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PLANT TISSUE CULTURE

A new economical storage technique for strawberry (*Fragaria × ananassa Duch.*) *in vitro*

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Abstract

Strawberry plants grown *in vitro* are typically stored and maintained on agar containing Murashige and Skoog (MS) media and sucrose as a carbohydrate source. This method of storing strawberry plants *in vitro* is expensive and time consuming, requiring sub-culturing onto fresh media every 2 to 3 mo. This study aimed to establish the viability of using a substrate alternative as an economical replacement for MS media, for both long-term storage *in vitro* and ease of transfer (*ex vitro*). A protocol was developed for strawberry *in vitro* using commercially available sterilized peat pellets (Jiffy-7® pellets), to optimize culture conditions and tissue culture practices. Suitability of the peat substrate was measured by the plant's overall response to culture *in vitro* and subsequent health and survival *ex vitro* following deflasking. Included in this study was a comparison of the use of vented *vs* non-vented tissue culture vessels for their effect on plant development and survival *in vitro* and *ex vitro*. The results show that strawberry plants can be grown and stored under *in vitro* conditions in vented vessels without sub-culturing for up to 3 yr or more. This equates to an approximately eightfold more efficient technique, significantly decreasing cost of storing and maintaining strawberry plants *in vitro*. Furthermore, plants grown in the peat substrate did not multiply, and had more established secondary roots than those grown in the conventional MS media.

Keywords Tissue culture · Photoautotrophy · Murashige and Skoog · Sub-culturing · Peat substrate

Introduction

Maintaining germplasm *in vitro* is important for strawberry breeding programs in the event of losses of mother stock plants grown in pots, and for the storage of elite accessions and cultivars. For the propagation and *in vitro* storage of strawberry plantlets, a conventional Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium is typically used (Rashid 2010; Quiroz *et al*. 2017; Naing *et al*. 2019; Phillips and Garda 2019). This media contains the following ingredients: MS basal medium, sucrose, and a gelling agent. Modifications to the medium by addition of growth regulators or hormones can be used for crops including strawberry to increase root and shoot development (Phillips and Garda 2012; Saad and Elshahed 2012); however, MS media free of hormones has proven suitable for general plant development in strawberry (Debnath and Teixeira da Silva 2007). By not requiring a plant growth hormone, *in vitro* strawberry is an ideal species for investigations into substrate substitutions, such as peat pellets.

Alternative substrates such as peat provide an opportunity to significantly decrease subculturing frequency. Strawberry plants grown on MS media require subculturing onto fresh media every 1 to 3 mo for best plant health and survival (Moradi *et al*. 2011; Harugade *et al*. 2014; Naing *et al*. 2019) which makes it an expensive and time-consuming exercise. Additionally, short intervals between subculturing increases the chance of somaclonal variation (Bairu *et al*. 2011), and the chance of contamination due to operator error.

Plants grown under high humidity in vessels and low light intensity typical in plant culture rooms (Ziv 1986) can have problems adjusting to the lower relative humidity *ex vitro*, mainly due to rapid water loss through the stomata. Comparisons of stomatal functions have been made between plantlets grown under photoautotrophic and photomixotrophic conditions. Under photoautotrophic conditions, stomatal size and density, transpiration rate, and wax content of the leaves all increase, and plantlets have normal and a

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controlled transpiration rate compared to plantlets grown under photomixotrophic conditions (Zobayed *et al*. 1999). Stomatal malfunction has been identified as one of the main reasons for desiccation when plants are transferred from an *in vitro* to an *ex vitro* environment (Ghashghaie *et al*. 1992; George *et al*. 2008). Changes to the plant's condition from mixotrophic (can use a mix of different sources of energy and carbon) to photoautotrophic (manufactures its own food by photosynthesis) can take several days after transfer *ex vitro* (George *et al*. 2008). Transferring plants from *in vitro* to *ex vitro* is less stressful to plants grown photoautotrophic due to the plant's photosynthetic capability and functioning stomata, thus limiting water loss.

