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Tropical Agricultural Research

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Development of Liquid Culture System for Rapid Multiplication of *Gyrinops walla*

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ARTICLE INFO

Article history:
Received: 30 July 2022
Revised version received: 17 October 2022
Accepted: 08 December 2022
Available online: 01 January 2023

Keywords:
Agarwood
Gyrinops walla
Liquid cultures
Rapid multiplication

Citation:
Kaushalya, D.B.R., Eeswara, J.P. and Jayasinghe , L. (2023). Development of liquid culture system for rapid multiplication of *Gyrinops walla*. Tropical Agricultural Research, 34(1): 43-51.

DOI:
http://doi.org/10.4038/tar.v34i1.8603

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ABSTRACT

Agarwood is an expensive resinous compound, produced by *Gyrinops walla* as defence response to external damages. The tissue culture technique is the best alternative for both rapid multiplication and product synthesis. This study demonstrated an efficient method of rapid multiplication using liquid cultures for *G. walla*. MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L IBA, 100 mg/L Myoinositol and 30.0 g/L of sucrose, and 3.0 g/L Phytagel (Sigma-Aldrich) was used as the control (T0). The effect of shoot growth on liquid culture on the same medium was investigated without inert support (T1) and kept on a shaker, with the support of filter paper bridges (T2), rigid support (T3), wet foam (T4), and a double layer of liquid and semi-solid medium (T5). At the end of the sixth week, growth rates and cell doubling times were calculated. Results showed significant differences between treatments at α=0.05 probability level ($P< 0.0001$). All liquid cultures except T3 stimulate the biomass. T1 had the highest growth rate (0.38 g/week) and the shortest cell doubling time (1.82 weeks) of *G. walla* (11-13 shoots from one shoot), but it also had hyperhydricity, which was overcome by combining liquid cultures with a flexible matrix or using a double-layer system. The nature of the matrix was found to be a vital factor in achieving the desired benefits of the liquid cultures. The use of a matrix is unnecessary if T5 is used to achieve the desired results.

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INTRODUCTION

Gyrinops walla, of the family Thymalaceae, commonly known as *Wallapatta*, is believed to be an endemic plant, and it is the only agarwood producing tree growing in Sri Lanka (Subasinghe and Hettiarachchi, 2015). Agarwood is a high-priced perfumery resin produced by species of four genera: *Aquilaria, Gyrinops*, *Aetoxylon* and *Gonystylus* as a defence reaction against fungal invasions (Li *et al.,* 2012).

The high demand and price of agarwood resins leads to extensive exploitation of natural populations of Wallapatta in the tropical rain forests of Sri Lanka, resulting in increased tree mortality, a reduced growth rate, and a decrease in the percentage of adult trees that produce seeds (Dharmadasa, 2013). Therefore, it has been promoted and encouraged to establish commercial / semi-commercial Wallapatta plantations for agarwood resin production. Although seed propagation is a reliable method, it is insufficient due to a lack of seed production, low seed viability, a low germination rate, delayed rooting, and the long juvenile period required for seed production. Plant tissue culture techniques are considered to be the best option for rapid multiplication and production of agarwood oil compared to the conventional method (Faizal *et al.,* 2021) because more secondary metabolites can be obtained from plantlets raised through tissue culture (Saikia *et al*., 2012). In spite of the merits, plant tissue culture also has certain demerits, such as a higher cost of production, slow growth rates and differentiation of cultures due to the use of conventional gelling agents; it also requires skilled labour which limits the use and exploitation of this technique (Mbiyu *et al*. 2012). Hence, it is needed to develop strategies to reduce the cost of production and thereby increase production efficiency. According to Mohaidib (2010), solidifying agents, which are added to increase the viscosity of media in order to keep explants upright without submerging in media, account for 70% of the cost of tissue cultured plant media.

The development of liquid culture systems can be used to reduce this cost component. In addition, better availability of nutrients and water, which is a result of lower resistance to diffusion due to closer contact between explants and media, results in higher growth and proliferation rates (Kim et al., 2003). Furthermore, dilution of plant exudates, homogeneous conditions throughout the culture, and a decrease in culture transfer frequency are also considered merits of liquid cultures (Mehrotra *et al*., 2007). However, continuous submerged conditions may create hyperhydricity in explants (Dutta and Prasad 2016), which can be overcome with the support of an inert matrix. Therefore, this study was planned and conducted to evaluate the effects of liquid and semi-solid cultures on growth and proliferation with the objective of developing an effective culture system for rapid multiplication of *Gyrinops walla* using a liquid culture system.

