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Short Communication

An Improved Ink-acetic Acid Technique for Staining Arbuscular Mycorrhizas of Citrus

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Abstract

Trypan blue is usually applied to stain arbuscular mycorrhizas of higher plant roots, whilst it is harmful for human health. The objective of the study was to exploit a safe, non-toxic, and cheap technique for staining mycorrhizal structure of plants such as citrus (*Citrus unshiu* grafted on *Poncirus trifoliata*). The 1–2 cm root segments were cleared with 10% KOH for 90 min at 95ºC, blanched with H_2O_2 (10%) for 15 min, acidified with 0.2 mol/L HCl for 10 min, stained with 5% black, blue or red ink prepared with 5% acetic acid for 3–8 min, and destained with 5% acetic acid. It is clear that both 5% blue ink-acetic acid solution and 0.05% trypan blue solution showed better color contrast and visible mycorrhizal structures. The mycorrhizal colonization of roots stained with 5% blue ink-acetic acid solution for 3–5 min was not significantly different, compared with 0.05% trypan blue staining for 5 min. On the other hand, the mycorrhizal colonization was higher in the roots stained with the 5% blue ink-acetic acid or 0.05% trypan blue than with the other ink-acetic acid staining. The results suggest that use of trypan blue for arbuscular mycorrhizal staining could be replaced with 5% blue ink-acetic acid solution for 3–5 min, at least in citrus roots.

Keywords: Arbuscular mycorrhizal fungi; Blue ink; Citrus; Mycorrhizal staining; Root mycorrhizal colonization

Introduction

Arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota, can establish the symbiosis with most terrestrial plants, namely, arbuscular mycorrhiza (AM). The symbiosis usually improves the uptake of water and mineral nutrients of the host plant in exchange for photosynthetically fixed carbon (Bucher *et al*., 2009). Several experiments have shown that inoculation with AMF can help the host plant for the uptake of mineral nutrients, especially P, promote plant growth, and enhance tolerance of abiotic and biotic stresses (Smith & Read, 2008; Feddermann *et al*., 2010; Miransari, 2010; Wu & Zou, 2011). In addition, AMF are an important component of natural and agricultural ecosystems, which influences plant diversity and community structure (Bainard *et al*., 2011).

A widely used procedure for staining arbuscular mycorrhizal colonization in root tissues was developed by Phillips and Hayman (1970) using trypan blue. Trypan blue, an acid azo dye, is listed by the International Agency for research on Cancer as a possible carcinogen. Contact with trypan blue may cause mild irritation, gastro-intestinal discomfort, even nausea and vomiting. Long-term exposure of trypan blue may induce retinal damage (Veckeneer *et al*., 2001). If the trypan blue solution is poured into water condition, water and soils will be polluted. In this case, a safe and non-toxic technique for arbuscular mycorrhizal staining will be exploited to replace trypan blue used mycorrhizal researches.

Although other researchers have proposed the use of acid fuchsin or chlorazol black E, the staining reagents are still suspected carcinogens (Gaur & Varma, 2007). Mauler-Machnik and Nass (1990) have developed a simple staining technique with an ink-25% acetic acid solution for infection by *Pseudocercosporella herpotrichoides* in wheat. An ink-vinegar solution was successfully used to stain mycorrhizal fungal structures clearly visible (Vierheilig *et al*., 1998). The objective of the present study was to find a viable and non-toxic arbuscular mycorrhizal stain with an ink-acetic acid solution for replacing trypan blue in citrus.

Materials and Methods

The citrus tree used here was 23-years-old *Citrus unshiu* cv. Guoqing-1 grafted on *Poncirus trifoliata*, which belongs to a campus orchard of Yangtze University, Jingzhou, China. The root samples of five similar citrus trees were collected at 5–15 cm soil depth within a 1.5 m canopy radius in May, 2010 and stored by FAA at 4°C until analysis.