Early research in the 1980s revealed that plants *in vitro* can re-develop photosynthetic ability (become photoautotrophic) when micropropagated using little or no sugar (Fujiwara 1987; Fujiwara *et al*. 1988; Kozai *et al*. 1988; Hdider and Desjardins 1994). For many species, using sugar-free media has advantages *in vitro*, encouraging plant growth, limiting fungal and bacterial contamination, and increasing the success of later transfers *ex vitro* (Fujiwara *et al*. 1988; Nguyen *et al*. 2016). Conventional micropropagation uses sucrose (sugar) in the culture medium as a carbohydrate source. A high sugar content in the culture medium can reduce the photosynthetic ability and impair stomatal function (Kozai *et al*. 1991; George *et al*. 2008). Kaul and Sabharwal (1971) found that the inclusion of sucrose in the culture medium decreased the chlorophyll content of leaves compared to those grown with lower or no sugar. Fujiwara *et al*. (1988) also found that strawberry growth and development was greater when cultured without sugar under photoautotrophic conditions.

In this study, several experiments were conducted to determine if a substrate without sugar could improve efficacy while maintaining plant health *in vitro*, by examining (1) growth and health of plants grown in a peat substrate *vs* MS media; (2) the effect of culture on peat in vented *vs* non-vented vessels on plant development and survival *in vitro* and *ex vitro*; and (3) plant health and survival after long-term storage in peat.

Materials and Methods

Peat Pellet *vs* MS Media

This experiment compared two substrate treatments: peat pellets *vs* the control MS media, to evaluate the viability of peat as a substrate substitute. For the peat substrate treatment, Jiffy-7® peat pellets (Jiffy Growing Solutions, Zwijndrecht, Netherlands) were chosen as they are assembled within a mesh case, simplifying preparation and transfer *ex vitro*. Jiffy pellets contain 100% natural compostable and biodegradable sphagnum peat (http://wwwjiffypot.com). The pellets were soaked in distilled water for up to 30 min, after which they were placed into 300-mL plastic vented vessels (Techno Plas, Adelaide, Australia) and the lid vents covered with a gas permeable tape (3M™ Micropore™, Sydney, Australia). Both substrates were autoclaved @121 °C for 30 min.

Nineteen healthy *in vitro* strawberry plants, derived from lateral buds of cultivar Red Rhapsody, were grown in 120-mL glass culture vessels *in vitro* on a medium containing 2.215 g L^{-1} MS basal medium with vitamins (PhytoTech Labs, Lenexa, KS) 20 g L^{-1} sucrose and 4 g L^{-1} Gellan Gum, CultureGel™ (PhytoTech Labs). These cultures were maintained in a growth room under Philips Master LED tubes (color temperature of 6500K) with a 12-h photoperiod, at 26 °C ± 2 °C. After 2 mo in culture under sterile conditions, plant roots and older leaves were removed, leaving the three youngest leaves intact. Plant weight was recorded, and each plant placed into either glass vessels containing MS media (eight plants) or peat pellets in plastic vessels (11 plants) (Fig. 1). All cultures were then placed into a growth room under Philips Master LED tubes (color temperature of 6500K) with a 12-h photoperiod, at 26 °C ± 2 °C.

Four mo after sub-culturing into their treatment substrates, half of the plants in each treatment were destructively sampled to evaluate plant growth and health, determined by plant weight, leaf color, leaf number, and root development. Survival and dry weight (after incubation at 60 °C for 24 h) were also recorded. The remaining plants were transferred *ex vitro* into Uncut Kwik Pot 42 Cell Trays (cell dimensions: 45mm L × 45mm W × 75mm D) containing steam-sterilized river sand and coir (1:1) seedling mix supplemented with standard fertilizer Scotts Osmocote® (Osmocote Exact Standard, Heerlem, Netherlands) at approximately 30 g per tray. The trays were placed under two layers of shade cloth in a glasshouse. After 1 wk, plants were hardened off by gradually removing one layer of shade cloth, and at 2 wk covers were completely removed. One mo after planting into trays, plants from both treatments were evaluated on leaf and shoot numbers produced, and root development.