MATERIALS AND METHODS

Plant materials

G. walla shoots grown *in-vitro* on Murashige and Skoog (1962) medium supplemented with 1.0 mg/L Benzyl Amino Purine (BAP) and 0.1 mg/L Indole- 3 Buteric Acid (IBA), 3% sucrose, and solidified with phytagel (Selvaskanthan *et al.,* 2020) were used as basic plant material for explants.

Experiment

The treatments tested for culture and their details are given in Table 1. Murashige and Skoog (1962) medium supplemented with 1.0 mg/L BAP and 0.1 mg/L IBA was used in all treatments.

Clumps of in-vitro grown *G. walla* shoots were separated into single shoots, and three shoots (approximately 0.4 g) were transferred to a glass jar filled with 30.0 mL of MS liquid medium supplemented with 1.0 mg/L Benzyl Ammino Purine (BAP) and 0.1 mg/L Indole-3-Buteric Acid (IBA) without inert support (T1), with the support of filter paper bridges (T2) or rigid support (T3) or wet foam (T4) or 15.0 mL of solid medium with 15.0 mL of liquid medium (T5). 30.0 mL of MS medium solidified with phytagel and supplemented with 1.0 mg/L Benzyl Amino

Purine (BAP) and 0.1 mg/L Indole-3-Buteric Acid (IBA) was used as the control, T0 (Table 1). Liquid cultures without inert support were kept on a shaker at 80 rpm. The pH of the media was adjusted to 5.8 prior to autoclaving at 1210C for 20 min.

The explants were placed in sterile culture vessels and incubated at 26 ^{0}C with a light intensity of 1000 lux using white fluorescent lamps, a photoperiod of 16 hours, and an 8 hour dark cycle.

Data collection

Two replicates per treatment were harvested at weekly intervals until the 8th week after establishing the cultures. Micropropagation success was determined by taking fresh weights, measuring height, and counting the number of leaves and shoots. In liquid cultures, the number of deformed leaves was also counted (Alturki, 2014). Growth curves for each culture method were developed using fresh weights. Growth rates and doubling times were calculated by regressing the growth during the exponential phase against time.

Experimental design and statistical analysis

The experiment was conducted according to a Completely Randomized Design (CRD). Each treatment was replicated 18 times. The parameters fresh weight and height were subjected to analysis of variance (ANOVA) and their means were separated by the Dunnet test, considering the semi-solid culture method as the control. Per-shoot count data, including numbers of buds, leaves, and abnormal leaves per shoot, were analyzed using the Kruksal-Wallis test.

Table 1: Treatment combinations (MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L IBA) used in the experiment.

Treatment	Physical condition	Type of support	Positioning of *ex-plants*
T0	Semi solid	-	Upright
T1	Liquid	-	Submerged
T2	Liquid (30.0 mL)	Filter paper bridge	Upright
T3	Liquid	Rigid support (Plastics)	Upright
T4	Liquid	Wet foam	Upright
T5	15.0 mL ml Liquid	15.0 mL ml semi solid	upright

