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In vitro propagation of *Gyrinops walla* Gaetner 'Walla patta', a vulnerable agarwood producing species in Sri Lanka

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Abstract

Aloeswood or Agarwood has long been a perfumery commodity traded between Mediterranean Region and Southeast Asia since B. C. era. Oil or chips obtained by destructive harvesting of several Thymeleaceous genera including *Aquilaria* and *Gonystylus* are expensive and such ingredients are high in demand in the global market. The recent discovery of *Gyrinops walla* as a potential producer of market-quality agarwood in mature damaged woods and branches, intensified illicit felling and exportation of *G. walla* that led to it being in the verge of extinction from Sri Lankan flora. The sustainable utilization of *G. walla* undoubtedly enhances the foreign exchange of the country and the non-destructive utilization *G. walla* through tissue culture-based techniques is the only option available for sustainable exploitation and conservation of the vulnerable *G. walla* species. Micropropagation of *G. walla* was achieved by varying concentrations of BAP and NAA on MS medium to produce shoot and root with leaf-derived callus, respectively. The phytohormone concentrations of 3.0 mg/l BAP and 2.0 mg/l NAA proved the optimum concentrations for shoot and root induction, respectively. The callus turned to green and produced fewer buds. Roots were protruded out from the calli in the root induction medium. The findings of the study led to conclude that the micropropagation was viable in potential as an *in vitro* system for sustainable utilization and conservation endeavours of *G. walla.*

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1. Introduction

Gyrinops walla (family Thymeliaceae) commonly known as 'Walla patta', is an endemic species growing in the wet zone of Sri Lanka (Townsend, 1981). *G. walla* is mainly distributed in forest areas at lower elevations (below 1525m from sea level) of the Western, Sabaragamuwa (southern margin, adjacent to the Sinharaja rain forest) and Southern Provinces of the country (Gunatilleke *et al*., 2005). The tree is small to medium in size with numerous branches and slender, wiry twigs (Dassanayake *et al*., 1981). The bark is smooth, thin and strongly fibrous and the color varies from gray to brownish gray. The wood is pale yellow in color and the intensity of the color varies with the amount of resin present in the wood.

"Walla patta" gained popularity in 2012, with the discovery of its similarity to the commercially valued agarwood, a fragrant resinous wood used in perfumery and medicine related industries, obtained from the trunk and branches of *Aquilaria* and *Gonystulus.* Agarwood is naturally formed in the heartwood as a result of defence mechanism against the invasion of certain fungal species of Ascomycetes. The characteristic fragrance of agarwood is reported to be due to the presence of sesquiterpenoids and chromone derivatives (Takemoto *et al.*, 2008). Following the discovery,

large scale smuggling of *G. walla* from Sri Lanka has been practiced until it was banned from exportation in 2012 (Dharmadasa *et al*., 2013). It is now considered threatened according to International Union for Conservation of Nature (IUCN) Red List Categories in 2012 (Ministry of Environment, 2012). Artificially induced Agarwood requires destructive harvesting such as drilling and insertion of chemicals into the stem of the mature trees. As *G. walla* is the only representative *Gyrinops* species for agarwood in Sri Lanka (Subasinghe and Hettiarchchi, 2016) and the declining population of *G. walla* in the country due to illicit felling for agarwood, destructive harvesting is not suitable in obtaining agarwood. Thus, it is necessary to conserve this valuable species using biotechnological methods such as micropropagation to assist in the conservation, improvement and development.

Successful protocols have been established for micropropagation of agarwood producing species in family Thymelaeaceae. Shoot tip culture method and nodal culture methods has been used to propagate *Aquilaria crassna* (Van Minh, 2001) and *Aquilaria hirta* (Hassan *et al*., 2011) in Murashige and Skoog (MS) medium supplemented with various concentrations of 6-Benzylaminopurine (BAP). Numerous studies have been conducted to micro-propagate *Aquilaria agallocha* using shoot tips (Meng-Ling *et al*., 2005) and shoot buds (Debnath *et al*., 2013). *In vitro* propagation, rooting of micro-shoots and acclimatization of *Daphne* species (a distinctly related species of *Aquilaria*) were also known to be propagated *via* micro-propagation where medium composed of Woody Plant Media, mineral salts, MS microelements and a set of vitamins, supplemented with NAA and calcium gluconate was found to be appropriate (Hanus-Fajerska *et al*., 2012). Attempts have been made to produce plant propagules *via* micropropagation in *Gyrinops* species. Multiplication of bud under effect of IBA, BAP and Kinetin was conducted for *Gyrinops versteegii* in *vitro* (Hidayat, 2011; Yelnititis, 2014). Callus induction has been reported for *Gyrinops walla* (Buddhapriya and Senerath, 2016) using leaf disc explants. However, so far, no records on the indirect organogenesis of *Gyrinops walla* were found in the literature.

