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Organic Additives Improves the *in Vitro* Growth of Native Orchid *Vanda helvola* Blume

Devina DAVID[1], Roslina JAWAN[2], Hartinie MARBAWI[2], Jualang Azlan GANSAU[2*]

[1]*University Malaysia Sabah, Faculty of Sustainable Agriculture, Locked Bag No.3, 90509 Sandakan Sabah, Malaysia; ddevina@ums.edu.my*
[2]*University Malaysia Sabah, Faculty of Science and Natural Resources, UMS Road, 88450 Kota Kinabalu Sabah, Malaysia; roslinaj@ums.edu.my; hartinie@ums.edu.my; azlanajg@ums.edu.my (*corresponding author)*

Abstract

In vitro seed germination has been proven to be the most efficient technique to propagate orchid. The application of this aseptic technique has contributed to conservation of many endangered orchid species. In this study, undehisced capsules of *Vanda helvola* Blume were collected from Orchid Conservation Centre in Lagud Sebrang Agriculture Park, after 120 days from hand pollination and aseptically cultured on three types of basal media such as Murashige and Skoog (MS), Knudson C (KC) and Vacin and Went (VW). After 90 days of culture, 66.40 ± 4.14% of seeds successfully germinated on KC medium. The effect of organic additives such as tomato juice, coconut water, peptone and yeast extract at different level of concentrations in KC basal medium were also tested on seed germination and seedling development of this native orchid. After 90 days of culture, over 90% of seeds were tremendously germinated on KC medium supplemented with 10% or 15% (v/v) of tomato juice. The incorporation of peptone at 0.1% (w/v) in KC basal media promoted rapid development of protocorm to seedling. Seedlings on this treatment produced an average of three leaves and two roots after 90 days of culture and were successfully acclimatized.

Keywords: basal media, conservation, *in vitro* seed germination, protocorm, Orchidaceae

Introduction

Orchidaceae belongs to the largest family of flowering plants in the world, with an estimation of 800 genera and 20,000 species around the world. It was estimated that 2,500-3,000 of orchid species are found in Borneo (Chan *et al.,* 1994). *Vanda helvola* Blume is an epiphytic orchid, with a vast distribution in Borneo, but mostly found at Mt Kinabalu and Tambunan areas. The flower has a coppery red mountain form and it flowered at regular intervals throughout the year.

The illegal activity on orchid collection from the natural habitat has resulted in nearly extinction for many native orchids including *V. helvola*. Since this orchid was listed in Appendices II of the regulations formulated by the Committee for International Trade in Endangered Species Wild Fauna and Flora (CITES 2014), an effective strategy becomes essential to conserve and multiple this orchid species, for conservation as well as for horticultural purposes.

Plant tissue culture techniques have been proven as an important tool for orchid micropropagation, as well as to conserve many rare or endangered orchid species (Harrison and Arditti, 1978; Vij *et al*., 1994; Shimora & Koda, 2004; Zeng *et al.,* 2012). As for *Vanda* sp. the *in vitro* seed germination protocol has been widely used for mass propagation of superior varieties such as *V. joaquim* (Rao and Advhani, 1964), *Vanda* hybrids (Mathews and Rao, 1980; Johnson and Kane, 2007), *V. tessellata* (Roy and Banerjee, 2002), *V. teres* (Sinha and Roy, 2004), *V. coerulea* Grif ex. Lindl (Roy *et al.,* 2011) and *V. dearei* (Jualang *et al.,* 2014).

The incorporation of organic additives such as potato extract, tomato juice, banana, coconut water and peptone into the growth medium has influenced the protocorm and seedling development of some epiphytic orchids as reported previously in *V. roxburgii* (Islam *et al.,* 2011), *Cymbidium penduculum* (Saranjeet and Buthani, 2012), *Phalaenopsis* hybrids (Shekarriz *et al.,* 2014) and *V. dearei* (Jualang *et al.,* 2014).