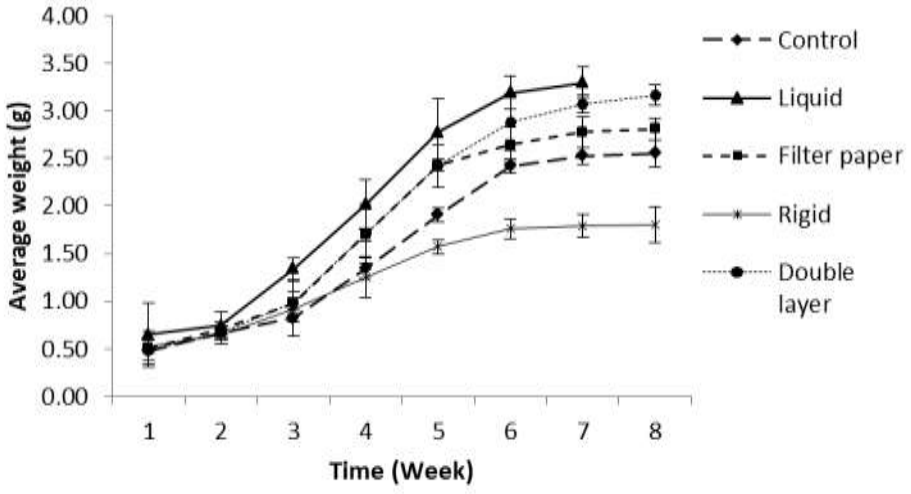

Figure 1: Fresh weights of *in-vitro* grown shoot of *G.walla*, as affected by semi-solid and liquid culture condition with and without inert support during shoot multiplication stage

RESULTS & DISCUSSION

Effect of culture system on growth and multiplication of *Gyrinops walla* shoots

The results showed a typical sigmoidal pattern of growth for all treatments except microshoots supported by wet form (Figure 1). During the first two weeks, microshoots grown on all treatments except wet foams showed very slow growth (lag period) and reached the exponential phase of growth at the end of the second week, reaching the stationary phase at 6 weeks after establishment. This result implies that one or more nutrients may be limiting to growth at 6 weeks after establishment, and therefore the subculturing interval for the cultures can be set at 6 weeks after culture establishment.

The plants grown on wet foams with liquid (T4) MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L IBA wilted and died within three days (Plate 1) indicating that growing microshoots on wet foams is not feasible. Therefore, this treatment was not considered for the data analysis.

Furthermore, growth data obtained at the sixth week was subjected to an analysis of variance (ANOVA) procedure, and the results showed significant differences between treatments at α=0.05 probability level ($P< 0.0001$). Results indicated that the fresh weight of *G. walla* grown on the liquid cultures was significantly higher compared to the semi-solid medium (Table 2) except for liquid cultures with rigid support (T3).

It was observed that the growth of the cultures established on rigid supports was poor due to the shoots' inability to increase the stem diameter. The place where shoots were connected to the rigid support was very thin and week. Initially, this system also showed growth comparable to the other treatments, but subsequently declined after 3rd weeks (Figure 1). In this system, microshoots were loosely attached to the holes made in the plastic at the time of

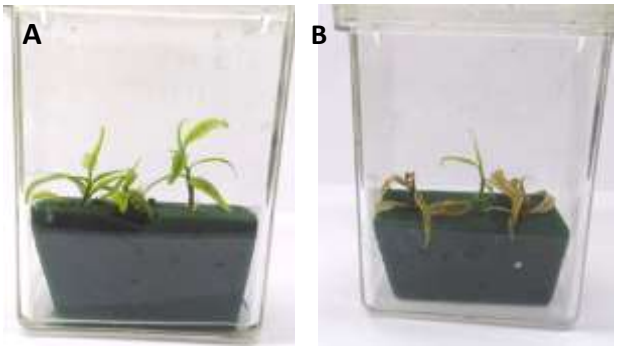

Plate 1: Effect of wet foam on the growth of *G.walla* micro shoots. A-Just after establishment, B-Three days after establishment

Table 2: Comparison of fresh weight of *G,walla* micro shoots grown on liquid media with and without inert support compared to semi-solid medium by Dunnet's test

Treatment Comparisons	Difference Between Means (g)	Simultaneous 95% Confidence Limits		Significance
T1 Vs T0	1.132	0.913	1.350	***
T2 vs T0	0.726	0.496	0.956	***
T3 vs T0	-0.755	0.973	0.536	***
T5 vs T0	1.102	0.809	1.287	***

culturing. However, with the growth, microshoots became tightly attached to the support, suppressing the growth since the support was not as flexible as filter paper bridges or the semi-solid medium. The cultures in the double-layer system also showed significantly higher growth compared to the semi-solid medium. Even though growth was similar to the control at the beginning, by the third week after culture establishment the growth rate had become higher in this treatment. It was observed that the rigidity of the bottom solidified layer in a double-layer system became loose with time and that there was no clear demarcation between the upper and lower layers any more. It may occur due to the continuous and long-term exposure of the bottom solidified layer to the upper liquid layer. However, they do not mix completely, but with the loosening of the rigidity of the bottom layer, shoots do not get attached firmly to the medium, and nutrients are accessible more to the shoots compared to a solid medium, resulting in a higher growth rate.

