IN VITRO SHOOT MULTIPLICATION AND ROOTING OF SHOOT TIP EXPLANTS OF *DIMORPHORCHIS LOWII*: AN ENDEMIC ORCHID OF BORNEO

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ABSTRACT

Five complex additives were evaluated for multiple shoot formation and rooting of *Dimorphorchis lowii* namely banana homogenate, coconut water, peptone, tomato juice and yeast extract. The basal medium used was Knudson C. Shoot tips grown from protocorms of *D. lowii* seeds were used as explants. The best performances for shoot multiplication after 6 months were seen in cultures with 150 ml L^{-1} coconut water when explants were placed horizontally with the maximum percentage of explant forming shoot (100 ± 0.00), the highest number of shoots per explant (13.83 ± 6.12), and the maximum length of shoot (mm) (38.86 ± 11.90). However, the maximum percentage of survival was best seen in explants that were positioned vertically (97.50 ± 7.91). The maximum percentage of shoots forming root (100.00 ± 0.00) was best seen in 2g L^{-1} peptone after 4 months of culture. Coconut water at 150 ml L^{-1} gave the highest number of roots per shoot (3.00 ± 1.44) and the highest root length (15.18 ± 7.69) was observed in 2 g L^{-1} yeast extract. Rooted plantlets were able to grow into normal plantlets in ex vitro condition after four weeks of acclimatization. This is the first report for in vitro shoot multiplication and rooting of *D. lowii*. The protocol developed could be used as an alternative method for large-scale production of this species.

Keywords: Orchidaceae, complex additives, explant position, *in vitro*

INTRODUCTION

Dimorphorchis lowii is an orchid species that is gaining much interest over the years because of its unique dimorphic flowers, which means it has two different flowers on one spike. It has the potential to be marketed as a high value potted or basket flower because of its unique, beautiful and fragrant flowers. This orchid is endemic to Borneo and is also one of the rarest epiphytic orchids found principally in the altitude of 600 to 1800m on Mount kinabalu, Mount Tombuyukon and Mount Trus Madi in Sabah (Crib & Bell 2008). Because of the increasing popularity, very rare (included in CITES-Appendix II) thus difficult to locate, it is important to establish a technique for multiplication to produce a large number of plantlets and subsequently reintroducing this plant back to its natural habitat. *Dimorphorchis* orchids, just like other monopodial orchids are vegetatively propagated through the top cutting of the mother plant. However, propagation through this method is damaging to the stock plant and apart from that only one new growth can be produced. *In vitro* asymbiotic seed germination offers an alternative to this vegetative propagation. But this technique also has the disadvantage as germinated seedlings are always not true-to-type (Arditti 2008). Therefore *in vitro* propagation *via* multiple shoot formation provides means for the production of uniform seedlings of *Dimorphorchis* orchids. George et al. (2008) reported that the production of plants from shoots has proven to be the most generally applicable and reliable method of true-to-type *in vitro* propagation. Currently, only one study on *in vitro* seed germination of *D. lowii* has been documented. Hence, the present study was initiated to induce formation of multiple shoots in *D. lowii*. Despite being an important technique for mass propagation, *in vitro* technique sometimes results in somaclonal variation in the plantlets therefore it is necessary to maintain the genetic integrity of the plant especially when working with species for the purpose of conservation (Sarasan et al. 2006; Pence 2010). Benson et al. (2000) and Karp (1995) reported that the addition of plant growth regulators in the culture media could induce genetic variation. Therefore the *in vitro* technique developed for conservation purposes should minimize the use of plant growth regulator by substituting it with complex additives such

as banana homogenate, coconut water, peptone, tomato juice and yeast extract. These are the most common additives used and they were reported to have a significant contribution to the micropropagation of many orchid species (Arditti 1993; George et al. 2008). Type of carbon source also plays an important role in the *in vitro* orchid propagation. It acts as an energy source and it can affect the growth of *in vitro* plant. Thus, in this study the effect of banana homogenate, coconut water, peptone, tomato juice and yeast extract and type of carbon source on multiple shoot formation and rooting of *D. lowii* were evaluated.

MATERIALS AND METHODS

Protocorms developed from seed cultures were transferred into ½ strength MS medium containing 20 g L^{-1} sucrose and 2 g L^{-1} yeast extract. This served as the staging medium for explant source. After 5 to 6 months, multiple shoots from these cultures were cut and separated individually and used as explants.

