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Full Length Research Paper

In vitro propagation of white-flower mutant of *Rhynchostylis gigantea* (Lindl.) Ridl. through immature seed-derived protocorm-like bodies

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Rapid *in vitro* propagation of white-flower mutant of *Rhynchostylis gigantea* (Lindl.) Ridl. was established for the first time through 4 month old immature seed-derived protocorm-like bodies (PLBs). 75% of PLBs developed from immature seeds cultured on half-strength MS medium with 0.05 mg·l^{-1} 6-BA and 0.2 mg·l^{-1} NAA within 70 days upon transfer to MS medium with 2.0 mg/l^{-1} BA and 1.0 mg·l^{-1} NAA were induced to produce callus. Maintenance of the callus was achieved by subculturing the callus at an interval of 2 months on medium having the same level of growth regulators. The callus turned up to protocorm-like bodies upon transfer to hormone-free MS medium after 30 days of culture and later developed to shoots with 2-4 leaves. Transfer of the emerged shoots to half strength MS medium containing 100 g·l^{-1} banana homogenate facilitated root development and shoot growth. Plantlets established *ex vitro* exhibited 95% survival.

Key words: Plant regeneration, protocorm, *Rhynchostylis gigantea* (Lindl.) Ridl., somatic embryogenesis.

INTRODUCTION

Somatic embryogenesis provides a suitable system for efficient micropropagation of rare plants and for production of transgenic plants. There are only a few recent reports that provide evidence for direct or indirect somatic embryogenesis in orchids: Oncidium (Chen et al., 1999; Chen and Chang, 2000a, b, 2002, 2004), Cymbidium (Cheng and Chang, 1998), Cleisostoma (Temjensangba, 2005) and Phalaenopsis (Ishii et al., 1998). So far, there have been two reports on the micropropagation of *Rhynchostylis gigantea* (Lindl.) Ridl. wild type (Li and Xu, 2006; Wen and Zhang, 2008). Wild type of R. gigantea was characterized with purple labellum and other white petals that have purple spots. Because of its full-bodied aroma and long florescence, excessive exploitation of wild R. gigantea for commercial demand made this species endangered (Figure 1 A). Recently, we found a natural mutant of *R. gigantea* that the petals and labellum are all white (Figure 1B). In order to efficiently protect the germ plasm resources of *Rhynchostylis* Blume, supply enough materials for future study and commercial demand, we established an efficient regeneration system of *Rhynchostylis* from immature seeds through somatic embryogenesis.

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Abbreviations: 6-BA; N6-benzyladenine, **MS;** Murashige –Skoog (1962) medium, **NAA;** α-naphthaleneacetic acid and **PLBs;** protocorm-like bodies.

MATERIALS AND METHODS

Plant materials

Four-month-old immature capsules of white flower mutant of *R. gigantea* were surface disinfected with 70% (v/v) ethanol for 1 min and 0.1% (w/v) $HgCl_2$ for 10 min followed by four rinses with sterile water. After absorbed water with sterile filter paper, the immature seeds were used as explants.

Medium composition and culture conditions

The basal medium was consisted of MS mineral salts and vitamins added with sucrose 30 g·l^{-1} and agar 6.0 g·l^{-1}. The banana was mashed before being added into the M7. The pH was adjusted to 5.6 with 1 mol l^{-1} NaOH or 1 mol l^{-1} HCl prior to autoclaving at 121ºC for medium supplemented with 0.05 mg l^{-1} BA in combination with 0.2

Figure 1A. Flower of *R. gigantea* (Lindl.) Ridl. Flower of mutant of *R. gigantea* (Lindl.) Ridl.

Figure 1B. Flower of *R. gigantea* (Lindl.) Ridl. Flower of wide type of *R. gigantea* (Lindl.) Ridl.

15 min. The growth regulators (BA and NAA) were filter-sterilized before being added to the autoclaved culture media. Contents of media used in this experiment were listed in Table 1. Cultures were incubated at (25 ± 2)℃ under 14 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 45 - 50 μmol m^{-2} s^{-1}.

Somatic embryo induction

The exiguous white seeds were transferred to half-strength MS

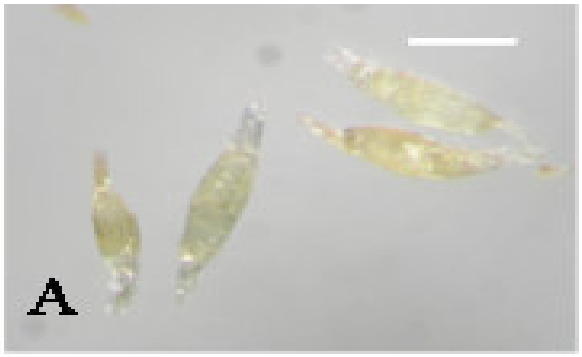

Figure 2A. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Expanded immature seeds after 25 days culture (bar 1000 μm).

mg l^{-1} NAA (M1) to induce protocorm. The seeds in each bottle were about 300 and the total bottles were 16. The number of seeds and protocorms were counted under stereomicroscope (Nikon C-DSD230) at the end of 70 days. The induction rates of protocorm were the means of 16 repeats. Then the protocorms were transferred to MS basal medium plus 2.0 mg l^{-1} BA in combination with 0.2, 0.5, 1.0 or 2.0 mg l^{-1} NAA (M2-M5) to induce somatic embryos. Every 18 ± 2 protocorms were cultured per bottle and 10 bottles for each treatment.

Shoot induction and plant development

Somatic embryos were transferred to shoot induction medium (M6) and incubated for 3 months. The isolated shoots with 2 real leaves were transferred to half-strength hormone-free MS medium added with 100 g l^{-1} banana (M7) to develop into plantlets.