2. Materials and Methods

2.1 In vitro bud induction

G. walla calli were initiated according to Munasinghe *et al*., (2020). Two grams of and leaf derived calli were sub-cultured on solid MS medium supplemented with 30.0 g/l sucrose, 100.0 mg/l myo-inositol, 8.0 g/l agar-agar, 1.0 g/l charcoal and different concentrations of BAP (ranged between 05 – 3.0 mg/l) (Meng-Ling *et al*., 2005). A total of five culture plates were prepared with three calli in each and repeated for each combination of medium components. The cultures were maintained at temperature of 25±2ºC and 75%±10% relative humidity under 16 hours photoperiod using cool-white fluorescent lamps (photon flux density – 20 - 50 µmol/m^2/s irradiance) as described by Jo *et al*., (2008). The observations were made and recorded for were; i) effect of growth hormones on establishment of shoot regeneration in terms of percentile establishment, ii) average shoot length (mm) of multiplied micro shoots and iii) average number of shoots per explant.

2.2 In vitro rooting

Shoots of varying length (ranging between 0.8 – 6.0 mm) were taken at different stages of sub-culturing and transferred in to half strength MSM containing 400 mg/l activated charcoal and NAA (ranging between 0.5 – 3.0 mg/l) for root induction (Norazlina *et al*., 2010). However, due to the necrosis of shoot induced calli, to study the effect of NAA on rooting, *G. walla* calli were used. Consequently, a total of five culture plates were prepared with three calli in each and repeated for each combination of medium components. The cultures were incubated at 25 ± 2 ºC and 75% ± 10% relative humidity under 16-hour photoperiod using cool-white fluorescent lamps (photon flux density – 20 - 50 µmol/m^2/s irradiance) for 60 days. Following four weeks of incubation, observations were made for were; i) per cent rooting i.e, based upon number of micro shoots forming roots among total cultured shoots, ii) numbers of roots per micro shoot and iii) root length (mm).

2.3 Direct organogenesis

The explants for direct organogenesis were obtained from shoot apex with emerging leaves from six-month-old *G. walla* plants and were severed into 1.5 –2.0 cm using sterilized scalpel under a dissecting microscope. Sterilized severed explants were inoculated in MSM supplemented with 30.0 g/l sucrose, 100.0 mg/l myo-inositol, 8.0 g/l agar-agar and 1 g/l charcoal with varying concentrations of BAP in combinations as indicated in Table 1 (Saikia and Shrivastava, 2015). The cultures were incubated at 25 ± 2 ºC and 75% ± 10% relative humidity under 16-hour photoperiod using cool-white fluorescent lamps (photon flux density – 20 - 50 µmol/m^2/s irradiance) for 60 days. Three replicates were used for each treatment and each replication contained three explants. The medium that produce maximum number of multiple shoots was used for the subsequent stages of multiplication and maintenance.

2.4 Statistical analysis

The descriptive statistics mean and Standard Deviation (SD) were calculated for the data obtained. The inferential statistics ANOVA and Tukey's Honest Test were performed to compare the means. All statistical analyses were performed using SAS (Ver. 9) (2008).

3. Results

3.1 Shoot induction

After placing 2 g of growing callus in MS medium supplemented with BAP alone, it was subjected to illumination for stimulation of shoot regeneration. At the beginning of culture under illumination, white callus turned to yellow, gradually changing it into pale to light green (Figure 1). Adventitious buds were developed after 4 weeks' cultivation and *ca*. 7 weeks later, the buds were developed into dumpy and twisted micro shoots, which served as shoot primordia from callus (Figure 2). Few buds like protuberances were developed with maximum induction (70 %) in treatment medium S1 (0.5 mg/l BAP) followed by treatment medium S3 (1 mg/l BAP) and other treatments showed a poor response (<50%) (Table 3). With the increase of incubation period, shoot buds were developed into multiple shoots; however, even single explant could respond only negligibly to bud induction in hormone-free MS medium (S1) i.e. control. The highest number of shoots was observed in treatment medium S2 (0.5 mg/l BAP) with an average number of 2.2 shoots per explant. The maximum average shoot height (7.0 mm) was recorded in treatment S2 followed by S3 and S4 respectively. The lowest average shoot height (0.8 mm) was observed in S6 (Table 1).