Therefore, the current study was carried out to develop and establish an efficient protocol for *in vitro* propagation, by studying the effect of basal media and organic additives on *in vitro* seed germination and seedling development of *V. helvola*.

Materials and methods

Capsule source and sterilization

Undehisced capsules of *Vanda helvola* Blume were collected from Orchid Conservation Centre in Lagud Sebrang Agriculture Park, after 120 days from hand-pollination. Capsules of *V. helvola* were gently scrubbed with soap solution then rinsed and left under running tap water for 30 min. The capsules were surface sterilized by immersion in a 30% (v/v) of Clorox® solution added with two drops of Tween 20 and agitated for another 20 min. The capsules were then rinsed five times with sterilized distilled water, followed by dipping in 95% (v/v) ethanol for 10 s and passed briefly through flame.

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Fig. 1. (a) Flowering plant of *Vanda helvola* Blume; (b) Orchid capsule

Effect of basal media on seed germination

Three basal media were assayed for their effectiveness in promoting seeds germination of *V. helvola*. Seeds were aseptically inoculated on Murashige and Skoog (MS) (Murashige and Skoog, 1965), Knudson C (KC) (Knudson, 1946) and Vacin and Went (VW) (Vacin and Went, 1949) basal media and cultures were maintained at 25 ± 2 °C under continuous light.

Effect of organic additives on seed germination

The effect of organic additives on seed germination was tested using KC as basal medium. The medium was individually supplemented with tomato juice or coconut water at 10, 15 and 20% (v/v) and peptone or yeast extract at 0.1, 0.2 and 0.3% (w/v), respectively. Tomato juice was prepared by cutting the freshly ripe tomato into smaller pieces (without skin and seed) and grounded in a mixer. Coconut water was obtained from young and tender fruit purchased from local market. The coconut water was filtered prior using. Peptone and yeast extract were obtained from Bacto™ (Beckon, France). Basal medium devoid additive served as control and all treatments were maintained at 25 ± 2 °C under continuous light.

Effect of organic additives on seedlings development

To see the effectiveness of organic additives on seedlings development, 60 days-old of protocorm derived from *in vitro* germination was used as explant in this study. A total of 15 protocorms were selected and cultured on every petri dish containing KC basal media supplemented with different organic additives such as tomato juice or coconut water at 10, 20 or 40% (v/v); peptone or yeast extract at 0.1, 0.2 and 0.4% (w/v). All treatments were maintained at 25 ± 2 °C under continuous light.

Acclimatization of V. helvola seedlings

Seedlings of *V. helvola* at 2.5 cm height and above, with well-developed rhizomes and shoots were taken out from flasks and washed thoroughly under tap water to remove traces of agar-gelled medium. Seedlings were treated with 0.5% (w/v) fungicide for 15 min. They were then planted in pots containing brick pieces and coconut husks (1:2) and mulched with moss (*Sphagnum* sp.). The brick pieces and coconut husk were autoclaved-sterilized prior to potting, while *Sphagnum* mosses were treated with 0.5% (w/v) fungicide for 15 min. The plants were initially covered with a polythene sheet for one month to maintain high humidity and were irrigated twice a week with tap water.

Data Collection

For seed germination study, observation and data collection were carried out up to 90 days of culture with an interval of 10 days. The percentage of germinated seeds was calculated by dividing the number of germinated seeds by the total number of cultured seeds (Shimora and Koda, 2004).

For seedling development study, the percentage of leaf and root formation was recorded. The length of leaf (third leaf) and the longest root were measured in millimetre (mm) every month.

Statistical analysis

Experiments were carried out in a completely randomized design (CRD). Every treatment in all experiments consisted of 10 replicates. Data were analyzed by SPSS (Statistical Package for Social Science) software and subjected to analysis of variance (ANOVA). The mean values were compared using Duncan's Multiple Range Test (DMRT) at $p < 0.05$ significance level.