The period between two and six weeks after establishment was identified as the exponential or logarithmic phase of the growth based on the growth curves. Then the growth of the cultures was plotted on a semi-logarithmic graph (Figure 2) and the growth rates obtained by regressing the logarithmic growth against time are given in (Table 3).

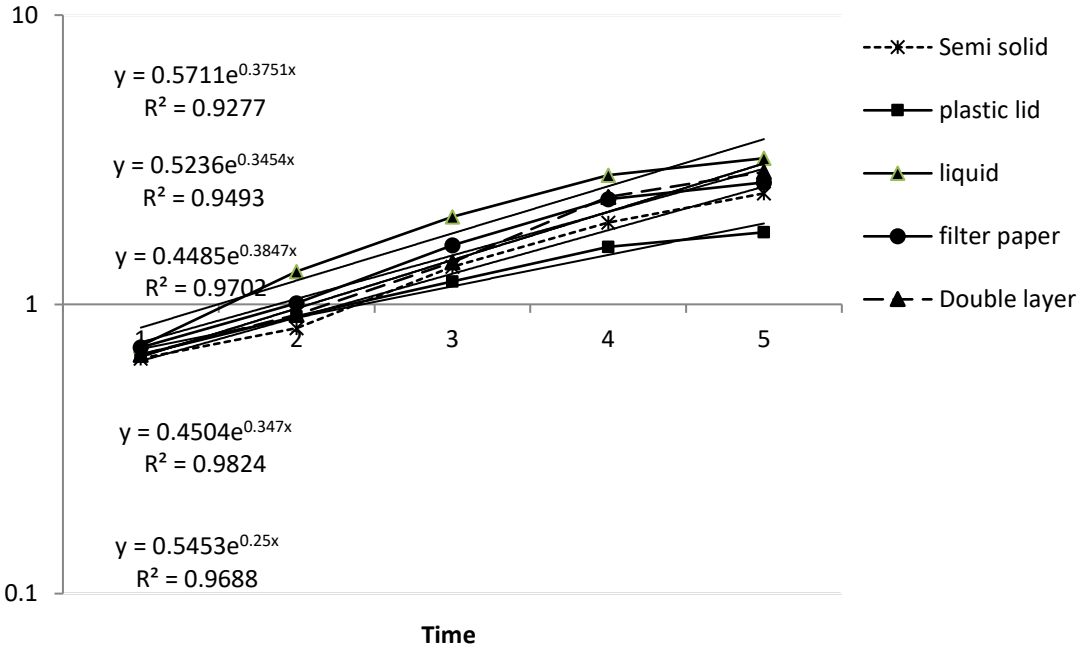

Figure 2: Growth rates of *in-vitro* grown *G. walla* shoots as affected by semi-solid and liquid culture conditions with and without inert support during shoot multiplication stage

Table 3: Fresh weight, growth rates and cell doubling times of *in-vitro* grown *G.walla* micro shoots under semi-solid and liquid culture conditions with and without inert support during shoot multiplication stage

Treatment	Fresh Weight (g)	Growth Rate (g/week)	Cell doubling time (weeks)
T0-Semi solid medium	2.42	0.34	2.03
T1- Liquid medium without support	3.20	0.38	1.82
T2- Liquid medium with filter paper bridge	2.64	0.35	1.98
T3-Liquid medium with rigid support	1.78	0.25	2.77
T4-Double layer system	2.92	0.37	1.87

At the beginning, the pattern of weight increase was similar across all treatments. However, three liquid culture systems (only liquid and liquid supported by filter paper) and the double-layer system showed higher growth than semi-solid medium in terms of weight increase. When considering three liquid systems, the highest growth was observed in liquid cultures without inert support compared to the other two liquid systems supported by filter paper bridges or a rigid supporter. The double-layer system had the second highest weights. Initially, the double layer system showed slow growth and was similar to the semi-solid medium. However, by the fourth week after establishment, shoot growth on the double-layer medium had surpassed that on the semi-solid medium. In the double-layer system, the bottom consisted of a layer of solidified MS medium, and over that, a layer of liquid medium was included. With the passage of time, the solid layer becomes less rigid, resulting in better availability of nutrients compared to the conventional solidified system. Liquid medium without supports showed the highest growth. In this system, microshoots were submerged in culture media and were shaking at 100 rpm, exposing a larger surface area to be in contact with the nutrient medium. Thus, the availability of nutrients and phytohormones as well as the increased amount of oxygen may result in higher growth compared to the microshoots grown on the other two liquid systems, which absorb nutrients through the cut surface only. In agreement with the results of the present study, Kim et al. (2003) reported better availability of nutrients and water in liquid cultures, as a result of lower resistance to diffusion and closer contact between the micro-shoots and the media.

