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TECHNICAL NOTE

A modified staining technique for arbuscular mycorrhiza compatible with molecular probes

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Abstract The effects of the different steps of the root staining on the arbuscular mycorrhizal (AM) fungal rDNA extraction and amplification have been assessed. The results obtained using molecular techniques are compared with those obtained from fresh, non-stained leek roots. A modified staining procedure that eliminates heating, the use of hydrochloric acid and trypan blue, has been proved to be the most adequate to observe the AM colonisation in different plant species with/without lignified roots allowing at the same time the subsequent rDNA extraction and amplification from the stained roots. The staining technique decreased the sensitivity of the process and a higher number of roots had to be used to obtain enough material for a positive amplification. The extraction and amplification process was reliable up to 3 days after staining. A week after staining, the amplification was not dependable and after 2 weeks there was no amplification from stained material.

Keywords Arbuscular mycorrhizal fungi · Fungal detection · DNA extraction · Nested PCR · Staining techniques

Introduction

The ubiquitous arbuscular mycorrhizal (AM) fungi are an integral component of any soil system and their effects on plant growth and ecosystem resilience are well documented. The existence of the symbiosis in a plant is determined by the presence of intraradical specific structures, recognisable after clearing and staining the roots. The development of molecular techniques has been a step forward in mycorrhizal studies and several taxa can now be determined through specific primers. The combination of both methodologies would allow the attainment of concrete information in natural and in agricultural ecosystems to further understand the ecological specificity and differential efficiency of this symbiosis. The staining techniques conventionally used are modifications of the original technique of Phillips and Hayman (1970). Although some authors have extracted and amplified DNA from roots stained by this technique (Van Tuinen et al. 1998), in our experience, the chemicals used, especially HCl, may hamper DNA extraction and amplification and thus the yield of positive results can be low and random. To avoid this difficulty, many authors divide the plant roots in two batches; one for colonisation assessment after staining and the other for molecular studies (Gollotte et al. 2004). Ishii and Loynachan (2004) modified the Koske and Gemma (1989) technique by eliminating the acidification step with HCl, keeping the clearing step with 10% KOH, and the staining with trypan blue. The roots used were from 4-week seedlings, allowing for good microscopic results from this simplified method, that would be difficult to use in woodier roots and the trypan blue might also produce artefacts in the DNA extraction and amplification (Kwok et al. 2004). A modification of these techniques was proposed by Vierheilig et al. (1998) in order to eliminate from the process the chemicals that created a higher health risk: the stains, trypan blue and acid fuchsin, lactic acid and HCl. The authors used young seedlings roots as a model, and no DNA extraction was attempted from stained roots.

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In this work, we have started from the modifications by Kormanik and McGraw (1982) and Koske and Gemma (1989) of the Phillips and Hayman (1970) procedure and integrated different changes (Vierheilig et al. 1998; Ishii and Loynachan 2004) to develop a staining technique that permits the microscopic observation with an acceptable resolution of herbaceous and woody plant roots and is compatible with molecular studies of the same root fragments. This method enables us to determine both colonisation percentages, from the observation of the stained roots, and the identity of the colonising fungi from the molecular studies.

Materials and methods

Plantlets from two species with different root characteristics were inoculated with *Glomus intraradices* Schenck & Smith BEG72. The plants used were leek (*Allium porrum* L) and olive (*Olea europaea* L). Leek plants were grown from seed for 3 months before harvesting. Olive plants cv "Arbequina" were 3-month-old rooted cuttings obtained from a commercial nursery (Agromillora Catalana S.A.); plants were inoculated at re-potting and grown for 6 months before harvesting. The growing medium was a pasteurised sandy soil, quartz sand and sphagnum peat (Floratorf Floraguard GmbH) mixture in a proportion of 3:2:1 (*v*/*v*). The resulting texture of the mix had 76% sand, 14% silt and 1% clay. The pH was 7.5, with 1% organic matter, a CEC below 10 meq/100 g and 8 mg/kg of Olsen extractable P. The colonisation of young leek roots by *G. intraradices* can be observed, under the dissecting microscope, without recourse to staining, due to the yellow pigment that accumulates in mycorrhizal roots (Bothe et al. 1994; Schliemann et al. 2008) and to the detection of the intraradical spores of the fungus in the non-suberised leek roots. These features were used to assess the impact of the chemicals and procedures used in the different steps of the staining techniques on the fungal DNA extraction and amplification, using non-treated leek roots as a reference.

