Fluorescent Staining of Arbuscular Mycorrhizal Structures Using Wheat Germ Agglutinin (WGA) and Propidium Iodide

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Abstract

The colonization of a host plant root by arbuscular mycorrhizal (AM) fungi is a progressive process, characterized by asynchronous hyphal growth in intercellular and intracellular spaces, leading to the coexistence of diverse intraradical structures, such as hyphae, coils, arbuscules and vesicles. In addition, the relative abundance of intercellular and intracellular fungal structures is highly dependent on root anatomy and the combination of plant and fungal species [1, 2]. Lastly, more than one fungal species may colonize the same root, adding a further level of complexity. For all these reasons, detailed imaging of a large number of samples is often necessary to fully assess the developmental processes and functionality of AM symbiosis. To this aim, the use of rapid and efficient staining methods that can be used routinely is crucial.

We herein present a simple protocol to obtain high detail images of both overall intraradical fungal colonization pattern and fine morphology, in AM root sections of *Lotus japonicus*. The procedure is based on tissue clearing, fluorescent staining of fungal cell walls with fluorescein isothiocyanate-conjugated wheat germ agglutinin (WGA-FITC) and the combine counter-staining of plant cell walls with propidium iodide (PI). The resulting images can be acquired using traditional or confocal fluorescence microscopes and used for qualitative and quantitative analyses of fungal colonization, of particular interest for the comparison of mycorrhizal phenotypes between different experimental conditions or genetic background.

Key words AM symbiosis, *Lotus japonicus,* roots, wheat germ agglutinin, propidium iodide, confocal microscopy, fluorescence.

1. INTRODUCTION

The detection and quantification of AM fungal colonization in host plant roots has proven a central step in the research of this plant symbiosis and a wide range of microscopy-based techniques have been developed to this aim [3, 4]. These approaches include large scale quantification of root system colonization, relative abundance of different intraradical structures such as hyphae, arbuscules or vesicles, the use of vital dyes to highlight fungal metabolic activity [5]. Some of the most common methods to visualize intraradical symbiotic structures include tissue clearing and subsequent staining with cotton blue [6] or chlorazol black E [7, 8]. Alternative nontoxic staining protocols were developed more recently, such as the ink and vinegar method [9]. Such methods normally achieve an acceptable definition of fungal colonization at single cell resolution, but the fine details of intracellular structures may result blurred. Under this respect, the introduction of fluorescent dyes has increased image contrast and resolution [10], especially when combined with image acquisition in confocal microscopy. Acid fuchsin is a red fluorescent dye that produces detailed images of fungal and plant cell walls [7, 11 – 13], with the additional advantage of an intense pink color under brightfield illumination (useful for the rapid screening of colonized root segments and low magnification imaging), and the disadvantage of high toxicity.

The use of wheat germ agglutinin (WGA) conjugated with a fluorophore is a very reliable alternative with a unique efficiency in resolving the finest details of fungal morphology [14]. WGA is a lectin that specifically binds chitin [15], the most abundant component of fungal cell wall [3]. Because of its high specificity, and the ability to bind chitin in both fresh and fixed samples, WGA-based protocols have been used to investigate fungal wall morphology in many studies of whole or sectioned samples, with both pre- and post-embedding staining [16 - 19].

WGA can efficiently be conjugated with different markers, making it a very versatile marker of fungal walls in both electron [16] and fluorescence microscopy [20]. While in the first case the lectin is conjugated with colloidal gold particles (with a diameter ranging between 5 and 30 nm) [18, 21, 22], small fluorophores such as fluorescein isothiocyanate (FITC), tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) or the wide range of Alexa fluorophores have been used as WGA tags for fluorescence microscopy in an increasing number of publications [3, 14, 19, 23 – 27]. This wide range of fluorescent WGA conjugates