For the effect of complex additive on shoot multiplication experiments, shoot tip explants were cultured on KC medium (Knudson 1946) in two different orientations, the horizontal placement and vertical placement. Eight explants per flask were used with four explants cultured horizontally on one side and the other four were cultured vertically on the other side of the 100 ml wide-mouth erlenmeyer flask. The medium was supplemented with 20 g L^{-1} sucrose with different concentrations of complex additives. There were five complex additives used, which included ripe tomatoes (100, 150 and 200 ml L^{-1}), banana (var. berangan) (25, 75 and 125 ml L^{-1}), coconut water (100, 150 and 200 ml L^{-1}), peptone (1, 2 and 5 g L^{-1}) and yeast extract (2 g L^{-1}). KC medium free of complex additive served as control. Ripe tomato and banana were cut into small sections and made into juice (tomato) and homogenate (banana) using a blender (Panasonic, MX 798S) prior to measuring the volume using volumetric cylinder. Coconut water was extracted from young coconut and filtered with cheese cloth to remove unwanted debris from the coconut. Peptone and yeast extract were in powder form (Bacto™ Beckon, France) and were weighed accordingly. All complex additives were added in the medium prior to autoclaving.

For the effect of type of carbon source and its concentration on shoot multiplication experiment, explants were cultured horizontally on KC medium supplemented with 150 ml L^{-1} coconut water. There were three types of carbon sources used (fructose, glucose and sucrose) at concentration of 10, 20 and 40 g L^{-1}. KC medium without carbon source served as control. Data collection and sub-culturing on both experiments were done at four-week intervals for a duration of 6 months. In rooting experiment, individual shoots (3 to 6 mm in length) each with two to three expanded leaves were detached from the shoot clumps that were previously cultured in the staging medium and used as explants. For the effect of type of complex additives on root formation experiment, five complex additives were investigated. Ripe tomatoes (100, 150 and 200 ml L^{-1}), banana (var. berangan) (25, 75 and 125 ml L^{-1}), coconut water (100, 150 and 200 ml L^{-1}), peptone (1, 2 and 5 g L^{-1}) and yeast extract (2 g L^{-1}). The preparation of complex additives was detailed in the shoot multiplication experiment. Explants were cultured on KC medium supplemented with 20 g L^{-1} sucrose. For the effect of type of carbon source and its concentration on root formation, three types of carbon sources (fructose, glucose and sucrose) at concentration of 10, 20, and 40 g L^{-1} were used. Explants were cultured on KC medium supplemented with 150 ml L^{-1} coconut water. Observations were recorded every four weeks for a duration of 4 months. Agar powder was added to the culture medium at a concentration of 8 g L^{-1} for the purpose of solidifying the medium. The pH of the medium was adjusted to 5.3 ± 0.02 prior to autoclaving for 20 minutes at 15 psi, 121 °C. Cultures were maintained at 25 ± 2°C under 24 hd^{-1} photoperiod with a PPF of 20 – 50 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Philips, Malaysia). All the experiments were carried out in a complete randomized design (CRD) with 10 replicates per treatment, with 4 explants per replicate.

In shoot multiplication experiment, percentage of survival, percentage of explant forming shoots, mean number of shoots per explant and mean length of shoot (mm) were recorded. In the rooting experiment, percentage of survival, percentage of shoot forming roots, mean number of roots per shoot, mean length

of root (mm) were recorded. The results were expressed as mean ± SD (Standard deviation). Data were subjected to analysis of variance (ANOVA) and means were compared by the Duncan's multiple range test at $P < 0.05$ using the SPSS ver. 20 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Effect of complex additives on multiple shoot formation

Among the five types of complex additives tested, 150 ml L^{-1} coconut water was found to be the most effective in promoting shoot multiplication from shoot tip explants when explants were cultured horizontally (Fig.1). Protocorm-like bodies (PLBs) developed at the base surface of shoot tip explants after a month in culture and these grew into clumps of shoot buds. Within 1 to 2 months, these shoot buds grew and developed into multiple shoots. The newly developed shoots continued to form other new shoot buds when sub cultured every month for a duration of 6 months. In cultures containing 2 g L^{-1} yeast extract, 25 ml L^{-1} banana homogenate and 100 ml L^{-1} tomato juice, some of these green protuberances remained undeveloped. In 150 ml L^{-1} coconut water, number of shoots increased by the fourth month and at the final subculture (month 6), it increased significantly (13.82 shoots) (Table 1). Coconut water was able to induce the growth of new shoots (and some roots) as compared to medium without this complex additive (control). Medium with 150 ml L^{-1} coconut water with the explants positioned horizontally also produced the highest percentage of shoot formation (100%), and the maximum length of shoot (38.86 mm). However, the maximum percentage of survival was seen in explants that were positioned vertically (97.5%) (Table 1).

