Improved in vitro rooting in liquid culture using a two piece scaffold system

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Abstract

Plant tissue culture techniques have been used to propagate horticultural crops at a commercial scale for more than three decades. However, due to the high cost it is generally only used for high value crops. To increase production efficiency and make micropropagation viable for a wider range of species, new approaches to address key steps of the process with high labor inputs need to be evaluated. For this study, a two-piece scaffold system was designed, prototyped using 3D printing, and tested to physically hold plants upright thereby facilitating liquid based rooting. This system was evaluated with Malus domestica, Betula lenta, and Musa sp. using static liquid culture as well as rocker based temporary immersion system and compared to rooting in semi-solid based medium as is commonly practiced. Significantly, earlier rooting was observed in all three species in liquid when compared to semi-solid culture system, and plants cultured in liquid on the rocker generally performed better than those in static liquid. In addition to quicker, more uniform rooting, reducing labor requirements, and preventing root damage. This newly designed system is simple, easy to use, will help to improve efficiency, and reduce the cost of micropropagation.

KEYWORDS
3D printing, in vitro rooting, micropropagation, plant tissue culture, prototyping, root-stand design

1 | INTRODUCTION

Micropropagation protocols are highly influenced by the physical state of the medium, with significant implications for mass production. Most commercially important crops such as vegetables, medicinal plants, fruit trees, and flowering plants can be vegetatively propagated using tissue culture. However, micropropagation can be a labor intensive and time-consuming task depending on the nature of the species. Semi-solid medium is commonly used for micropropagation to provide support for the developing plants even though liquid culture has been shown to provide better growth for many species [1–4] can reduce subculturing by allowing media to be replenished, and is more compatible with automation. Another problem associated with micropropagation using semi-solid media during the rooting phase is that the gel must be manually washed from the delicate roots to prevent microbial growth in the greenhouse/growth facility. This process is not only time consuming, but and can damage the fine roots and increase the chances of infection. Over all, high cost and low efficiency limit the commercialisation of micropropagation to high value plants and unique applications [5].

A liquid medium provides several advantages over a semi-solid medium. It is convenient to handle, easier to
replenish, provides high nutrient diffusion rates, and reduces oxidative stresses on the explant [6]. As a result, the use of liquid media is generally more productive and leads to higher multiplication rates compared to conventional gel based methods of micropropagation. Comparative studies have shown that liquid is better than semi-solid medium for a number of species [1–4]. Rooting in a liquid medium is particularly advantageous and cost effective because gel does not need to be carefully washed from the roots. However, an advantage of semi-solid culture during the rooting phase is that it provides support to ensure that only the base of the plant is exposed to auxins. This is important to induce rooting while promoting healthy foliar growth. A challenge in liquid culture systems is keeping individual micro-shoots in an upright position during rooting, which can lead to abnormal foliar development. There are several reports for in vitro rooting in liquid medium using test-tubes, small flasks, or artificial support like glass beads or coir [7,8]. However, these approaches tend to require large numbers of vessels, space, and labor for cleaning support materials and glass making them difficult to implement at commercial scale.

The rooting stage in micropropagation is important as it directly affects greenhouse survival and acclimatization success. It would be beneficial to develop a system that supports explants in an upright position during the rooting phase to exploit the quicker rooting response observed in liquid culture systems. The objective of the current study was to develop and test a two-part scaffold system to provide support for liquid based rooting.

This was accomplished by using iterative design and 3D printing to develop a system that is compatible with commercially available culture vessels. The two-piece scaffold forms a grid into which shoots can be placed and held upright (Figure 1). The two pieces of the grid can be easily separated after growth and allow the plants to be gently removed without damage to the roots. This system was tested using static liquid culture or in combination with a temporary immersion rocker system and compared to semi solid culture for three species, banana (Musa spp.), apple (Malus domestica), and cherry birch (Betula lenta).