Results and discussions

Capsule and seed characterization

Flower of *V. helvola* varies from dull pale yellow – brown to coppery red with dull yellow (Fig. 1a). Pollination of orchid flowers was done manually and the green capsules harvested after 120 days from hand pollination. Weight and length of capsule were determined at 12.56 g and 9.5 cm respectively (Fig. 1b). Seeds of *V. helvola* were minute, dust-like, ranging from 200 μm to 300 μm in length. Seed at this age was yellowish and the testa (seed coat) was transparent. The characterization of capsule and seed of *V. helvola* were comparatively smaller than *V. dearei* as previously reported by Jualang *et al.* (2014).

Effect of basal media on in vitro seed germination

Among the three types of basal media, KC medium was found superior for germination of *V. helvola* seeds, compared to MS and VW media. Seeds sown on KC medium swollen within 28 days, developed into protocorms stage by day 60. Observation after 90 days of culture revealed that the germination percentage was significantly high in KC basal medium, with 66.40 ± 4.14% (Table 1). It was followed by MS medium with 18.90 ± 4.01% and VW medium yielded the lowest germination percentage at 9.70 ± 1.89%.

The *in vitro* seed germination of *V. helvola* was obviously affected by the type of basal medium. According to Arditti and

Table 1. Effect of basal media on germination of *Vanda helvola* seeds after 30, 60 and 90 days of culture

Basal media	Germination response (day)	[y]Germination percentage (% ± SD) by days		
		30	60	90
KC	28	12.10 ± 2.07[a]	33.70 ± 1.56[a]	66.40 ± 4.14[a]
MS	29	1.90 ± 0.74[b]	10.30 ± 1.57[b]	18.90 ± 4.01[b]
VW	35	0.30 ± 0.67[c]	6.90 ± 1.72[c]	9.70 ± 1.89[c]

[y] Data are means of 10 replicates. Mean followed by the same letter (column) did not differ significantly at $p < 0.05$ according to Duncan multiple range tests. SD: Standard deviation; Basal media KC (Knudson C, 1946); MS (Murashige & Skoog,1962); VW (Vacin & Went, 1949)

Table 2. Effect of organic additives on *Vanda helvola* seeds germination, cultured on KC basal medium

Organic additive	Concentration	Germination response (day)	[y]Germination percentage (% ± S.D)		
			30 days	60 days	90 days
Control	0	28	12.10 ± 2.08[e]	33.70 ± 1.57[e]	66.40 ± 4.14[e]
Tomato juice (%, v/v)	10	23	42.80 ± 2.53[c]	81.60 ± 1.35[a]	91.20 ± 1.55[a]
	15	23	44.30 ± 3.53[c]	83.90 ± 2.47[a]	92.30 ± 1.42[a]
	20	21	47.90 ± 4.31[b]	83.10 ± 3.18[a]	80.60 ± 1.90[b]
Coconut water (%, v/v)	10	38	0[h]	9.20 ± 3.08[g]	19.80 ± 2.49[j]
	15	38	0[h]	11.70 ± 3.56[fg]	24.20 ± 4.02[i]
	20	35	0[h]	13.80 ±3.12[f]	26.50 ± 4.74[i]
Peptone (%, w/v)	0.1	28	3.40 ± 1.78[g]	47.30 ± 5.12[c]	73.20 ± 2.97[d]
	0.2	28	8.20 ± 1.48[f]	49.50 ± 4.77[d]	74.90 ± 2.08[cd]
	0.3	28	7.10 ± 1.10[f]	52.50 ± 2.99[d]	76.10 ± 2.02[c]
Yeast extract (%, w/v)	0.1	15	44.80 ± 4.08[c]	74.20 ± 2.44[b]	62.10 ± 3.03[f]
	0.2	15	39.10 ± 4.89[d]	73.90 ± 1.72[b]	58.10 ± 3.14[g]
	0.3	14	53.50 ± 4.09[a]	73.10 ± 3.62[b]	49.20 ± 3.01[h]

[y] Data are means of 10 replicates. Mean followed by the same letter (column) did not differ significantly at $p < 0.05$ according to Duncan's Multiple Range Tests. SD – Standard deviation.

