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Short Communication

Development of a Protocol for *In- vitro* Establishment of G*yrinops walla*

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ABSTRACT: *Gyrinops walla is a fragrance-producing resinous tree growing in the natural forests of Sri Lanka. There has been a very high demand for G. walla, resulting over-exploitation of the species. Tissue culture techniques can be used to conserve this species and to produce bioactive secondary metabolites responsible for the fragrance. The establishment of the aseptic culture is considered as the critical stage of in-vitro culture. The present study was carried out to develop a protocol for the establishment of in vitro cultures of G. walla using the explants of leaf, shoot tips and axillary buds. Among the treatments, the best results in producing uncontaminated, green and alive cultures were obtained when the explants of shoot tips and leaves (80% and 100%, respectively) were treated with 10% (v/v) of bleach (NaOCl) solution with two drops of tween-20. Immature axillary buds from the 3rd to 5th nodes from shoot tips produced the best explant in producing the aseptic culture. Surface sterilization with 10% (v/v) bleach coupled with 0.2% of $HgCl_2$ for 5 min reduced the percentage of contaminated cultures by 20% and improved the percentage of uncontaminated green and live cultures of axillary bud to 73%.*

Keywords*: Aseptic in- vitro culture, contamination, Gyrinops walla*

INTRODUCTION

Gyrinops walla, a member of the family Thymelaeaceae, is commonly known as Walla Patta in Sinhala and Sri Lankan Agarwood in English. It is an endemic, fragrance-producing resinous plant grown in the wet and intermediate zones of Sri Lanka (Jayaweera, 1982).
Agarwood extracted from *Gyrinops* species has its unique fragrance and therefore, widely used as an ingredient in traditional medicine (Chen *et al*., 2011), perfumery industries (Chaudhari, 1993), cosmetic industries (Qi and He, 2005), incenses and in aroma therapy (Barden *et al*., 2000). The higher international demand, economic value and lucrative market for *G. walla* in the Middle Eastern countries have created large scale illegal harvesting and smuggling of *G. walla* from tropical rainforest areas in Sri Lanka, which has resultant increase in tree mortality, reduced growth rate, and decreased the percentage of adults trees that reproduce (Dharmadasa *et al*., 2013). Therefore, *G. walla* was classified as endangered species and has been protected under the Flora and Fauna Protection Ordinance (Amendment) No 49 of 1993 in Sri Lanka, since 2004. It also was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 2005.

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Propagation by seeds is usually a reliable method for *G. walla*. However, this method is inadequate to meet the present demand of seedling supplies, due to lack of seed production, low seed viability, low germination rate, delayed rooting of seedlings and long lifecycle. Therefore, there is an urgent need to apply modern technologies for the conservation of existing species as well as for the preservation of its germplasm. Application of plant tissue culture technique may be the best alternative for propagation as well as production of bioactive secondary metabolites of agarwood and oil, within a short cultivation time (Walker *et al*., 2002) than the conventional methods.

Odutayo *et al*.(2007) reported that the establishment of aseptic *in vitro* plantlets from the woody perennial plant sources presents the major challenge in the micropropagation compared to herbaceous plants, mainly due to the contamination and exudates from cut woody stems (Preece, 1991). Therefore, development of a protocol to establish the aseptic *in vitro* cultures of *G. walla* is a vital step, which determines the success of the propagation by plant cell and tissue culture techniques.

METHODOLOGY

One year-old seedlings of *G. walla*, received from the Divisional Forest Office at Ratnapura, Sri Lanka were used to obtain the explant materials. They were grown in pots containing the mixture of compost, soil and sand (1:1:1 v/v/v ratio) and were maintained at 27±2 ^{o}C and 12 h light/12 h dark period inside the glass house at the Agricultural Biotechnology Centre, Faculty of Agriculture, University of Peradeniya, Sri Lanka.

Preparation of mother plants

Mother plants were prepared for the collection of explants and were pre-treated with 0.1% w/v of redoxylmetalyxyl once a week where spraying was continued for four weeks prior to collecting the explants in order to reduce the contamination during the establishment of *in vitro* cultures.

