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Micropropagation of Chang Daeng (*Rhynchostylis rubrum*) by embryogenic callus

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Young shoot (approx. 10 mm) gave the highest percentage of multiple shoot formation (100), number of shoots (10 shoots/explant) and embryogenic callus (EC) formation (56%). Proliferation of the calli in both Vacin and Went (VW) and New Dogashima medium (NDM) supplemented with 2% sucrose and 15% coconut water (CW) under light or dark condition was nearly the same. The color of calli in VW in both conditions was light yellow or cream while NDM provided bright yellow or yellowish green. Calli subcultured on NDM medium under light condition turned to green and then produced the highest percentage formation (79.8) and number of mature somatic embryos (9 embryos/callus). Within 1 month, those embryos germinated in the presence of AC in the culture medium. The shoots at length more than 3 cm with an average number of 2-3 roots were successfully transferred to soil. This empirical technique could be useful for micropropagation as well as genetic transformation in this plant.

Abbreviation: EC: embrygenic callus, VW: Vacin and Went, NDM: New Dogashima Medium, SE: somatic embryo, CW: coconut water, AC: activated charcoal

Key words: Chang Daeng, *Rhynchostylis rubrum*, micropropagation, embryogenic callus

Introduction

The orchid genus includes a large number of species. Not only their economical interest but they also present ecological interests by the diversity. *Rhynchostylis* is a genus of about 4 to 5 species from Thailand. Many attractive of *Rhynchostylis* orchid have become commercially important in potted plant industries. These species are monopodial orchid originated in Southeast Asia (Thailand, Lao and Vietnam) and need to be protected from the danger of extermination through deforestation. Therefore, a rapid multiplication in commercial scale by micropropagation of this specie is required.

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In vitro propagation via protocorms-like bodies (PLBs) of *R. gigantea* through young pod culture has been firstly reported by Vajarabhaya and Vajarabhaya (1970). Since then, there are few reports on microproapagation of *Rhynchostylis* using buds, leaf primodia (Vij *et al*., 1984) and thin cell layer (TCL) (Bui van Le *et al*., 1999) in *R. gigantea*, leaves or root in *R. retusa* (Vij *et al*., 1987). However, that method can not be used for commercial micropropagation effectively.

There are very few reports on callus cultures in orchids, this might be due to the slow growth and a necrotic tendency of orchid callus (Kerbauy, 1984; Kerbauy, 1991; Philip and Nainar, 1986; Colli and Kerbauy, 1993; Begum *et al*., 1994). Begum *et al*. (1994) reported that globular compact calli were induced from inner tissue of *Cymbidium* PLB, but these structures could not be subcultured, turned brown and died after 2 months of incubation. Chang and Chang (1998) successfully obtained calli from pseudobulbs, rhizomes, and root explants and maintained these in subculture as well as regenerated plants from callus in *Cymbidium ensifolium*, a terrestrial orchid species; however, this study did not focus on callus induction from explants. Plant regeneration from callus of orchid is usually achieved through PLB formation, a process that is suggested to involve somatic embryogenesis (Begum *et al*., 1994; Steward and Mapes, 1971; Ishii *et al*., 1998; Chen and Chang, 2000).

Recently, embryogenic callus (EC) from actively growing parts such as shoot tip or shoot tip of flower stalk have been successfully used by some workers for micropropagation and breeding of *Phalaenopsis* orchids (Ishii *et al*., 1998; Tokuhara and Mii, 2001), *Oncidium* (Chen and Chang, 2000), *Cymbidium* (Chang and Chang, 1998). These results suggest that the embryogenic callus could be utilized as the materials for commercial propagation of *Rhynchostylis.* So far, there are no reports on the induction of callus in *Rhynchostylis.* We, therefore, design to investigate the effects of culture media, culture environment and activated charcoal on callus or embryogenic callus induction and plant regeneration in *Rhynchostylis rubrum*.

Materials and methods

Plant material and callus induction

Rhynchostylis rubrum in vitro grown six-month-old-plantlets, of approximately 1-5 cm and native from Thailand, were used for explant source. Single plant was placed on 15 ml agar-solidified medium containing Vacin and Went (VW) mineral salts (Vacin and Went 1949), supplemented with 0.65% agar-agar, 2% sucrose, 15% coconut water (CW) and 0.1% activated charcoal

(AC). The pH of the medium was adjusted to 5.7 before sterilisation by autoclaving for 15 min at 120^{o}C.