In addition to the weight measurements, the increase in height was also measured to identify the best system for micropropagation and secondary metabolite production for *G. walla.* Similar to the increase in weight, a sigmoidal growth pattern was observed for the increase in height for all five treatments (Figure 3). Plant height also reached its stationary phase at the end of the 6th week. Interestingly, the double layer system produced longer shoots compared to the control ($P<0.0001$) and this may occur due to better availability of nutrients and phytohormones as well as the fact that they were maintained in upright positions, which may express the apical dominance in shoots. Even though cultures maintained on liquid culture without support showed better growth in terms of weight gain, the increase in height was significantly lower compared to all other treatments ($P<0.001$). Though nutrient availability was similar in other liquid systems, the second longest shoots were produced in a filter paper supported liquid system. In the liquid system without support, micro-shoots were submerged and continuously shaken, thus losing the expression of apical dominance as well as the response to gravity (Dutta and Prasad, 2016).

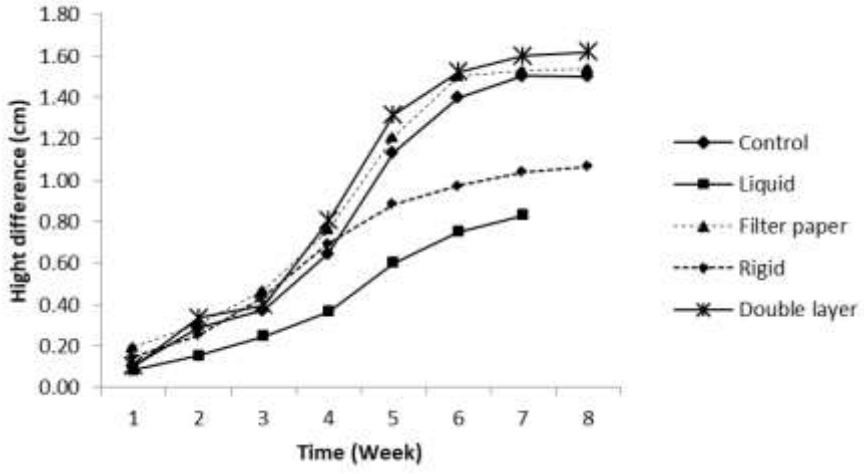

Figure 3: Average height difference of *in-vitro* grown *G.wallla* shoots as affected by semi-solid and liquid culture conditions with and without inert support during shoot multiplication stage

The number of multiplied buds was counted at the end of the sixth week, and the data was analyzed using the Kruskall-Wallis test. Results showed that production of axillary buds on liquid media without support was 13±1.2 and with a filter paper bridge, it was 11±0.89 both were significantly higher ($P<0.001$) than in the semi-solid (7±0.75) and the liquid media with rigid support (3±0.95) (Figure 4). The results of the present study clearly indicate that liquid cultures significantly increase the number of buds as a result of a better supply of nutrients. The most axillary buds (13±1.2) were found in liquid cultures without support, indicating that submerged micro-shoots were constantly shaken to increase axillary bud multiplication and growth while decreasing apical dominance.

Compared to a semi-solid medium, the proliferation response was significantly higher in liquid systems. The highest bud/shoot production was found in liquid medium (Plate 2). In this system, new buds were initiated near each leaf axil, followed by bud elongation. The higher contact of the surface area with the medium under submerged conditions and the increased ventilation may enhance the proliferation rates in this system. It has been reported that continuous shaking promotes lesser expression of apical dominance and leads to enhanced induction and proliferation of axillary buds (Mehrotra et al., 2007). Proliferation occurs primarily from the enlarged base in the semi-solid medium. The lowest proliferation was observed in liquid medium combined with rigid support. In this system, even though the shoots were loosely attached to the support initially, with growth it becomes tight and creates a grove on the stem, reducing the subsequent growth.

