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AGAROSE-PARAFFIN DOUBLE EMBEDDING ASSISTED WITH 3D PRINTING FOR HIGH THROUGHPUT RICE ROOT SECTIONING

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SUMMARY

Anatomical analysis of the plant tissues is an important technique used in plant research for studying the process of plant growth, development, adaptation to environmental stress as well as for characterizing gene localization. In Vietnam, most anatomical analysis for plant material was manually conducted which is time-consuming and labor-intensive. In this report, we present an innovative method that can deliver high-quality rice root sectioning. To optimize the efficiency of the conventional paraffin sectioning technique, the agarose-paraffin double embedding was assisted with 3D-printing technique for designing the specific mold which allow to fix from 3 to 12 rice roots in agarose. Molds consist of a lower section with grooves for firmly holding the rice roots in the right place, the additional middle sections allowing up to 3 layers of root samples to be inserted and an upper section to hold the samples before pouring the embedding. The agarose blocks obtained from the molds were then fixed in the formalin solution, then dehydrated through a gradient concentration of ethanol before being impregnated through a series of mixtures of paraffin/xylene and finally embedded in the paraffin. Anatomical sections of 10 μm of thickness were cut with a microtome, spread on glass slides, and then used for staining and collecting the images. As a result, the agarose-paraffin double embedded tissue blocks were more easily orientated and better solidified for rapid and high-quality sectioning. The sections also showed no interference of agarose with staining, as result, the tissues were much clear. In conclusion, this method represents an innovative technique for high throughput plant sectioning which could be applied to different types of plant tissues.

Keywords: agarose-paraffin block, anatomical analysis, double embedding, rice root sectioning, histological analysis, 3D printing mold.

INTRODUCTION

The anatomical analyses of plant tissues are an important approach for studying the process of plant growth, development, adaptation to environmental stress as well as for characterizing gene localization (Zhou *et al.*, 2014; Wachsman *et al.*, 2015; Augstein, Carlsbecker, 2018). Various anatomical analyses have been carried out in *Arabidopsis*, in rice or many other plants, for different types of tissues such as root, stem, leaf, or flowers (Dolan *et al.*, 1993; Burton *et al.*, 2012; Péret *et al.*, 2009; Lartaud *et al.*, 2015).

The root system is an important part of a plant that is responsible for water and mineral uptake, anchoring, and drought avoidance. In *Arabidopsis* - a model plant, many anatomical studies have been conducted to describe the process of lateral root initiation and development (Dolan *et al.*, 1993; Dubrovsky *et al.*, 2006). In rice (*Oryza sativa* L.), the root system includes seminar root,

crown root, and lateral root in which crown roots take the majority part (Rebouillat *et al.*, 2009). Since anatomical analysis is a laborious experiment, the sectioning technique still needs to be improved and optimized, especially for high-throughput phenotyping experiments (Atkinson, Wells, 2017).

To obtain a thin section, plant samples need to be embedded in solidified materials such as agarose or paraffin before sectioning by a microtome or a vibratome. The role of solidified materials is to form the block containing plant tissues which will be easily orientated and sufficiently solid for sectioning (Lhotáková *et al.*, 2008, Atkinson, Wells, 2017). Paraffin has been used for many years for sectioning the plant tissues (Ruzin, 1999). In this process, plant samples will become hardened by replacing the intracellular water with paraffin. The long and laborious procedure of paraffin embedding consists of many steps such as fixation, dehydration, infiltration, and mounting could result in a very thin and high-quality section of plant tissues (Zelko *et al.*, 2012; Metusala, 2017). Another solidified material that was also successfully applied in anatomical technique is agarose (Ron *et al.*, 2013). Agarose-embedding was used for the fresh specimens, which does not require a long procedure of fixation and infiltration, therefore it took less time and labor than using paraffin material. Also, thanks to the longer solidification duration, the handling step to stabilize the sample in agarose is much easier than paraffin embedding. Nevertheless, the plant tissues embedded in agarose are not sufficiently hardened for very thin and high-quality sectioning (Ron *et al.*, 2013). Therefore, combining the advantages of both solidified materials, the agarose-paraffin double embedding method was developed in which the tissue sample is firstly impregnated in agarose for stabilizing and subsequently in a block of paraffin. This combined method is suitable for the small and fragile tissues since agarose helps to stabilize and hold the sample and make the sample easily orientated in paraffin wax.

In this paper, we applied an agarose-paraffin double embedding method to deliver a high-quality section. Also, new 3D-printing molds were designed for increasing the throughput of tissue sections compared to the conventional sectioning methods.