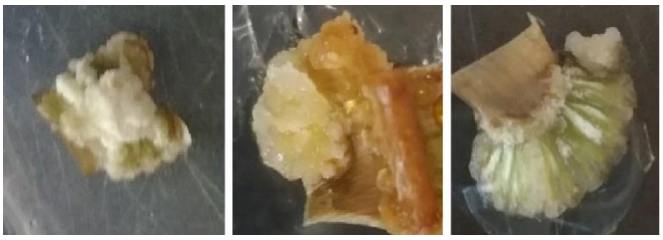

Figure 1: Changes of calli under illumination.

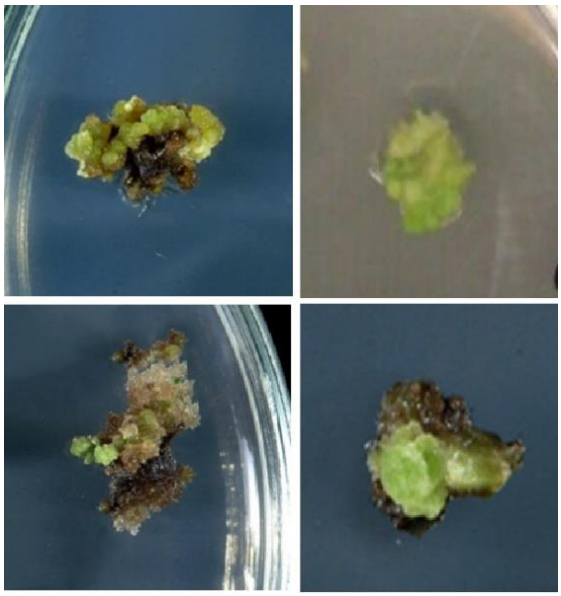

Figure 2: Developed shoot buds in *G. walla* callus.

Table 1: Effect of BAP on shoot regeneration *G. walla* callus in MS media

Treatment	BAP (mg/l)	Shoot regeneration %	Average number of shoots per explant	Average shoot height (mm)
S1	0.0	0.00(0.00)^{e}	0.00 (0.00)^{f}	0.00 (0.00)^{f}
S2	**0.5**	**70.00 (5.24)^{a}**	**2.2 (0.3)^{a}**	**7.0 (0.7)^{a}**
S3	1.0	56.00 (2.91)^{b}	1.4 (0.2)^{b}	4.0 (0.7)^{b}
S4	1.5	26.00 (6.96)^{c}	1.0 (0.3)^{d}	1.4 (0.4)^{d}
S5	2.0	27.00 (7.17)^{c}	1.2 (0.2)^{c}	1.6 (0.2)^{c}
S6	3.0	24.00 (6.59)^{d}	0.8 (0.2)^{e}	0.8 (0.2)^{e}

The mean values are followed standard deviation within parenthesis. The same letter along the columns indicates no statistically significant difference at $p \leq 0.05$.

3.2. Root regeneration

To complete plant organogenesis, shoot explants of *G. walla* were subjected to stimulate for rooting. However, while micro shoots initiated calli were transferred to the root regeneration medium, micro shoots started to turn brown and finally leading to the necrosis precluding from continuation of plant regeneration (Figure 3). Consequently, *ca.* 2 g of

fresh callus, which were incubated under dark were used to observe the effect of NAA on root regeneration. Within 14 -21 incubation days, white to pale yellow micro roots were developed from the callus and unlike in the case of shoot regeneration, the callus was turned into pale yellow instead of green (Figure 4). The maximum roots formation (42.0 %) was observed in ½ MS rooting medium treated with 2 mg/l NAA (R5) whereas the minimum root formation (10 %) was recorded in ½ MS medium supplemented with 0.5mg/l NAA (R2) (Table 2). The maximum and the minimum average root lengths observed in R5 and R2 were 12.8 mm and 5.6 mm, respectively and were statistically significant ($p < 0.05$).

Figure 3: Necrosis of bud induced calli

Figure 3: Roots developed in *G. walla* calli

Table 2: Effect of NAA on root regeneration of *G. walla* callus in MS media

Treatment	NAA (mg/l)	Root regeneration %	Average number of roots per explant	Average root length (mm)
R1	0.0	0.00 (0.00)^{e}	0.00 (0.00)^{e}	0.00 (0.00)^{f}
R2	0.5	10.00 (1.58)^{d}	1.00 (0.3)^{c}	5.60 (1.6)^{e}
R3	1.0	29.00 (1.87)^{b}	1.00 (0.3)^{c}	9.40 (1.5)^{c}
R4	1.5	41.00 (3.31)^{a}	1.40 (0.2)^{b}	10.40 (0.9)^{b}
R5	**2.0**	**42.00 (4.90)^{a}**	**1.60 (0.2)^{a}**	**12.80 (1.1)^{a}**
R6	3.0	25.00 (2.73)^{c}	0.80 (0.2)^{d}	6.4 (1.8)^{d}

The mean values are followed standard deviation within parenthesis. The same letter along the columns indicates no statistically significant difference at $p \leq 0.05$.