The staining techniques conventionally used are modifications (Kormanik and McGraw 1982; Koske and Gemma 1989) of the method proposed by Phillips and Hayman (1970). These procedures include several stages that need to be modified to lower the impact of the chemicals on the fungal DNA and also to decrease health risks. A step by step system was undertaken to determine which processes negatively affected the DNA extraction using leek as a model plant and testing modifications of these techniques and also of the non-toxic method developed by Vierheilig et al. (1998). The DNA amplification was assessed in treated leek roots against a positive control of fresh non-stained colonised leek roots. Roots from olive and leek plants were used to assess the results of the staining technique under the dissecting microscope. Olive plants stained with different techniques were used to assess the success of DNA amplification in lignified roots. To determine the dependability of the technique, a leek root system, where colonisation was evident due to the yellow pigment acquired, was split in two halves; one was stained and five and ten 1-cm root pieces, chosen under the microscope, of either stained or fresh colonised roots, were

Table 1 Step-by-step assessment of the staining process with respect to microscopic visualisation of the symbiosis and DNA extraction and amplification in AM-colonised leek roots

Clearing treatment	Pre-staining treatment	Staining	De-staining	Microscopic visualisation	DNA extraction And PCR amplification
None	None	None	None	Faint, but positive	Positive
KOH 10% 15 min at 90°C	1% HCl 10 min	None	None	Faint	Negative
KOH 10% overnight	None	None	None	Faint	Negative
KOH 1% 120 min at 70°C	None	None	None	Faint	Negative
KOH 1% overnight	None	None	None	Faint	Positive
KOH 1% overnight	1% HCl 10 min	None	None	Faint	Negative
KOH 1% overnight	75% Acetic acid 10 min	None	None	Faint	Negative
KOH 1% overnight	5% Acetic acid 5 min	None	None	Faint	Positive
KOH 1% overnight	5% Acetic acid 5 min	0.05% Trypan blue in lactoglycerol	Lactoglycerol	Positive	Negative
KOH 1% overnight	5% Acetic acid 5 min	10 min at 90°C in 5% Black Shaeffer ink in 5% acetic acid	Acetoglycerol	Positive	Negative
KOH 1% overnight	5% Acetic acid 5 min	24 h in 5% Black Shaeffer ink in 5% acetic acid	Acetoglycerol	Positive	Positive

used to extract and amplify DNA. The process was replicated five times for each concentration of root pieces, fresh or stained. To assess the shelf life of the stained roots in respect to DNA extraction and amplification, the molecular probes were carried out in leek roots 1 day, 3 days, 1 week and 2 weeks after staining. To normalise the DNA extraction step and because in many instances roots obtained from soil carry as many PCR inhibitors as the soil itself (Kennedy et al. 2007), fungal DNA extraction was done using the Power Soil DNA isolation kit (MoBio Laboratories Inc., Carslbad, CA, USA). Root pieces were crushed with a micro-pestle in the extraction buffer, and the following steps were done according to the manufacturer's instructions. Primary PCR was performed with the eukaryote specific primers LSU0061(LR1)/LSU0599 (NDL22) (Van Tuinen et a. 1998), with 2 µl of the DNA root extract as template, 2 µl of a 10 µM solution of each primer and 10 µl of Eppenndorf Master Mix 2.5× (Eppendorf AG, Hamburg Germany) in a total volume of 25 µl. PCR conditions were: initial denaturation at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min; the last cycle was followed by a final extension at 72°C for 7 min. Amplicons were diluted 50 times and then used as templates in a nested PCR with specific primers for the Glomerales: FLR3/FLR4 (Gollote et al. 2004). Nested PCRs were carried out in 25 µl volume and the reaction conditions differed from the primary PCR conditions on the annealing phase that was done at 60°C instead than at 54°C and on the length of the final extension phase at 72°C changed to 10 min instead of 7 min. PCR products were visualised and separated by electrophoresis in 2% agarose gels stained with SYBR Safe® dye, following the producer's protocol. In all the molecular probes, a previously extracted DNA from leek roots inoculated by *G. intraradices* BEG 72, subjected to the nested PCR amplifications and sequenced to verify the fungal identity, was used as a positive control.

