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Surface sterilization and micropropagation of *Ludisia discolor*

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ABSTRACT

The jewel orchid *Ludisia discolor*, found in many parts of Asia, is valued due to its appearance and medicinal benefits. Existing surface sterilization protocols were found to be not successful in initiating *in vitro* cultures of *L. discolor* in this study. Hence, a surface sterilization protocol, incorporating the use of 0.4% (w/v) mercury chloride, was developed to address this issue. This method yielded the lowest contamination (7.7%) and best growth at 22.5%. Nodal segments of *L. discolor* produced the best growth when placed in half-strength semi-solid MS medium with 0.2% (w/v) activated charcoal, 8% (w/v) Mas banana cultivar homogenate, 3% (w/v) sucrose, 3.5 g L^{-1} Gelrite, 1.0 mg L^{-1} 1-naphthaleneacetic acid, and 0.1 mg L^{-1} thidiazuron, a confirmation of the protocol promoted by Shiau et al. (2005). All *in vitro* cultures of *L. discolor* were successfully acclimatised under low light conditions using coconut coir, coconut husk and peat moss.

1. Introduction

The Orchidaceae, composed of about 20,000 orchid species, is the biggest family of flowering plants (Dressler, 1993; Moreira and Isaias, 2008). Orchids are known for their diversity and unique adaptations in their anatomy and morphology (IUCN/SSC Orchid Specialist Group, 1996). The jewel orchid *Ludisia discolor* can be found in various tropical locations within the Asia Pacific region (Hawkes, 1970; Shiau et al., 2005; Teo, 1978). *L. discolor* is valued in horticulture for its dark-coloured, golden-streaked and velvety-textured foliage (Sumathi et al., 2003; Watson, 2011). The plant is small in stature (10–15 cm in length), found in shaded, covered and damp areas of forests, and reproduce through seed production, with seedlings maturing after two to three years of growth (Hawkes, 1970; Shiau et al., 2005). Wild accessions of *L. discolor* are becoming scarcer due to whole plant harvests and lax management rules (Lim et al., 2013; The Agrobiodiversity Initiative in the Lao PDR, 2010). Trade data indicate that wild *L. discolor* are exported to China for between US$1–9/kg in China and US$40/kg in Korea (The Agrobiodiversity Initiative in the Lao PDR, 2010). Hence, there is a great need in sourcing alternative methods of boosting the populations of *L. discolor* in Malaysia.

Micropropagation has emerged as a popular tool in mitigating pressure on natural populations of the jewel orchids while allowing quick production of these orchids for economic purposes, as seen in the case of *Anoectochilus formosanus* (Shiau et al., 2002; Shih et al., 2005). Current micropropagation methods for *A. formosanus* favour the use of shoot tip or nodal explants from adult plants. Such tissues are available throughout the year and can be propagated exponentially compared to seed cultures (Ket et al., 2004). Micropropagation of L. *discolor* was conducted previously via nodal segments (Teo, 1978) and seed germination (Chou and Chang, 1999). Shiau et al. (2005) successfully propagated *L. discolor* through asymbiotic *in vitro* germination of seeds produced from artificial cross-pollination using pollinia and ovules of aged flowers.

A major concern in plant tissue culture is the occurrence of *in vitro* contamination. Plant contaminations can manifest as the natural presence of bacteria or fungi upon the surfaces and natural openings of the target tissue (Webster et al., 2003), or as plant diseases caused by pathogens such as bacteria, fungi, mycoplasm, nematodes, viroids and viruses (Lizarraga et al., 1986). Both commercial and research plant

Abbreviations: $HgCl_2$, mercury chloride; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid; NaOCl, sodium hypochlorite; PFD, photon flux density; TDZ, thidiazuron.

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tissue culture laboratories may lose from 3 to 15% of *in vitro* cultures in their possession to bacterial and fungal contaminations (Leifert et al., 1989; Yadav and Singh, 2011). In order to reduce the occurrence of such contaminations and boost plant survival, the highest priority is placed on the development and pairing of both surface sterilization recipes and efficient aseptic techniques prior to their execution on the target explant (Srivastava et al., 2010; Yadav and Singh, 2011).