2 | MATERIALS AND METHODS

2.1 | 3D printed Root-scaffold

A two-piece rooting scaffold was produced using AW3D HD2X or AXIOM 3D printers (Airwolf3D, CA, USA) and 2.85 mm Polycarbonate (PC) filament (Fly Thinking Material, China). All units were designed using Fusion 360 (Autodesk, United States) software and exported as gcode files. Several iterations of the design were produced (not shown) until the final version was decided upon based on fit in the vessel and ease of use. The dimension of the final root-stand design was 235 × 85 × 80 mm (l × b × h) with a thickness of 2.75 mm (Figure 1).

2.2 | In vitro cultures of cherry birch, apple rootstock and banana

In vitro-grown cherry birch (Betula lenta), apple (Malus domestica cv. Geneva 41), and banana (Musa sp.) plantlets were obtained from the germplasm collection at the Gosling Research Institute for Plant Preservation (GRIPP), University of Guelph, and cultured on previously optimized media for multiplication and later for rooting as described below.

In vitro cherry birch shoots were multiplied on medium comprised of DKW (Driver and Kuniyuki Woody Medium) [9] basal salt mixture with vitamins (PhytoTechnology, Shawnee Mission, KS, USA), 5 µM 6-benzylaminopurine (BA), and 3% sucrose. The in vitro grown shoots were then transferred to rooting medium containing DKW basal salt mixture with vitamins, 20 µM indole-3-butyric acid, and 3% sucrose [10]. In vitro apple shoots were multiplied on DKW basal salt mixture with vitamins, 2.2 µM BA, and 3% sucrose. The in vitro grown shoots were then transferred to rooting medium containing DKW basal salt mixture with vitamins, 2.5 µM indole-3-butyric acid, and 3% sucrose. In vitro banana shoots were multiplied on MS (Murashige and Skoog medium) [11] basal salt mixture with vitamins (PhytoTechnology, Shawnee Mission, KS, USA), 30 µM BA, 5 µM Kinetin, and 3% sucrose. The in
vitro grown shoots were then transferred to rooting medium containing MS basal salt mixture with vitamins, 10 µM naphthaleneacetic acid (NAA), and 3% sucrose.

All media were solidified using 2.2 g/L phytagel (PhytoTechnology) added after the pH was adjusted to 5.75. The culture vessels were then heat sterilized at 121°C at 118 kPa for 20 min. For plant multiplication, autoclaved culture media (50 mL) were dispensed into Magenta® GA7 boxes. After transfer of two explants onto culture media, magenta boxes were sealed with Micropore tape (Fisher Scientific, Canada). The shoots were maintained in the culture room at 25 ± 2°C under a 16 h photoperiod with a light intensity of approximately 40 µmol·m⁻²·s⁻¹ provided by cool white fluorescent lamps (Osram Sylvania, Mississauga, ON).

2.3 | In vitro rooting using 3D printed root-stands

Three culture systems were evaluated viz. rocker based temporary immersion system in combination with the root stand (RL), stationary liquid culture system in combination with the root stand (SL), and semi-solid culture using the media described above (SS). For the treatments testing the rootstand (RL and SL), the two-piece root-stand was inserted in the culture vessels [12] (We Vitro, ON, Canada) before being autoclaved (Figure 1A), and 60 mL sterile media was dispensed in each vessel. The media for liquid treatments was the same as described above with the omission of the gelling agent. Twelve, four-week-old shoots of each plant (cherry birch, apple and banana), were transferred to each culture vessel containing respective rooting media. For the RL treatment, vessels were maintained on a rocker set to 30 sec/cycle thereby immersing the shoots with an interval of 25 sec between cycles. Stationary liquid cultures were kept beside semi-solid cultures on normal culture shelves. All media were supplemented with 3% sucrose and all chemicals and plant growth regulators were purchased from PhytoTechnology, Shawnee Mission, KS, USA. In case of semi-solid media, they were solidified with 2.2 g/L phytagel (PhytoTechnology) added after the pH was adjusted to 5.75 and then autoclaved for 20 min at 121°C and 118 kPa. Autoclaved semisolid culture media were then dispensed into culture vessels. After transfer of explants into culture media, vessels were sealed with Micropore tape (Fisher Scientific, Canada). Plants and plant materials were maintained in a growth room at 25°C and 16 h light/8 h dark photoperiod (40 µmol m⁻² s⁻¹) provided by cool white fluorescent lamps (Osram Sylvania, Mississauga, ON).