MATERIALS AND METHODS

3D printing molds

The 3D models of embedding molds are designed according to Atkinson (Atkinson, Wells, 2017) with some modifications to adapt to rice root study such as mold size, number of groove, number of mold layer…) by using Fusion 360 software (Autodesk, USA). Polylactic acid (PLA) molds were produced using Fused Deposition Modeling (FDM) 3D printer (Stratasys, Ltd., USA) in Fab-Lab at University of Science and Technology of Hanoi.

Plant materials and sample collection

In this study, *O. sativa cv.* Kitaake and the transgenic rice containing one specific promoter fused with the *GUS* coding sequence were used. The rice seeds were germinated on half-strength Murashige and Skoog medium (Duchefa, Netherlands) in test tubes and grown in the phytotron at 28°C with 14 h/10 h of day/night period. The plant tissues including the stem base, seminal root, crown roots and lateral root were carefully collected from 7 day-old plantlets.

Agar-paraffin double embedding

Embedding in agarose

The 3% agarose (w/v) is prepared for the embedding medium. Agarose was completely melted by microwave and kept at 50°C. After being cut from the rice plants, rice root samples are placed immediately into the mold grooves using curved blunt tip tweezers. Next, the clamp is gently pressed on top of the grooves to stabilize the roots in place. The molds are then filled with agarose and let cool down to the room temperature for 1 h (Fig. 2A).

Fixation, Dehydration, clearing, infiltration and mounting

The agarose-embedding molds containing rice root were fixed in the FAA solution (formaldehyde 3.7%, acetic acid 5% and ethanol 50%) in the vacuum chamber for 3 h with the replacement of solutions every hour. This procedure lets the air bubbles get out from plant tissues for better infiltration of paraffin into plant tissues. For the dehydration process, the sample is further shifted into the series dilution of ethanol from 30% to 100% (v/v) with the replacement of solutions every hour. Next, the samples were incubated in an ethanol-butanol mixture with the increasing concentration of butanol until 100% before incubating in the same solvent overnight to increase the paraffin infiltration. This was then followed by the sample transfer to a series of solutions in which the concentration of butanol is gradually replaced by xylene for clearing. Finally, for paraffin infiltration, xylene was gradually replaced by molten paraffin at 60°C. After 48 to 72 h of embedding in paraffin, the samples were adequately solidified for the mounting process. In this step, the modular tissues embedding center Myr EC350 (Thermo Scientific, USA) was used for rapid and better handling.

Sectioning, staining and image collection

A vibrating HM 340E Rotary Microtome (Thermo Scientific, USA) was used for plant tissue sectioning. A cross-section of 10 μm was produced, placed onto glass slides, next, dried at 50°C for a few hours. Slides can be used for immediately staining or can be stored at room temperature for further analysis.

Before staining, the slides containing plant tissues must be de-paraffinized in 100% xylene solution (Merck, USA). Next, samples were slowly rehydrated by putting them into a series of gradient concentrations of ethanol. The slides are immersed in ethanol of 96%, 85%, 75%, 50% and 30%, respectively. Each immersion takes place for 5 min. Samples are removed from ethanol and rinsed in deionized water for 10 min, before being incubated in 1% Safranin O (Sigma Aldrich, USA) for 5 min. Then the slides are re-rinsed with deionized water for an additional 5 min until completely drying for further analysis. Regarding transgenic plants containing the *GUS* gene, the GUS staining was carried out before fixing in formalin solution. Therefore, 10 μm sections on glass slides were produced for collecting images. Light microscopy was carried out with an inverted microsystem Zeiss Axio Vert. A1 (Zeiss, Germany). Images were collected with a Zeiss Axiocam 105 color camera (Zeiss, Germany) and analyzed with a digital imaging Zen software.

RESULTS AND DISCUSSION

3D printing molds: design and production

Molds were designed to consist of a lower section with grooves for firmly holding the rice roots in the right place, which suspends the roots over a well filled with agarose. These grooves are spaced at a suitable distance so that slices of all samples in the same row are fitted within the width of a microscope glass slide (Fig. 1A). Molds are assembled by fitting an upper section (Fig. 1B) to hold the samples before pouring the embedding medium. To increase the throughput of embedding, the specific mold is designed with two additional middle sections (Fig. 1C), which can be stacked, allowing three layers of root samples to be prepared (Fig. 1D). A biodegradable thermoplastic using Polylactic Acid (PLA) polymer was used as the material for printing the molds. This material is widely used in additive manufacturing for creating prototypes since it is inexpensive and easy-to-use (Wasti, Adhikari, 2020). Therefore, PLA is suitable to make low-cost molds for high-throughput sectioning of rice root anatomy.