The most important part of any experiment involving plant tissue culture is the seasonal source of the plants and the target surface-sterilization method; wastage of plant cultures, labour and time may result from errors performed at this stage (Srivastava et al., 2010; Yadav and Singh, 2011). Common sterilants used in the surface sterilization procedure in plants are ethanol, sodium or calcium hypochlorite, and mercury chloride. Sodium hypochlorite (NaOCl) is typically found in commercial bleach solutions diluted down to 5.25%. It has been advocated that any commercial bleach mixture intended for surface sterilization should contain a minimum of 0.5% NaOCl (Lizarraga et al., 1986). Mercury chloride ($HgCl_2$) is an effective sterilization agent used for reducing contamination in *in vitro* plant cultures and stimulating seed germination (Gopal et al., 1998; Yadav and Singh, 2011). Different plant species and parts may require different concentrations and immersion periods of $HgCl_2$ for effective decontamination (Yadav and Singh, 2011). Mercury chloride has been used as a sterilant for many species of orchids. The concentrations and timing of $HgCl_2$ used may vary according to the species, but 0.1% $HgCl_2$ is commonly applied for between 1-8 min on seed capsules, rhizome sections and nodal segments in some orchid species (Basker and Narmatha Bai, 2010; Billore et al., 2017; Gangaprasad et al., 2000; Mahendran and Narmatha Bai, 2009; Sheelavantmath et al., 2000). Apart from causing damage to the environment, $HgCl_2$ is also known to be volatile at room temperature and cause mercury poisoning, hence being extremely toxic to the person handling the chemical (Lizarraga et al., 1986).

At present, no improved tissue cultures protocols exist in the case of *L. discolor*. Hence, this study aimed at developing a surface-sterilization and micropropagation method for the jewel orchid *L. discolor*.

2. Materials and methods

2.1. Sample selection

Wild L. *discolor* plants were sourced from Sungai Enam, Temenggor, Perak, Malaysia. The orchids were found amidst the leaf litter and on rocks located in the forest understorey (Fig. 1).

Voucher specimens of *L. discolor* were deposited at the herbarium of Universiti Sains Malaysia and given the accession number L/C 006. The orchid was identified by Dr. Rahmad Zakaria of the School of Biological Sciences, Universiti Sains Malaysia and Mr. Ong Poh Teck of the Forest Research Institute Malaysia (FRIM).

2.2. Surface sterilization and micropropagation of L. discolor

The protocol was adapted from a method designed for single node shoot sections of *L. discolor* (Arditti and Ernst, 1993; Teo, 1978). Details of each treatment are described in Table 1. All solutions were prepared with autoclaved distilled water. The $HgCl_2$ solution was prepared by mixing the required amount of the chemical (AnalaR, BDH Limited, Poole, England) with 200 mL sterile distilled water and a drop of Tween® 20 (Table 1). The 40% bleach solution was prepared by mixing 80 mL of Clorox® (5.0% NaOCl) with 120 mL sterile distilled water and a drop of Tween® 20.

The plants were first scrubbed and rinsed under running tap water. Visible debris and dead plants parts were discarded. The plants were gently agitated for 20 min in a solution composed of 2 mL dishwashing soap (Care, Malaysia), 300 mL distilled water, and three drops of Tween® 20 (Honeywell Specialty Chemicals Seelze GmbH, Germany) as a surfactant. Whole stems were separated from the leaves, petioles and roots. The plants were then gently agitated three times in 300 mL sterile distilled water, followed by a brief rinse in 95% ethanol for 30 s. The plants were introduced into the laminar flow cabinet and rinsed in sterile distilled water.

