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Original Article

Micropropagation of Chang Daeng (*Rhynchostylis gigantea* var. Sagarik) by embryogenic callus

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Abstract

Efficient propagation of Chang Daeng through embryogenic callus was investigated. 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 one 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.

Keywords: Chang Daeng, *Rhynchostylis rubrum*, micropropagation, embryogenic callus

1. Introduction

Orchids are not only important in terms of economical aspects but also attractive in terms of ecological issues, like the diversity of species. *Rhynchostylis* is a genus of monopodial growth habit and native to Southeast Asia (Thailand, Lao, and Vietnam) including about 4 to 5 native species in Thailand. Many attractive *Rhynchostylis* orchids have become commercially important in potted plant industries. These species needed to be protected from the danger of extermination through deforestation. Therefore, a rapid multiplication in commercial scale by micropropagation of this species is required.

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In vitro propagation via protocorm-like bodies (PLBs) of *R. gigantea* through young pod culture has been firstly reported by Vajarabhaya and Vajarabhaya (1970). Since then, there have been few reports on microproapagation of *Rhynchostylis* using buds and leaf primodia (Vij *et al.*, 1984) and thin cell layer (TCL) (Bui van Le *et al.*, 1999) in *R. gigantean*, and leaves and roots in *R. retusa* (Vij *et al.*, 1987). However, these methods cannot be used for commercial micropropagation effectively. There are very few reports on callus cultures in orchids, which might be due to the slow growth and a necrotic tendency of orchid callus (Kerbauy, 1984; Philip and Nainar, 1986; Kerbauy, 1991; 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 two months of incubation. Chang and Chang (1998) successfully obtained calli from pseudobulbs, rhizomes, and root explants of

Cymbidium ensifolium, a terrestrial orchid species, maintained these calli in subculture, and succeeded to obtain regenerated plants from the calli in; however, this study did not focus on callus induction from explants. Plant regeneration from callus of orchid is usually achieved through PLB formation, which is suggested to involve somatic embryogenesis (Steward and Mapes, 1971; Begum *et al.,* 1994; Ishii *et al.*, 1998; Chen and Chang, 2000).

Recently, embryogenic callus (EC) has been successfully obtained from actively growing parts such as shoot tip and shoot tip of flower stalk by some workers for micropropagation and breeding of *Phalaenopsis* orchids (Ishii *et al.*, 1998; Tokuhara and Mii, 2001), Oncidium (Chen and Chang, 2000), and Cymbidium (Chang and Chang, 1998). These results suggest that the embryogenic callus could be utilized as the materials for commercial propagation of *Rhynchostylis*. However, 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.*

2. Materials and Methods

2.1 Plant material and callus induction

In vitro grown six-month-old-plantlets with approximately 1-5 cm height of *Rhynchostylis rubrum*, which is native to Thailand, were used for explant sources. Each plantlet was placed on 15 ml agar-solidified medium containing Vacin and Went (VW) mineral salts (Vacin and Went 1949), supplemented with 0.65% agar, 2% sucrose, 15% coconut water (CW), and 0.1% activated charcoal (AC). The pH of the medium was adjusted to 5.7 before sterilization by autoclaving for 15 min at 120°C.

2.2 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, and (2) VW. Those media were supplemented with sucrose, CW and AC as mentioned earlier. The pH of the media was adjusted to 5.7 before autoclaving. The effects of two culture media on callus proliferation were investigated in the presence or absence of AC and under light or dark conditions. The cultures were incubated at 26± 2°C. In case of culturing under light condition, fluorescent lamps at 10 μmol/m^2/s were applied for 14 hrs/day. After 3 and 5 months of culture, proliferation and development of the callus were recorded.

2.3 Statistical analysis

Approximately 50 young plantlets 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 with three replications and each replication consisted of 10 to 30 calli. Completely randomized design was employed to determine statistical difference among the treatments.