Figure 1. Strawberry (*Fragaria*×*ananassa* Duch.) plants cultured in Murashige and Skoog medium (*top*) and peat (*bottom*).

For all experiments conducted, plant health *in vitro* and after transfer *ex vitro* were evaluated according to leaf color on a scale of 1 to 4, where 1 = pale brown or dead, 2 = pale yellow/green, 3 = light green or patchy, and 4 = dark green (healthy). Evaluation of root development was on a scale of 1–5, where 1 = less than five roots, 2 = five to 10 roots, 3 = 10 to 15 roots, 4 = 15 to 20 roots, and 5 = more than 20 roots. Data was analyzed using GenStat Release 16.1, ANOVA, general analysis of variance, significant at the $P = 0.050$ level.

Vented *vs* Non-vented Vessel

Plant growth in peat substrate in vented and non-vented vessels was compared to evaluate the effect of gas exchange and lowered humidity within the vessels on health *in vitro* and survival *ex vitro*. Twenty plants of the strawberry cultivar Meadowsong cultured on MS media were sub-cultured into peat pellets in 300-mL vessels. All vessels were capped with lids with a hole (vent) (approximately 4 mm). The lids of the vented treatment were covered with a gas permeable tape while control vessel vents were covered with two layers of duct tape to restrict gas and moisture exchange. Ten plants were included in each treatment. After 15 wk, plants were evaluated as described previously. Five-wk *ex vitro* survival rates and dry weight of plants from both treatments were measured.

Plant Storage Survival in Peat Substrate

Experiment 1 (22 to 36 Mo in Peat)

In this experiment, *in vitro* plants of the cultivar Red Rhapsody were tested for plant health and survival without sub-culturing after long-term storage at two intervals. The plants were sub-cultured onto peat in vented vessels as previously described. During the experiment, vessels were moistened intermittently with approximately 10 to 20 mL sterile water only if the peat pellet looked dry. This was dependent on the individual peat pellet; some vessels had extra water added once over the course of the experiment, while many did not require any additional water.

Ten plants were evaluated at 22 mo and three plants at 36 mo after sub-culturing. Following this time, the stem was cut at the point where it emerged from the peat substrate surface. The top section was sub-cultured onto MS media to determine if transfer back onto MS after extended storage on peat could be successful, and to determine levels of contamination from fungi and bacteria and to check for off-types.

Experiment 2 (8 Mo in Peat)

In this study, long-term survival and storage of 35 *in vitro* plants of four different breeding accessions in peat substrate were assessed after 8 mo without sub-culturing. Plants were sub-cultured from an MS media and placed into peat pellets in vented vessels as previously described. The genotypes were accessed from the Australian Strawberry Breeding Program, and included accessions 2014-009-121 (five plants), 2014-009-159 (five plants), 2014-049-104 (10 plants), and 2014-051-151 (15 plants). Plants were evaluated for health (leaf color), presence of off-types, and contamination. Also assessed was the plant's response to transfer back onto MS media after being stored for 8 mo on peat substrate, as per experiment 1 (36 mo in peat) above.

Results

Peat *vs* MS Media

All plants from MS and peat pellet treatments appeared healthy, with a leaf color rating of 4 (dark green and healthy) after 4 mo *in vitro* (Table 1). One mo *ex vitro*, the plants cultured in peat rated slightly higher (4) for leaf color than those in MS (3.5). Significant differences in leaf and shoot numbers, and root ratings were observed between treatments 1 mo after transfer *ex vitro* into trays. Plants in MS showed a higher multiplication rate of shoots (mean shoot number of 4), and more leaves and roots (ratings of 25.5 and 4.25 respectively) than those in peat. In comparison, plants cultured on peat displayed no shoot multiplication, with a mean shoot number of 1.0, a mean leaf number of 12.71, and root number of 2.71. The plants grown in peat had shorter primary roots and more secondary roots compared to plants grown in MS media (data not shown).