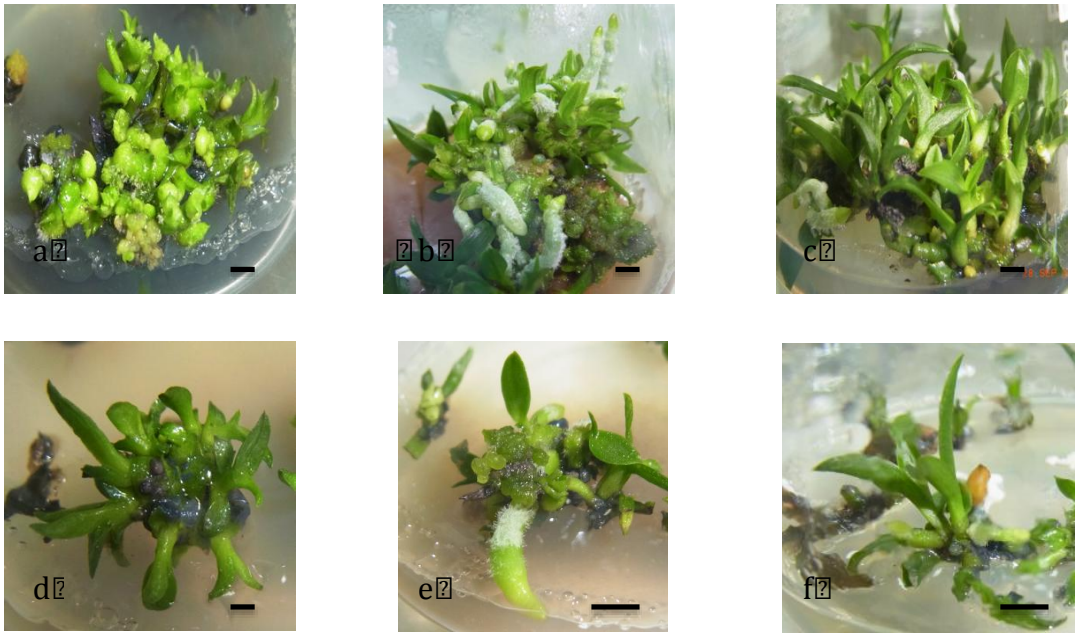

Fig. 1. The effect of complex additives on shoot development of *Dimorphorchis lowii* shoot tip culture when placed horizontally in Knudson C medium after 6 months. (a) 2 g/L Yeast extract; (b) 25 ml/L Banana homogenate; (c) 150 ml/L Coconut water; (d) 2 g/L Peptone; (e) 100 ml/L Tomato juice, and (f) Control. (Bar scale 5 mm)

Table 1. The effect of complex additives on shoot multiplication of *Dimorphorchis lowii* when placed vertically and horizontally on KC medium after 6 months of culture