Collection of explants

Axillary buds (nodal segments containing unopened buds), shoot tips and leaves were used as the explants for the *in vitro* culture of *G. walla*. For the shoot tips and axillary bud cultures, 5 to 8 axillary buds along with shoot tips were collected and for the leaf cultures, immature leaves (7 weeks after emergence) were excised from the healthy lateral branches of mother plants. Freshly collected leaves and nodal segments along with shoot tips were washed thoroughly under slow running tap water for 10 min followed by washing in teepol solution for 1 minute and then were rinsed thoroughly for another 10 min.

Sterilization methods

Experiment 1: Effect of 10% bleach on surface sterilization of leaf, axillary buds and shoot tips

Explants of leaf, axillary bud and shoot tips were surface sterilized separately using 10% (v/v) of Clorox, a locally available commercial bleach solution (containing the active ingredient of NaOCl) with 0.6 ml of Polyethylene Glycol Sorbitan Monolaurate (Tween-20) for10 min. Sterilizing solutions with explants were gently stirred manually during the

sterilization period. This procedure was repeated with 10% commercial bleach solution without tween-20 for another 10 min. Thereafter, leaves, shoot tips and nodal segments were rinsed three times using sterile deionized-distilled water and each rinse was lasted approximately for 1 min.

Due to inadequate numbers of shoot tips, there was a need to establish aseptic cultures of axillary buds, which could be adequately collected from the mother plants of *G. walla*. With this intention, a preliminary study was conducted to find out the effect of maturity level on establishment of aseptic cultures of axillary buds. Immature axillary buds (3^{rd}, 4^{th} and 5^{th} nodes from shoot tips) and green matured axillary buds (6^{th}, 7^{th} and 8^{th} nodes from shoot tip) were taken and subjected to surface sterilization as done in the experiment 1 and then were treated with 0.1% mercuric chloride ($HgCl_2$) for 5min, separately.

Experiment 2: Effect of different concentration and exposure time of $HgCl_2$ on establishment of aseptic axillary bud cultures

According to the results of preliminary study on maturity level, immature nodal segments were collected and subjected to different concentrations of $HgCl_2$ treatments at different exposure time. In treatments 1, 2 and 3 (T1, T2 and T3), the nodal segments were surface sterilized with 10% bleach solution and then dipped in 0.1% $HgCl_2$ for 5, 10 and 15 min respectively. In treatments 4, 5 and 6 (T4, T5 and T6), after the surface sterilization with bleach solution, the axillary buds were immersed in 0.2% of $HgCl_2$ for 5, 10 and 15 min, respectively.

Establishment of cultures

All tissue culture techniques were conducted under aseptic conditions in a laminar flow cabinet (Labgard Class II, Type A/B3). Shoot tips and axillary bud explants were prepared by trimming the cut surfaces and were about 12-15 mm in length. Leaves were cut into approximately 12 mm x 12mm pieces containing midrib and two veins at each side of the mid rib. All three explants were cultured on Murashige and Skooge medium (Murashige and Skooge, 1962) supplemented with 3% (w/v) of sucrose and 1mg/L Benzyl Amino Purine (BAP) and solidified with 0.3% (w/v) Phytagel (Sigma, UK) and the pH of the medium was adjusted to 5.8 with 1 mol/L HCl or NaOH before autoclaving at 120 °C for 20 min at 15 psi. Each culture tubes contained 10 ml of the culture medium. Inoculation was done as one explant per culture tube. Leaf cultures were incubated in dark cabinet at 26±2 °C and axillary bud and shoot tip cultures were incubated at 16 hours of light (flux density) at 25±2 °C of temperature with 75% of relative humidity.

Each treatment was replicated fifteen times. Cultures were continuously observed and the number of contaminated cultures, uncontaminated green and alive cultures, and uncontaminated cultures that have turned brown were recorded. Readings were summarized at the end of the third week, and chi-squared goodness of fit test was performed to analyze the data using Minitab 18 statistical package.