Results

The modifications reported are the synthesis of the results observed in the staining treatments of leek and olive root systems. These modifications are listed for each basic step of the staining process compared with the conventional techni-

Table 2 Staining variations used for detecting AM colonisation in woody roots allowing subsequent DNA extraction and amplification

Clearing	Bleaching	Acidification	Staining	De-staining	Microscopic visualisation	PCR amplification	Lane number
KOH 1% overnight	None	5% Acetic acid 5 min	5% Black Shaeffer ink in 5% acetic acid 24 h	acetoglycerol	Negative		
KOH 1% overnight + 1% KOH at 70°C 2 h	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	0,05% Trypan blue in acetoglycerol 24 h	acetoglycerol	Positive	Negative	2
KOH 1% overnight + 1% KOH at 70°C 2 h	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 24 h	acetoglycerol	Negative		
KOH 1% overnight + 1% KOH at 70°C 1 h	30% H2O2 + NH3 1 h	5% Acetic acid 1 h	0,05% Trypan blue in acetoglycerol 24 h	acetoglycerol	Positive	Negative	4
KOH 1% overnight + 1% KOH at 70°C 1 h	30% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 24 h	acetoglycerol	Negative		
KOH 1% overnight + 1% KOH at 70°C 1 h	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 48 h	acetoglycerol	Positive	Negative	3
KOH 1% overnight + KOH 1% 70°C 1 h	10% H2O2 1 h	5% Acetic acid 1 h	0,05% Trypan blue in acetoglycerol 24 h	acetoglycerol	Positive	Negative	7
KOH 1% overnight + KOH 1% 70°C 1 h	10% H2O2 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 48 h	acetoglycerol	Positive	Negative	8
KOH 1% overnight	30% H2O2 + NH3 1 h	5% Acetic acid 1 h	0,05% Trypan blue in acetoglycerol 24 h	acetoglycerol	Positive	Negative	9
KOH 1% overnight	30% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 48 h	acetoglycerol	Positive	Negative	10
KOH 1% overnight	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	0,05% Trypan blue in acetoglycerol 24 h	acetoglycerol	Positive	Negative	5
KOH 1% overnight	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 48 h	acetoglycerol	Negative		
KOH 1% overnight	10% H2O2 + NH3 1 h	5% Acetic acid 1 h	5% Black Shaeffer ink in 5% acetic acid 72 h	acetoglycerol	Positive	Positive	6

Lane numbers refer to the lanes in the staining gel reproduced in Fig. 2.