3D printing molds increases the throughput of rice root sectioning

Since the thin and fragile rice root needs to be well orientated and embedded one by one in the paraffin, this process is extremely difficult, time-consuming and labor-intensive. The agarose-paraffin double embedding assisted with

specific molds has overcome these technical issues since the fresh tissues have already been organized lying down, the agarose blocks were easily taken off from the molds (Fig. 2B). The excess part of the rice root from the agarose block was trimmed and subsequently fixed in the formalin solution (Fig. 2C, 2D, 2E). The conventional method could deliver 2 strips with about 20 rice root cross-sections (Fig. 2F). Meanwhile, the double embedding reached 4 strips per glass slide with a twice higher number of crown root cross-sections (about 50 crown roots cross-sections per glass slide) (Fig. 3G). This showed a significant increase in throughput over conventional methods (2G).

The quality of sectioning

To test whether agarose could interfere with the staining process, GUS histochemical staining was performed with both cross and longitudinal sections of rice root. Thanks to the good orientation of root tissue in agarose-paraffin block during the mounting process, the sections were properly produced in a transverse or longitudinal direction. The GUS staining of a sample from the agarose-paraffin double embedding technique was illustrated in Fig. 3. Each tissue layer such as epidermis (ep), exodermis (ex), cortex (co), endodermis (en), parenchyma (pa), central cylinder (cc) and lateral root primordium (pr) was very clearly observed.

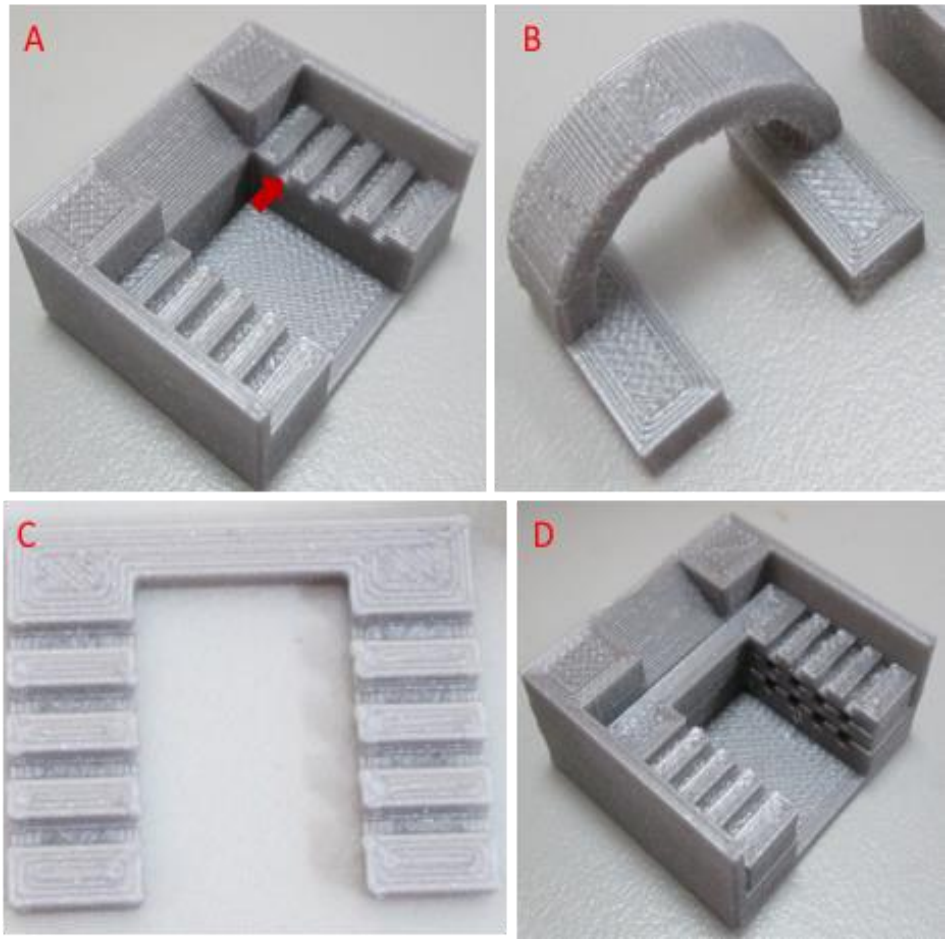

Figure 1. The 3D printed molds used for embedding roots in agarose medium. (A) The lower section the grooves (red arrows) (B) The upper section for holding the samples. (C) The middle section. (D) The lower section designed for adding 3 middle sections for embedding up to 12 rice roots.