allows their use in combination with additional fluorescent dyes, by optimizing spectral separation. Fluorescent WGA staining protocols provide unrivalled image contrast and detailed imaging of fungal morphology. Such advantages are counterbalanced by the typical disadvantages of fluorescence-based protocols. Exposure to direct light should be prevented throughout sample preparation, to reduce signal fading; background autofluorescence of root tissues may overlap with the fluorophore emission spectrum; fluorescence quenching and photobleaching may progressively reduce signal intensity upon long periods of observations [27]. With due precautions, this approach has anyway proven very successful in a wide range of plants [3], including rice, maize, soybean, legumes, onion, sunflower. Furthermore, the combined use of other compatible fluorescent markers for plant cell walls has also been introduced to provide general details of root anatomy and highlight its alteration during AM colonization [3, 19].

Herein, we describe a rapid staining method based on the combined use of FITC-conjugated WGA and propidium iodide (PI) on fixed, sectioned and cleared sections of *Lotus japonicus* roots inoculated with *Funnelliformis mosseae*; we have direct experience of the effectiveness of this method in other AM fungal and plant associations (e.g. *Gigaspora margarita, G. rosea; Medicago truncatula, Daucus carota*), suggesting that it can easily be adapted to most mutualistic and non-mutualistic plant-fungus interactions.

2. MATERIALS

2.1 Equipment and material

1. Petri dishes
2. Vibratome
3. Microscope slides
4. Coverslips
5. Light-blocking box to store mounted microscope slides during and after staining
6. Fluorescence or confocal microscope equipped with an objective of at least 40X magnification.

2.2 Plant material

1. AM colonized roots of *Lotus japonicus* (MG20), as described in [19].

2.3 Buffers and other solution

1. 0.01 M Phosphate buffered saline (PBS), pH 7.2.
2. 70% ethanol
3. Fixative buffer: dissolve 1% formaldehyde in hot PBS (90°C) by stirring until the solution is clear. Add 10% dimethyl-sulfoxide (DMSO).
4. Embedding agarose medium: 5 % agarose (Type II-A, Medium EEO, Sigma Aldrich) dissolved in boiling distilled water.
5. Clearing solution: dilute commercial bleach 1:10 in PBS
6. FITC-WGA solution: dissolve (10 mg/mL) FITC-WGA (Invitrogen) in PBS.
7. PI solution: dissolve (100 ng/ml) propidium iodide (PI) in PBS. A 10X or 100X stock solution can be used.
8. Antifade reagent (e.g. ProLong, Immunological Sciences).

3. METHODS

3.1 Plant growth conditions

1. Plants of *L. japonicus* (MG20) - grown in pots containing sterile quartz sand and watered with Long Ashton solution [28] – were mycorrhized with the AM fungus *Funneliformis mosseae* [19].

3.2 Root sample preparation

1. 0.5 to 1 cm long AM-colonized root samples from 8-wk-old pot-grown legume plants are harvested and incubated overnight in fixative buffer at 4°C (see Note 1 and 2).
2. Samples are subsequently washed three times in PBS for 5 min to remove excess fixative.
3. Fixed roots are embedded in 5% agarose inside a 4 cm-diameter Petri dish. Following solidification, a blade is used to prepare agarose blocks containing the samples (see Note 3 and 4).
4. Agarose blocks are sliced using a Vibratome to obtain 100µm-thick sections that are collected in a Petri dish containing PBS.

3.3 WGA staining

1. Sample slices are placed on microscope slides (previously cleaned with 70% ethanol, rinsed in distilled water and air-dried) and incubated for 5 min in clearing solution, by covering most of the microscope slide with a large drop (see Note 5).
2. After gently removing the clearing solution with the help of filter paper, sections are washed three times for 10 min with phosphate buffered saline.
3. Slides are then incubated for 2 hours at room temperature with FITC-WGA solution.
4. After removing the WGA solution, samples are washed three times for 10 min in PBS.
5. PBS is replaced with a few drops of PI solution (approximately 50µl) and an equivalent volume of antifade reagent. Slides are then cover-slipped and stored at 4°C in light-proof boxes containing wet filter paper to prevent slide drying (see Note 6 and 7).

