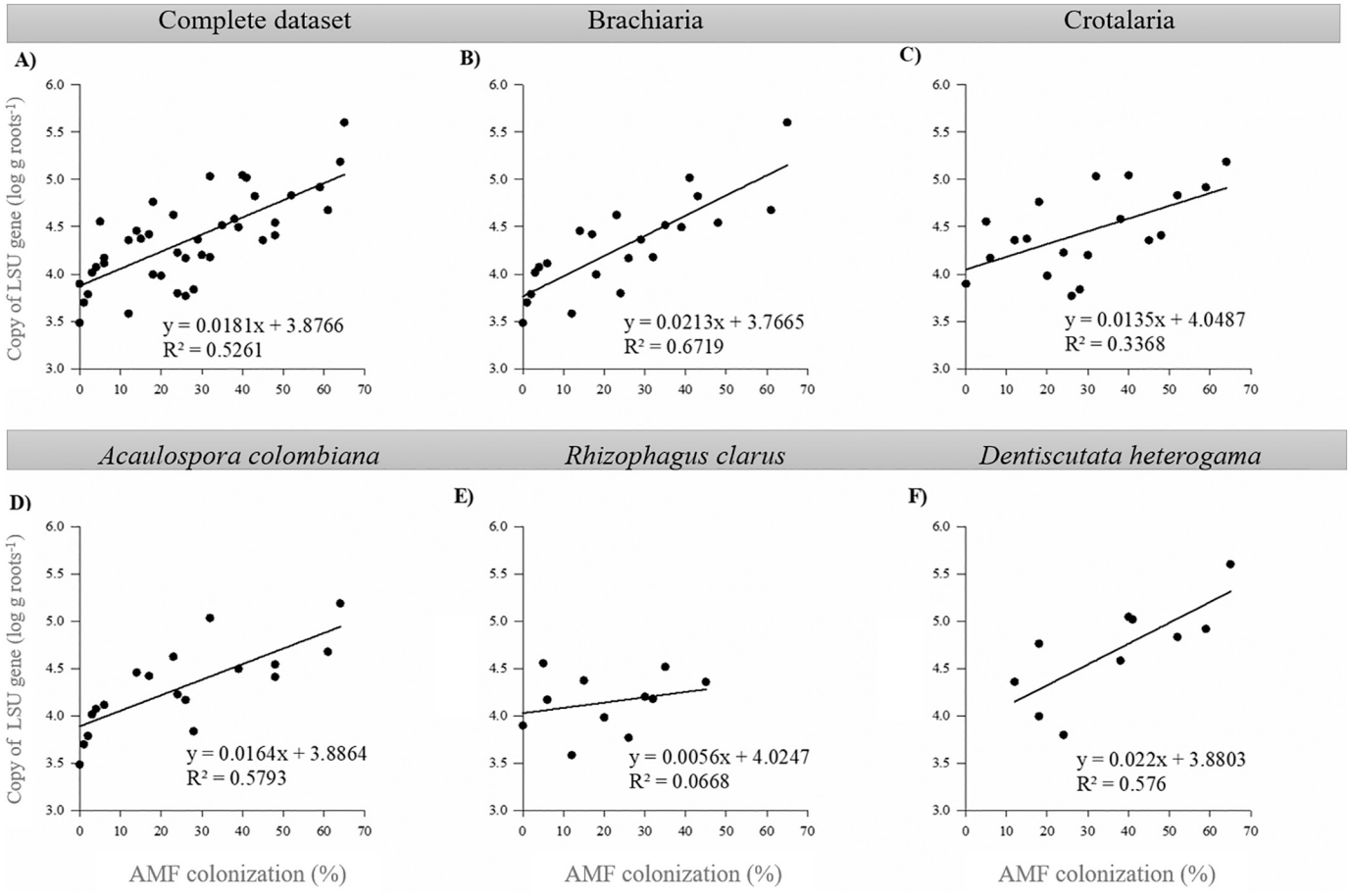

Fig. 1. Linear relationship between the abundance of the gene copies of Glomeromycete large ribosomal subunit (LSU) rDNA sequences, using FLR3 and FLR4 primers in a qPCR reaction, and mycorrhizal colonization (%), using microscope in roots of brachiaria (*Urochloa brizantha* cv. Marandu) and crotalaria (*C. juncea* L.) separated according to follow: A) Complete dataset; B) Brachiaria (*U. brizantha* cv. Marandu) and C) Crotalaria (*C. juncea* L.); D) *Acaulospora colombiana;* E) *Rhizophagus clarus;* F) *Dentiscutata heterogama.*

two species to achieve the same amount. At harvest, 20 days after the start of the experiment, roots were washed; one part was preserved in 70% alcohol (for microscopy) and another part was kept at −20 °C (for qPCR).

