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RESEARCH

In vitro rooting of *Gyrinops walla* in activated charcoal-containing semi-solid culture and filter paper-bridged liquid culture systems

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ABSTRACT

Plant tissue culture is the best option in producing planting material for cultivation programmes of *Gyrinops walla*. Perfection of *in vitro* rooting protocol for this species is the most decisive step in micropropagation. In the present study the effect of physical state of substrate, different concentrations of sucrose, IBA and NAA in media containing activated charcoal on *in vitro* rooting was investigated. In experiment-1, 0.2% (w/v) activated charcoal was added in to the ½ MS medium fortified with 30 or 40 g/L sucrose and varying concentrations of IBA or NAA (0, 0.1, 0.5, 1.0 and 2.0 mg/L). In experiment-2, filter paper-bridged liquid medium was used with 40 g/L sugar and varying concentrations of IBA or NAA (0, 0.1, 0.5, 1.0 and 2.0 mg/L). The treatments were arranged in completely randomized design with three replicates. Percentage of rooted microshoots and number of roots per shoot were recorded. The ½ MS semi-solid medium with 0.2% activated charcoal, 40 g/L sucrose and 0.1 mg/L NAA resulted in the significantly highest rooting percentage (50%)while the significantly highest number of roots (6.0) were noted in ½ MS charcoal added (0.2%) basal medium with 40 g/L sucrose and 0.5 mg/L NAA. Shoots supported with filter paper bridge on ½ MS liquid medium, supplemented with 0.5 mg/L IBA, had the highest rooting percentage (66.7%) and the highest mean number of roots per shoot (3.0) ($P<0.05$). Results confirmed that *in vitro* rooting of *G. walla* could be perfected by optimizing the culture medium and its constituents.

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INTRODUCTION

Gyrinops walla (agarwood) is classified as an endangered forest species in Sri Lanka. Felling of agarwood indiscriminately for its fragrance material in natural habitats has seriously hampered natural regeneration of *G. walla*, thus, threatening the survival of this species. Seeds are commonly used for propagation of *G. walla*. At present, *G. walla* seeds are scarce making it difficult to meet the demand of seedling supplies. The natural regeneration of this plant species is also slow due to poor rate of seed germination, low seed viability, and high rate of seedling mortality in natural habitats (Subasinghe and Hettiarachchi, 2013). While taking firm steps for conserving the existing trees from extinction, promising systems for sustainable supply of seedlings, mass propagation, and population regeneration and growth are necessary to be developed for using *G. walla* in commercial scale. Plant cell and tissue culture technology has opened up possibilities for adoption in all these disciplines in addition to the improvement and conservation of this tree species (George and Sherrington, 1984; George, 1993). Therefore, micropropagation is considered as the best and sustainable option for mass scale multiplication of *G. walla*.

Rooting of *in vitro* multiplied shoots is the most pivotal *in vitro* step of micropropagation of woody perennial plants, and difficulties in *in vitro* rooting have long remained as a major challenge in the micropropagation of woody species. Harada and Murai (1996) reported that the success of entire *in vitro* cycle depends mainly on the formation of adventitious roots *in vitro* as it determines the higher survival percentage of plants during acclimatization. Rooting media should be optimized with the physical state of substrate, type of auxins used and its concentration, and the constituents added to the culture media for achieving successful *in vitro* rooting (Swamy et al., 2002).

Micropropagation protocols are highly influenced by the physical state of the medium, with significant implications for mass production. Fotopolous and Sotiropoulos (2005) reported that the rooting ability of *in vitro* shoots was affected by mineral concentration of the medium. Many studies on *in vitro* rooting of woody perennial plants have reported that half strength of MS (½ MS) medium was the best to use for rooting of micro shoots (Saxena and Bhojwani, 1993; Purohit et al., 1994; Thirunavoukkarasu et al., 2010; Moitreyee and Karuna, 2015). *In vitro* rooting was highly influenced by the different types and concentrations of auxins used. Growth and morphogenesis in *in vitro* cultures are regulated by the interaction and balance between the auxins available in the medium. Indole-3-Butaryc Acid (IBA) and Naphthaleneacetic Acid (NAA) are the most efficient auxins for root initiation in micropropagation (Singh and Chand, 2003; Khan et al., 2008). Moreover, the addition of activated charcoal to a culture medium is a widely recognised practice in plant tissue culture, mainly to improve growth and/or promote morphogenesis in many plant species (George, 1993; Pan and Van Staden, 1998).