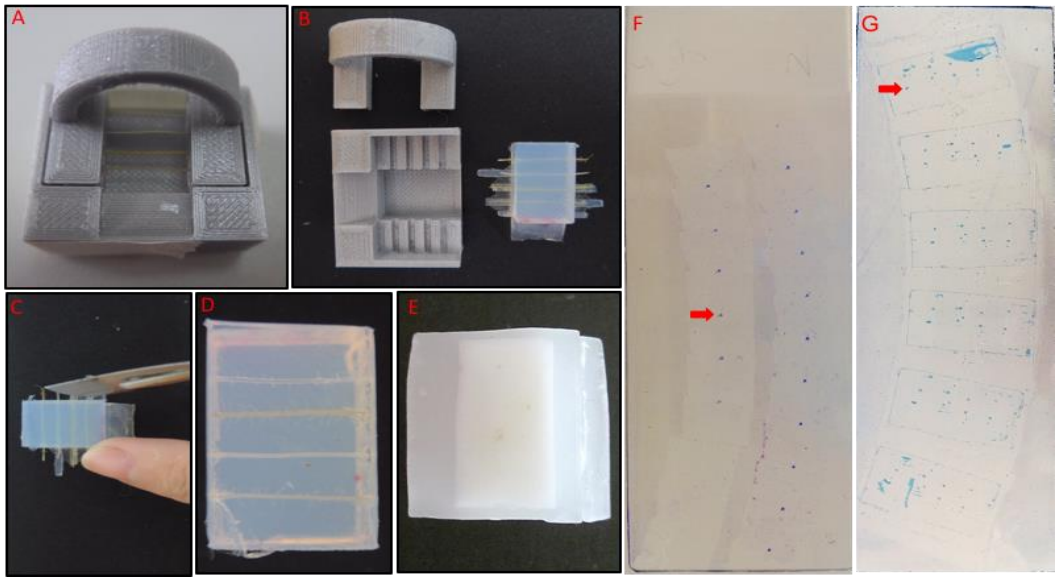

Figure 2. The agarose embedding process. (A) Assembled molds with root samples embedded in agarose. (B) The agarose block is easily removed intact from the mold. (C) The excess portions are trimmed using a scalpel. (D) Agarose block prepared for fixation in Formalin. (E) Agarose block embedded in paraffin ready for sectioning by a microtome. (F) Microscopic slide with 2 strips of slices in paraffin. (G) Microscopic slide with a strip of agarose-paraffin block sections containing 8 distinct root slices.

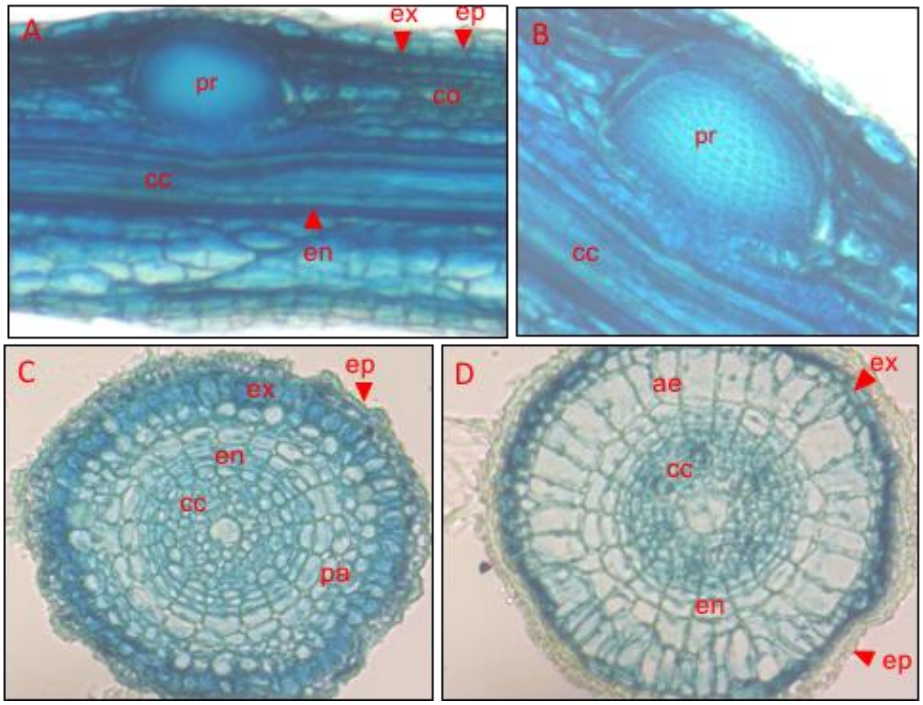

Figure 3. The GUS staining of a sample from agarose-paraffin double embedding technique. Longitudinal (A, B) and cross-sections (C, D) of GUS staining of 7-day-old rice root at 10X (A, C) and 40X (B, D) objective lens. Abbreviations as: ep, epidermis; ex, exodermis; co, cortex; en, endodermis; pa, parenchyma; cc, central cylinder, pr, lateral root primordium; ae, aerenchyma.