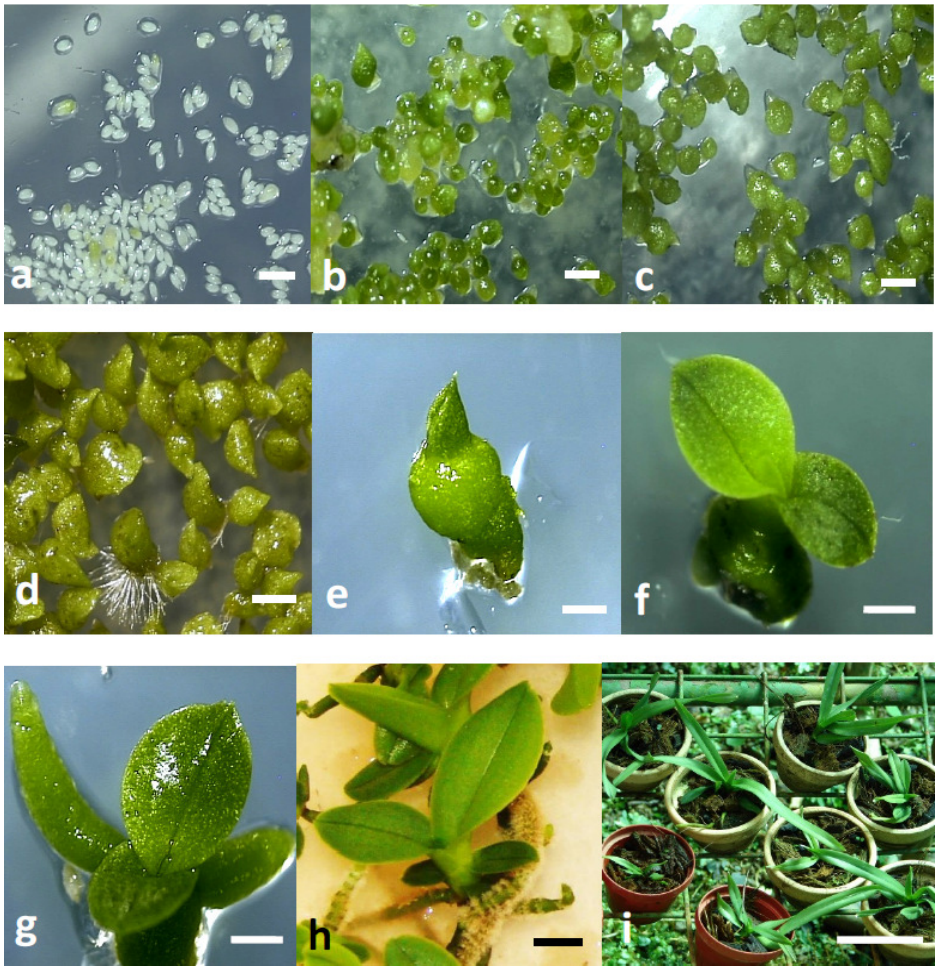

Fig. 2. Seedling development stages of *Vanda helvola*. (a) Seeds 10 days of culture. (b) Embryo enlarged after 20 days of culture. (c) Germinated seeds after 30 days of culture. (d) Protocorm at 40 days of culture. (e) Initiation of shoot from protocorm. (f) Protocorm with two leaves. (g) Established seedling with two leaves and two roots. (h) Seedling treated with 40% (v/v) tomato juice. (i) Acclimatized seedlings of *Vanda helvola* at Sabah Agriculture Park. Bar (a – h) 1.0 mm; (i): 5 cm

Table 3. Effect of organic additives on *Vanda helvola* seedlings development, after 90 days of culture on KC basal medium