Experiments were repeated twice for all three plant species. Observations were recorded for days to root, numbers of root, root length, and shoot height for all three plant species.

For acclimatization, in vitro rooted shoots were removed from the semi-solid culture media and gently washed under running tap water. Rooted shoots from liquid media were washed thoroughly before removing from the culture vessels. Rooted shoots were separated in the tray by pulling the top piece of the root-stand out followed by taking them off of
the second piece of the root stand (Figure 1). Rooted plantlets were transplanted into 18-cell trays filled with Sunshine Professional growing mix 4 (Sun Gro Horticulture Canada, Vancouver, BC). Plants were kept in a mist bed (24°C for 16 h light and 20°C for 8 h darkness, over 85% humidity) for 10 d, and then moved to standard greenhouse conditions (24°C for 16 h light and 20°C for 8 h darkness, light intensity of 110 µmol m⁻² s⁻¹). The survival rate of the transplanted plants was recorded after 3 weeks.

2.4 | Statistical analysis

The data from three plant species were subjected to one-way analysis of variance separately using JMP Pro 11.0.0 software (SAS Institute, Cary, NC, USA). All statistical analyses were conducted using JMP version 10 (SAS Institute, Cary, NC, USA). Rooting data and plant species were analyzed using a factorial analysis of variance to evaluate the significance of the main effects and the interaction effects. The mean values were compared using pairwise Tukey’s test at \( \alpha = 0.05 \) significance level and the data is represented as mean ± SE. Treatments showing statistically significant difference are indicated by different letters in the graph.

3 | RESULTS AND DISCUSSION

3D printing has been used to develop prototypes for in vitro cultures in a very cost-effective manner by enabling rapid iterative development of new culture systems directly by researchers [12]. The present study provides a detailed demonstration of a 3D printed scaffold system to facilitate in vitro rooting in a liquid culture system. The root-stand depicted in Figure 1 was developed as an insert for a commercially available culture vessel (We Vitro, ON, Canada). PC was used to enable heat sterilization. The PC root-stands were successfully printed with minimal warping or delamination.

Cherry birch, apple and banana were selected representing woody and herbaceous plants with semi-solid rooting protocols optimized a priori. Liquid based rooting using the root-stand promoted significantly earlier root initiation than on semi-solid media in all three species with the exception of cherry birch rooted in stationary liquid (Figures 2D and 3). In general, plants cultured using the root-stand with liquid culture systems performed the best in respect to the number of roots and root length compared to semi-solid culture system, however, root length was not significantly different in banana from the semi-solid culture system (Figure 2A, B). This is similar to previous findings where higher numbers of roots were reported in banana, teak, and other plants in liquid medium with a coir substrate compared to conventional agar-gelled media [7]. The current results suggest that the benefits previously observed using a coir substrate are at least
in large part due to the absence of a gelling agent rather than a dark environment or other confounding factors. All plants were observed normal without any sign of hyper-hydricity and this may be due to size of the culture vessels and shorter culture period.

For cherry birch and apple, 100% plants rooted early in the rocker based liquid culture system after three weeks. This system also produced plants with significantly more roots compared to stationary liquid and semi-solid medium, presumably due to increased aeration (Figures 2A and 3). Apple root length was significantly higher in the stationary liquid and rocker based liquid cultures as compared to semi-solid culture (Figure 2B). Previous studies have found that liquid culture systems result in higher rooting efficiency provided that the plants are in a standing position [13,14]. The root-stand provides a simple method to stand the shoots in an upright position in relatively large vessels so only the lower end of the shoot remains submerged to facilitate improved liquid rooting at a relatively large scale.

