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SHORT COMMUNICATION

A novel method to scale up fungal endophyte isolations

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Studies involving fungal endophytes very often rely on surface-sterilisation of plant samples to enable endophyte isolations. However, surface-sterilisation can be very time-consuming, potentially limiting the number of samples processed. To overcome this limitation, a novel method was developed to bulk surface-sterilise multiple plant tissue samples simultaneously and separately. The method relies on 24 perforated Falcon™ tubes, each containing a sample, sequentially transferred through a series of containers holding the sterilants. The samples that can be surface-sterilised using this method include roots, stems and leaves or entire seedlings. This method increased our throughput by a factor of 24 relative to conventional surface-sterilisation methods.

Keywords: surface-sterilisation; endophyte; entomopathogen; colonisation; isolation; biological control

Fungal endophytes are an inconspicuous group of fungi that live asymptomatically inside healthy plant tissues for all or part of their life cycle (Petrini, 1991; Wilson, 1995). Their cryptic nature presents challenges to the ecologists, mycologists, plant pathologists and entomologists who work with them. Of particular interest to those involved in biological control of insect pests and plant diseases is determining the fate of introduced fungal entomopathogens as endophytes, including their distribution, abundance and interactions with other endophytes within the plant. Several methodological obstacles exist in studies involving fungal endophytes (Hyde & Soytong, 2008; Sun & Guo, 2012), including the processing time required to isolate them. The conventional isolation method involves surface-sterilisation of plant samples (e.g. leaves, stems and roots) for subsequent cultivation and isolation of endophytes on artificial media (Schulz, Guske, Dammann & Boyle, 1998). A survey of fungal endophyte-related articles published in 2014 reveals that surface-sterilisation usually takes between 4–6 minutes (and sometimes 15 minutes) per plant sample (Higgins, Arnold, Coley, & Kursar, 2014; Jaber & Salem, 2014; Murphy, Doohan & Hodkinson, 2015; Terhonen, Keriö, Sun & Asiegbu, 2014), potentially influencing sampling intensity and the resulting estimations of endophyte colonisation levels. As illustrated by Parsa, Ortiz, and Vega (2013), this time consuming method typically

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involves the sequential transfer of a single plant sample through several Petri dishes holding sterilants or sterile distilled water (Figure 1a).

Surface-sterilisation of host plant samples is an integral component of fungal endophyte work, and is used to remove epiphytic fungi and other microorganisms from the surfaces of plant tissues. The sterilants used to remove these surface microorganisms generally include dilute sodium hypochlorite and ethanol. The time required in each sterilant can be determined in a pilot study and usually varies depending on the host plant tissue, age, sensitivity and thickness (Fröhlich, Hyde & Petrini, 2000; Hyde & Soytong, 2008; Schulz & Boyle, 2005; Schulz et al., 1998), and must be sufficient to sterilise the surface but not destroy the tissue (Schulz & Boyle, 2005). Any fungi that are subsequently isolated from the plant samples are assumed to be endophytic, so it is crucial to have confidence in the surface-sterilisation process.

Here, we describe a bulk surface-sterilisation device that can be used to surface-sterilise up to 24 plant samples separately and simultaneously. To illustrate its use, we have provided an example protocol that was used to successfully surface-sterilise root samples of cassava (*Manihot esculenta* Crantz). However, this device can be used to process whole seedlings as well as leaves, stems and roots of any plant. This method includes the use of plant sample imprints to confirm surface-sterilisation has been effective.

The bulk surface-sterilisation device consists of a series of five plastic containers (300 mm length × 195 mm width × 135 mm height) each of which holds 4 L of solution. The first container holds 0.5% sodium hypochlorite diluted in sterile distilled 0.05% Triton X-100; the second contains 70% ethanol diluted in sterile distilled water and the third, fourth and fifth contain sterile distilled water (Figure 1b). The device requires a modified lid that fits the containers and in which 24 holes (30 mm diameter) are cut to accommodate 24 BD Falcon™ tubes (50 mL; BD Biosciences, Bedford, MA) (Figure 1d). Approximately 50 holes (1.5 mm diameter) have been drilled into each Falcon™ tube to allow for rapid entry and exit of the different solutions (Figure 1c). Each tube contains a single sample (e.g. a leaf, a piece of root, a seedling, etc.) trimmed if required to ensure the sample is submerged during the sterilisation process. Labels can be placed on the lids of the Falcon™ tubes in order to identify the sample.

A pilot study was conducted to determine the appropriate concentrations of sterilants and timings to be used to effectively surface-sterilise cassava roots (unpublished data). During these pilot studies, it was found that a pre-wash in Triton X-100 (0.05%) for 3 minutes assisted in preparing the samples for the sterilisation process. After the pre-wash, the timings used for 60 mm lengths of cassava roots in the sterilants were: (1) sodium hypochlorite (0.5%) for 3 minutes; (2) ethanol (70%) for 1 minute and (3) three rinses in sterile distilled water for 15 seconds each.

The pre-wash in Triton X-100 is carried out on an Innova™ 2000 Platform Shaker (New Brunswick Scientific Co. Inc., Edison, New Jersey) and the other containers are set up in the laminar flow cabinet (Figure 1b) and agitated by hand. The samples are moved from one container to the next container by removing the lid in order to lift up all 24 tubes at the same time, allowing it to drain for a few seconds, and then transferring it to the next container. After the final rinse in sterile distilled water, the lid (with 24 tubes attached) is removed and placed into an empty (sterile) container, wherein the samples can drain. As a result of adopting this method, our sample sterilisation throughput increased by a factor of 24 relative to using the conventional method (Parsa et al., 2013; Figure 1).