With these considerations, the present study was undertaken with the aim of optimizing the *in vitro* rooting protocol by studying the effects of physical state of substrate, concentrations of sucrose and auxins on *in vitro* rooting of *G. walla*.

METHODOLOGY

Well elongated, vigorous shoots were selected for *in vitro* rooting experiments 24 weeks after establishment, and shoots were transferred to different rooting media under the aseptic conditions in a laminar flow cabinet (Labgard Class II, Type A/B3).

Experiment 1: Effect of different concentrations of auxins and sucrose in activated charcoal-containing semi-solid medium on *in vitro* rooting of *G. walla*

Rooting media (½ MS) were prepared according to the formulation of Murashige and Skoog (1962) at half of its concentration. Activated charcoal (0.2 % w/v) was added to the ½ MS medium with the supplementation of two different sugar concentrations (30 or 40 g/L) and varying levels (0.1, 0.5, 1.0 and 2.0 mg/L) of IBA or NAA (Table 1). The ½ MS

medium added without 0.2 % (w/v) activated charcoal was used as the control treatment. A 0.2 % activated charcoal was used in the present study based on the results reported from previous studies (Jouira et al., 1998; Nirmal Babu et al., 2003; Le Saos and Hourmant, 2001). The pH of the medium was adjusted to 5.8 with 1.0 mol/L using HCl or NaOH, followed by addition of 0.2% (w/v) Phytagel (Sigma, UK) prior to solidifying, and then the medium was autoclaved at 120 °C at 15 psi for 20 minutes.

Experiment 2: Effect of physical state of substrate and different concentrations of auxins on *in vitro* rooting of *G. walla*

A ½ MS liquid medium was prepared without adding a gelling agent and based on the results of the previous study (Selvaskanthan et al., 2021), and 40 g/L sucrose was chosen to supplement with 0.1, 0.5, 1.0 and 2.0 mg/L IBA or NAA (Table 2). A ½ MS liquid medium without any auxin was used as the control treatment. To support the microshoots in the liquid media, sterilized filter papers were folded and inserted into the culture vessels like a bridge, and the microshoots were placed in the middle of the filter paper bridge. All the tissue culture procedures were done under a laminar flow cabinet (Labgard Class II, Type A/B3).

Culture conditions

All the cultures were incubated under 1000 lux light intensity using white fluorescent lamps, with a photoperiod of 16 hours light and an 8 hour dark, at 25±2 °C temperature and 75% relative humidity.

Data Collection

In both experiments, all treatments were replicated thrice with four micro shoots in each. Numbers of roots/shoots were recorded and rooting percentages were calculated at weekly interval.

Table 1: Treatments with combinations of sucrose and auxins (IBA and NAA) in activated charcoal-containing medium

Treatment codes	0.2 % activated charcoal-containing MS medium		
	Sucrose (g/L)	IBA (mg/L)	NAA (mg/L)
R1	30	0.1	0
R2	40	0.1	0
R3	30	0.5	0
R4	40	0.5	0
R5	30	1	0
R6	40	1	0
R7	30	2	0
R8	40	2	0
R9	30	0	0.1
R10	40	0	0.1
R11	30	0	0.5
R12	40	0	0.5
R13	30	0	1
R14	40	0	1
R15	30	0	2
R16	40	0	2
R17*	30	0	0

Note: Treatment codes (R1 to R17* control) were given to different *in vitro* rooting treatments in activated charcoal-added (0.2% w/v) medium except R17, supplemented with different combinations of sucrose and auxins (IBA or NAA) as given in the table

