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Efficient Adventitious Shoot Regeneration from Shoot Tip Culture of *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple), a Rare Thai Orchid Species

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Abstract

Shoot tip culture was applied to *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple) to increase the number of orchids that have a low population in natural habitats. The effects of sucrose concentrations, plant growth regulators (PGRs), and activated charcoal (AC) in different culture media on protocorm-like body (PLB) induction, multiple shoots, root formation, and ploidy level stability were investigated. The effects of various concentrations of sucrose (0 - 20 g L^{-1}) incorporated in 5 liquid media; Vacin and Went, 1949 medium (VW), VW medium supplemented with 150 ml L^{-1} coconut water (modified VW), Schenk & Hildebrandt, 1976 medium (SH), Murashige and Skoog, 1962 medium (MS), and half strength MS medium (1/2 MS) were studied. The highest PLBs (7.9 pieces) and survival (92.6 %) were observed in modified VW liquid medium supplemented with 10 g L^{-1} sucrose without plant growth regulators after 8 weeks of culture. Shoot and root induction were observed in SH agar medium supplemented with PGRs. The supplementation of 0.1 mg L^{-1} TDZ on SH agar medium induced the highest shoot formation at 73.3 shoots per explant, while in SH agar medium supplemented with NAA induced root formation, the highest root formation was at 4.2 mm. After 12 weeks of culture, SH agar medium showed the release of phenolic compounds. AC was used to inhibit phenolic exudates and to induce root formation in SH agar medium. The combination of 1 g L^{-1} AC and 1.0 g L^{-1} NAA showed an optimum root formation at 51 % and rooting from a shoot at 2.3 roots per shoot. A similar pattern of ploidy level was observed by flow cytometry from the leaves of both stock plantlets and regenerated plantlets. There was no difference in the morphology of stock plantlets and regenerated plantlets.

Keywords: *Rhynchostylis gigantea*, shoot tip culture, plant growth regulators

Introduction

Over 25,000 species of orchids belong to the Orchidaceae family, which is the largest family of flowering plants (Angiosperms) [1]. They can grow in various environments; however, morphological characteristics depend on the climate of their habitat [2]. The genus *Rhynchostylis* contains 4 species. Three of the 4 species, namely: *R. gigantea, R. coelestis*, and *R. retusa*, are widely distributed in Thailand. This genus is found in northern Thailand, northern Myanmar, and Indochina. *R. gigantea* (Lindl.) Ridl. has a variety of flower colors. Amethyst-purple flowering *R. gigantea* is found in the least number in natural habitats [3]. The attractive amethyst-purple flower color of *R. gigantea* makes it a popular commercial orchid for its species and hybrids.

Orchid tissue culture technique was developed by the initial work of Morel [4] and applied to gain more mass clonal production. Shoot tip culture is the method that is used for increasing the clonal

propagation, not only for commercial reasons, but also for conservation. Multiple rates of protocorm-like body (PLB) production and plantlet development without mutation are the most desirable for tissue culture. The main factors that regulate PLB and plantlet regeneration are nutrient media, carbon sources, plant growth regulators, and activated charcoal (AC) [4-6]. Common nutrient media that are used for orchid tissue culture are Vacin and Went, and Murashige and Skoog [7-9]). However, other factors are important, because using only nutrient media is not sufficient to regenerate plantlets in low CO_2 concentrations and light intensities in *in vitro* conditions. Carbon supplementations are required [10]. Sucrose is often used as a carbon source in cell and tissue culture media. Plants can utilize some sugars, such as monosaccharide or polysaccharide. Alternative carbon sources are sugar alcohol (known as polyol), such as sorbital, glycerol, manitol, etc., which have been applied for tissue culture procedures [11]. The induction of shoots and roots was stimulated by plant growth regulators in natural and synthetics (organic additives, such as coconut water), which were successfully used for the propagation of *Phalaenopsis* [12], *Paraphalaenopsis serpentilingua* [13], and *Dendrobium nobile* var. Emma white [14]. PGRs are considered to have an increased effect on cell division, elongation, and differentiation [15]. Auxins and cytokinins are efficient plant growth regulators which stimulate the induction of roots and shoots, respectively. NAA and BA are commonly supplemented in orchid culture media [16,17]. Meanwhile, TDZ is an interesting cytokinin for enhancing PLB proliferation and shoot regeneration in *Phalaenopsis* and *Vandaceous* species [18,19]. AC may also be added to stimulate growth and development [20]. The advantage of AC for orchid tissue culture may result from the absorption of organic compounds [21] and provide dark conditions for culture medium root induction [22]. Moreover, genetic stability of orchid tissue culture is one of the most important criteria for conservation and economic interests. The most efficient method to stabilize DNA ploidy is flow cytometry [23].