Table 1. Mean strawberry (*Fragaria*×*ananassa* Duch.) plant growth parameters for peat substrate *vs* Murashige and Skoog medium at 4 mo *in vitro* and 1 mo after transfer *ex vitro*

Treatment	Initial weight (g)	Dry weight (g) 4 mo *In vitro*	Leaf color 4 mo *In vitro*	Leaf number 1 mo *Ex vitro*	Root rating 1 mo *Ex vitro*	Shoot number 1 mo *Ex vitro*	Leaf color 1 mo *Ex vitro*
Peat vented	0.05	0.13	4	12.71^{b}	2.71^{b}	1.00^{b}	4.0
MS media (non-vented)	0.04	0.23	4	25.50^{a}	4.25^{a}	4.00^{a}	3.5

Superscripts denote statistically significant differences at $P < 0.01$

Vented *vs* Non-vented Vessel

Although there were no significant differences in leaf color and leaf number for plants in vented *vs* non-vented vessels, there was a significant difference in the root rating at 15 wk *in vitro* (Table 2). At 15 wk *in vitro*, plants from the non-vented treatment displayed shorter and more tightly clumped roots, showing a root rating of 3.1. The vented treatment displayed longer roots (root rating of 4.2), with many protruding through the peat casing (Fig. 2*a*). Visually there were differences in plant vigor, with plants in vented vessels having better leaf and root development (Fig. 2*b*). After 5 wk *ex vitro*, there was no significant difference in plant dry weight or survival rates, and all plants survived transplant well.

Plant Storage Survival in Peat Substrate

Experiment 1 (22 to 36 Mo in Peat)

At 22 mo after being cultured on peat substrate without subculturing, the 10 plants were green and healthy (Fig. 3*a*). Even after being stored on peat substrate for 36 mo all assessed plants still had green leaves (Fig. 3*b*). All plants evaluated for their long-term storage ability on peat substrate *in vitro* at 22 and 36 mo were successfully sub-cultured back into MS media. One plant exhibited fungal contamination 1 mo after transfer onto MS media, which may be attributed to operator error. No off-types were evident.

Table 2. Mean strawberry (*Fragaria*×*ananassa* Duch.) growth parameters for plants in vented *vs* non-vented vessels at 15 wk *in vitro* and 5 wk after transfer *ex vitro*

Treatment	Weight (g) 15 wk *In vitro*	Leaf color 15 wk *In vitro*	Leaf number 15 wk *In vitro*	Root rating 15 wk *In vitro*	Dry weight (g) 5 wk *Ex vitro*	% Survival 5 wk *Ex vitro*
Non-vented	0.328	4	12.9[a]	3.1[a]	0.05[a]	100
Vented	0.583	4	14.1[a]	4.2[b]	0.20[a]	100