Complex Additives (ml L^{-1} and g L^{-1})		Percentage of survival (%)	Percentage of explant forming shoots (%)	Mean number of shoots per explant	Mean length of shoot (mm)
Vertical explant placement on media					
Control		67.50^{abcd}	2.99^{i}	1.03^{ef}	3.96^{efg}
Coconut water (CW)	100 ml L^{-1}	92.50^{ab}	60.00^{bcdef}	4.65^{def}	15.52^{de}
	150	97.50^{a}	73.33^{abc}	8.51^{bc}	26.30^{bc}
	200	80.00^{abc}	47.50^{bcdefgh}	7.70^{cd}	20.88^{cd}
Tomato juice (TJ)	100	70.00^{abc}	51.67^{bcdef}	3.36^{ef}	6.68^{efg}
	150	23.80^{fgh}	28.33^{defghi}	0.77^{ef}	1.46^{fg}
	200	59.38^{bcdef}	42.71^{bcdefgh}	2.13^{ef}	5.78^{efg}
Banana homogenate (BH)	25	75.00^{abc}	34.26^{cdefghi}	2.16^{ef}	6.63^{efg}
	75	65.00^{abcde}	13.33^{ghi}	1.31^{ef}	4.58^{efg}
	125	77.50^{abc}	22.50^{efghi}	1.98^{ef}	6.88^{efg}
Peptone (P)	1 g L^{-1}	78.30^{abc}	40.63^{bcdefghi}	2.47^{ef}	7.25^{efg}
	2	84.38^{abc}	39.58^{bcdefghi}	2.39^{ef}	7.70^{efg}
	5	60.00^{abc}	75.00^{ab}	2.81^{ef}	7.69^{efg}
Yeast extracts (YES)	2	52.78^{cdefg}	47.22^{bcdefgh}	2.44^{ef}	5.64^{efg}
Horizontal explant placement on media					
Control		50.00^{cdefg}	37.50^{bcdefghi}	2.38^{ef}	6.60^{efg}
Coconut water	100 ml L^{-1}	70.00^{abc}	62.50^{bcdefg}	4.37^{def}	12.14^{efg}
	150	75.00^{abc}	100.00^{a}	13.83^{a}	38.86^{a}
	200	67.50^{abcd}	72.50^{abc}	11.93^{ab}	33.60^{ab}
Tomato juice	100	25.00^{gh}	48.15^{bcdefgh}	2.78^{ef}	4.74^{efg}
	150	2.00^{h}	10.00^{hi}	0.40^{f}	0.70^{g}
	200	21.88^{gh}	4.17^{i}	0.67^{ef}	1.25^{fg}、
Banana homogenate	25	57.50^{bcde}	62.50^{bcdef}	4.78^{de}	11.44^{ef}
	75	62.50^{bcde}	27.50^{efghi}	3.23^{ef}	8.28^{efg}
	125	35.00^{defgh}	38.33^{bcdefghi}	2.90^{ef}	6.47^{efg}
Peptone	1 g L^{-1}	50.00^{cdefg}	56.25^{bcdef}	2.44^{ef}	7.44^{efg}
	2	68.75^{abc}	58.33^{bcde}	3.09^{ef}	8.41^{efg}
	5	52.50^{abcde}	64.58^{bcd}	4.67^{def}	9.83^{efg}
Yeast extracts	2	33.33^{efgh}	18.52^{fghi}	1.04^{ef}	2.00^{fg}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's Multiple Range Test. Treatments were conducted in 10 replicates.

From this study, it showed that coconut water has a significant effect on shoot multiplication of *D. lowii*. Results showed that 150 ml L^{-1} coconut water was the best additive for shoot multiplication. With the adding of this complex additive, it gives the highest percentage of survival, the highest percentage of shoot formation, the highest number of shoot produced, the highest shoot length, the highest number of leaf produced and the highest length of the longest leaf. Coconut water is a complex additive, which contains many nutritional and/or hormonal substances (Chugh et al. 2009). It has been successfully used alone or in combination with plant growth regulator in the propagation of many orchids. Pyati (2002) reported that the addition of coconut water to Murashige and Skoog medium, induced 78% of nodal explants of *Dendrobium macrostachyum* to produce axillary buds, which eventually grew into axillary shoots after a month in culture. The same result was also found in half strength MS medium supplemented with coconut water in combination with 1.0 mg L^{-1} BA and 2.0 mg L^{-1} NAA to be suitable for shoot bud formation and proliferation of *Paphiopedilum hangianum* (Zeng et al. 2013). The success of shoot regeneration from protocorms using coconut water as an additive was also reported by Roslina (2010). Their research finding showed that coconut water could promote the induction of shoot of *Vanda dearie* after 20 days of culture. The position of the explants also played an important role in ensuring higher number of multiplication. In this study, by placing the explants horizontally, it produced an optimum result (100% in shoot formation, 13.83 shoots and 38.86 mm in shoot length) as compared to vertical position. George et al. (2008) reported that generally, when explants were cultured horizontally, a greater surface of the explant was in contact with the medium and the availability of nutrient and growth regulators to those parts of the explant increased.