Similar to other orchids, *Rhynchostylis* has been used for a long time, especially for seed culture [24-27]. Other parts of *R. gigantea* have been used for clonal propagation, such as transverse thin cell layers [28] and young shoots [29]. Among them, shoot tips were less reported for *in vitro* culture. Therefore, this study investigates the culture conditions needed for high regeneration of plantlets via shoot tip cultures of *R. gigantea* (amethyst-purple).

Materials and methods

Effects of media and sucrose concentrations on protocorm-like body formation

Shoot tips from 5-month-old *in vitro* seedlings of *R. gigantea*, 5 - 7 cm high, were excised to 2 - 3 mm under a stereo microscope. Shoot tips were placed on agar without sucrose for 1 d, and then healthy shoot tips, which did not turn to a brown color, were selected. Completely randomized design (CRD) was used to study the effects of 15 treatment combinations (**Table 1**). For each treatment, 10 shoot tips were cultured with 3 replications. The culture was based on 5 different liquid media; Vacin and Went (VW) medium, Vacin and Went supplemented with 150 ml L^{-1} coconut water (modified VW) medium, Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt), Murashige and Skoog (MS) medium (Murashige and Skoog), half strength Murashige and Skoog (1/2 MS) medium, and all media supplemented with sucrose (0, 1 and 2 % (w/v)). Cultures were maintained at standard conditions; 25 ± 2 °C, under white fluorescent light (Philips, Thailand) at an intensity of 37 μmol m^{-2} s^{-1} for 16 h per d. The cultures in the liquid medium were shaken in a rotary shaker at a speed of approximately 110 rpm. After 8 weeks of culture, survival rate and PLB formation were examined.

Effects of α-napthaleneacitic acid (NAA), benzylaminopurine (BA), and thidiazuron (TDZ) on growth and formation of PLBs, shoots, and roots

Based on the varying effects of basal media on maximum PLB formation, modified VW medium and SH medium supplemented with 1 % sucrose were selected for further examination of the effects of PGRs on PLB multiplication and subsequent plantlet development. About 3 mm diameter *R. gigantea* PLBs were cultured in modified VW (**Table 2**) and SH basal medium (**Table 3**) supplemented with 1 % sucrose, and subcultured every 4 weeks at pH 5.3 and 5.8, respectively. There were, in total, 21 treatments, with 4 replications for each medium. There were 3 PLBs in each bottle. Supplementation of α-

napthaleneactic acid (NAA) (0.5 and 1 mg L^{-1}), benzylaminopurine (BA) (1, 2 and 4 mg L^{-1}), and thidiazuron (TDZ) (0.1, 0.5 and 1 mg L^{-1}), individually and in combinations, were examined and compared with control (modified VW and SH medium without PGRs). Cultures were maintained at standard conditions. Numbers of shoot and root formations were observed after 12 weeks of culture.

Effects of α-napthaleneacitic acid (NAA) and activated charcoal on root induction

Shoots from PGR treatments in SH agar medium, supplemented with 0.1 mg L^{-1} TDZ, were used and cultured in SH medium, supplemented with NAA and AC. The purpose was to determine the morphogenetic effects of NAA and AC on subsequent plantlet growth. Modified VW did not produce plantlets, but induced PLB formation; meanwhile, SH medium induced shoots and roots. About 5 mm of regenerated tiny shoots without roots were cultured in SH agar medium, supplemented with 1 % sucrose at pH 5.8 as a control. For the treatments, SH agar medium was supplemented with 0.5 and 1 mg L^{-1} NAA in combination with 1 g L^{-1} AC. Cultures were maintained at standard conditions. Numbers of shoot and root formations were observed after 12 weeks of culture.

Plant regeneration and transfer to the saranhouse

After 8 months of culture, plantlets with 2 - 3 cm in height, 2 - 3 leaves, and 2 - 3 roots were removed from the bottles. The plantlets were washed thoroughly under running tap water and transplanted into plastic pots, 10 cm in diameter, filled with only charcoal chips. Twenty plantlets were grown in each pot, with 3 replications. All pots were then transferred to a saranhouse, of which conditions were 60 % sunlight at 30 - 35 °C. The survival rate was recorded after growing in the saranhouse for 12 weeks.