The experimental design was completely randomized, comprising ten treatments each with three replicates: red, black and blue ink (Hero, Hamed Warasta Group of Companies, Shanghai, China) stained respectively for 3, 5, and 8 min, and trypan blue (Sigma, USA) for 5 min as the

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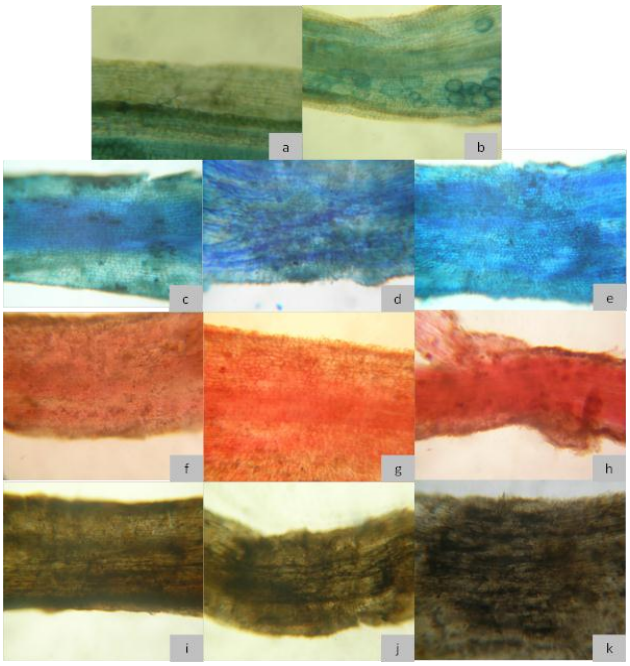

Fig. 1: Root mycorrhizal staining status of citrus roots. A and b were stained with trypan blue for 5 min, c, d and e with 5% blue ink-acetic acid respectively for 3, 5 and 8 min, f, g and h with 5% red ink-acetic acid for 3, 5 and 8 min, and i, j, and k with 5% black ink-acetic acid for 3, 5 and 8 min

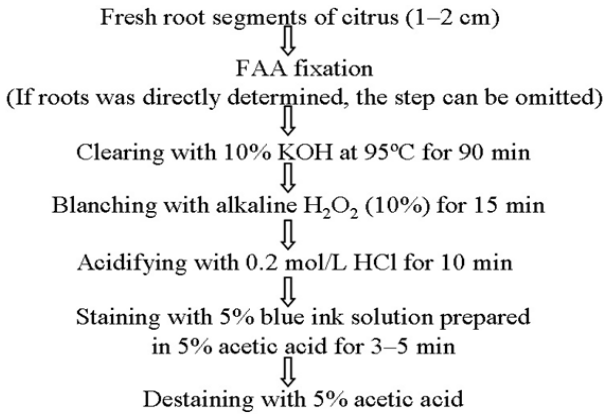

Fig. 2: A simple, non-toxic, and cost-effective blue ink-acetic acid staining procedure to visualize arbuscular mycorrhizas in citrus roots

control. For each repeat at least fifteen root segments were selected.

The fresh roots were chopped into 1–2 cm root segments, which were cleared with 10% KOH at 95ºC for 90 min, blanched with alkaline H_2O_2 (10%) at room temperature for 15 min, and acidified with 0.2 mol/L HCl at room temperature for 10 min. The treated root materials were processed with trypan blue and ink-acetic acid solution, respectively. Part of the root segments was stained with 0.05% trypan blue in lactophenol for 5 min (Phillips & Hayman, 1970). The other part was stained with 5% ink solution prepared with 5% acetic acid for 3, 5, or 8 min and destained with 5% acetic acid (Vierheilig *et al.*, 1998). Measurement of root mycorrhizal colonization was done after clearing and staining the roots. The stained root specimens were placed in a glass slide and examined by a light microscope (DME, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with a E5400 digital camera (Nikon Corporation Instruments Company, Tokyo, Japan). Estimation of AM colonization was assessed using the Wu *et al.* (2008) method as a proportion of the root length infected by AMF.

A one-way ANOVA was used to analyze the data with the 8.1 version of SAS software. Fisher's Protected Least Significant Difference was applied to compare the means at 5% level.

Results and Discussion

When the root segments were stained with trypan blue, the root mycorrhizas were stained and clearly visible intraradical hyphae and vesicles were found (Fig. 1a–b). Moreover, there was a better contrast observed in the trypan blue-stained roots. The result implies that trypan blue as a color reagent could obtain better staining. Therefore, trypan blue staining is generally used for mycorrhizal observation at low magnifications.

On the other hand, trypan blue is mutagenic and carcinogenic. Mycorrhizal researchers also selected acid fuchsin, cotton blue, chlorazol black E, and wheat germ agglutinin as the mycorrhizal stains, which are azo dyes and cannot be used as less-toxic replacements (Vierheilig *et al.*, 2005). Inks are non-toxic and are recommended as baby safe grade. In the present study, after staining with ink-acetic acid solutions, the colonized roots represented different effects on color contrast, hyphae, vesicles, and arbuscules. In general, the staining with blue ink-acetic acid exhibited better color contrast and visible mycorrhizal structures (Fig. 1 c–e), and using the red ink-acetic acid and black ink-acetic acid as the color reagent showed flat contrast and illegible hyphae (Fig. 1 f–k).