3. Results and Discussion

3.1 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 terms of both percentage (100) and number of shoots (10) formed from single shoot (Table 1). Multiple shoot formation decreased as cultured single shoot were longer or larger. The shoots larger than 30 mm (in height) failed to develop multiple shoots. Many orchid 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 of those organs 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

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 explants (mm)	Multiple shoot Formation (%)	Number of shoots (±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

shoot bud formation (Bui van Le *et al.,* 1999). However, proliferation of shoots in the present study was achieved by applying only CW, which is known to contain natural cytokinins (Nasib *et al.,* 2008). It is possible that a single shoot as explant in this study could synthesize its own auxin and addition of CW to the medium might have improved the ratio of auxin and cytokinin for multiple shoot formation. There have been no reports on developmental stage of protocorm or shoot on multiple shoot induction in tissue culture of orchid. It suggests that younger tissues (10-20 mm shoots) have higher meristematic activities, suggests that younger tissues (10-20 mm shoots) have higher meristematic activities, especially in lateral or axillary buds. Under an optimum ratio of auxin and cytokinin those buds in younger shoots (10-20 mm) gave rise to multiple shoots while older shoots (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 the present study, effect of the kind of culture media on PLB formation was not investigated. However, our 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 absence of sucrose. In almost all case, calli of orchids were induced from PLB or PLB segment (Begum *et al.,* 1994; Chang and Chang, 1998; Ishii *et al.,* 1998; Chen and Chang, 2000). In our preliminary experiment, many explants of *Rhynchostylis rubrum* including PLB have been investigated for callus induction but all of the explants died (data not shown). In the present study, we first showed that intact single shoot is the suitable explant for callus induction. An optimum size for the highest callus induction (56%) was revealed to be the single shoot at 10 mm in height, followed by those from 20 mm shoots (22%). The shoots higher than 20 mm failed to developed calli (Table 1). All calli arose from the crown portion (between stem and rhizome). The characteristics of the calli were nodular, not compact but friable and pale yellow to yellow in color (Figure 1). In the previous studies, both auxin and cytokinin were required for callus induction from all cultured explants (Begum *et al.*, 1994; Chang and Chang, 1998; Chen and Chang, 2000). In our study, those PGRs were 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 in *Phalaenopsis*. The result may suggest that CW, sucrose together with AC could improve callus formation in the present study on *Rhynchostylis rubrum.*

3.2 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 under light and dark conditions (CE) was nearly the same (Figure 2) but the color of them was quite different. The color of calli in VW under both CE was light yellow or cream, while NDM provided bright yellow calli. However, transfer of the culture from dark to 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 on VW medium (Figure 3A). On the other hand, the nodular structure in calli subcultured on ND medium under light condition turned green and then produced mature somatic embryos (Figure 3B). The highest percentage formation (79.8) and number (9) of nodular structure were obtained in this culture medium (Table 2). Within one month, the protuberances continued to grow and became isolated into individual green somatic embryos when dispersed in liquid medium (Figure 3C).

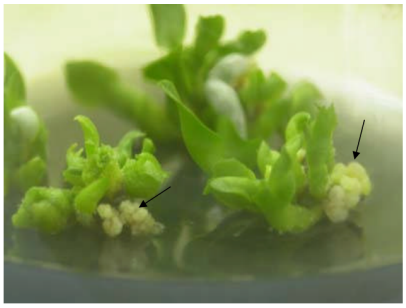

Figure 1. Nodular calli obtained from the crown portion (arrows) of vitro-plant of *Rhynchostylis rubrum* after 4 weeks of transferred onto agar-solidified VW medium supplemented with 2% sucrose, 15% CW and 0.2% AC.