Table 2: Treatment codes for different liquid medium compositions provided with filter paper bridge

Treatment codes	Concentrations of auxin	
	IBA (mg/L)	NAA (mg/L)
L1	0.1	0
L2	0.5	0
L3	1	0
L4	2	0
L5	0	0.1
L6	0	0.5
L7	0	1
L8	0	2
L9	0	0

Note: Treatment codes (L1 to L9) were given to different *in vitro* rooting treatments in ½ MS filter paper bridged liquid medium supplemented with different concentrations of IBA or NAA

Statistical analysis

One-way analysis of variance (ANOVA) was conducted on the variable number of roots per shoot using Minitab 17 and the mean values were compared using pairwise Tukey's test at P =0.05 and the results were presented as mean ± SE.

RESULTS AND DISCUSSION

Experiment 1: Effect of different concentrations of auxins and sucrose in activated charcoal-containing semi-solid medium on *in vitro* rooting of *G. walla*

Adventitious root initiation was observed at the 5th week after transferring shoots into the rooting medium. As presented in Table 3, the highest rooting percentage (50.0 %) was recorded in 0.2 % (w/v) activated charcoal added ½ MS medium, fortified with 40 g/L sucrose and 0.1 mg/L NAA (R10). The highest mean number of roots/shoots (6.0; P<0.05) was developed in the ½ MS charcoal added medium supplemented with 40 g/L sucrose and 0.5 mg/L NAA (R12), but the roots were horizontal and fragile. In ½ MS medium complemented with 0.2 % (w/v) activated charcoal, 40 g/L sugar and 1.0 mg/L IBA (R6) or 1.0 mg/L NAA (R14), the callus formation was observed at the base of the shoots. The roots developed from the callus were thick but broke during sub-culturing of shoots.

In vitro rooting was not successful as expected. Rooting was not observed in the control treatment (R17) and also in the other rooting media supplemented with 0.1 mg/L IBA (R5), 2.0 mg/L IBA (R7 and R8) and 2.0 mg/L NAA (R15 and R16). In activated charcoal added medium (Figure 1a), a prominent growth enhancement was observed compared to the control treatment (Figure 1b).

Difficulties in rooting *in vitro* have long remained a major challenge in the micropropagation of woody species (Harada and Murai, 1996). Reduced macronutrient concentration in the growth medium could exert a favourable effect on micropropogation because the concentration of macronutrients like nitrogen needed for root formation is much lower than that is required for shoot formation and growth (Driver and Suttle, 1987). Therefore, half strength of MS medium was used in the present study. Many studies on *in vitro* rooting of woody perennial plants have reported that half strength of MS (½ MS) medium was best to use for rooting of micro shoots (Saxena and Bhojwani, 1993; Purohit et al., 1994; Thirunavoukkarasu et al., 2010; Moitreyee and Karuna, 2015). Types of auxin used and their concentrations play an important role in root formation (Swamyet al., 2002). Moreover, the beneficial effects of activated charcoal on *in vitro* rooting can be attributed to its adsorptive properties (Pan and Staden, 1998).

Table 3: Effect of auxins and sucrose at varying concentrations in activated charcoal-added medium on *in vitro* rooting

Treatments	% of Rooting	Number of roots/shoot
R1 (30 g/L sucrose, 0.1 mg/L IBA)	0	0c
R2 (40 g/L sucrose, 0.1 mg/L IBA)	25.0	1.3bc±0.14
R3 (30 g/L sucrose, 0.5 mg/L IBA)	16.7	1.3bc±0.38
R4 (40 g/L sucrose, 0.5 mg/L IBA)	25.0	1.7bc±0.14
R5 (30 g/L sucrose, 1.0 mg/L IBA)	0	0c
R6 (40 g/L sucrose, 1.0 mg/L IBA)	16.7	1.7bc±0.38
R7 (30 g/L sucrose, 2.0 mg/L IBA)	0	0c
R8 (40 g/L sucrose, 2.0 mg/L IBA)	0	0c
R9 (30 g/L sucrose, 0.1 mg/L NAA)	0	0c
R10 (40 g/L sucrose, 0.1 mg/L NAA)	50.0	3.7ab±0.14
R11 (30 g/L sucrose, 0.5 mg/L NAA)	25.0	1.7bc±0.29
R12 (40 g/L sucrose, 0.5 mg/L NAA)	33.3	6.0a±0.66
R13 (30 g/L sucrose, 1.0 mg/L NAA)	8.3	1.0bc±0.43
R14 (40 g/L sucrose, 1.0 mg/L NAA)	33.3	3.0abc±0.25
R15 (30 g/L sucrose, 2.0 mg/L NAA)	0	0c
R16 (40 g/L sucrose, 2.0 mg/L NAA)	0	0c
R17 (30 g/L sucrose)	0	0c