Effect of complex additives on root formation

Out of the various complex additives tested for rooting, the most effective to promote root production (3.00 roots) after 4 months in culture was seen in cultures added with 150 ml L^{-1} coconut water (Table 2; Fig. 2). In cultures added with 2 g L^{-1} peptone, the best performance in terms of the percentage of rooting (100%) and percentage of survival (95%) were observed after 4 months in culture. The highest root length (15.18 mm) was observed in cultures added with 2 g L^{-1} yeast extract. In this experiment, 150 ml L^{-1} coconut water, 2 g L^{-1} peptone and 2 g L^{-1} yeast extract were equally important in ensuring an average of 95% survival, a 100% of rooting was observed in 2 g L^{-1} peptone. Therefore, based on the overall results, 150 ml L^{-1} coconut water and 2 g L^{-1} peptone were the best two complex additives to promote rooting. From this study, it was found that coconut water exhibited a positive effect in promoting both shoot and root formation. Coconut water is known to be effective for enhancing the development of cultured cells and tissues because it possesses a wide spectrum of growth factors, and has been successfully used in orchid production (Baque et al. 2011). Baque reported that coconut water proved as the best complex additive for the enhancement of fresh and dry biomass, number of roots, leaf area as well as development of healthy plantlets of *Calanthe* hybrids. Vijayakumar et al. (2012) also reported that the addition of 150 ml L^{-1} coconut water in the MS medium induced shoot formation and root initiation of *Dendrobium aggregatum*. The addition of organic nitrogen compounds such as peptone into basal medium containing inorganic salts is also widely used in *in vitro* propagation of orchids (Arditti & Ernst 1993). This organic nitrogen compound has been reported as supplements of polypeptides or free amino acids to enhance growth of seedlings and also seed germination of *Paphiopedilum rothschildianum* (Chyuam et al. 2010).

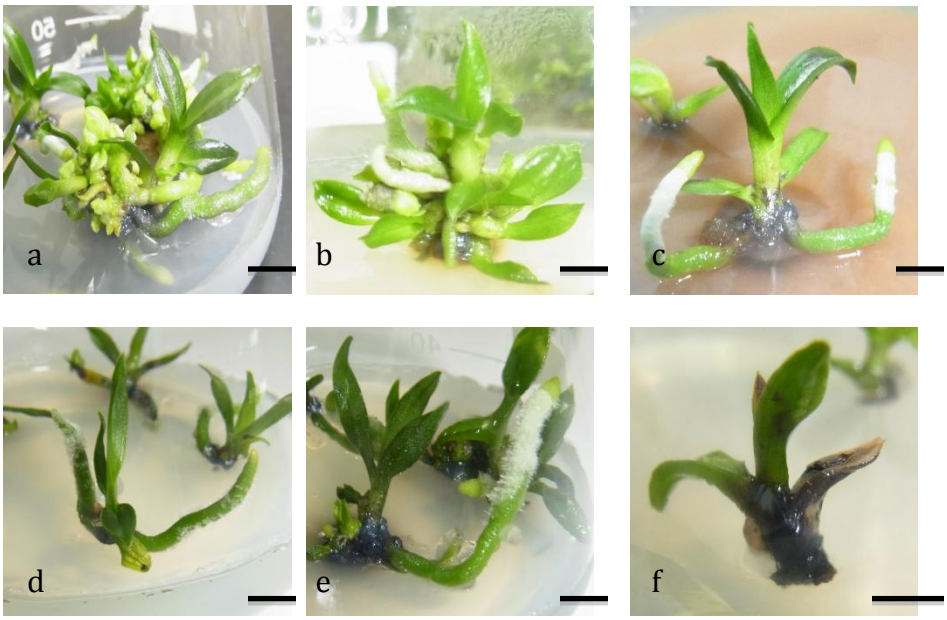

Figure 2. The effect of complex additives on root formation of shoot tip culture of *Dimorphorchis lowii* cultured in KC medium after 4 months in culture. (a) 150 ml L^{-1} coconut water, (b) 150 ml L^{-1} tomato juice, (c) 125 ml L^{-1} banana homogenate, (d) 2 g L^{-1} peptone, (e) 2 g L^{-1} yeast extract, (f) Control (Bar scale 5mm).

Table 2. The effect of complex additives on root formation of *Dimorphorchis lowii* cultured on KC medium after 4 months of cultures

Complex additives (ml/L and g/L)		Percentage of survival (%)	Percentage of shoots forming root (%)	Mean number of roots per shoot	Mean length of roots (mm)
Control		82.50^{a}	0.00^{h}	0.00^{e}	0.00^{h}
Coconut Water	100 ml/L	95.00^{a}	72.50^{abc}	2.21^{ab}	8.90^{bc}
	150	87.50^{a}	76.67^{abc}	3.00^{a}	7.54^{bcd}
	200	77.50^{a}	10.00^{fg}	0.70^{cd}	3.25^{efg}
Tomato juice	100	87.50^{a}	49.17^{cde}	1.53^{bc}	3.65^{defg}
	150	95.00^{a}	66.67^{bc}	1.33^{bc}	4.62^{def}
	200	95.00^{a}	37.50^{def}	0.77^{cd}	3.98^{def}
Banana homogenate	25	95.00^{a}	20.83^{fg}	0.85^{cd}	2.13^{fg}
	75	75.00^{a}	31.06^{ef}	1.08^{cd}	1.48^{fg}
	125	85.00^{a}	59.16^{bcd}	1.63^{cd}	4.60^{def}
Peptone	1 g/L	82.50^{a}	74.17^{abc}	1.48^{bc}	6.93^{cde}
	2	95.00^{a}	100.00^{a}	1.43^{bc}	10.99^{b}
	5	72.50^{a}	30.83^{ef}	0.63^{cd}	1.70^{fg}
Yeast extract	2	88.89^{a}	82.41^{ab}	1.55^{bc}	15.18^{a}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's Multiple Range Test. Treatments were conducted in 10 replicates.