Determination of DNA content using flow cytometry (FCM)

To analyze their ploidy stability, nuclei were isolated by chopping the leaves (0.5×0.5 cm) of 1-year-old *in vitro* regenerated plantlets and comparing them with stock plants (starting plantlet material) and control plantlets (cultured in SH medium without PGRs), developed from all treatments in 1,000 µl of Otto I buffer, suspensions filtered through nylon mesh, and centrifuged, 10 suspended in a new 200 µl batch of Otto I. After being mixed with Otto I, 400 µl of Otto II was added; following the addition of 50 µg L^{-1} propidium iodide, 50 µg L^{-1} RNase, and 2 µl L^{-1} ß-mercaptone, it was mixed and ready to be run in flow cytometry (BD FACS Canto Flow Cytometer). All chemicals used in this study were of analytical grade.

Data collection and statistical analysis

Experiments were performed in a completely randomized design (CRD) with 3 replications. Each treatment had 10 PLBs/replication. PLB formation from shoot tips was evaluated after 8 weeks of culture. Morphogenesis was expressed as a percentage of survival and number of PLBs per explant. Regenerated plants were evaluated after 12 weeks of culture. Morphogenesis was recorded as the number of shoots and the length of the leaf and root. The experimental data were analyzed using ANOVA. Mean values were compared using Duncan's multiple range test (DMRT) at $P \leq 0.01$.

Results

Effects of media and sucrose concentrations on protocorm-like body formation

After 8 weeks of culture, 5 different liquid media, namely, VW, modified VW, SH, MS, and 1/2 MS supplemented with 0, 1 and 2 % sucrose concentrations, showed highly significant differences on survival and PLB formation (**Table 1**). Modified VW supplemented with 1 % sucrose gave the highest survival of 92.6 %, but was not significantly different within its medium, followed by SH medium supplemented with 1 % sucrose. In addition, modified VW supplemented with 1 % sucrose gave the highest PLB formation of 7.9 pieces, followed by modified VW without sucrose (3.8 pieces) and SH medium with 1 % sucrose (3.5 pieces).

Table 1 Effects of 5 different liquid media supplemented with different sucrose concentrations on survival and PLB formation from *R. gigantea* (amethyst-purple) shoot tips after 8 weeks of culture.

Medium	Sucrose (%)	Survival (%)	PLB formation (piece of PLBs)
VW	0	55.0 ± 11.4^{b}	1.5 ± 0.7^{b}
	1	65.0 ± 6.9^{ab}	2.6 ± 1.8^{a}
	2	70.0 ± 9.2^{a}	2.3 ± 1.8^{ab}
Modified VW	0	92.3 ± 5.3^{a}	3.8 ± 1.3^{b}
	1	92.6 ± 5.1^{a}	7.9 ± 3.6^{a}
	2	79.3 ± 7.7^{a}	2.9 ± 1.3^{b}
SH	0	13.8 ± 6.5^{b}	1.9 ± 0.8^{b}
	1	79.3 ± 7.7^{a}	3.5 ± 1.8^{a}
	2	76.7 ± 7.85^{a}	2.7 ± 1.4^{ab}
MS	0	26.9 ± 8.87^{b}	2.3 ± 0.9^{ab}
	1	42.1 ± 11.63^{ab}	2.6 ± 1.5^{a}
	2	59.1 ± 10.72^{a}	1.4 ± 0.7^{b}
1/2 MS	0	25.0 ± 8.33^{b}	1.7 ± 1.3^{b}
	1	64.0 ± 9.79^{a}	2.6 ± 0.9^{a}
	2	71.8 ± 7.88^{a}	1.9 ± 1.0^{ab}

Data presented as means ± standard deviation (Std. Deviation). Means with different letters within columns are significantly different according to Duncan's Multiple Ranges Tests (DMRT) at $P \leq 0.01$.

Table 3 Effects of NAA, BA and TDZ supplemented to SH agar medium on survival, PLB formation, number of shoots per PLB, leaf length, and root length from *R. gigantea* (amethyst-purple) PLBs after 12 weeks of culture.