For Treatments 1 and 2 (Table 1), the plants were agitated in 40% Clorox® (Wash A), after which the plants were rinsed three times in 200 mL sterile distilled water. Next, the plants were sliced into nodal segments measuring 1.5–2 cm in length with a pair of forceps and scalpel upon a piece of sterile filter paper (Whatman No. 1, 9 cm; GE Healthcare Life Sciences, England). After this step, the segments were subjected to a second Clorox® wash (Table 1, Clorox® Wash B). The segments were rinsed three times in 300 mL sterile distilled water, disinfected for the final time in 95% ethanol for 30 s, and then rinsed five times in sterile distilled water prior to their subculture.

For Treatments 3–6 (Table 1), the plants were agitated in $HgCl_2$ solution for 10 min following their introduction into the laminar flow cabinet, and rinsed five times in 200 mL sterile distilled water. The plants were then sliced into their components with a pair of sterile forceps and scalpel. The explants were then disinfected for the final time in 95% ethanol for 30 s, followed by rinsing five times in sterile distilled water prior to their subculture.

All surface-sterilized segments were dried on sterile filter paper prior to subculture in boiling tubes containing 15 mL of the following semi-solid media which were selected based on previous studies involving the micropropagation of the jewel orchids: T1 (Shiau et al., 2005), T2, T3 or T4 (Chou and Chang, 2004). The composition of each medium is listed in Table 2. All cultures were incubated at 25 ± 2 °C under 16 h photoperiod using cool white fluorescent lamps (150 μmol m^{-2} s^{-1}).

2.3. Acclimatization of L. discolor

The acclimatization of *in vitro L. discolor* plantlets was conducted as described by Ket et al. (2004) and Shiau et al. (2005). All *in vitro* plants

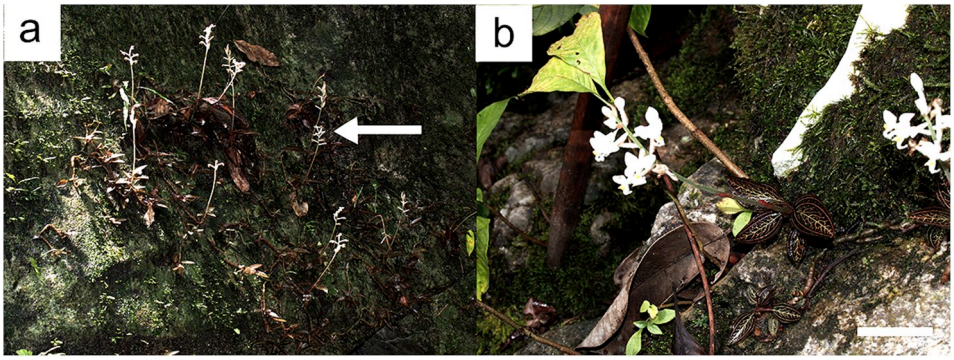

Fig. 1. (a) The growth habit of *L. discolor* (arrow). (b) The orchid colony grew on damp mossy rocks. Bar = 5 cm.

Table 1
Different surface-sterilization treatments applied for *L. discolor*.

Sterilising Agent	Treatment	Number of ethanol rinses	Wash A		Wash B	
			Conc.	Duration (mins)	Conc.	Duration (mins)
Clorox®	1	2	40% v/v	15	40% v/v	15
	2	2	40% v/v	25	40% v/v	10
$HgCl_2$	3	2	0.10% w/v	10	–	–
	4	2	0.25% w/v	10	–	–
	5	2	0.40% w/v	10	–	–
	6	2	0.50% w/v	10	–	–

Table 2
Composition of media used in this study.