RESULTS AND DISCUSSION

Three different explants of *G. walla* were used in this study to develop protocol on their successful establishment of *in vitro* cultures. In the Experiment 1, none of the cultures were contaminated when the leaf explants were subjected to surface sterilization using 10%

commercial bleach solution and tween-20 [Plate I(a)] and callus induction was observed in all cultures [Plate I(b)]. When the axillary buds were surface sterilized with 10% bleach solution alone, all the cultures were contaminated within seven days of culturing [Plate I(c)]. Satisfactory result was obtained with the surface sterilization of shoot tips using 10% of commercial bleach solution. Among the 93.33% of uncontaminated shoot tip cultures, 80% were green and alive [Plate I(e)] and 13.33% of cultures turned brown [Plate I(f)].

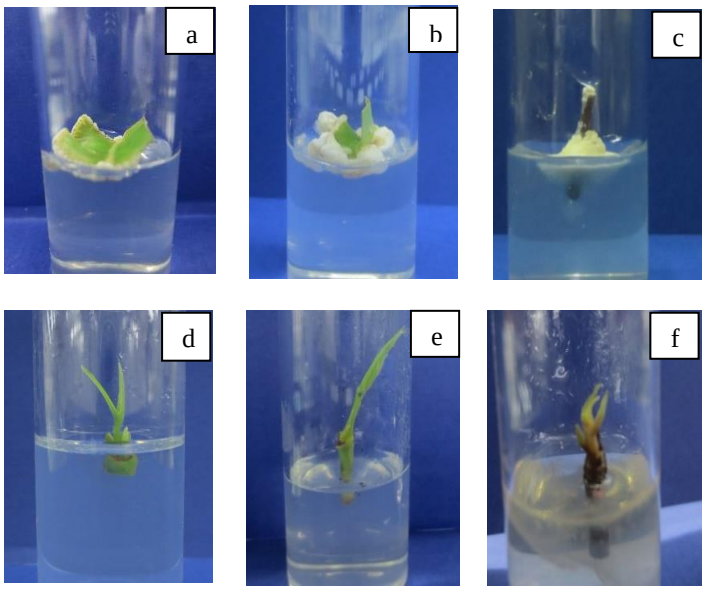

Plate I. Effect of different sterilization techniques on *in vitro* cultures of leaf, axillary bud and shoot tip explants

(a) uncontaminated, green and alive culture of leaf, (b) callus induction from uncontaminated leaf explant, (c) contaminated culture of axillary bud, (d) uncontaminated, green and alive culture of axillary bud, (e) uncontaminated growing shoot tip culture, (f) browning of shoot tip culture

Surface of the explants carries a wide range of microbial contaminants. To avoid this source contamination, the explants must be thoroughly surface sterilized before inoculating them in to the culture medium. Clorox is a commercial bleach solution, which is locally available in markets, and it contains sodium hypochlorite as the active ingredient. Sodium hypochlorite is a widely used and an effective killer of pathogens; even micromolar concentrations are enough to reduce contaminations of fungi and bacteria at time intervals between 20 - 45 min (Oyebanji *et al*., 2009). Tween-20 is a commonly used surfactant to enhance the effectiveness of sterilization procedure (Colgecen *et al*., 2011). Essentially, surface sterilization of woody explants for *in vitro* culture is comparatively a difficult step in culture initiation as endophytic contaminants are not killed by surface sterilization alone (Webster *et al*., 2003). The woody plants appear to have microbial contamination within the vascular system and other tissues (Smith, 2000). This may have attributed to the total contamination of axillary bud cultures when they were subjected to surface sterilization alone.

Contamination of explants depends on plant species, physiological stage of the plant and environmental related factors. Among these factors, physiological stage of explants is one of

the important factors determining the success of sterilization and establishment of the aseptic culture. Results of the preliminary experiment showed that the % of uncontaminated green and alive explants were higher (33.33%) when immature axillary buds (3^{rd} to 5^{th} nodes from shoot tips) were surface sterilized and treated with 0.1% $HgCl_2$ for 5 min. The green matured axillary buds (6^{th} to 8^{th} nodes from shoot tip) totally failed to produce uncontaminated cultures. Webster *et al*. (2003) also reported that young explants produce more uncontaminated cultures than the matured explants.