While the entire banana shoots developed roots in all three culture systems, there was a significant difference in the time it took. All banana plants in the rocker based liquid culture system rooted within 17 days, while plants growing on stationary liquid took 31 days and shoots on semi-solid medium took 35 days. Similar trends were observed in apple and cherry birch (Figure 2D). Plants with more and longer roots can perform better in the greenhouse as well as in the field [13,14]. Plants coming from the rocker based liquid culture system acclimatized well to greenhouse conditions with normal growth. Apart from rooting, acclimatization can also be more effective from liquid media than in agar-gelled media [7]. Plants rooted earlier in the rocker based liquid culture system, had a 100% survival rate, and grew normally. The use of liquid rooting on the rocker system would shorten the overall culture time by two to three weeks for field transplanting and could be a significant benefit for producers.

Root elongation in vitro is mainly influenced by the substrate and medium [15,16]. Some of the problems with agar as a substrate have already been stated like gel strength, mineral composition, mineral availability, inhibitory compounds that affect the plant growth and development [16–18]. Roots of apple and cherry birch produced in gelled medium were thick and had fewer secondary roots, similar to reports on white spruce in agar based medium [19]. Gelled media
may physically constrain root elongation and roots become thicker by radial expansion of cells [20]. In radiate pine, plantlets with roots that elongated in agar withered and turned black when transferred to soil [21]. In another woody species, Acacia, roots produced in agar lacked both root hairs and fully developed vascular systems, while roots produced in liquid medium were normal [22]. The liquid medium produces the highest rooting percentage and shoots length for *Boswellia serrata* Roxb [14]. The concentration and availability of oxygen in liquid cultures is a major issue due to continuous immersion of explants. However, the gaseous environment of the medium can be improved either by agitation, bubbling or, in stationary systems, by placing the tissues in direct contact with air [23]. Several reports have suggested the use of porous support material, such as paper, beads, foam, rockwool or vermiculite [16,23–25]. However, the use of these kinds of support material require extra efforts for cleaning, handling and recycling the materials, and they generally need to be manually washed from the roots. In contrast, the root-stand is very simple to handle, autoclavable and facilitates liquid based rooting without the need for a substrate (We Vitro, ON, Canada). The root-stand has now been injection moulded for commercially available in the market with a very economic rate. The most important feature of the root-stand is two separate pieces which helps in minimizing the physical damage of the roots while taking out from the culture medium or vessels. No physical contact with the roots is made since there is no porous material like gel or rockwool has to be removed. There is minimal damage to the roots/root hairs during acclimatization which can be a major factor for higher survival and plant growth.

4 | CONCLUDING REMARKS

In conclusion, the root-stand described here improved rooting for all three species tested and represents a simple and efficient method for in vitro rooting in liquid media. 3D printing was successfully used to prototype and test the root-stand to improve plant tissue culture systems. This root-stand can help overcome issues with in vitro rooting in semi-solid or stationary liquid culture system by keeping the shoot in upright position, providing good gas exchange, and the ability to remove the rooted shoots without disturbing the roots. Early rooting, especially in woody species, using this root-stand will be valuable for commercial producers.

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AUTHOR CONTRIBUTIONS

Mukund R. Shukla and A. Maxwell P. Jones conceptualized the root-stand and A. Maxwell P. Jones and Kevin Piunno designed the two pieces root-stand. Mukund R. Shukla conducted the experiments, collected the in vitro growth data, and prepared the manuscript. Mukund R. Shukla, Kevin Piunno, and A. Maxwell P. Jones analyzed the data and helped in the preparation of various parts of the manuscript. A. Maxwell P. Jones conceived the project and acquired its funding. Praveen K. Saxena and A. Maxwell P. Jones managed, organized, and supervised the study. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The root stand has since been commercialized by Kevin Piunno, the founder of We Vitro Inc. All other authors declare no competing interest.

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