3.4 Confocal microscopy

1. Imaging is obtained using either a fluorescence microscope equipped with appropriate filter sets for FITC and PI; alternatively, a confocal microscope can be used to excite both fluorophores at with 488nm (Ar laser) and record FITC (emission window at 500-550nm) and PI (600-700nm) fluorescence.
2. High-resolution images require the use of X40 or higher magnification immersion objectives.
3. Confocal Z -stacks of different total thickness and z-step can be acquired, depending on the dye penetration and image requirements. Indicatively, 40-50 optical sections with 1 µm z-step provide acceptable detail of arbuscule morphology. Maximum brightness projections are normally prepared from each z-stack (Fig. 1)

4. Notes

1 Fixation is optional: if not applied, a single 10 min rinse in PBS is recommended before clearing.

2 If necessary, fixed samples can be stored in PBS at 4°C for up to a few days.

3 Prepare the embedding agarose medium half an hour before use and keep it liquid by heating it in a boiling water bath at about 89.5°C.

4 Precise orientation of root segments during sectioning of either longitudinal or transverse sections is critical to obtain high quality images of both fungal morphology and root anatomy.

5 The optimal duration of tissue clearing in diluted bleach should be adjusted for each plant species. Short treatments may prevent the complete removal of cytoplasm residues and membranes, limiting WGA access to exposed chitin in fungal walls. Similarly, too long a treatment may cause partial degradation of the fungal wall and result in a similar loss of image contrast and resolution.

6 Even if several hundred µm-thick sections can be used, in most cases the dye penetration will be limited to the first couple of cell layers at the section surface.

7 Sample drying during any step of the procedure should be carefully avoided, to preserve tissue morphology.

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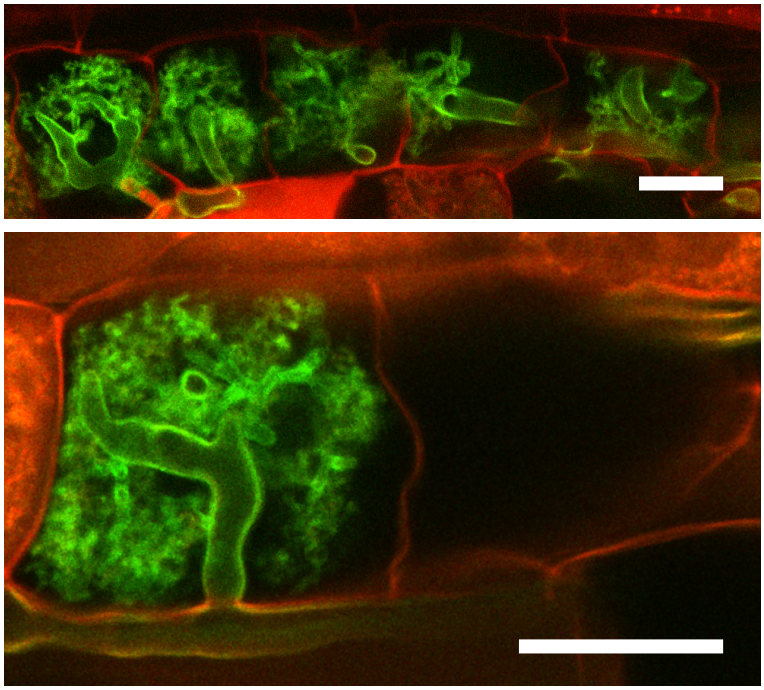

Fig. 1. Representative images of *L. japonicus* root samples colonized by *F. mosseae*. Fungal walls are stained with WGA-FITC (green) and plant walls with PI (red). Bars = 25µm.