In Experiment 2 (Figure 1), the immature axillary bud explants treated with 0.1% of $HgCl_2$ (T1, T2 and T3) showed a significantly lower (P<0.05) production of uncontaminated cultures than those treated with 0.2% of $HgCl_2$ (T4, T5 and T6). A higher percentage (P<0.05) of uncontaminated cultures (86.66%) were also observed when the axillary bud explants were treated with 0.2% of $HgCl_2$ for 10 and 15 min (T5 and T6) along with surface sterilization with bleach solution. However, percentage of browning was also high (P<0.05) in these two treatments (60% and 80% for T5 and T6, respectively). The highest percentage (73.33%) of green and alive cultures were achieved when the axillary bud explants were subjected to 0.2% $HgCl_2$ treatment for 5 min (T4), which significantly differed from all the other treatments as tested by chi-square (P<0.05), indicating it as the most effective sterilization treatment for axillary bud cultures [Plate I(d)].

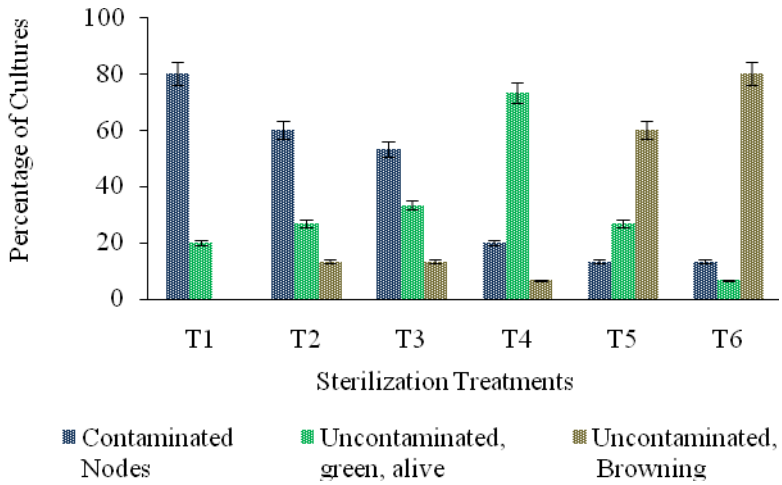

Figure 1. Effect of different sterilization treatments (T1, T2, T3: 0.1% of $HgCl_2$ for 5, 10, 15 min respectively;
T4, T5, T6: 0.2% of $HgCl_2$ for 5, 10, 15 min respectively) on establishing uncontaminated green and alive plantlets from axillary bud explants

The results of the present study showed that treatment with lower concentration of $HgCl_2$ (0.1%) had poor response, which could be due to that the plant used in the study is a woody plant. Moreover, higher concentration $HgCl_2$ (0.2%) with longer exposure (10 and 15 min) had an adverse effect on the cultured explants, which could be due to the chemical being phytotoxic to the plant tissues in such situations. According to Colgecen *et al*. (2011), the concentration and time of exposure to the treatment differ depending on the plant and different parts of plants due to their morphological characters such as softness or hardness of the tissue. Therefore, when $HgCl_2$ was used as a sterilizing agent, a balance between concentration and time of exposure should be standardized empirically to minimize explants

injury and achieve better survival (Srivastava *et al*., 2010).Thus, selection of explants from the healthy mother plants coupled with an effective sterilization method would avoid contamination of axillary bud cultures. Furthermore, standard methods should be followed in using and disposing $HgCl_2$ to avoid harmful effects to humans and contamination of soil or ground water.

CONCLUSIONS

The present study developed a protocol for establishing leaf, axillary bud and shoot tip explants for *in vitro* culture of *G. walla.* Among the treatments tested, surface sterilization with 10% (v/v) bleach solution produced the best results for the leaves and shoot tips explants. As the plant used in this study was woody in nature, surface sterilization only with bleach solution was not satisfactory for the axillary buds explants. However, the results revealed that the exposure to 0.2% of $HgCl_2$ for 5 min was an effective method of sterilization of the explants of immature axillary buds. This protocol fundamentally offers a way forward for successful *in vitro* propagation of *G. walla* by plant tissue culture techniques.

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