Organic additives (Concentration)		[y]Protocorms with leaves (% ± SD)	[y]No. of leaf per responsive explant (± SD)	[y]Protocorms with roots (% ± SD)	[y]No. of root per responded explant (± SD)	Leaf[m]		Root[n]
						[y]Length (mm ± SD)	[y]Width (mm ± SD)	[y]Length (mm ± SD)
Control		94.70 ± 4.92^{b}	2.30 ± 0.48^{cd}	89.40 ± 2.12^{b}	1.65 ± 0.47^{d}	6.00 ± 1.15^{d}	2.21 ± 0.25^{cd}	6.00 ± 1.15^{d}
Tomato juice (%, v/v)	10	97.30 ± 2.21^{ab}	1.90 ± 0.74^{de}	97.90 ± 1.85^{a}	2.25 ± 0.72^{bc}	8.20 ± 1.14^{c}	2.56 ± 0.21^{ab}	8.26 ± 1.09^{b}
	20	94.90 ± 3.07^{b}	2.58 ± 0.50^{bc}	96.00 ± 2.91^{a}	2.42 ± 0.62^{b}	9.30 ± 0.95^{b}	2.61 ± 0.23^{ab}	9.48 ± 1.48^{a}
	40	70.60 ± 4.65^{d}	1.71 ± 0.41^{ef}	76.90 ± 5.47^{c}	3.14 ± 0.51^{a}	6.12 ± 0.48^{d}	2.79 ± 0.47^{a}	9.58 ± 0.96^{a}
Coconut water (%,v/v)	10	84.20 ± 4.73^{c}	2.19 ± 0.54^{cd}	77.00 ± 4.27^{c}	1.82 ± 0.39^{d}	4.94 ± 0.60^{e}	2.13 ± 0.32^{cde}	1.8 ± 0.46^{e}
	20	80.80 ± 6.56^{c}	2.05 ± 0.55^{de}	40.90 ± 3.45^{d}	1.06 ± 0.41^{e}	4.61 ± 0.85^{e}	2.16 ± 0.33^{cde}	1.8 ± 0.41^{e}
	40	58.60 ± 6.80^{e}	1.29 ± 0.44^{fg}	0^{f}	0^{g}	2.60 ± 0.52^{f}	1.96 ± 0.25^{de}	0^{f}
Peptone (%, w/v)	0.1	99.50 ± 1.08^{a}	3.10 ± 0.74^{a}	98.70 ± 1.34^{a}	1.97 ± 0.41^{d}	10.97 ± 0.75^{a}	2.37 ± 0.16^{bc}	6.86 ± 0.54^{c}
	0.2	94.20 ± 4.66^{b}	2.91 ± 0.30^{ab}	88.50 ± 6.19^{b}	1.80 ± 0.63^{d}	10.84 ± 0.69^{a}	2.37 ± 0.20^{bc}	6.58 ± 0.61^{c}
	0.4	32.30 ± 4.08^{f}	2.21 ± 0.34^{cd}	14.40 ± 3.13^{e}	0.60 ± 0.19^{f}	2.85 ± 0.51^{f}	2.04 ± 0.22^{de}	1.26 ± 0.24^{e}
Yeast extract (%, w/v)	0.1	83.70 ± 3.33^{c}	2.28 ± 0.52^{cd}	0^{f}	0^{g}	5.98 ± 0.55^{d}	2.11 ± 0.10^{de}	0^{f}
	0.2	80.90 ± 4.09^{d}	1.54 ± 0.28^{g}	0^{f}	0^{g}	5.51 ± 0.60^{e}	1.95 ± 0.18^{e}	0^{f}
	0.4	0^{g}	0^{h}	0^{f}	0^{g}	0^{g}	0^{f}	0^{f}

[y] Data are means of 10 replicates. Mean followed by the same letter did not differ significantly at $p < 0.05$ according to Duncan's Multiple Range Tests. SD: Standard Deviation; [m] the length of leaf was recorded from the third leaf of seedling ; [n] the length of root was recorded from the longest root formed