Plant growth regulator (mg L^{-1})			Survival (%)	PLB formation (piece of PLBs)	Number of shoots per PLB	Leaf length (mm)	Root length (mm)
NAA	BA	TDZ					
-	-	-	61.1 ± 8.2^{abc}	3.0 ± 1.1^{bc}	$48.1 \pm 3.7^{b-f}$	4.1 ± 0.2^{a}	4.2 ± 0.4^{a}
-	1	-	80.6 ± 6.7^{a}	3.7 ± 0.8^{bc}	50.6 ± 3.3^{bcd}	$2.6 \pm 0.1^{c-g}$	2.0 ± 0.7^{ab}
-	2	-	58.3 ± 8.3^{abc}	4.4 ± 0.6^{abc}	53.7 ± 4.4^{bcd}	$2.7 \pm 0.1^{c-g}$	2.0 ± 0.1^{ab}
-	4	-	27.8 ± 7.6^{c}	5.6 ± 1.3^{ab}	$38.2 \pm 3.9^{d-f}$	$2.5 \pm 0.1^{d-g}$	–
0.5	-	-	61.1 ± 8.2^{abc}	3.0 ± 0.5^{bc}	$41.3 \pm 2.1^{c-f}$	4.1 ± 0.2^{a}	4.1 ± 0.2^{a}
1	-	-	52.8 ± 8.4^{abc}	2.9 ± 0.4^{bc}	$44.3 \pm 3.5^{c-f}$	3.5 ± 0.1^{b}	2.8 ± 0.3^{ab}
-	-	0.1	63.9 ± 8.1^{ab}	1.7 ± 0.5^{c}	73.3 ± 6.2^{a}	$2.5 \pm 0.1^{d-g}$	–
-	-	0.5	63.9 ± 8.1^{ab}	2.8 ± 0.4^{bc}	61.9 ± 3.3^{ab}	2.3 ± 0.1^{fg}	–
-	-	1	52.8 ± 8.4^{abc}	4.8 ± 0.8^{abc}	$45.9 \pm 3.7^{b-f}$	$2.6 \pm 0.1^{c-g}$	–
0.5	1	-	80.6 ± 6.7^{a}	4.7 ± 0.5^{abc}	$49.0 \pm 3.1^{b-e}$	3.0 ± 0.1^{c}	3.4 ± 0.3^{ab}
0.5	2	-	58.3 ± 8.3^{abc}	5.4 ± 0.8^{ab}	33.2 ± 3.0^{ef}	$2.7 \pm 0.2^{c-g}$	–
0.5	4	-	50.0 ± 8.5^{abc}	7.3 ± 0.8^{a}	31.8 ± 3.3^{f}	$2.6 \pm 0.1^{c-g}$	2.0 ± 0.10^{ab}
1	1	-	58.3 ± 8.3^{abc}	4.5 ± 0.6^{abc}	$43.7 \pm 2.7^{c-f}$	$2.8 \pm 0.1^{c-f}$	1.5 ± 0.3^{b}
1	2	-	77.8 ± 7.0^{a}	4.4 ± 0.6^{abc}	$42.8 \pm 4.0^{c-f}$	2.9 ± 0.1^{cde}	–
1	4	-	36.1 ± 8.1^{bc}	4.9 ± 0.7^{abc}	31.3 ± 4.8^{f}	2.3 ± 0.2^{g}	–
0.5	-	0.1	52.8 ± 8.4^{abc}	4.4 ± 0.7^{abc}	53.8 ± 3.7^{bcd}	2.9 ± 0.1^{cde}	–
0.5	-	0.5	72.2 ± 7.6^{a}	5.0 ± 0.6^{ab}	56.2 ± 3.7^{bc}	$2.6 \pm 0.1^{c-g}$	–
0.5	-	1	80.6 ± 6.7^{a}	5.8 ± 0.7^{ab}	51.7 ± 5.1^{bcd}	2.4 ± 0.1^{efg}	–
1	-	0.1	66.7 ± 8.0^{ab}	3.2 ± 1.0^{bc}	$45.5 \pm 2.9^{b-f}$	2.9 ± 0.1^{cd}	–
1	-	0.5	55.6 ± 8.4^{abc}	5.3 ± 0.6^{ab}	$40.1 \pm 4.1^{c-f}$	2.4 ± 0.1^{efg}	–
1	-	1	55.6 ± 8.4^{abc}	5.3 ± 1.1^{ab}	$37.1 \pm 5.3^{d-f}$	2.4 ± 0.1^{efg}	–

Data presented as means ± standard deviation (Std. Deviation). Means with different letters within columns are significantly different according to Duncan's Multiple Ranges Tests (DMRT) at $P \leq 0.01$.