When compared with root mycorrhizal colonization, it showed that the root colonization and the staining was similar between trypan blue for 5 min and blue ink-acetic acid for 3–5 min (Table I). The result suggests that the use of 5% blue ink-acetic acid for 3–5 min could replace trypan blue as the staining reagent for mycorrhizal staining. Once the roots were stained with the 5% blue ink-acetic acid in 8 min, the mycorrhizal colonization would be significantly decreased (Table I). Therefore, both the ink species and the staining time are vital for the staining procedure used here.

In the present study, black and red ink-acetic acid solution could stain mycorrhizal structures of citrus roots (Table I). However, the mycorrhizal colonization stained by black and red ink-acetic acid solution was significantly lower than that stained by trypan blue. The result might

Table 1: Root mycorrhizas after staining with trypan blue, red ink, black ink and blue ink in roots of citrus

Staining agent	Stained time (min)	Root colonization (%)	Stained result	Comment
Trypan blue	5	32.96a	Fungus stained; better contrast; clearly visible intraradical hyphae, vesicles, etc. (Fig. 1a–b)	Suitable
5% blue ink-acetic acid	3	32.34ab	Fungus stained; good contrast; visible intraradical hyphae (Fig. 1c)	Suitable
	5	32.25ab	Fungus stained; good contrast; visible intraradical hyphae, arbuscule (Fig. 1d)	Suitable
	8	21.67c	Fungus stained; flat contrast (Fig. 1e)	Not suitable
5% red ink-acetic acid	3	28.00b	Fungus stained; flat contrast; illegible intraradical hyphae (Fig.1f)	Not suitable
	5	10.36e	Fungus stained (Fig. 1g); flat contrast; illegible hyphae	Not suitable
	8	12.67de	Fungus stained (Fig.1 h); flat contrast; illegible hyphae	Not suitable
5% black ink-acetic acid	3	17.33cd	Fungus stained (Fig. 1i); flat contrast; illegible hyphae	Not suitable
	5	9.12e	Fungus stained (Fig. 1j); flat contrast; illegible hyphae	Not suitable
	8	9.57e	Fungus stained (Fig. 1k); flat contrast; illegible hyphae	Not suitable

Note: Means followed by the same letter within a column are not significantly different at $P<0.05$

ascribe to the fact that bad color contrast and illegible hyphae, vesicles and arbuscules occurred in the roots stained by black and red ink-acetic acid solution. As a result, the staining figures bring the researchers to difficultly distinguish the mycorrhizal structures. Therefore, black and red ink-acetic acid solution cannot be used to stain mycorrhiza, at least in citrus roots. Interestingly, Vierheilig *et al*. (1998) found adequate staining of mycorrhizal ryegrass roots with black ink from Carrefour, Pelikan, Shaeffer or Cross Company and red ink from Park Company. Further study will conduct to evaluate the staining technique with ink-acetic acid solution in other plants.

In addition, trypan blue (28.6 dollars/5 g, Sigma) is a more expensive biochemical reagent than ink (0.8 dollars/60 mL, Hero). Meanwhile, in bleaching, trypan blue staining method used lactophenol (mixture of lactic acid, glycerol, phenol & distilled water) as bleaching solution, but the improved ink-acetic acid staining method 5% acetic acid. It seems that blue ink-acetic acid staining was a cheaper procedure for staining arbuscular mycorrhizas than trypan blue. It is known that trypan blue hampered DNA extraction and amplification from the stained roots (Pitet *et al*., 2009). Further experiments are need to clarify whether the blue ink-acetic acid affects DNA extraction and amplification from the stained roots.

Conclusion

We strongly propose the use of 5% blue ink-acetic acid solution for 3–5 min to replace trypan blue to stain arbuscular mycorrhizas of citrus roots, which can give the same color contrast, visible mycorrhizal structures and mycorrhizal colonization. The procedure of clearing and staining root mycorrhizas using the improved blue ink-acetic acid method was briefly outlined in Fig. 2. The other ink-acetic acid solutions combined with different staining times were not suitable to stain arbuscular mycorrhizas, at least in citrus roots.

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