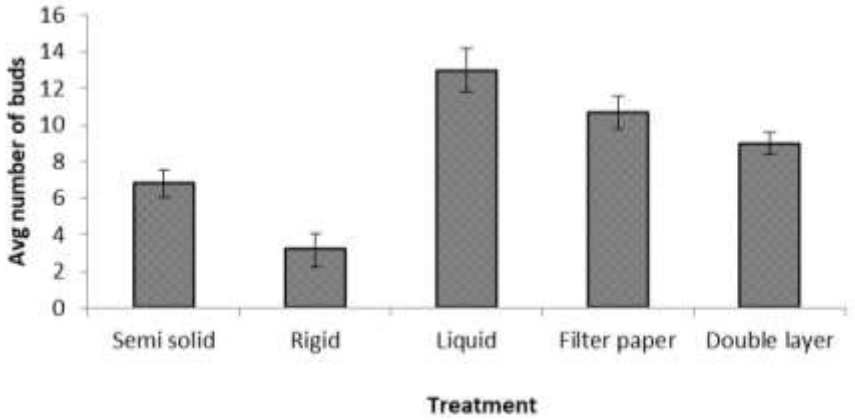

Figure 4: Proliferation response of different culture system at the end of sixth week, (Number of buds/shoot of *in-vitro* grown *G. walla* shoots as affected by semi-solid and liquid culture conditions with and without inert support)

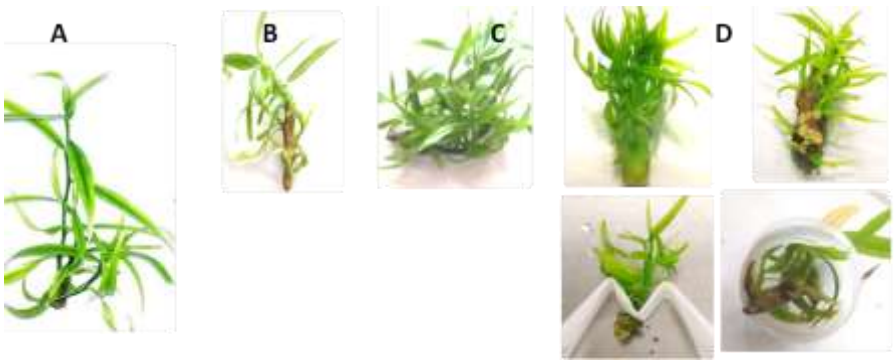

Plate 2: The effect of semi-solid and liquid culture conditions (with and without inert support) on *G,walla* shoot multiplication. Note A. semi-solid, B. liquid medium, C. Double layer, D. filters Paper Bridge, E. rigid support

There are no studies related to the liquid culture of *G. walla*. Similar results have been reported for the other species in terms of higher biomass accumulation and multiplication in liquid systems (Mehrotra et al., 2007). Even though the purpose of the solidifying agent in culture media is to provide support and keep the plant in an upright position, reduction of growth as well as multiplication under increased agar concentrations has been reported for many species (Hussein et al., 2014) In terms of growth and multiplication, liquid culture is promising. However, maintaining shoots continuously in liquid cultures without supports leads to hyperhydricity, abnormal leaf morphology, fragile leaves, and high leaf loss, whereas a liquid system with support facilitates the production of shoots of similar quality to that of a semi-solid medium without hyperhydricity.

CONCLUSIONS

A higher multiplication rate of *G. walla* shoots, 11-13 from one shoot, could be achieved by culturing ex-plants on liquid MS medium supplemented with 1 mg/L BAP and 0.1 mg/L IBA against the multiplication rate of 6-7 shoots on MS medium solidified with phytagel and supplemented with the same concentrations of BAP and IBA within 6 weeks. The development of hyperhydricity in liquid cultures can be overcome by combining them with a flexible matrix when liquid culture systems are used for propagation purposes.

ACKNOWLEDGEMENTS

Authors would like to thank the National Research Council (NRC), Sri Lanka for funding to carry out this study.

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