Effects of NAA and AC on root formation

Based on the previous results, SH agar medium was used to test the efficiency of NAA and AC for root induction. After 12 weeks of culture, highly significant differences in root formation, rooting from a shoot, and root length were observed, in **Table 4**. The supplementation of NAA and AC in SH agar medium was essential for root formation. For root formation in all of treatments, control and a combination of 1 mg L^{-1} NAA with 1 g L^{-1} AC gave the highest levels, at 53.1 and 51 %, respectively. The addition of AC and the combination of NAA and AC showed an increase in the number of roots compared with the control condition. AC can enhance root length, while the supplementation of NAA into SH agar medium decreased the root length. Meanwhile, the combination of NAA and AC showed an increase in root length compared with supplementing NAA alone (**Table 4** and **Figure 1**).

Table 4 Root formation, rooting from a shoot, and root length of *R. gigantea* (amethyst-purple) on SH agar medium supplemented with different concentrations of NAA and activated charcoal after 12 weeks of culture.

Treatment		Root formation (%)	Rooting from a shoot (roots per shoot)	Root length (mm)
NAA (mg L^{-1})	Activated charcoal (g L^{-1})			
-	-	53.1 ± 3.1^{a}	2.0 ± 0.1^{ab}	9.0 ± 0.1^{ab}
0.5	-	38.6 ± 3.1^{b}	1.5 ± 0.1^{b}	4.0 ± 0.0^{c}
1.0	-	39.7 ± 4.8^{ab}	1.6 ± 0.2^{b}	4.0 ± 0.0^{c}
-	1.0	40.5 ± 4.0^{ab}	2.2 ± 0.1^{a}	11.0 ± 0.1^{a}
0.5	1.0	41.0 ± 2.8^{ab}	2.0 ± 0.0^{ab}	8.0 ± 0.2^{bc}
1.0	1.0	51.0 ± 3.8^{b}	2.3 ± 0.1^{a}	10.0 ± 0.1^{ab}

Data presented as means ± standard deviation (Std. Deviation). Means with different letters within columns are significantly different according to Duncan's Multiple Ranges Tests (DMRT) at $P \leq 0.01$.

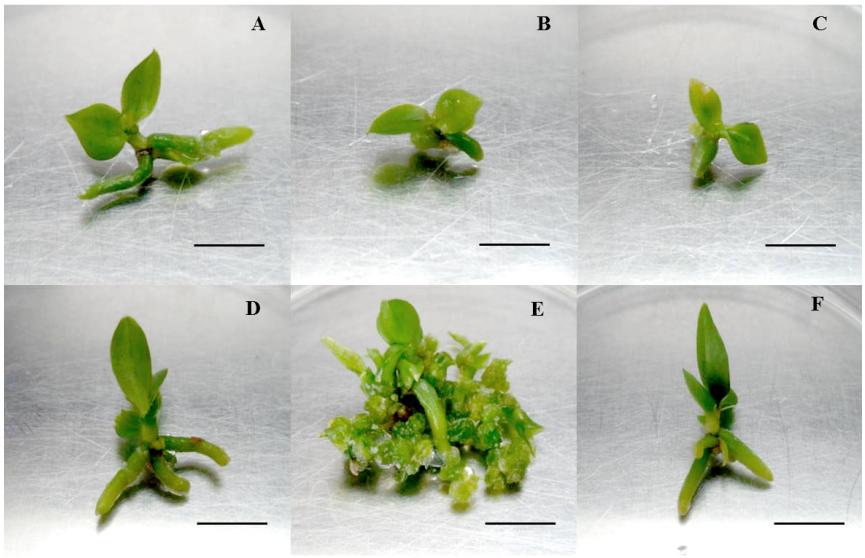

Figure 1 Development of plantlets of *R. gigantea* (amethyst-purple) from tiny shoots in SH medium within 12 weeks after culture: (A) SH agar medium without PGRs , (B) SH agar medium supplemented with 0.5 mg L^{-1} NAA, (C) SH agar medium supplemented with 1.0 mg L^{-1} NAA, (D) SH agar medium supplemented with 1 g L^{-1} activated charcoal, (E) SH agar medium supplemented with 0.5 mg L^{-1} NAA and 1 g L^{-1} activated charcoal, and (F) SH agar medium supplemented with 1.0 mg L^{-1} NAA and 1 g L^{-1} activated charcoal. Scale bar = 1 cm.