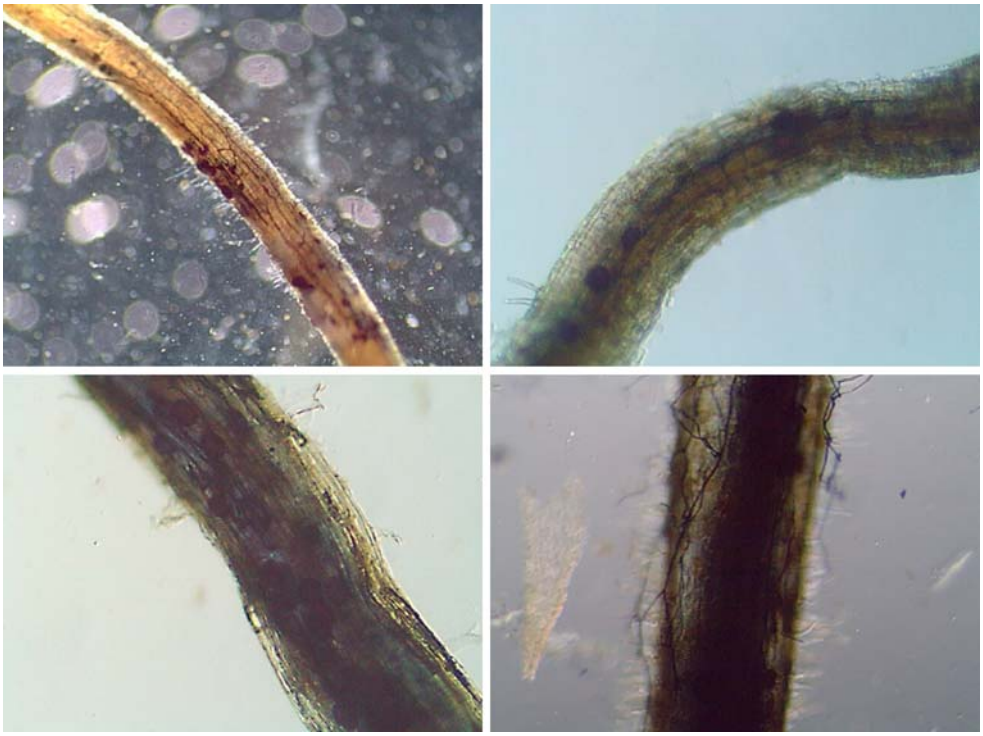

Fig. 1 Olive roots stained with Black Shaeffer ink

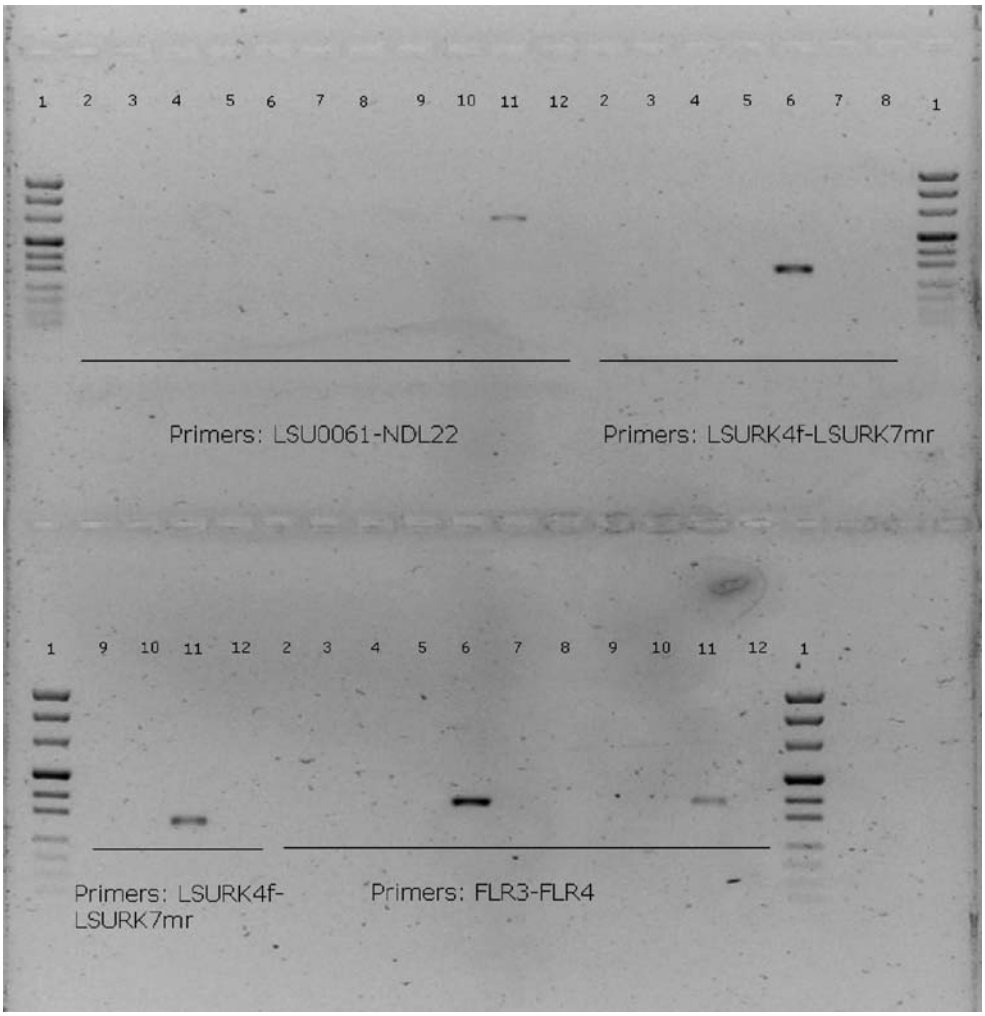

Fig. 2 Primary and nested PCR detection of sequences from stained olive roots using the primers LSU0061 and NDL22 and FLR3 and FLR4, after staining with different methods. Each *lane* corresponds to a staining treatment specified in Table 2 except for lane *1*: DNA ladder and lane *11*: positive control

ques. In Table 1, the variations done to obtain a clear positive visualisation of the leek root colonisation together with a positive PCR amplification are reported. In Table 2 are listed the combinations tried to achieve the staining of olive roots (Fig. 1) and a positive PCR amplification result (Fig. 2).