Aside from GUS staining reagent (Sigma-Aldrich, USA), two other staining reagents which are commonly used in plant histology including Safranin O (Sigma-Aldrich, USA) and Blue Methyl (Sigma-Aldrich, USA) were utilized to check whether they interfere with agarose. In this case, 7 day-old rice stem bases were collected and also double embedded by agarose-paraffin as previously conducted with GUS staining technique. Our results in Fig. 4 showed that in both cases, the tissues were well observed. In rice, the crown roots are initiated from the pericycle which is the cell layer adjacent to the endodermis (Coudert *et al.*, 2013). This cell layer is thin in size and similar to endodermis in the structure which makes it difficult to be recognized. In our cross-sections, the pericycle was well characterized (Fig. 4A, D, E), from what the young primordium was initiated.

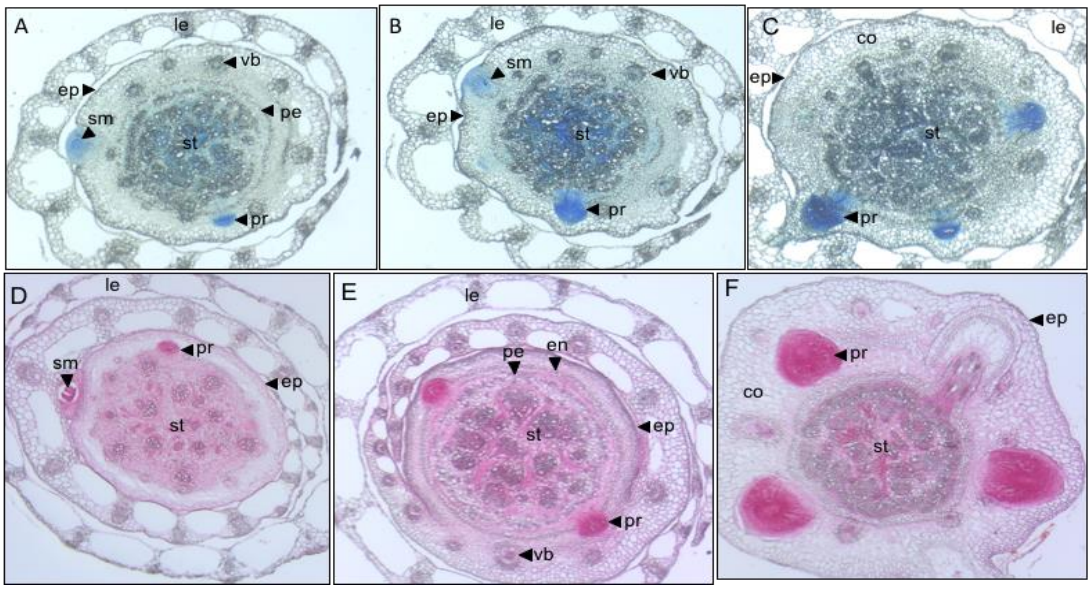

Figure 4. The Methyl blue (A, B, C) and Safranin O (D, E, F) staining of rice stem base cross sections from agarose-paraffin double embedding technique. Abbreviations as: le, leaf; ep, epidermis; sm, shoot apical meristem; vb, vascular bundle, co, cortex; en, endodermis; pe, pericycle; pr, crown root primordium; st, stele.

From the above results, we could conclude that the agarose embedding did not interfere with the penetration of fixatives or other chemical reagents in the staining and paraffin infiltration process. This technique resulted in high-quality sectional images and could be applied for further studies in rice.

CONCLUSION AND PERSPECTIVES

In this paper, we combined both improved techniques including agarose-paraffin double embedding assisted with 3D printing technology for high throughput rice root sectioning. First, we were successful in designing and producing the 3D mold that can significantly increase the output of root sectioning from 20 rice root cross-sections per cut to around 50 cross-sections each cut. Besides, the double embedding technique with agarose-paraffin has facilitated the manipulation of tissue samples, reducing time and labor. In the future, we hope to optimize this technique to apply to different types of plant tissues.

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