Plant regeneration and transfer to the saranhouse

The plantlets, 6 months old and 2 - 3 cm in height, with 2 - 3 leaves and 2 - 3 roots (**Figure 2A**) were transferred from the bottles. The plantlets were washed thoroughly under running tap water and transplanted into plastic pots with charcoal chips. All pots were then transferred to a saranhouse. The survival rate after growing in the saranhouse for 12 weeks was 100 % (**Figure 2B**). The leaves and roots of plantlets were used to measure the growth. Significant differences in root length after 12 weeks are shown in Table 5, from 1.9 to 2.2 cm. The number of leaves, leaf lengths, and number of roots were increased, but not significantly different, in 12 weeks, from 3.5 to 3.7, 1.6 to 1.8, and 1.9 to 2.2 cm, respectively.

Table 5 Number of leaves, leaf lengths, number of roots, and root lengths of *R. gigantea* (amethyst-purple) plantlets at 0 and 12 weeks after growing in the saranhouse.

Growth (week)	Number of leaves	Leaf length (cm)	Number of roots	Root length (cm)
0	3.5 ± 0.1^{a}	1.6 ± 0.1^{a}	3.7 ± 1.2^{a}	1.9 ± 0.1^{b}
12	3.7 ± 0.1^{a}	1.8 ± 0.1^{a}	3.9 ± 1.0^{a}	2.2 ± 0.1^{a}

Data presented as means ± standard deviation (Std. Deviation). Mean value within the column followed by the same letters are not significantly different at $P \leq 0.01$ according to simple T-Test; n = 60.

Figure 2 Plant regeneration and transfer to the saranhouse: (A) Plantlets regenerated on SH agar medium without plant growth regulators and (B) 12-week-old plantlets in the saranhouse. (Scale bar: A and B = 2 cm).

Ploidy stability analysis using flow cytometry (FCM)

Flow cytometry histograms were obtained from the 3 youngest leaves harvested from 1-year-old plants. For a typical DNA histogram of the material, 2 peaks were observed; the first peak represented the G1 phase (unreplicated, 2C), and the second represented the G2/M phase (replicated, 4C) of the cell cycle [30,31]. Similar patterns of ploidy levels were observed by flow cytometry of 1-year-old *in vitro* regenerated plantlets compared with stock plants (started plantlet material) and control plantlets (cultured in SH medium without PGRs) (**Figure 3**).