Effect of carbon source on multiple shoot formation

Among the three types of sugar (Table 3, Fig. 3a and 3b), sucrose at 10 g L-1 produced the highest percentage of survival and length of the longest leaf with 85.71% and 9.54 mm respectively. Meanwhile, 20 g L^{-1} glucose exhibited the highest mean number of shoots (4.65), mean length of shoots (16.79 mm) and mean number of leaves (10.75). The highest shoot formation (82.29%) was obtained in 20 g L^{-1} sucrose. Inhibitory effects on shoot multiplication and proliferation were observed in media containing 4 g L^{-1} fructose where no shoots were produced. An increase in carbon source concentration from 10 g L^{-1} to 20 g L^{-1} caused an increase in the amounts of total carbohydrates and starch therefore lots of energy to help promote shoot multiplication and proliferation. However, when carbon source was increased to 40 g L^{-1}, it reduced the number of shoot production. This is in agreement with the study done by Wotavová-Novotná et al. (2007), where they observed that sucrose concentrations above 20 g L^{-1} inhibited shoot growth of *Dactylorhiza majalis* and *D. incarnate*. Conversely, in a study done by Ferreira et al. (2011) on *Dendrobium* orchid, an increase in sucrose from 40 to 60 g L^{-1} was advantageous in increasing the number of shoots produced per explant as it contained higher amount of carbohydrate and starch. So generally, different concentration of carbon sources perform differently depending on the species of plants (Arditti and Earnst 2008; Nambiar et al. 2012; Mamun et al. 2014). In this study, sucrose and glucose at 20 g L^{-1} performed better than fructose. There was no significant different between 20 g L^{-1} sucrose and 20 g L^{-1} glucose in terms of the parameters observed therefore sucrose would be more favorable as it is widely used in *in vitro* propagation of orchid. Additionally, glucose is more expensive than sucrose and the use of glucose will only be preferred for micropropagation where it produces clear advantageous result (Thorpe et al. 2008). A study done by Mamun et al. (2014) stated that 20 g L^{-1} sucrose with the combination of potato extract gave the best response in the production of shoots in *Dendrobium* orchid and in medium with no sucrose, explants failed to produce shoot formation. Sucrose is generally more favorable not just in orchid propagation but also for other plants. According to Thorpe et al. (2008) in their study on carrot *in vitro,* sucrose was found to be the best source of carbon followed by glucose, maltose and raffinose.

Table 3. The effect of carbon source on shoot development of *Dimorphorchis lowii* cultured horizontally on KC medium supplemented with 150 ml L^{-1} coconut water after 6 months of culture

Carbon source (g L^{-1})		Percentage of survival (%)	Percentage of explant forming shoots (%)	Mean number of shoots per explant	Mean length of shoots (mm)
Control		37.50^{bc}	25.00^{bcde}	2.45^{abc}	4.65^{cd}
	10	58.33^{ab}	38.89^{bcd}	2.90^{abc}	10.93^{abc}
Fructose	20	50.00^{b}	51.85^{abc}	1.97^{bc}	7.15^{bcd}
	40	12.50^{c}	0.00^{e}	0.00^{c}	0.00^{d}
	10	68.75^{ab}	55.21^{abc}	1.75^{bc}	7.31^{bcd}
Glucose	20	59.38^{ab}	51.04^{abc}	4.65^{a}	16.79^{a}
	40	10.00^{c}	22.22^{cde}	0.65^{c}	1.30^{d}
	10	85.71^{a}	57.14^{ab}	1.86^{bc}	7.64^{bcd}
Sucrose	20	84.38^{a}	82.29^{a}	4.03^{ab}	14.48^{ab}
	40	37.50^{bc}	16.67^{de}	1.08^{c}	4.08^{cd}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test. Treatments were conducted in 10 replicates.