(a) **(b)**

Figure 1: *In vitro* plantlets of *G. walla* in activated charcoal-added (0.2% w/v) ½ MS medium supplemented with 40 g/L sucrose and 0.5 mg/L NAA (a) and in the control (b) at 6th week after transferring

Carbon in the form of activated charcoal is often used in plant tissue culture to improve cell growth and development (Pan and Van Staden, 1998). Influence of activated charcoal in growth and development may be attributed to the adsorption of inhibitory substances from the culture medium (Fridborg et al., 1978; Theander and Nelson, 1988), drastic decrease in the phenolic oxidation (Teixeria et al., 1994) and alteration of pH of the growth medium to optimal levels for morphogenesis during root initiation (Owen et al., 1991). Activated charcoal has a very fine network of pores with a large inner surface area, on which many substances can be adsorbed, playing a critical role in establishing a darkened environment in the medium to simulate soil conditions (Dumas and Monteuuis, 1995). Light passing through a solidified medium is reduced and/or kept away from the rooting zone. This could promote some physiological reactions which occur in the dark. It is a generally recognized that auxins are metabolised slowly in the dark than in the light

(Norton and Boe, 1982). Therefore, darkness is beneficial for rooting, especially during the inductive phase.

The most crucial impact of adding activated charcoal to the culture medium is the drastic alteration in concentrations of plant growth regulators (PGRs) and other organic supplements in the basal culture medium (Fridborg et al., 1978; Ebert and Taylor, 1990; Ebert et al., 1993; Nissen and Sutter, 1990), vitamins (Weatherhead et al., 1978; Weatherhead et al., 1979; Pan and Van Staden, 1998) and metal ions like Cu^{2+} and Zn^{2+} (Van Winkle et al., 2003), and subsequently influencing the plant regeneration processes (Druart and Wulf, 1993). Fridborg and Eriksson (1975) also suggested that activated charcoal removes growth regulators, particularly auxins, from the medium.
Non-selective adsorption might be the possible reason for suppression of root induction in activated charcoal added media in this study. This observation is supported by the findings of Buendia-Gonzalez et al. (2007), who reported that in micropropagation of *Prosopis laevigata*, root induction was suppressed by the addition of activated charcoal. On the contrary, micropropagated shoots of *Enseteven tricosum* were successfully rooted on ½ MS medium supplemented with IBA, BAP and 1.0 g/L activated charcoal (Negash et al., 2001). Moreover, the addition of activated charcoal to a culture medium is a widely recognised practice in plant tissue culture, mainly to improve growth and/or promote morphogenesis in many plant species (George, 1993; Pan and Van Staden, 1998).