Plantlet regeneration

Nodular calli from young vitro-plant were transferred to two different basal media; (1) New Dogashima Medium (NDM) (Tokuhara and Mii, 1993) which was developed for the culture of several orchids, (2) VW. Those media were supplemented with sucrose, CW and AC as mention earlier. The pH of the media was adjusted to 5.7 before autoclaving. To investigate the optimum conditions for callus proliferation the effects of two culture media in the presence or absence of AC and culture environment (light and dark) were carried out. The cultures were incubated at 26±2^{o}C. In case of culturing under light condition, fluorescent lamps at 10 μmol/m^{2}/s was applied for 14 h/day. After 3 and 5 month of culture, proliferation and development of the callus were recorded.

Statistical analysis

Approximately 50 young plants were used for callus induction in each size of the plants (1-5 cm). Observations were carried out once a week. The results were scored according to the average percentage of plant-forming callus. In case of plantlet regeneration, each treatment was done for three replications and each replication consisted of 10 to 30 calli. Completely randomized design was employed to determine statistical difference between the treatments.

Results and discussion

Influence of size of vitro-plant on multiple shoot and callus formation

The youngest shoot (approx. 10 mm) gave the best result in proliferation of shoots in term of both percentage (100) and number of shoots (10) formed from single shoot (Table 1). Multiple shoot formation decreased according to the longer or larger of cultured single shoot. The shoot larger than 30 mm (in height) fail to develop multiple shoots. Many orchids species require auxins and/or cytokinins for induction of both shoot and protocorm-like bodies (PLBs). The ratio of those plant growth regulators for development depends on the species (Arditti and Ernst, 1993). For *in vitro* regeneration of *R. gigantea* via PLBs, NAA at 0.1 mg/l was first reported by Vajarabhaya and Vajarabhaya (1970). In case of thin cell layer of this species, NAA (1 mg/l) in combination with BA (3

mg/l) were required for a large number of shoot bud formation (Bui van Le *et al*., 1999). However, proliferation of shoots in this present study required only cytokinin in form of CW. It is possible that single shoot as explant in this study could synthesize its own auxin. Addition of CW to the medium might improve the ratio of auxin and cytokinin for multiple shoot formation.

An optimum size for the highest multiple shoot induction was obtained from single shoot at 10 mm in height, followed by those from 20 mm shoots. The shoots higher than 30 mm failed to develop multiple shoot (Table 1). There have no reports on developing stage of protocorm or shoot on multiple shoot induction in tissue culture of orchid. It suggests that younger tissues (10-20 mm shoots) contain higher meristematic activities, especially lateral or axillary buds. Under an optimum ratio of auxin and cytokinin those buds give rise to mutiple shoots while older shoot (more than 20 mm in height) developed roots at those buds. Appropriate mineral composition of the media is essential for the successful PLB formation. Tokuhara and Mii (1993) reported that NDM gave the best results in PLB formation in *Phalaenopsis*. In this present study, culture media were not investigated. However, preliminary result revealed that NDM was inferior to VW (data not shown). Ishii *et al.* (1998) also reported that VW supplemented with 20% CW was suitable for PLB multiplication in *Phalaenopsis*. Richard Schaffer "Santa Cruz" in the absent of sucrose.

Table 1. Percentage of callus formation from various sizes of vitro-plants of *Rhynchostylis rubrum* on agar solidified VW medium supplemented with 2% sucrose, 15% CW and 0.2%AC for 6 weeks.

Size of plants (mm)	Multiple shoot formation (%)	No. of shoot (± SD)	Callus formation (%)	No. of callus/ shoot clump
10	100	10±1.6	56.6±13.1	1-3
20	62.5	5.6±0.5	22.3±2.1	1
30	27.4	1.0±1.4	0	0
40	0	0	0	0
50	0	0	0	0
>50	0	0	0	0