Root samples, kept in ethanol, were clarified with 10% KOH, stained with blue ink (Vierheilig et al., 1998) and a total of 30 root fragments (~1 cm root length) were analyzed under the microscope (BX40; Olympus, Japan), varying from 0%, where no fungal structures were observed, to >90%, for root segments fully colonized by the AMF (Trouvelot, 1986). A total of 26 samples of brachiaria and 24 samples of crotalaria were selected based on AMF colonization percentage (Table S1), to contemplate a gradient of colonization, from 0% to the highest percentage of colonization observed in the samples (65% for brachiaria and 64% for crotalaria).

Root DNA was extracted from ~0.1 to 0.4 g of selected root samples, kept at −20 °C, using a DNeasy™ Plant Mini Kit (Qiagen, Germany),

according to the manufacturer instructions. AM fungi qPCR assays were run using a Applied Biosystems Step One Real-time PCR System (Thermo Fisher Scientific, Singapore), with a SYBRGreen detection, and the set of primers FLR3 and FLR4 (Table S2), in the same conditions as described by Gollotte et al. (2004). Standard curves were obtained using tenfold serial dilutions of purified PCR (10^3 to 10^9 copies) containing the targeted gene. The reliability of the standard curves was controlled by verifying reproducibility of the Ct values, the quality of the dilution series and the efficiency (94.05%). The specificity of the primers were confirmed by melting curves analysis.

Correlation between the percentage of colonization obtained by microscopy and qPCR was determined and the adjustment of the regression was obtained (SigmaPlot 12.5). This approach considered either the complete dataset (three AMF and two plants) or data separately per plant or AMF species.

Considering the complete dataset, the correlation coefficient (r) between the abundance of the gene by qPCR and the mycorrhiza colonization obtained by microscope was 0.7253 (Table 1), and showed a $R^2 = 0.5261$ (Fig. 1A). This correlation could be impacted by the fact that root samples were split in two subsamples before analyzes. Even though the root systems were equally separated during sampling, spatial heterogeneity may affect the correlation between the analysis (Gamper et al., 2008). Redecker (2000) used the same dyed sample to extract the DNA, but even in this case, the sample representativeness can be questioned from 1 to 2 cm fragments.

When correlations were analyzed separately for each plant species, a better correlation between microscopy and qPCR was observed for brachiaria ($r = 0.8197$, $R^2 = 0.6719$) in comparison to crotalaria ($r = 0.5803$, $R^2 = 0.3368$) (Fig. 1B). This result indicates that the visibility of roots structures are determinants of the efficacy of methods for AMF quantification, as found by Alkan et al. (2004). These authors observed a better correlation between the microscopic and qPCR analysis in tomato than in medicago roots, and attributed this result to the difficulty in visualizing AMF structures in medicago roots.

Separating the data according to the AMF species, *Acaulospora colombiana* and *Dentiscutataheterogama* showed a better correlation compared to *Rhizophagusclarus* inoculation (Fig. 1D, E, F). Many studies have shown that depending on the set of primers, specific groups of mycorrhizal fungi are not amplified with the same intensity, which could explain this result. Similar observations were made by Jansa et al. (2008) using different primers (LR1 and FLR2), with good correlation between methods for all AMF species inoculated in medicago and leek roots, excepted for *G. mosseae*.

In this study, even if spores of specific AMF groups were inoculated, roots can be also colonized by endogenous AMF presented in the soil which explained our choice to target communities and not specific species. Thonar et al. (2012) studied the establishment 19 AMF isolates in maize, medicago and leek root by qPCR using the combination of specific primers. The correlation between microscopy and qPCR was not observed for some AMF species, most likely, due to the presence of other AMF taxa, for which qPCR markers are not yet available.

To conclude, our data showed that qPCR allowed differences in mycorrhization to be highlighted. Therefore, the set of primers FLR3 and FLR4 seem to be suitable for the rapid diagnosis of mycorrhization, showing better correlation for brachiaria compared to crotalaria, and under inoculation of *A. colombiana* and *D. heterogama*.

Authors' contributions

BA devised the project, conducted the experiment and wrote the first draft of the manuscript. YFR performed the qPCR analyses in the laboratory. All authors contributed to subsequent revisions, read, and approved the final manuscript.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106382.

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