Medium	Composition
T1 (Shiau et al., 2005)	Half-strength Murashige and Skoog (Murashige and Skoog, 1962) basal medium, 0.2% (w/v) activated charcoal, 8% (w/v) Mas banana cultivar homogenate, 3% (w/v) sucrose, 3.5 g L^{-1} Gelrite, 1.0 mg L^{-1} 1-naphthaleneacetic acid (NAA), and 0.1 mg L^{-1} thidiazuron (TDZ).
T2 (Chou and Chang, 2004)	Knudson C (Knudson, 1946)
T3 (Chou and Chang, 2004)	Modified Knudson C
T4 (Chou and Chang, 2004)	MS basal medium, 3 g L^{-1} tryptone, 30 g L^{-1} sucrose, 2.75 g L^{-1} Gelrite (pH 5.2)

were removed from their culture vessels and rinsed under running tap water to remove external debris and Gelrite residue (Fig. 2). The plantlets were transferred to perforated plastic pots filled with pre-soaked peat moss, coconut coir and coconut husks (1:1:1). The ends of the nodal sections, or rooting regions of the nodal segments, were buried in the moist soil substrate.

The shade house was double-layered with 65% shading nets, giving rise to a maximum of 22.4% of natural lighting at noon within the shade house. The highest and lowest photon flux density (PFD), following a 13/11 natural photoperiod, was recorded at 445 µmol m^{-2} s^{-1} and 5 µmol m^{-2} s^{-1} during the afternoon and sunrise/sunset respectively, with the average PFD calculated at 66.2 µmol m^{-2} s^{-1}. The temperature within the shade house was maintained at 27 ± 5 °C throughout the day. The plants were irrigated twice weekly with tap water.

2.4. Statistical analyses

The study employed a factorial design. All treatments consisted of a minimum of nine replicates containing a single explant. Each treatment was repeated twice, hence having a minimum of 27 replicates in total. Data collected in Section 2.2 were categorised into three groups: contamination percentage; survival percentage, i.e. the percentage of non-contaminated explants that appeared to be green and viable after the surface sterilization treatment; and growth percentage; i.e the percentage of non-contaminated explants that displayed growth and proliferation after the surface sterilization treatment. Data were recorded after one month of culture. Means were analysed with two-way ANOVA and differentiated using Tukey's HSD test, with the probability value set at 0.05. All segments that were successfully surface-sterilized were transferred to fresh media.

Fig. 2. Rooted L. *discolor* plantlet after being removed from *in vitro* conditions.

3. Results

3.1. Surface sterilization and micropropagation of L. discolor

There was a significant reduction in contamination when nodal segments of *L. discolor* were subjected to Treatment 2 (48.8%, Table 3), when compared to Treatment 1 (86.7%, Table 3). However, both treatments, involving the use of Clorox®, did not produce any post-sterilization growth.

On the other hand, treatments involving $HgCl_2$ significantly reduced contamination percentages when compared to treatments involving Clorox® (Table 3). No significant differences were observed between the contamination percentages of Treatments 3–6 (Table 3). However, Treatment 5 produced 63.1% survival and 22.5% growth, which was higher than the other treatments. Hence, all *ex vitro* nodal segments of *L. discolor* were subsequently treated with 0.4% (w/v) $HgCl_2$ for optimal results in terms of asepticity and regrowth of the nodal segments.

In terms of medium effectiveness, medium T1 produced the highest growth (19.6%, Table 4) when compared to media T2, T3 and T4 (3.6, 3.8 and 8.8% respectively; Table 4). Two-way ANOVA between surface sterilization techniques and medium types indicated that no interactions existed between the two treatments in terms of the survival and regrowth (Table 5). Hence, medium T1 was selected for subsequent subcultures of nodal segments of *L. discolor*. Subsequent subcultures of *L. discolor* in this medium yielded 100% growth at all times.

All surface-sterilized nodal segments ultimately produced single or multiple shoots that developed into plantlets with leaves and roots (Fig. 3). The cultured nodal segments either grew directly from the

Table 3
Effect of surface sterilization on the survival and growth of nodal segments of *L. discolor*.

Sterilization Agent	Treatment	Contamination (%)	Survival (%)	Growth (%)
Clorox®	1	86.7 ± 2.6 c		–
	2	48.8 ± 7.7 b		–
$HgCl_2$	3	21.1 ± 5.4 a	56.1 ± 6.6a	12.5 ± 5.9 ab
	4	24.2 ± 7.6 a	18.9 ± 4.6b	6.8 ± 2.9 b
	5	7.7 ± 3.3 a	63.1 ± 6.0a	22.5 ± 6.7 a
	6	10.5 ± 5.0 a	17.1 ± 4.3b	2.6 ± 1.8 b

Values represent M ± SE (n ≥ 27). Means with the same alphabets are not significantly different.