Clearing

The conventional clearing methods use heating, for up to 121°C, in an aqueous solution of KOH (from 2.5% to 10%). In the modified method, roots were left overnight in 1% KOH to achieve an acceptable clearing. The use of 10% KOH or the heating of 1% KOH resulted in no DNA amplification of colonised leek roots.

Rinsing and bleaching

Cleared roots are washed several times with tap water and left for 1 h with an alkaline solution of H_2O_2 (from 3% to 10%). The standardised use of a higher titre hydrogen peroxide (20%, 30%), or of hypochlorite (Kormanik and McGraw 1982; Koske and Gemma 1989) in fresh colonised leek roots, results in no amplification. Vierheilig et al. (1998) do not bleach roots and achieve good results in 7-week-old herbaceous plants; for older and woodier roots (olive), bleaching is essential to see the intra-radical fungal structures.

Acidification

Cleared roots, bleached if necessary, are left 1 h in a 5% solution of acetic acid (equivalent to household white vinegar). The use of HCl, even at 0.1% and for a short period, results in no amplification from colonised leek roots.

Staining and de-staining

Roots acidified with 5% acetic acid solution were stained in a 5% Black Shaeffer ink in a 5% acetic acid solution at ambient temperature for 72 h and de-stained in a solution of acetic glycerol (glycerol 500 ml:H_2O 450 ml and 5% acetic acid solution 50 ml).

No DNA amplification from colonised leek roots was obtained with the use of a 0.01% fuchsin–lactic acid staining solution (Kormanik and McGraw 1982) or of a 0.05% trypan blue acidic glycerol solution (Koske and Gemma 1989) or boiling the roots in a 5% ink 5% acetic acid solution (Vierheilig et al. 1998).

The conventional staining techniques developed by Kormanik and McGraw (1982) and Koske and Gemma (1989) did not result in DNA amplification from colonised leek roots. Variations of these techniques show that the use of 10% KOH and 1% HCl, trypan blue and heating over

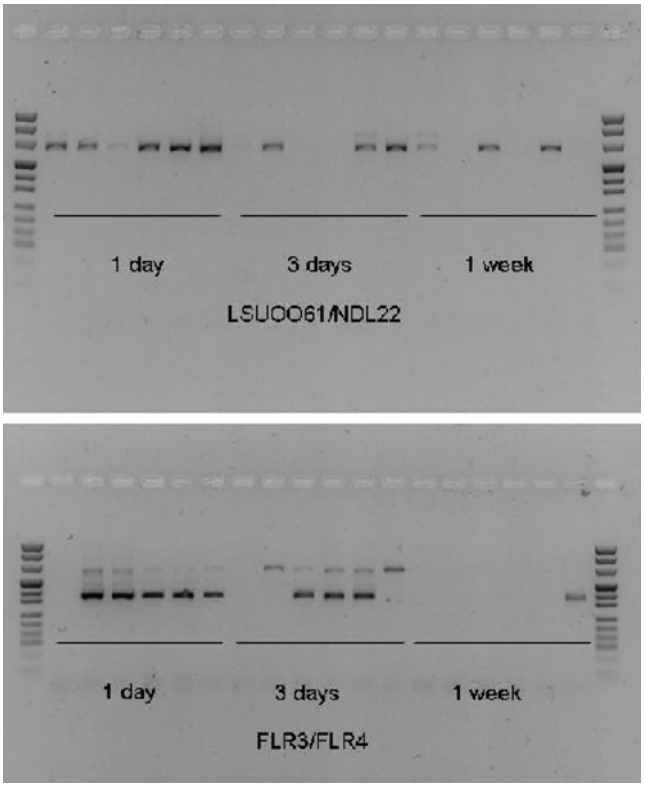

Fig. 4 Primary and nested PCR detection of sequences from stained leek roots using the primers LSU0061 and NDL22 and FLR3 and FLR4, respectively, 1 day, 3 days and 1 week after staining