Superscripts denote statistically significant differences at $P < 0.01$

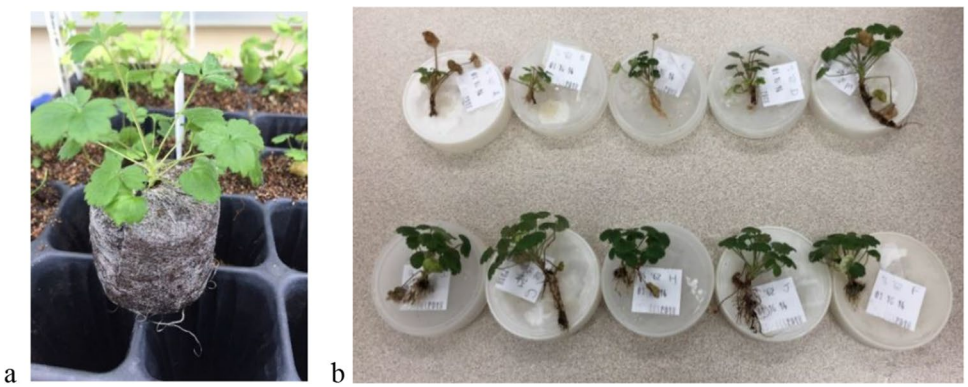

Figure 2. Effect of vented *vs* non-vented vessel on strawberry (*Fragaria*×*ananassa* Duch.). (*a*) Roots of plant in vented treatment protruding through peat casing. (*b*) Strawberry plants from non-vented treatment *top row*, and vented treatment *bottom row.*

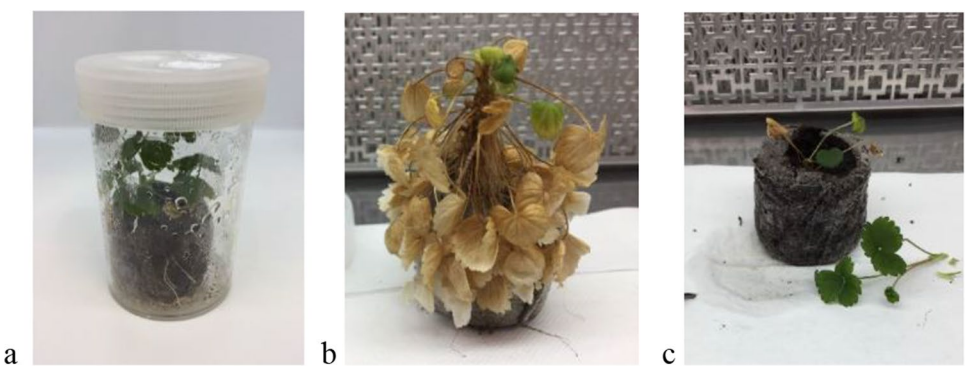

Figure 3. Strawberry (*Fragaria*×*ananassa* Duch.) plant health and survival after long-term storage in peat. (*a*) Strawberry plant at 22 mo stored in vitro on peat. (*b*) Strawberry plant at 36 mo stored *in vitro* on peat. (*c*) Plant cut back at 8 mo and ready to be transferred back onto Murashige and Skoog medium.

Experiment 2 (8 Mo in Peat)

All plants in this experiment had a health rating of 4 (dark green leaves) after 8 mo of culture on peat pellets. Plants that were subsequently sub-cultured back onto MS media (Fig. 3*c*) exhibited no fungal or bacterial contamination, and no off-types were observed after 1 mo. In this instance, the genotypes subjected to the long-term culture treatment on peat substrate did not appear to be poorly affected by the length of culture, up to 8 mo.

Discussion

The protocol developed here for growing *in vitro* strawberry plants on peat substrate is ideal for long-term storage and transfer *ex vitro*. The necessary period between sub-culturing is significantly lower, with an approximately eight-fold decrease in the number of sub-cultures required, resulting in a substantial increase in efficiency and costs. In comparison, the current MS gel-based media requires sub-culturing once every 1 to 3 mo, as the nutrient level in the media is slowly depleted. The savings on resources and time are a prominent advantage of adopting this new protocol.

Strawberry plant growth *in vitro* was noticeably different when cultured in peat pellets under photoautotrophic conditions. Plants in peat remained single stemmed and did not multiply as much as those in MS. Strawberry root development improved when cultured in peat pellets and in vented vessels. Although root numbers were significantly higher for plants in MS media (mean root ratings of 4.25 in MS and 2.71 for peat treatment), due to greater shoot multiplication, plants in peat had a more mature root system (more secondary roots). Additionally, the roots of peat plants are further enhanced when stored in vented vessels. This was demonstrated by an increase in the root rating of plants in peat in vented vessels 1 mo *ex vitro.* Overall strawberry growth and development was greater for plants grown in peat in vented vessels. These findings confirm the findings of Fujiwara *et al*. (1988) who also found strawberry growth and development was greater when cultured without sugar under photoautotrophic conditions.