Table 4
Effect of medium type on the survival and growth of nodal segments of *L. discolor*.

Medium type	Survival (%)	Growth (%)
T1	42.0 ± 6.0a	19.6 ± 5.4 a
T2	37.7 ± 5.9a	3.6 ± 2.5 b
T3	30.8 ± 5.8a	3.8 ± 2.6 b
T4	36.2 ± 5.8a	8.8 ± 3.8 ab

Values represent M ± SE (n ≥ 27). Means with the same alphabets are not significantly different.

Table 5
Analysis of variance for the survival and growth of nodal segments of *L. discolor* as a function of surface sterilization technique and medium type.

Variable and source	*df*	*MS*	*F*	*p*
Survival of nodal segments of *L. discolor*				
Surface sterilization treatment	3	3.970	20.976	0.000
Medium type	3	0.113	0.597	0.617
Surface sterilization treatment*medium type	9	0.277	1.464	0.162
Error	256	0.189		
Growth of nodal segments of *L. discolor*				
Surface sterilization treatment	3	0.364	4.804	0.030
Medium type	3	0.382	5.045	0.020
Surface sterilization treatment*medium type	9	0.060	0.786	0.629
Error	206	0.076		

Values are statistically significant when $p \leq 0.05$.

original segment (Fig. 3a and b) or through an extension from a node point (Fig. 3c and d). Some nodal segments were observed to produce roots (Fig. 4). Successfully surface-sterilized nodal segments were sub-cultured into fresh medium.

3.2. Acclimatization of L. discolor

All *in vitro L. discolor* plantlets were successfully acclimatised using coconut coir, coconut husk and peat moss (Fig. 5). No plant mortality was observed during the acclimatization period. All acclimatised plants displayed growth morphology similar to that observed in *in vitro* plants. It was observed that *in vitro* rooting was not necessary for the orchid plant to survive the acclimatization process as new roots were formed in the plants after two weeks of acclimatization.

4. Discussion

4.1. Surface sterilization and micropropagation of L. discolor

Different explants arising from various species and sources of explants may require contrasting surface sterilization treatments. A balance must be achieved between the strength of the sterilant and the duration of exposure for different target species and explants. Bleach is a popular method of sterilising *ex vitro* plants, and has been successfully

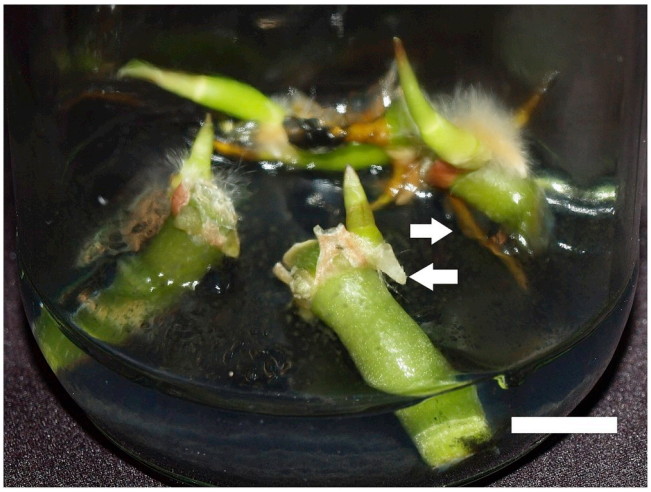

Fig. 4. (a) Shoot and root (arrows) formation in *in vitro L. discolor*. Bar = 1 cm.