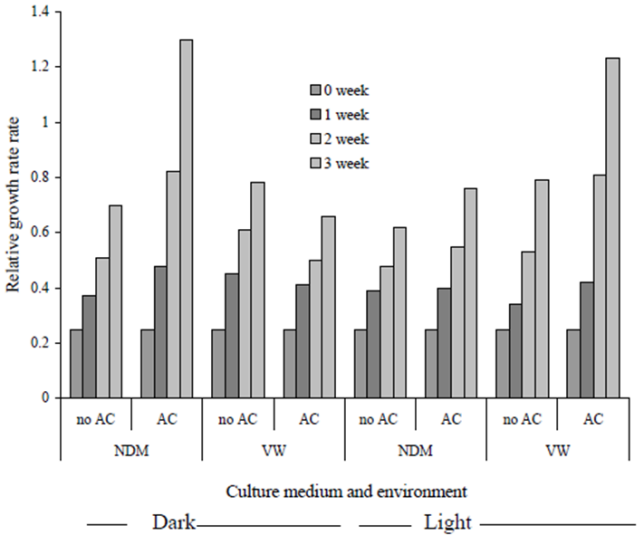

Figure 2. Proliferation of callus in term of increase in fresh weight after culture on two different media in the presence or absence of AC under light or dark condition for 3 weeks.

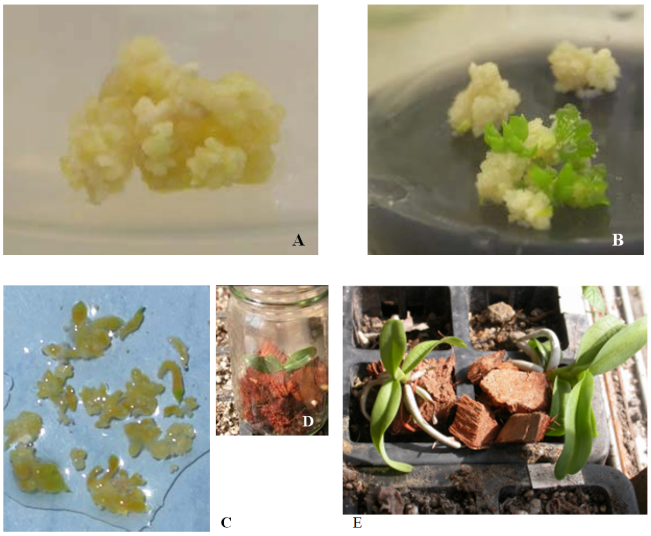

Figure 3. Development of somatic embryos after transfer to light condition for one month and germination of them to complete plantlet. The culture medium was NDM supplemented with 2% sucrose, 15% CW and 0.2% AC, (bar = 5 mm). A: Formation of opaque somatic embryos (SE), B: Individual SE was isolated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

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 results were obtained from embryogenic callus proliferation in *Phalaenopsis*. The calli of *Phalaenopsis* could be well multiplied on PGR containing medium under dark condition (Chen and Chang, 2000). Moreover, Ishii *et al.* (1998) reported that sucrose was the key factor for callus induction whereas CW promoted PLB formation. In other orchids, carbon sources have been also reported to play significant role on plant regeneration from callus/embryogenic callus (Ishii *et al.*, 1998; Chen and Chang, 2000; Chang and Chang, 1998). Unfortunately, there have been no reports of CW 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 formation, and plantlet formation from callus (Bui van Le *et al.*, 1999; Chen and Chang, 2000). To improve the proliferation and regeneration of plantlet through somatic embryogenesis in *Rhynchostylis rubrum,* it might be necessary to test the effect of sucrose concentration on the callus proliferation and plant regeneration. So far, from the results obtained by adding 2% sucrose to NDM or VW medium in this study, it seems to be sufficient enough to proliferate callus and for subsequent plantlet regeneration like that reported in culturing leaf-segment of *Phalaenopsis* (Ishii *et al*., 1998). In the present study, we have succeeded in establishing a 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 embryogenic callus could be maintained efficiently on the ND 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.

3.3 Transplantation

The shoots of >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 (Figure 3D,E) before their transfer to greenhouse.

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	Characteristics of mature embryo
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

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Abbreviation

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