Effect of sucrose concentration on *in vitro* rooting

Carbohydrates are required by plant cells as carbon resources, act as energy for growth and biosynthetic processes, and may influence *in vitro* rooting (Ferreira et al., 2011). The most common carbohydrate used in micropropagation is sucrose, at a concentration of 30 g/L as recommended by Murashige and Skoog (1962). Presence of sucrose in the culture medium contributes to the process of cell division in the root apical meristem, which causes an increase in root length. Higher concentration of sucrose (40 g/L) than the recommendation (30 g/L) of Murashige and Skoog (1962) with reduced nitrogen strength by half, showed better *in vitro* root induction in the present study. Similar results were reported by Gabryszewska (2015), where sucrose at concentrations of 40- 50 g/L strongly stimulated the number of roots per micro-plants (5.8-6.0) in the medium with a reduced level of nitrogen salts by half and quarter in *Helleborus niger*. Gabryszewska (2015) reported that the high level of nitrogen salts (100 % according to MS medium) and a low level of sucrose (10-30 g/L) in the medium exerts a strong suppression of root induction and root development. Similar to the results of the present study, Martins et al. (2015) reported that salts and sucrose concentrations used for *in vitro* propagation of *Billbergiaze brina* (Bromeliaceae) had a positive impact on rooting showing a quadratic relationship with sucrose concentration in the medium. In the same study, the root growth was the best at 43.3 g/L sucrose.

Effect of types and concentrations of auxins on *in vitro* rooting

Auxins have been shown to be effective inducers of adventitious roots in several woody species (De Klerk et al., 1999). Ameena et al. (2009) reported that root induction of *Ficus anastasia* was higher in half strength MS medium supplemented with 0.1 mg/L IBA or NAA. In *Ulmus* species, ½ MS media fortified with NAA was found to be the best in producing the highest percentage of roots and long roots formation (Durkovic and Misalova, 2009). Similar to the results of the present study, Moitreyee and Karuna (2015) also reported that ½ MS media supplemented with ≥1.0 mg/L NAA and IBA developed calli at the base of shoots and roots. They further reported that the roots formed from these calli were thick, fragile, and easily breakable during sub-culturing. Similar drawback was reported in micro shoots of *Lonicera caerulea* developed *in vitro* (Karhu, 1997). Callus initiation at the base of microshoots was observed when they were cultured on ½ MS medium supplemented with higher concentration of IBA and NAA, which was in agreement with the results reported in

Aquilaria malaccensis (Moitreyee and Karuna, 2015), *Peganum harmala* (Saini and Jaiwal, 2000) and *Rotula aquatic* (Martin, 2003). Kataoka (1994) reported that roots growing in agar medium showed structural abnormalities and often lack of root hairs affecting their field establishment (Debergh and Maene, 1981).

Experiment 2: Effect of physical state of substrate and different concentrations of auxins on *in vitro* rooting of *G. walla*

Root initiation had started at the 4th week of transferring shoots in ½ MS liquid medium supplemented with 0.5 mg/L IBA (Figure 2a). The highest rooted shoot percentage (66.7 %) and the high mean number of roots per shoot (3.0; $P<0.05$) were recorded in ½ MS liquid media provided with filter paper bridge and fortified with 0.5 mg/L IBA (Table 4). None of the shoots were rooted in ½ MS medium supplemented with 2.0 mg/L IBA or NAA and in the control treatment. Similar results were reported by Selvaskanthan et al. (2021) with the same concentrations of IBA and NAA in semi-solid medium supplemented with 3g/L phytagel. Induction of calogenesis at the basal end of shoots was not observed in MS semi-solid basal media, supplemented with IBA, in the filter paper-bridged liquid medium and in none of the IBA containing media. Elongation of roots was also better in liquid media compared to semi-solid medium. *In vitro* rooted plantlets were transferred to auxin-free MS media for further root elongation (Figures 2b and2c).

Table 4: Effect of IBA and NAA at varying concentrations in ½ MS filter paper-bridged liquid medium supplemented with 40 g/L sucrose on *in vitro* rooting

Treatments	% of rooting	Mean number of roots/shoot
L1 (0.1 mg/L IBA)	25.0	1.3ab±0.20
L2 (0.5 mg/L IBA)	66.7	3.0a±0.35
L3 (1.0 mg/L IBA)	16.7	0.7ab±0.20
L4 (2.0 mg/L IBA)	0	0b
L5 (0.1 mg/L NAA)	16.7	1.0ab±0.35
L6 (0.5 mg/L NAA)	8.3	1.0ab±0.61
L7 (1.0 mg/L NAA)	25.0	1.7ab±0.20
L8 (2.0 mg/L NAA)	0	0b
L9 (control)	0	0b