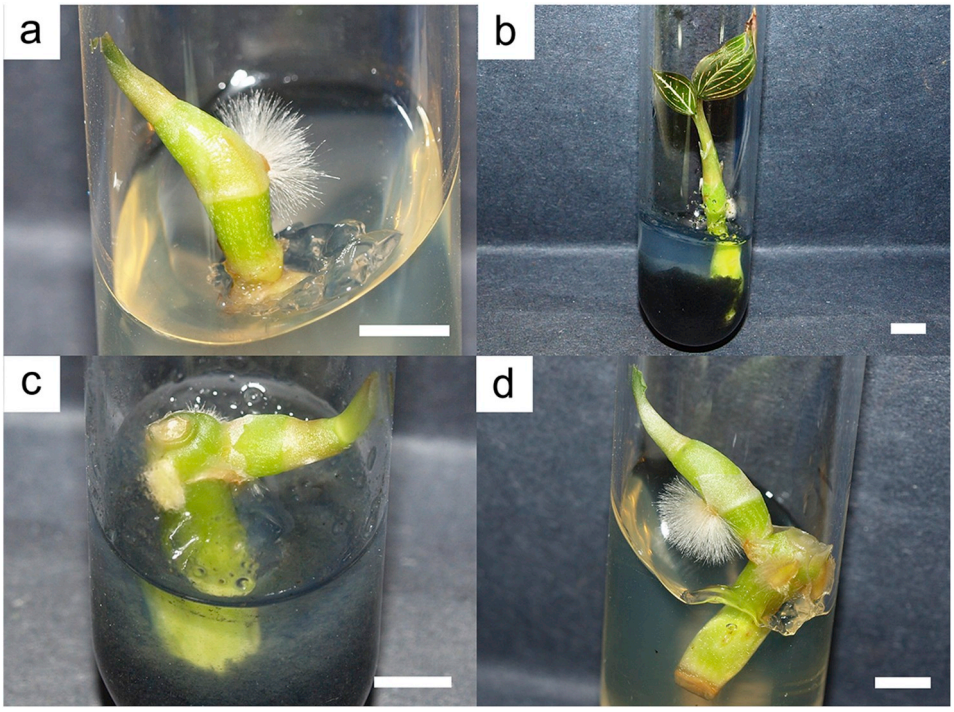

Fig. 3. The growth of surface-sterilized *L. discolor in vitro*. The cultured nodal segments produced growth directly from the original segment (a, b) or through an extension from a node point (c, d). Bar = 0.5 cm.

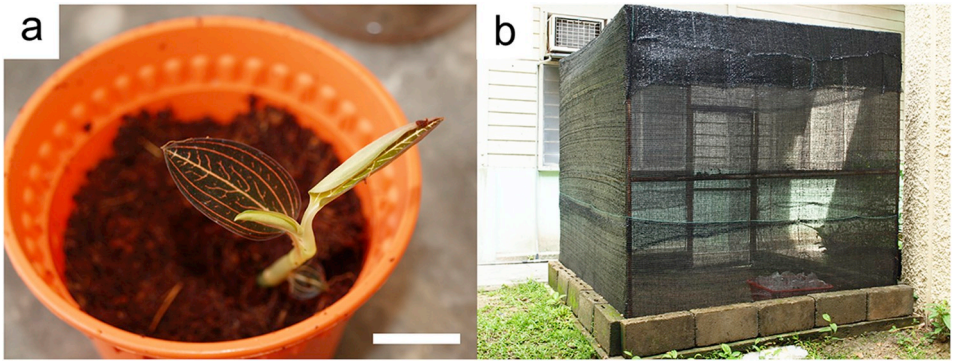

Fig. 5. (a) *In vitro* L. *discolor* acclimatised in coconut coir, coconut husk and peat moss. (b) The shade house (4 m^2) housing the orchids was designed to mimic the shady condition of the forest floor. Bar = 1 cm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

used to introduce many plants to *in vitro* conditions. Many species were also successfully treated with $HgCl_2$ at concentrations ranging from 0.05-0.1% (w/v), with the duration of the treatment adapted for each plant (Narayanaswamy, 1999; Yadav and Singh, 2011).