Effect of carbon source on root formation

Among the three carbon sources used for rooting study (Table 4), 10 g L^{-1} sucrose gave a 100% survival with the highest percentage (85%) of explant forming root with the longest shoot elongation of 5.03 mm. However the highest mean number of root (1.90) produced at 20 g L^{-1} glucose and the longest root was obtained in 10 g L^{-1} glucose although there was no significant difference between 10 g L^{-1} glucose with 10 g L^{-1} sucrose. Overall, 10 g L^{-1} sucrose (Fig. 3c and 3d) was found to be the best carbon source for rooting of *D. lowii*, followed by 20 g L^{-1} glucose. This is similar with the result obtained by Jitsopakul et al.,(2013) where10 g L^{-1} sucrose produced 100% root formation as well as the highest root number and plant height in shoot tips of *Vanda corulea*. In plant cell and tissue culture, sucrose is considered as an important carbon and energy source (Baque et al. 2011; Kumaraswamy et al. 2010) and without any carbon source supplemented in the medium shoots were not able to form roots as seen in control (Table 4). Ferreira et al. (2011) reported that the presence of sucrose in the culture medium contributed to the process of cell division in the root apical meristem, which caused an increase in root length. Apart from promoting root formations 10 g L^{-1} sucrose also could promote protocorm-like bodies growth in some orchids. Advina et al. (2014) and Jheng et al. (2006) reported that 10 g L^{-1} sucrose produced the highest increase of PLBs growth rate percentage in *Dendrobium* and *Oncidium* orchids respectively. Other reports also stated that sucrose concentration could affect the successful growth of plantlet during acclimatization stage. Faria et al. (2004) mentioned in their study that *V. cristata* plantlets obtained from protocorms cultivated in culture medium with a reduced sucrose concentration from 5 to 20 g L^{-1} showed a success rate of 80% when transferred to greenhouse conditions.

Table 4. The effect of carbon sources on root development of *Dimorphorchis lowii* on KC medium supplemented with 150 ml L^{-1} coconut water after 4 months of culture

Carbon source (g L^{-1})		Percentage of survival (%)	Percentage of explant forming root (%)	Mean number of roots per explant	Mean length of roots (mm)	Mean shoot elongation (mm)
Control		82.50^{ab}	0.00^{d}	0.00^{e}	0.00^{d}	3.03^{bcd}
Fructose	10	97.50^{a}	32.50^{bc}	0.51^{de}	2.91^{bc}	4.15^{ab}
	20	77.50^{abc}	69.17^{a}	1.20^{bc}	4.94^{ab}	3.59^{abc}
	40	47.50^{cd}	55.00^{ab}	0.94^{cd}	4.34^{ab}	2.92^{bcd}
Glucose	10	87.50^{a}	79.17^{a}	1.23^{bc}	6.98^{a}	4.34^{ab}
	20	77.50^{abc}	67.50^{a}	1.90^{a}	4.98^{ab}	2.98^{bcd}
	40	52.50^{bcd}	12.50^{cd}	0.28^{e}	0.68^{cd}	1.86^{d}
Sucrose	10	100.00^{a}	85.00^{a}	1.23^{bc}	6.31^{a}	5.03^{a}
	20	85.00^{a}	75.00^{a}	1.64^{ab}	6.40^{a}	3.78^{ab}
	40	42.50^{d}	52.50^{ab}	0.96^{d}	6.05^{a}	2.14^{cd}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test. Treatments were conducted in 10 replicates.

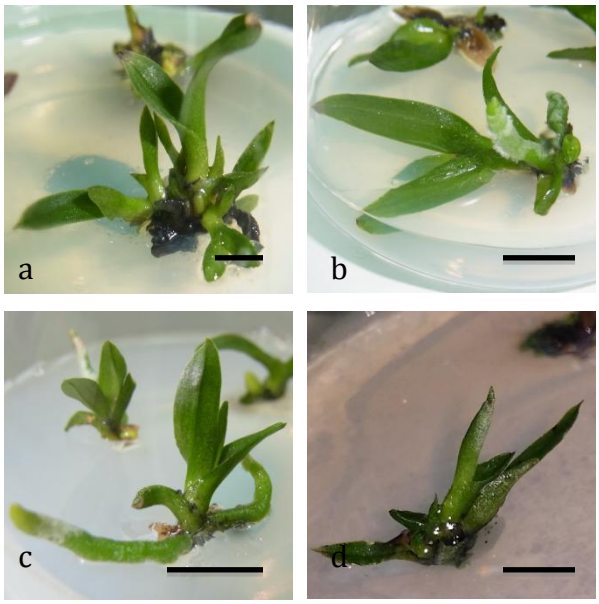

Figure. 3 (a) The effect of 20 g/L sucrose and (b) control on shoot multiplication and (c) the effect of 10 g/L sucrose and (d) control on rooting of *D. lowii* in KC medium added with 150 ml/L coconut water. (Bar scale=5mm).