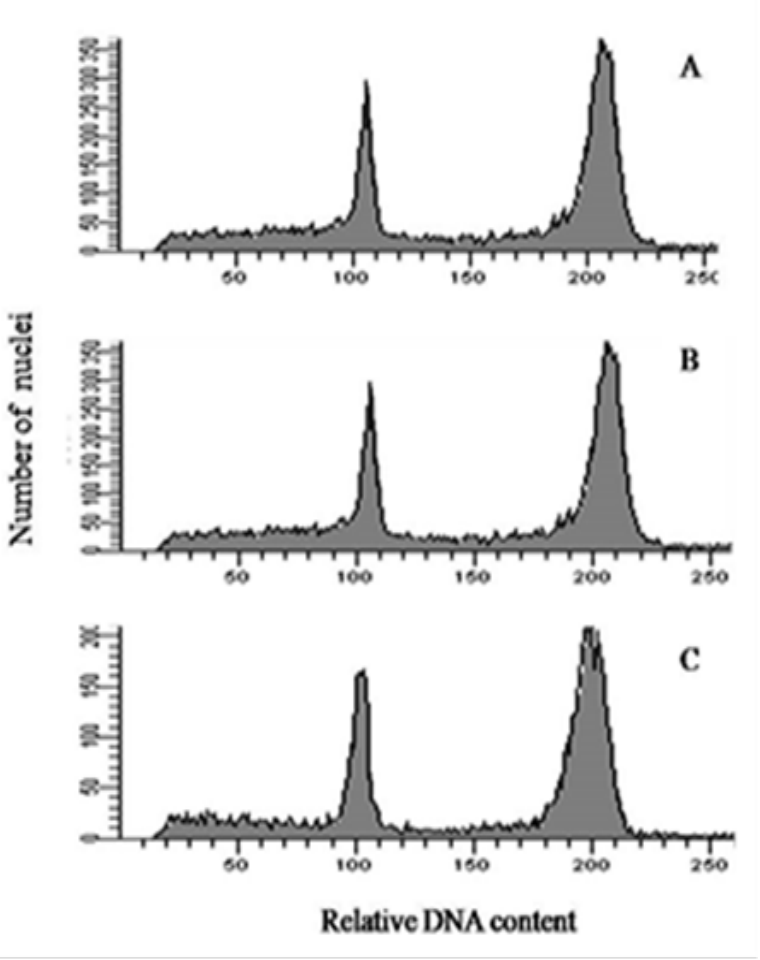

Figure 3 Flow cytometry histograms from the 3 youngest leaves of *R. gigantea* (amethyst-purple) showing ploidy levels: (A) 1-year-old started plantlet material, (B) 1-year-old regenerated plantlets cultured in SH agar medium without PGRs, and (C) 1-year-old regenerated plantlets cultured in SH agar medium supplemented with 1 mg L^{-1} NAA.

Micropropagation of *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple) was established by starting with shoot tips from *in vitro* plantlets (**Figure 4**). Protocorm-like bodies were induced by modified Vacin and Went with 10 mg L^{-1} sucrose. After PLB formation, SH agar medium supplemented with 0.1 mg L^{-1} was used to induce shoot formation. For root formation, SH agar medium with 1 mg L^{-1} NAA and 1 mg L^{-1} activated charcoal exhibited the highest number of roots and highest root lengths. However the observation of ploidy level by flow cytometry showed the same pattern of ploidy levels of regenerated plantlets compared with mother plants.

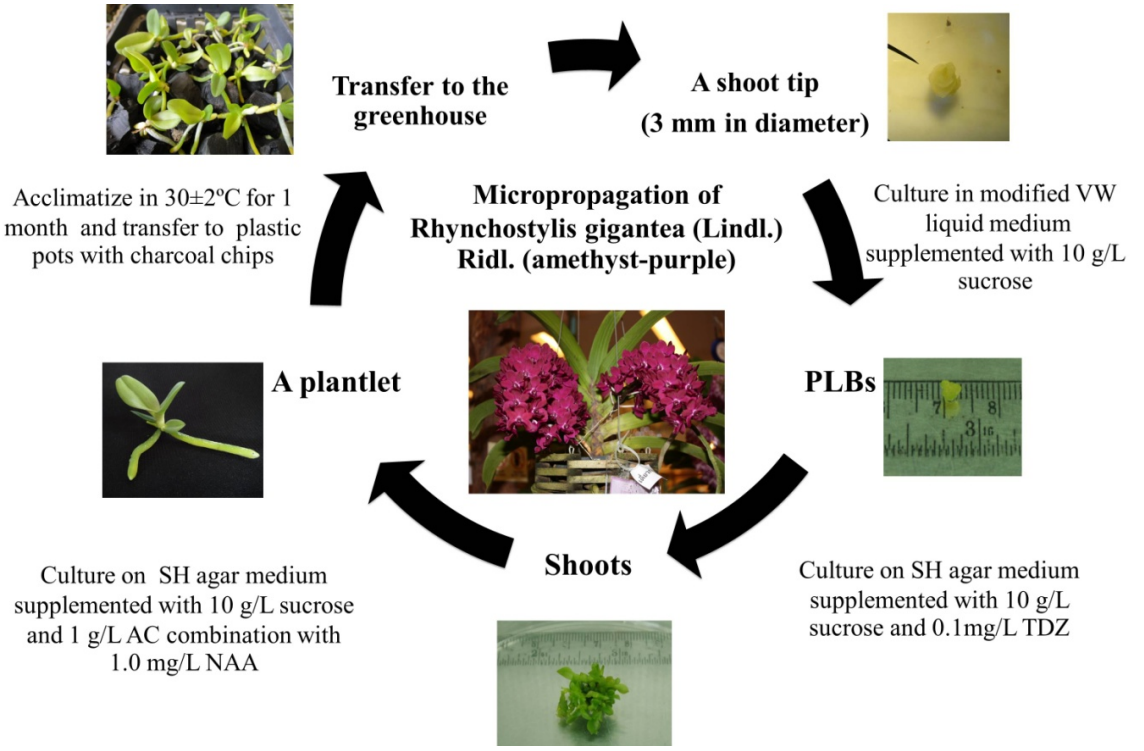

Figure 4 Established micropropagation protocol for *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple) from shoot tips.