Ex vitro L. discolor plants obtained in this study were initially surface-sterilized using a method designed for single node shoot sections of the orchid (Arditti and Ernst, 1993; Teo, 1978). However, the treatment was found to be inadequate. All surface-sterilized explants were contaminated within five days of the treatment. The treatment was then modified to incorporate the use of various concentrations (10–70%) of Clorox®, but this step did not produce any post-sterilization growth despite the complete elimination of contamination in some treatments (data not shown). The use of bleach was hence eliminated from this study. It was also found that agitation alone was insufficient in removing debris lodged within the contours of the plant segments, given the terrestrial nature of the plant. Hence, a scrubbing step was introduced in this study.

In this study, *L. discolor* was successfully surface-sterilized using 0.4% $HgCl_2$. Lower concentrations of $HgCl_2$ produced high contamination percentages, while the use of 0.5% $HgCl_2$ produced lower plant growth percentages. Growth obtained from the use of $HgCl_2$ could not be replicated when bleach was used as the sterilant. Mercury chloride is an accepted method of surface-sterilising many species of wild species and hybrids of orchids, especially in the case of endangered terrestrial orchids. A possible reason is that minimal numbers of samples are usually collected from the wild, hence requiring surface sterilization methods that are effective in recovering as many aseptic plants as possible with minimal damages to the explants, as was the main concern in the execution of this study. For the endangered *Ipsea malabarica*, treatment with $HgCl_2$ was deemed an integral step due to the rare nature of the plant (Martin, 2003). Hence, an 8–10 min immersion of the rhizomes in 0.1% $HgCl_2$ was adopted in the micropropagation of the orchid (Martin, 2003). In the case of *A. sikkimensis*, shoot cuttings located at the apical regions were washed under running tap water for 15 min and soaked in 1% solution of a commercial detergent, followed by passage through 70% ethanol and diluted Steriliq with 2% hypochlorite, and finally 0.1% $HgCl_2$ prior to culture on Woody Plant Medium (WPM; Gangaprasad et al., 2000). Freshly-harvested immature seed capsules of *Satyrium nepalense* were immersed in 0.1% $HgCl_2$ for 2 min, prior to flaming and excision (Mahendran and Narmatha Bai, 2009). Capsules from hand-pollinated *Geodorum densiflorum* were sterilized in 0.1% $HgCl_2$ for 8 min prior to culture on medium (Sheelavantmath et al., 2000). Parts of epiphytic orchids that were subjected to similar treatments included rhizomes of *Dendrobium* sonia (Billore et al., 2017) and undehisced capsules of *Eria bambusifolia* (Basker and Narmatha Bai, 2010), both of which required a 3-min immersion in 0.1% $HgCl_2$. Nodal segments of *Dendrobium macrostachyum* were surface-sterilized for 20 min in 2% NaOCl, followed a few steps later by a 1-min immersion in 0.5% $HgCl_2$ prior to culture on MS medium (Pyati et al., 2002), comparable to the method applied in this study.

Bleach was found to be an ineffective sterilant in this study. Bleach was found to be adequate for removing contaminants when applied at 1.4% for 1 min on leaves of *Aquilaria crasna* and *A. sinensis* (Nurul Hazwani et al., 2012; Okudera and Ito, 2009), and at 50% for 20 min on nodal segments and shoot tips of *A. hirta* (Hassan et al., 2011). However, bleach was not effective in the case of *A. malaccensis*. The authors postulated that plants harvested from unregulated environments and natural habitats may possess traits that differ from plants grown under controlled conditions, hence the requirement for a stronger sterilising agent (Nurul Hazwani et al., 2012). Since the L. *discolor* plantlets were sourced directly from the soil, the flora contained within the plants could be hardier in nature, hence requiring the use of $HgCl_2$ for effective surface sterilization and explant survival.