Acclimatization

Plantlets (approximately 2 to 3 cm in height) with 3 to 5 leaves bearing 2 to 3 roots were taken out from the culture flask and washed thoroughly under running water to remove the remaining agar. The micropropagated plantlets were acclimatized at 25 ± 2°C using cotton covering the lower part of plantlets. These cottons were moistened with one-half strength of MS macro salt solution. After 2 weeks, they were finally transplanted to the small clay pots containing cocoa peat and small pieces of charcoal and sphagnum moss (1:1:1). Rooted plantlets were able to grow into normal plantlets in *ex vitro* condition after four weeks of acclimatization (Fig. 4).

Schematic representation of micropropagation protocol of *D. lowii* is shown in Fig. 4. PLBs were induced from shoot tip explants cultured in KC medium with the addition of 150 ml L^{-1} coconut water. After month 6, clumps of multiple shoots were obtained and these shoots were ready to be separated and re-cultured in a rooting medium. The best rooting medium was KC medium added with 150 ml L^{-1} coconut water or 2 g L^{-1} peptone. After month 4, these shoots were rooted and ready to be acclimatized.

CONCLUSION

A protocol was established for *in vitro* propagation of *D. lowii* using *in vitro* shoot tips, which originated from seeds producing PLBs as the starting material. Shoot multiplication was best seen in KC medium supplemented with 150 ml L^{-1} coconut water when explants were positioned horizontally. The best rooting performance was also observed in shoot tips that were cultured in KC medium added with 150 ml/L coconut water or with 2 g L^{-1} peptone. In the carbon source study, 20 g L^{-1} sucrose was found to be the best choice for shoot multiplication and 10 g L^{-1} sucrose was the most efficient for rooting. The process of this rapid propagation is summarized in Fig. 4. It is hoped that this successful protocol for micropropagation using shoot tip culture can be applied in a commercial scale with the aim to mass-produce and to reintroduce this species in their natural habitat.

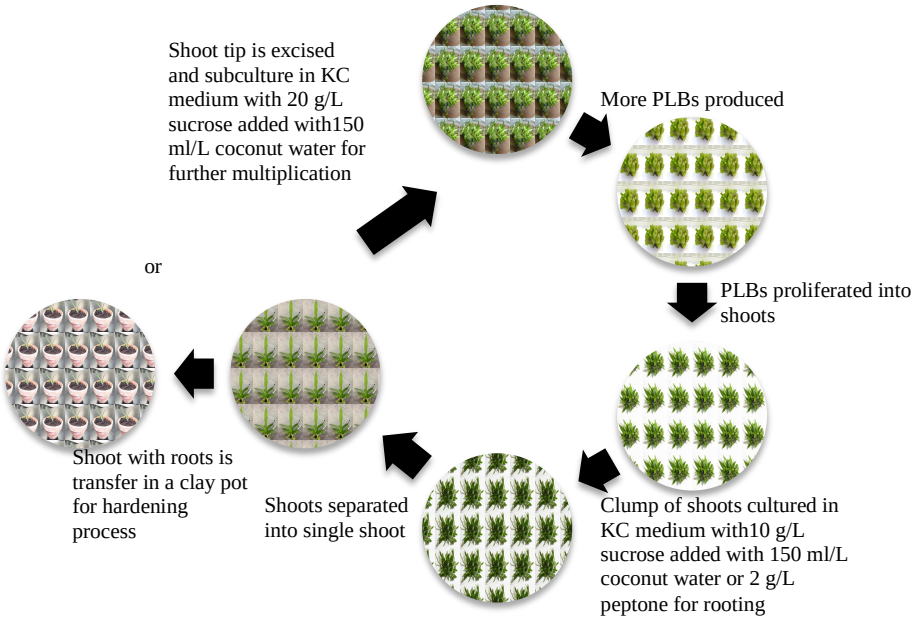

Fig. 4. The process of rapid shoot multiplication and rooting of *Dimorphorchis lowii* from shoot tip explants

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