Plants in peat pellets transferred *ex vitro* easily due to being enclosed in the pellet mesh, had less root damage, and adjusted faster to *ex vitro* conditions. This may possibly be a result of reduced water loss and transplant shock caused by stomatal malfunction as described by George *et al*. (2008), and by having an intact root system enclosed in the pellet. Plant roots from the MS treatments had some damage and breakage due to the removal of gel and handling.

An established root system is beneficial not only for plant health and growth *in vitro* but also for successful transfer *ex vitro*. Strawberry plants produce both primary and secondary (feeder) roots. The primary roots are the first roots produced and are long and can live for many yrs. The secondary roots tend to be short lived and make up the bulk of the root system. Secondary roots absorb water and nutrients for the primary roots to then transport into the plant crown (Darrow 1966). Sustaining a healthy root system is essential to obtain plant growth and health while in culture, and for transplant *ex vitro*. The results showed that plants grown in peat substrate in vented vessels produced shorter primary roots and had more established feeder roots than those grown in MS media. Roots of plants in the vented treatment were longer compared to the non-vented treatment, which were shorter and more tightly clumped. There was a significant difference in the root ratings between plants grown in peat and MS treatments. Plants grown in peat substrate had shorter primary roots and more secondary roots compared to those grown in the MS treatment.

Experience has shown that plants grown in peat pellets for extended durations benefit from the addition of sterile water if they dry out due to the vents. Peat dryness and plant dehydration is noticeable by lack of condensation on the sides of the vessels, the peat looks dry, and the plants may wilt. Peat hydration has been seen to depend on several variables including culture room temperature and humidity, and correct soaking times. If peat is looking dry, it is recommended to add enough sterile water to hydrate the pellet but not leave water pooling on the bottom of the container.

One limiting factor of using peat as a substrate is that bacterial contamination can be difficult to observe. These studies, however, did not observe any bacteria contamination after plants were sub-cultured back onto MS media. Being sucrose-free, peat pellets limit bacterial growth compared with MS media; however, the potential for contamination should be considered if using this protocol. In the long-term storage experiment 1 (22 to 36 mo in peat), one plant showed fungal contamination after transfer back to MS media after 22 mo on peat substrate. This low percentage of contamination is not uncommon for MS media and peat substrate cultures and may be due to operator error during transfer to MS media or by an irregular-shaped vented plastic vessel or lid.

Two strawberry cultivars and four different breeding accessions were tested on peat in these experiments due to the limited availability of plants; however, this allowed insight to how different cultivars would react to culture on peat. Testing other plant species in peat substrate could result in this protocol successfully being implemented by other industries. Peat pellets typically have a low pH, which is suitable for acid tolerant plants which includes strawberry. Further research to substrate alternatives such as coir peat is recommended. Coir has a neutral pH, similar water holding properties to peat pellets and is more sustainable. These and other parameters will need to be investigated further.

Conclusion

The adoption of peat substrate into our tissue culture laboratory has significantly increased efficiency of the long-term storage of plant material. Additionally, the transfer process from *in vitro* to *ex vitro* is simplified due to improved root development and readiness for the *ex vitro* environment. The peat protocol allows for an increased length of the sub-culturing cycles (approximately every 2 to 3 yr) while decreasing media expenses, both financial and in time. This equates to an efficient and effective technique. Applied to crops like strawberry, this system would be beneficial for the storage of germplasm collections, limiting the number of transfers, reducing the chance of contamination, the requirement to re-initiate into culture, and most importantly the chance of somaclonal variation.

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