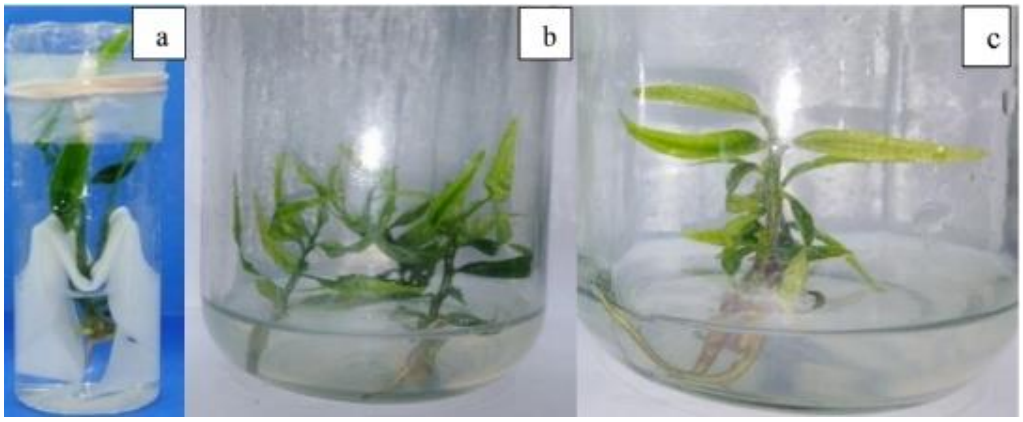

Figure 2: (a) Root initiation on filter paper-bridge in liquid medium containing ½ MS with 0.5 mg/L IBA at 4 weeks after inoculation; (b) rooted shoots transferred to MS semi-solid medium at 6 weeks after inoculation; (c)root elongation in MS semi-solid medium at 8 weeks after inoculation

Micropropagation protocols are highly influenced by the physical state of the medium, with significant implications for mass production and the consistency of the medium influences the *in vitro* organogenesis (Mengarda et al., 2009; Silva et al., 2012). Liquid media provide several advantages over a semi-solid media, especially during rooting stage. Further, liquid media are easy to wash off from the delicate roots to prevent damages of fine roots and microbial growth during acclimatization.

Mohammed and Vidaver (1988) found that liquid media is better for direct organogenesis compared to semi-solid medium. Suthar et al. (2011) reported that the best rooting response of 75 % was obtained when shoots were rooted on liquid medium in the *in vitro* propagation of *Boswellia serrate*. The superiority of liquid media is attributed with better aeration of the root primordial compared to semi-solid media. Blidar et al. (2011) reported that increased nutrient availability and selective uptake of nutrients through provision of constant access to the nutrients around the roots of wheat plantlets were optimized in filter paper-bridged liquid media.

On the contrary, easy access to the nutrients is restricted in semi-solid culture media due to agar. The minerals and organic compounds that are homogeneously trapped in the agar meshes in the fresh media are not replaced through diffusion once they have been consumed; and therefore, a gradient of concentrations of nutrients is created around the root systems in cultures with time. Moreover, another major drawback identified in preventing the use of a liquid culture system is the inability to keep individual microshoots in an upright position during rooting, which will lead to develop considerable number of abnormal roots. However, the use of filter paper bridges to maintain microshoots in upright position is considered as one of the cost-effective methods that could be effectively and efficiently used in *in vitro* rooting under liquid media.

CONCLUSION

Results confirmed that *in vitro* rooting of *G. walla* could be perfected by optimizing the culture medium and its constituents. The sugar and nitrogen concentrations of the liquid growth media affect the *in vitro* rooting of *G. walla.* According to the results, the liquid ½ MS medium supplemented with 40 g/L sucrose and 0.5 mg/L IBA can be recommended for *in vitro* rooting of *G. walla.* Further studies are recommended to enhance the rooting efficiency of *in vitro* generated shoots.

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