3.3. Direct organogenesis from shoot explants of G. walla

At the initial stage of the culture, the shoot explants were light green in colour for 4 - 5 days under given culture conditions. Subsequently, the explants turned into brown and became necrotic (Figure 5). The extended incubation for another 3 – 4 weeks with the same culture conditions showed no signs of formation of shoot primodia.

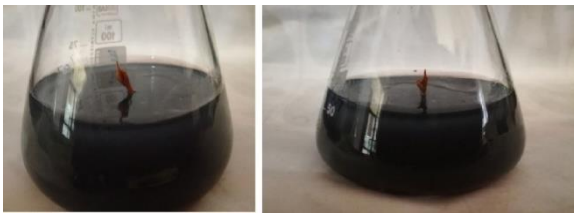

Figure 5: Necrosis of cultured shoots of *G. walla*.

4. Discussion

The leaf derived calli of *G. walla* indicated feasibility of inducing shoots and comparatively higher number of shoots were recorded in the medium supplemented with 0.5 mg/l of BAP. However, previous studies showed that the responsiveness of cytokinin to auxin ratio matters regarding the effective micropropagation of the

species in family Thymelaeacaea. Debnath *et al*., (2013) studied the effect of cytokinin to auxin ratio on micropropagation of *Aquilaria agallocha*, where they observed that in high cytokinin to auxin ratio, white callus turned green under illumination, while in higher ratio of auxin to cytokinin, yellow callus was observed, which finally become necrotic. Further, Debnath *et al*., (2013) have accounted for having higher shoot bud initiation with 4 mg/l BAP + 0.5 mg/l NAA. In the present study, shoot bud induction has been achieved with one hormone (BAP) instead of two hormones in combination. Difficulty in root induction of the shoots derived from calli, as mentioned in Section 2.2, lingered as a major challenge in the micropropagation of *G. walla.* In order to avoid such situations, it has been suggested that implementation of reducing (half-normal) strength of the medium components as the mineral concentration of the medium which affects rooting characteristics (Saikia and Shrivastava, 2015) and increasing the concentration of auxins (Meng-Ling *et al*., 2005). In the present study, it was observed that root induction percentage was higher in half strength MS (½MS) medium supplemented with 2 mg/l NAA compared to other concentrations. Root induction of certain species of family Thymelaeacea, such as *Aquilaria malaccensis* (Norazlina *et al*., 2010) and *Aquilaria agallocha* (Meng-Ling *et al*., 2005) have been achieved using full strength MSM + 4 mg/l IBA and ½MS + 5 µmol/l NAA, respectively. Tissue culture protocols, more specifically, plant micropropagation is time consuming, laborious and requires expensive chemicals. In the present study, shoot bud induced calli were unable to establish in root regeneration media and ultimately became necrotic. It is believed that nutrient deficiency or imbalance, hormonal imbalance, high relative humidity within culture vessels and hyperhydricity, higher ethylene production and poor ventilation of the *in vitro* culture vessel, lingered into necrosis of shoot induced calli in root regeneration media (da Silva *et al*., 2020). Although, the micropropagation of *G. walla* was not successful, it is noteworthy that in the present study, shoot and root inductions have been achieved with 0.5 mg/l BAP and 2 mg/l NAA, respectively, an indication of the possibility of achieving plantlets with modification. Direct organogenesis is considered as one of the most reliable method in plant regeneration due to upholding genetic resemblances to that of parent generation. However, during the study, it was unable to continue direct organogenesis of *G. walla* due to excessive production of phenolic compounds, leading to the necrosis of the explant. Several efforts have been taken to establish shoot induction of some species from family Thymelaeacea, such as *Aquilaria malaccensis* (Saikia and Shrivastava, 2015), *Aquilaria agallocha* (Meng-Ling *et al*., 2005), *Aquilaria microcarpa* (Sabdin *et al*., 2011), *Aquilaria crassna* (Mongkolsook *et al*., 2007) and *Daphne* sp. (Hanus-Fajerska *et al*., 2012) where remarkable results have been observed. In the present study, direct organogenesis was conducted with shoot apex with emerging leaves. Therefore, it is suggested that the differences in the regeneration potential of different explants need to be considered in plant regeneration (Murashige, 1974).

5. Conclusions

Root and shoot initiation of *G. walla* calli was achieved with MS media supplemented with 3.0 mg/l BAP and 2.0 mg/l NAA, respectively. However, due to time and resource constraints, it was incomplete. Plant regeneration of *G. walla* required to be attempted with varying cytokinin to auxin ratios and be continued up to acclimatization.

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