An optimum size for the highest callus induction (56%) was obtained from single shoot at 10 mm in height, followed by those from 20 mm shoots (22%). The shoots higher than 20 mm failed to develop calli (Table 1).All calli arose from the crown portion (between stem and rhizome). The characteristics of the calli were nodular, seem to be compact but friable and pale yellow to yellow in color (Fig. 1). Almost all callus induction from orchid tissue culture were obtained from PLB or PLB segment (Begum *et al*., 1994; Chang and Chang, 1998; Ishii *et al*., 1998; Chen *et al*., 2000). Many explants of *Rhynchostylis rubrum* have been investigated

for callus induction but all of the explants died (data not shown). Intact single shoot proved to be the best explant for callus induction. Both auxin and cytokinin are required for callus induction from all cultured explant (Begum *et al*., 1994; Chang and Chang, 1998; Chen *et al*., 2000). In our study, those PGR was not tested. VW supplemented with 2% sucrose, 15% CW and 0.2% AC was sufficient for yellow nodular callus formation. Ishii *et al.* (1998) reported the largest number of calli on medium containing CW in the presence of sucrose. The result may suggest that CW, sucrose together with AC could improve callus formation in *Rhynchostylis rubrum*.

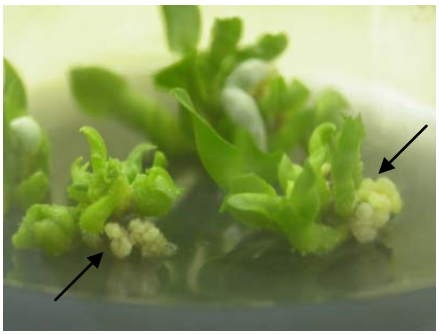

Fig. 1. Nodular calli obtained from the crown portion (arrows) of vitro-plant of *Rhynchostylis rubrum* after 4 weeks of transferred onto agar-solidified VW medium supplemented with 2% sucrose, 15% CW and 0.2% AC.

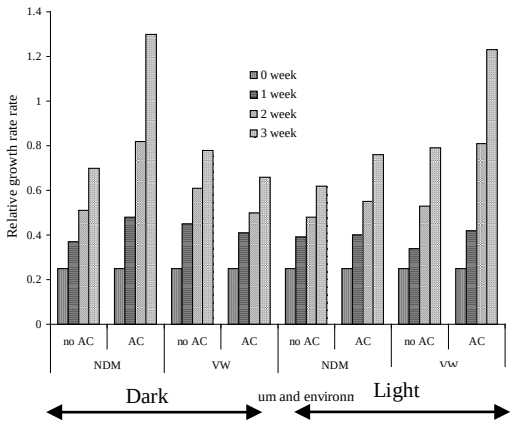

Fig. 2. Proliferation of callus in term of increase in fresh weight after culture on two different media in the presence or absence of AC under light or dark condition for 3 weeks.

Table 2. Effect of culture media (CM), culture environment (CE) and AC on development of green somatic embryo (SE) in embryogenic callus (EC) after 8 weeks of culture.

CE	CM	AC	Development of SE (%)	Number of SE	Characteristic of mature embryos
Dark	NDM	+	6.23	0.125	Hyperhydricity
		-	0	0	-
	VW	+	0	0	-
		-	0	0	-
Light	NDM	+	79.86	9	Normal
		-	6.23	0.125	Normal
	VW	+	18.75	0.75	Normal
		-	6.23	0.125	Normal