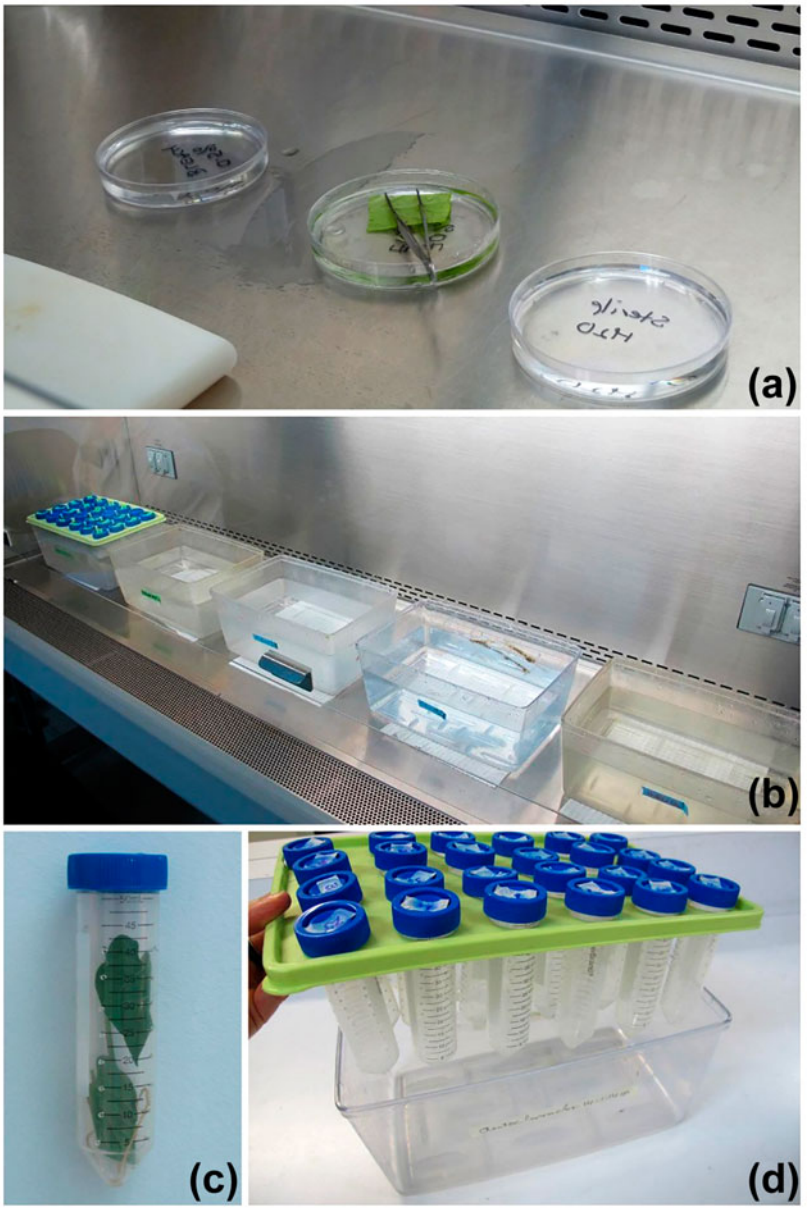

Figure 1. (Colour online) (a) Conventional surface-sterilisation of plant samples, showing a single sample immersed in a sterilant within a Petri dish. (b) Bulk surface-sterilisation device within a laminar flow hood. The first container on the left holds 24 Falcon™ tubes, each with a plant sample. The lid with 24 samples is transferred through the system so that the samples are simultaneously immersed in each of the sterilants followed by three rinses in sterile distilled water. (c) Plant sample (i.e. bean seedling) within a perforated Falcon™ tube. (d) Bulk surface-sterilisation container showing lid holding 24 Falcon™ tubes.

Before dissecting the samples, each 60 mm piece of cassava root is removed from its tube and allowed to dry for approximately 10 seconds on sterile paper towel. A plant sample imprint is then made to confirm the effectiveness of surface-sterilisation. To make an imprint, the sample is very gently pressed onto 75% potato dextrose agar media (PDA) (Difco™, Becton Dickson & Company, Sparks, MD) in 100 × 15 mm Petri dishes. Antibiotics can be incorporated into the medium to eliminate bacteria. The root sample is then dissected into three smaller fragments (8 mm long) and plated individually onto artificial media (e.g. PDA with antibiotics) in 60 × 15 mm Petri dishes. Plant sample imprints are monitored for presence of fungi for up to 14 days after processing. If fungi are found on an imprint, that 60 mm root piece is discarded from the data-set.

Surface-sterilisation of host plant samples is a time-consuming but essential component of endophyte studies. Improvements to its efficiency are warranted given that studies involving endophytes are dependent on methodology. The bulk surface-sterilisation device described here provides a method for multiple host plant samples to be surface-sterilised separately and simultaneously. This greatly increases the volume of data that can be collected in a given period of time when compared to previously used methods. This means plants can be sampled more intensively, likely improving the resulting estimations of fungal endophyte presence and distribution within plants for ecological studies, and studies of introduced fungal endophytes for biological control.

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