Ernst (1993), germination was considered difficult, as specific nutritional and environmental conditions are needed for *in vitro* germination of orchid seeds. Previous studies have revealed that seed germination of orchid depends on nitrogen source contained in the germination medium (Roy and Banerjee, 2002; Kauth *et al*., 2006; Stewart and Kane, 2006). In the present study, the total nitrogen content in MS medium (60.01 mM) is considerably higher than in KC (16.04 mM) and VW (8.97 mM). It was also observed that seeds of *V. helvola* prefer to germinate better in KC medium. This might be due to high water requirement for seed germination, so the elimination of mineral salt is advantageous (Rasmussen, 1995) or the genotype dependent (Johnson and Kane, 2007) of *V. helvola* as an epiphytic orchid. The efficacy of the KC medium for seed germination was also demonstrated earlier in Blue *Vanda* (Seeni and Latha, 2000) and *V. dearei* (Jualang *et al*., 2014).

Effect of organic additives on in vitro seed germination

The process of seed germination started when the embryo began to swell after 10 days of inoculation on KC medium containing 10% (v/v) of tomato juice (Fig. 2a). Colours of embryos varied from milk-white at the beginning of germination to bright green from 10 to 20 days of culture. Embryos were then enlarged by two times and occupied the whole seed coat (Fig. 2b). Within 23 days of culture, the embryos were finally discharged from the seed coat, moment when it was considered that the seeds germinated (Fig. 2c). According to Harrison and Arditti (1978) and Johnson & Kane (2007), a seed was considered germinated after the embryo discharged from the seed coat. The germinated seeds were then enlarged further to form a cone-shaped known as protocorm by approximately 40 days (Fig 2d). Some of the protocorms formed papillae and they remained at this stage up to 65 days. It was also observed that the protocorms were spherical and green.

The addition on KC basal medium of organic additives such as tomato juice, coconut water, peptone or yeast extract gave the earliest respond for seed germination on medium containing 0.3% (w/v) of yeast extract which germinated within 14 days of culture (Table 2). Even though seeds were germinated earlier in this treatment, the yield however decreased to 49.20 ± 3.01% as some of the protocorms turn to yellowish and later died after 90 days of culture. The supplementation of 10 or 15% (v/v) tomato juice on KC basal media had significantly increased the germination percentage to 91.20 ± 1.55% and 92.30 ± 1.42% respectively after 90 days of culture. Meanwhile, coconut water at 10, 15 or 20% (v/v) gave the lowest percentage of germination after 90 days of culture. The incorporation of suitable organic additives obviously increased the germination rate of *V. helvola* compared to KC medium alone. The development from seed to protocorm was fast and the size of protocorm was larger on medium containing tomato juice compared to other treatments. This result was supported earlier by Rao and Avadhani (1964) who concluded that the addition of tomato juice had a significant influence on seed germination and protocorm differentiation on *Vanda Joaquim*.

Effect of organic additives on seedling development

Protocorms of *V. helvola* responded variably when cultured on different organic additives for the first month of culture. The seedlings development process began with the formation of tiny leaf primordial at the apex of protocorm (Fig. 2e). First leaf initiated after 40 days of culture, followed by a formation of second leaf one month after that (Fig. 2f). The formation of first root started after 60 days of culture and it was followed either by the formation of a second root or the third leaf (Fig. 2g). After 90 days of culture, a seedling could produce up to three leaves and two roots.