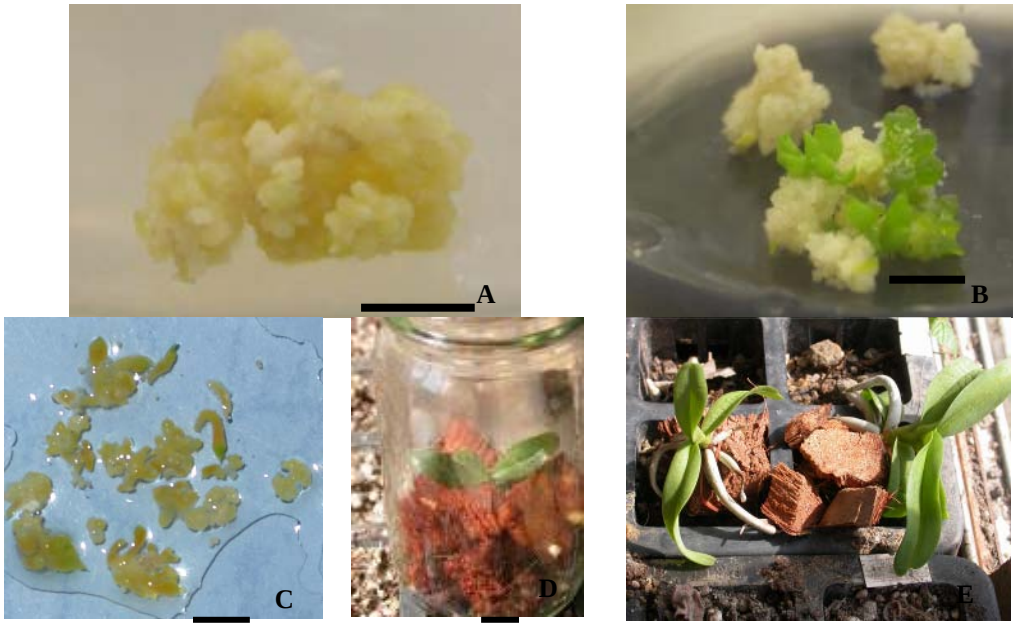

Fig. 3. Development of somatic embryos after transfer to light condition for one month and germination of them to complete plantlet. The culture medium was NDM supplemented with 2% sucrose, 15% CW and 0.2% AC. (bar=5 mm)
A: Formation of opaque somatic embryos (SE); B: Individual SE was isolated from embryonic mass and form green SE; C: Various stages of SE in liquid medium; D and E: Acclimatization of complete plantlets

Plantlet regeneration

Calli induced on VW medium containing 2% sucrose, 15%CW and 0.2%AC continue to proliferate after being transferred to both medium in two

different conditions of culture. Proliferation of the calli in both culture media (CM) and culture environments (CE) was nearly the same (Fig. 2) but the color of them was quite different. The color of calli in VW in both CE was light yellow or cream while NDM provided bright yellow. However, the culture in light conditions could induce the development of green somatic embryos in both CM. Surface appearance of callus changed to more compact, opaque and whitish yellow color and small protuberances were formed in the first 2-3 weeks of culture (Fig. 3A). On the other hand, the nodular structure in calli subcultured on NDM medium under light condition turned green and then produced a mature somatic embryo (Fig. 3B). The highest percentage formation (79.8) and number (9) of this structure were obtained in this culture medium (Table 2). Within 1 month, the protuberances continued to grow and became isolate into individual green somatic embryo when dispersed in liquid medium (Fig. 3C). In the presence of AC in CM, the best results in both percentage and number of green somatic embryos were obtained. These results suggest that development of green somatic embryos was greatly affected by AC in the medium and that the frequency of green somatic embryo formation was enhanced by light condition. Contrary to the results obtained from embryogenic callus proliferation in *Phalaenopsis* (Chen *et al*., 2000), the calli could be well multiplied on PGR containing medium under dark condition. Moreover, Ishii *et al*. (1998) reported that sucrose was the key factor for callus induction whereas CW promoted PLB formation. In another orchid, carbon sources have been also reported to play significant role on plant regeneration from callus/embryogenic callus (Ishii *et al*., 1998; Chen *et al*., 2000; Chang and Chang, 1998). Unfortunately, there have no reports of this substance on callus proliferation and plantlet regeneration in *Rhynchostylis* spp., including *Rhynchostylis rubrum*. On the other hand, plant growth regulators are considered to accelerate the growth and development of PLBs, callus and plantlet formation from callus (Chen *et al*., 2000; Bui van Le *et al*., 1999). To improve more proliferation and regeneration of plantlet through somatic embryogenesis in *Rhynchostylis rubrum* it is suggest that sucrose concentration should be tested. So far, from the results obtained by adding 2% sucrose to NDM or VW medium in this study, it seem to be sufficient enough to proliferate callus subsequent to plantlet regeneration like that report in culturing leaf-segment of *Phalaenopsis* (Ishii *et al*., 1998).

This simple, rapid and efficient system for embryogenic callus induction from young shoot and plant regeneration from callus through somatic embryo structures in *Rhynchostylis rubrum.* The high embryogenic callus could be maintained on the NDM medium with 2% sucrose, 15% CW and 0.2% AC without plant growth regulators. The presence of light conditions is generally

essential for embryo initiation and maturation. This empirical technique could be useful for micropropagation as well as genetic transformation in this plant.

Transplantation

The shoots >3 cm long bearing on average 2-3 roots were planted in 24-well-tray containing coconut husk and covered with a plastic film in order to maintain humidity during acclimatization for 3-4 weeks (Fig. 3) before their transfer to greenhouse.

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