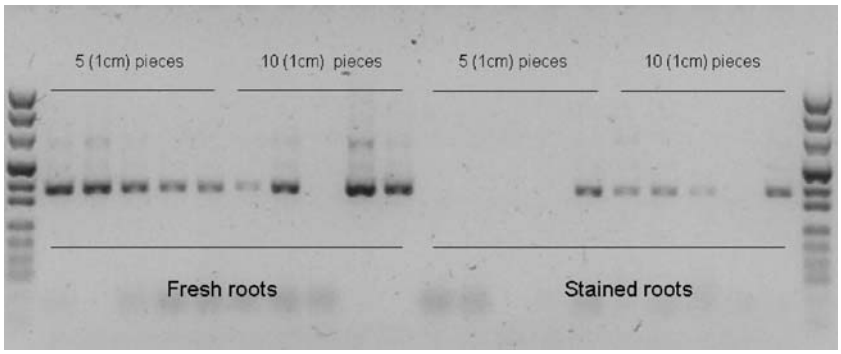

Fig. 3 Nested PCR detection of sequences from fresh and stained leek roots using the specific primers FLR3 and FLR4, 24 h after staining

90°C (Tables 1 and 2) yield no amplification of DNA from *G. intraradices*-colonised leek and olive roots. Amplification from leek roots was successful using a modified system based on Vierheilig et al. (1998) method, where clearing and staining were done at ambient temperature (instead of boiling) and de-staining was done with acetic glycerol. The microscopic visualisation after this procedure was good for leek roots; however, it did not allow the observation of the intraradical structures in the woody roots of olive, these roots retained a dark pigment that prevented the observation of the fungal structures in the root. A bleaching step was added using alkaline 10% H_2O_2 for 1 h which allowed for DNA amplification and for the microscopic differentiation between colonised and non-colonised woody roots (Table 2). To obtain a dependable DNA extraction and amplification, five 1-cm root pieces from fresh leek roots were enough; however, when the roots were stained, ten 1-cm pieces were needed to obtain similar results (Fig. 3). The stained roots DNA extraction and amplification was reproducible 3 days after staining and erratic 1 week after staining; 2 weeks after staining, the molecular probes did no longer give positive results (Fig. 4).

Discussion

The basic processes used to stain the roots involve cell wall and cell membrane chemical digestion to allow the penetration of the dyes. Trypan blue is a diazo-dye derived from toluidine and has been shown to have teratogenic effects in rats (Christie 1965) and to alter cell viability and gene expression in human retinal pigment epithelial cells (Kwok et al. 2004). Ishii and Loynachan (2004) report PCR amplification from *Glomus mosseae*-colonised roots after clearing with 10% KOH and staining with trypan blue in acetoglycerol, eliminating the acidification step with HCl; however, our results show that following clearing with 10% KOH and trypan blue staining there was no DNA amplification from *G. intraradices*-colonised stained roots. The quality of staining is linked to a good digestion of the cell walls (achieved with KOH), and with woody roots a step with H_2O_2 is also necessary to be able to see the stained fungal tissue inside the roots. The acidification step is important for the penetration of the dye, the hydrochloric acid can be changed to acetic acid and the stain still is of good quality. Ishii and Loynachan (2004) eliminated the acidification step and presumably got good staining in herbaceous seedlings; our results show very poor staining quality in older plants, even herbaceous, when the acidification step is eliminated. In 3-month-old leek roots, the differences between staining with 1% KOH, acetic acid and ink or staining with 10% KOH, HCl and trypan blue were negligible; however, in the case of lignified 9-month-old olive roots, the use of 1% KOH needed to be complemented by 10% H_2O_2 achieving a partial bleaching that permitted a laborious assessment of the percentage colonisation. The time delay between staining and DNA extraction is crucial and might account for failures in the PCR amplification. The use of a soil extraction kit was done to remove inhibitors and to simplify the methodology; however, it can also decrease the yield of DNA extracted, thus accounting for the need of five 1-cm pieces from fresh roots and ten 1-cm pieces from stained roots to obtain a specific PCR amplification. In our experimental conditions, the staining technique proposed allows for the staining and ensuing molecular assessment of mycorrhizal roots, and can be used in plants with lignified roots. The combined use of both techniques will be useful when assessing field samples to correlate, amongst other parameters, the presence and the intensity of the root colonisation with the identity of the symbionts.

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