The effect of organic additives on seedling development is presented in Table 3. The results revealed that the addition of organic additives had significantly enhanced the development of seedlings compared to control medium. It was observed that 99.50 ± 1.08% of protocorms treated in KC medium containing 0.1% (w/v) peptone successfully produced 3.10 ± 0.74 leaves after 90 days of culture with an average length of leaves at 10.97 ± 0.75 mm per responsive explant. The nutrients requirements for *V. helvola* growth varied from seed to seedling stage. From the

current findings, the addition of peptone at 0.1% (w/v) was beneficial in promoting the leaf and root formation of *V. helvola*. Previously, Nhut *et al.* (2008) described that peptone has been used as a source of carbon and nitrogen in plant tissue culture. The characteristic of peptone being water soluble protein hydrolase with very high amino acid content might also contribute to culture growth (Saranjeet and Bhuthani, 2012). The beneficial effect of peptone incorporated into basal media to improve the growth rate of *V. tessellata* was also reported earlier (Roy and Banerjee, 2002), or to increase uniformity and seedling development of *C. tuberosus* (Kauth *et al.,* 2006) and *in vitro* multiplication of *Phalaenopsis* hybrid (Shekarriz *et al.,* 2014).

The addition of tomato juice at 40% (v/v) promoted the highest number of roots. Explants cultured on this treatment produced 3.14 ± 0.51 roots, with average length of 9.58 ± 0.96 mm after 90 days of culture. Seedlings treated with tomato juice at concentrations of 10, 20 or 40% (v/v) however, were observed slightly yellowish compared to other treatments (Fig. 2h). This might be contributed by the bright red carotenoid pigment in tomato. In previous study by Rao and Avadhani (1964), tomato juice improves the rate of cell division, maximum vacuolation and early differentiation of the orchid embryos. Rao and Avadhani (1964) also added that seedlings treated with tomato juice could produce high number of roots 3 to 4 times longer, thin walled and branched. Several studies also demonstrated the effect of tomato juice on growth, however the finding gave low growth rate on *Phalaenopsis violacea* (Gnasekaran *et al.,* 2012) and *Dendrobium* hybrids (Nambiar *et al*., 2012).

Supplementation of coconut water or yeast extract was not significant on promoting the *V. helvola* seedlings development when compared to control medium, which is devoid of any additive. Even though coconut water is commonly used in orchid micropropagation, this finding however is in contrast with previous results that coconut water was found beneficial on seed germination of *Vanda coerulea* x *Ascocenrtum auranticum* (Kishor *et al*., 2006) and *Geodorum* sp. (Sheelavantmath *et al*., 2000). The incorporation of yeast extract was also not suitable for *V. helvola* development even though in previous study by Jawan *et al.* (2010) it was suggested that 0.2% (w/v) of yeast extract significantly increased the shoot formation of *Vanda dearie*, an endemic orchid of Borneo.

Acclimatization of V. helvola seedlings

The mean survival rate of *V. helvola* seedlings was 55.67% after six months of acclimatization with size reached up to 4.72 ± 0.22 cm of height and 7.00 ± 0.71 of leaf produced per explant. The seedlings were then shifted to former habitat at Orchid Conservation Centre in Lagud Sebrang Agriculture Park for further growth (Fig. 2i). The acclimatization of some orchids has been successfully demonstrated in *V. coerula* planted on charcoal chips and broken tiles (2:1) (Seeni and Latha, 2000; Malabadi *et al.*, 2004); *Dendrobium tosaense* planted on moss (*Sphagnum* sp.) (Lo *et al*., 2004) and *Ascocenda* 'Kangla' seedlings planted on brick:charcoal (2:1) and mulched with moss (*Sphagnum* sp.) (Kishor *et al.*, 2006).

Conclusions

An efficient protocol for *in vitro* seed germination and seedlings development of *V. helvola* was achieved using selected organic additives at suitable concentration. After 90 days of culture, over 90% of seeds germinated on KC medium supplemented with 10% or 15% (v/v) of tomato juice. The incorporation of peptone at 0.1% (w/v) in KC basal media promoted rapid development of protocorm to seedling. Seedlings on this treatment produced an average of three leaves and two roots after 90 days of culture and were successfully acclimatized. This protocol has a tremendous potential as a conservation tool to protect the local orchid species in Borneo.

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