In this study, surface-sterilized nodal segments of *L. discolor* grew best when placed in medium with 0.2% activated charcoal, 8% Mas banana cultivar homogenate, 1.0 mg L^{-1} NAA, and 0.1 mg L^{-1} TDZ. Subsequent subcultures of *L. discolor* in this medium yielded 100% growth at all times, a result that could not be replicated in the case of full-strength MS medium (data not shown). Banana homogenate and activated charcoal are common components in the asymbiotic germination of seeds of various orchid plants (Ernst, 1974; Shiau et al., 2002; Yam and Weatherhead, 1990). Li et al. (2015) found that more than 90% of *L. discolor* plantlets survived and thrived when subcultured in Hyponex medium (3 g L^{-1}) with 2.5% sucrose, 20 g L^{-1} mannitol, 1.0 mg L^{-1} NAA, 10% banana homogenate, 1.0 g L^{-1} activated charcoal and 1% carrageenan for 18 months. Tsui (1992) reported that dehisced seeds of *L. discolor* displayed high levels of germination when sown on medium composed of half-strength MS components and coconut milk (MS CM).

The addition of activated charcoal to the tissue culture medium in this study was found to be important in promoting the growth of *L. discolor* plantlets. Activated charcoal is postulated to assist in plant growth *in vitro* by absorbing inhibitory substances in the medium, such as polyphenols (Fridborg and Eriksson, 1975; Ket et al., 2004), or by reducing luminous flux hitting the base of the cultured explant. This allows the accumulation of cofactors or auxins integral in plant growth (Druart and Wulf, 1990; Ket et al., 2004). Activated charcoal also inhibits ethylene production (Ernst, 1975) and promotes aeration of the medium (Ernst, 1974), hence assisting in the growth and proliferation of the targeted plants (Ket et al., 2004).

4.2. Acclimatization of L. discolor

Shade conditions and high humidity levels were necessary in the survival of *L. discolor* plants when placed outdoors. No morphological abnormalities and phenotypic variations were detected among the

hardened plants. The orchids were observed to grow well when acclimatised in substrate containing peat moss, coconut coir and coconut peat (1:1:1). Rooting could be observed in *L. discolor* within two weeks of acclimatization. High survival was obtained for *A. formosanus* when the seedlings were acclimatised for two weeks in peat moss: vermiculite potting mixture placed in closed plastic containers under high humidity (Shiau et al., 2002). Jackson et al. (2003) attempted to induce a quicker method of inducing rooting in *ex vitro L. discolor* by placing rootless and three-leaved 6 cm terminal cuttings of the orchid plant in substrates composed of unmilled sphagnum moss or a commercial potting mix called BM1 (Berger Peat Moss, St. Modeste, Quebec, Canada). The team found that no significant differences were detected in root number when sphagnum was used. However, the sphagnum moss possessed a higher level of porosity and was able to retain the moisture in the substrate, under a humidity level set at 70% using a fine mist spray. Roots were able to be induced from the cuttings after four weeks (Jackson et al., 2003).

5. Conclusion

In this study, nodal segments of *L. discolor* were successfully surface-sterilized by incorporating 0.4% (w/v) $HgCl_2$ in the surface sterilization protocol, yielding the lowest contamination (7.7%) and the highest growth at 22.5%. The best growth was achieved when nodal segments of *L. discolor* were placed in semi-solid half-strength MS medium with 0.2% (w/v) activated charcoal, 8% (w/v) Mas banana cultivar homogenate, 3% (w/v) sucrose, 3.5 g L^{-1} Gelrite, 1.0 mg L^{-1} NAA, and 0.1 mg L^{-1} TDZ. The orchid plants were successfully acclimatised in a substrate composed of coconut coir, coconut husk and peat moss (1:1:1) under low light conditions.

Authors contribution statement

Rahmad Zakaria, Vikneswaran Murugaiyah and Sreeramanan Subramaniam provided the resources, ideas and supervision for the study as well as writing. Ranjetta Poobathy designed the methodology for the study, carried out the experiments and wrote the article.

Declaration of competing interest

No conflicts of interest were declared by the authors of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101380.

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