Discussion

The micropropagation of *Rhynchostylis gigantea* (Lindl.) Ridl. (amethyst-purple) from shoot tip culture, by utilizing culture media and PGRs, was studied. The same genotype was required for maintaining good characteristics. This micropropagation will help to reduce the demand for taking wild orchids from their natural habitat, and benefit conservation. The nutrient requirements for orchid cultures are different among species [32,33]. There are many different media used for tissue culture. Jitsopakul *et al*. [19] reported that 2 basal media (VW and MS) were used, and VW was found to be equally suitable for shoot tip cultures of *Vanda coerulea*. Carbon sources, as well as nutrient requirements are important factors for tissue culture; because light deficiency and low CO_2 present in tissue culture condition, carbon sources play an important role as energy sources and osmotic regulators [34]. Sucrose is the most frequently used sugar for *in vitro* culture, but other sugars, such as glucose, fructose, sorbitol, and maltose are also used [35,36]. The results showed that PLBs and shoots can be grown from shoot tips in 5 different media. Modified VW liquid medium supplemented with 10 g L^{-1} sucrose gave the highest number of PLB formations. Similarly, 1 % sucrose in modified VW liquid medium was reported to initiate PLBs from adventitious buds of *Phalaenopsis* [8].

The types, concentrations, and combinations of plant growth regulators play important roles during *in vitro* propagation of many orchid species [37]. Auxins and cytokinins are the 2 main groups of PGRs used in plant tissue culture. The different types and concentrations of PGRs were used to induce shoot and root formation. BA is one of the commonly used cytokinins. From the results, 1 - 2 mg L^{-1} BA induced shoots and PLB formation, but 4 mg L^{-1} BA decreased shoot formations of *R. gigantea*, while low concentrations of TDZ (0.1 - 0.5 mg L^{-1}) gave the highest number of shoots per PLB. TDZ was first reported to have cytokinin activities by Mok *et al*. [38]. In many orchids, TDZ was used to induce PLB

proliferation and shoot regeneration [39,40]. Nayak *et al*. [40] reported that the use of MS medium supplemented with 1 mg L^{-1} TDZ gave the highest frequency of shoot regeneration and shoot production, at 92.5 % and 25.8 shoots per PLB, respectively. The induction of orchid shoots from PLBs was reported in *Epidendrum radicans* [41], *Vanda coerulea* [42], and *Dendrobium chrysotoxum* [43] by using TDZ alone and in a combination with auxin. As a result, the efficiency of TDZ in shoot regeneration seems to be quite different among species [44]. In this study, the combination of NAA and AC affected the increase in the number of root formations from shoots. The results indicated that the combination of NAA and AC increase the number of root and root length more than by using NAA alone. NAA may not have a direct effect on the development of shoots, but may be effective, mostly through induction of roots. They increased the effects on cell division, elongation, and differentiation [15]. In addition, AC was added into the culture medium, and the results showed that 1 g L^{-1} AC gave the highest root length (11 mm). Thomas [45] reported that AC alone or in combination with auxins induced rooting of micropropagated shoots. AC also stimulated rooting by absorbing toxic substances and excluding light from the medium. Bhadra and Hossain [20] reported that AC enhanced the initiation of roots of *Geodorum densiflorum* (Lam.) Schltr. The ploidy level results of *R. gigantea* by flow cytometry analyses showed the same 3 patterns: 1. 1-year-old started plantlet material, 2. 1-year-old regenerated plantlets cultured on SH agar medium without PGRs, and 3. 1-year-old regenerated plantlets cultured in SH agar medium supplemented with 1 mg L^{-1} NAA. Sopalun *et al*. [46] reported that the pattern of ploidy level by flow cytometry of *Gammatophyllum speciosum* obtained from *in vitro* culture is the same as those grown in a saranhouse.

Conclusions

In summary, we have successfully established a protocol for the micropropagation of *R. gigantea* (Lindl.) Ridl. (amethyst-purple). The culture protocol to obtain plantlets required approximately 8 months from shoot tip culture. This work will be of benefit to *R. gigantea* (amethyst-purple) regeneration, and maintain its outstanding genotype for potted orchids and for breeding program.

Acknowledgements

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