RESULTS

Protocorm induction from immature seeds

Immature seeds from 4 month old capsules of the white flower mutant expanded after 25 days, differentiated into white early stage protocorm after 40 days of culture on M1 (Figures 2A -B). The induction rate of protocorms from 4 month old seeds was about 15% (Table 1). Most of the immature seeds turned brown and shrived. In this stage, the protocorms were immature, semitransparent, and only 0.5 - 1.0 mm. Each early stage protocorm would develop into a plantlet by subculture on MS basal medium for about 4 months (data not shown).

Somatic embryo induction

The early stage protocorms were transferred to MS medium supplemented with 2.0 mg l^{-1} BA in combination with different contents of NAA. After 2 months culture, the explants began to differentiated green protuberance (Figure 2C). As culture progressed, many finger-like and semi-transparent somatic embryos were formed from the explants (Figure 2D), and the number of somatic embryos per explants ranged from 5 to 40. The embryogenesis of early stage protocorms was 12 - 75% on medium added with different combination of BA and NAA (Table 1). The

Table 1. Induction rate of immature protocorm on M1 and the effects of different combination of BA and NAA on embryogenesis of protocorm.

No. of medium	Contents of medium (mg l^{-1})	Percentage of immature protocorm formation	Embryogenesis of protocorm (%)
M1	1/2MS + BA 0.05+NAA 0.2	15.7	-
M2	MS + BA 2.0+NAA 0.2	-	25.3
M3	MS + BA 2.0+NAA 0.5	-	40.3
M4	MS + BA 2.0+NAA 1.0	-	75.7
M5	MS + BA 2.0+NAA 2.0	-	41.1
M6	MS	-	-
M7	MS + banana 100 g l^{-1}	-	-

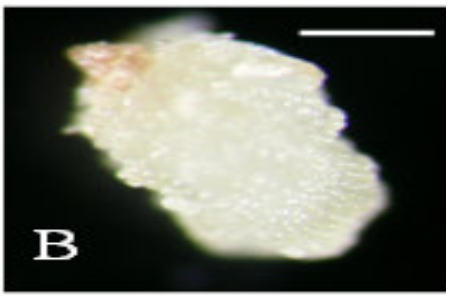

Figure 2B. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. White protocorm at early stage after 40 days culture (bar: 200 µm).

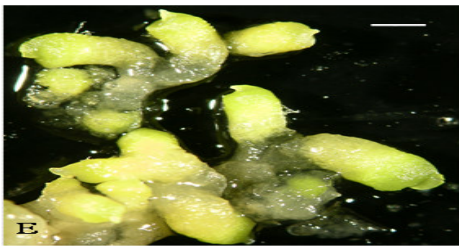

Figure 2E. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. Through somatic embryos. Isolated protocorm with one cotyledon on MS medium for 30 days (bar: 500 µm).

Figure 2C. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Embryogenic callus (bar: 200 µm, arrow mark protuberance that will develop into somatic embryo).

Figure 2D. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Fingerlike somatic embryos (bar: 500 µm).

greatest frequency of somatic embryos induction (75%) was on MS medium supplemented with 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA. Embryonic cultures could be maintained and proliferated by transferring to the same fresh medium every 2 months.

Shoot induction from somatic embryos

The fingerlike somatic embryos developed into protocorm-like bodies (PLBs) by culturing on MS basal medium for 30 days. The top of the somatic embryos expanded and differentiated one cotyledon (Figure 2E). As culture processed, many trichomes developed from the bottom of the protocorms and leaves differentiated form the top of the protocorms (Figures 2F and G). These trichomes disappeared gradually as the leaves developed. This process was similar to that of the development of seed-derived protocorms (data not shown). More than 90% of the somatic embryos developed into PLBs and germinated by culturing on MS basal medium for 2 months.

Plantlet development

Isolated shoots with 2 - 3 leaves were transferred to hormone-free MS medium added with 100 g l^{-1} banana. On this medium, shoot buds of R. gigantea grew well and rooted quickly. The leaves elongated to 1 - 3 cm and the

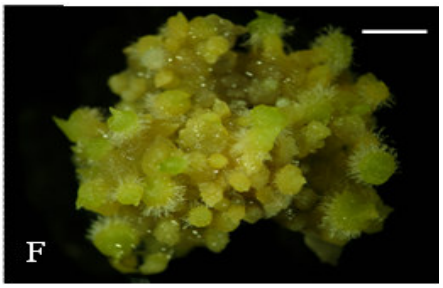

Figure 2F. Plant regeneration from imamture seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Trichomes developed from the protocorm on MS medium for 38days (bar: 2 mm).

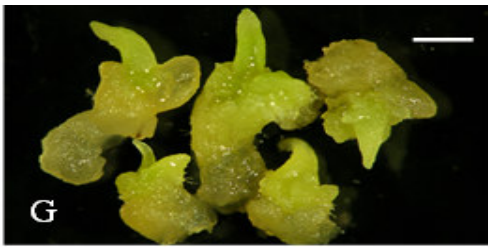

Figure 2G. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Isolated shoots with one real leaves cultured on MS medium for 45days (bar: 1mm).

Figure 2H. Plant regeneration from immature seeds of white flower mutant of *R. gigiantea* (Lindl.) Ridl. through somatic embryos. Plant- lets in bottle (bar: 1cm).

roots elongated to 0.5 - 2.0 cm by 80 days of culture shoot (Figure 2 H). Rooted plantlets were washed medium and planted in the mixture of coconut fibre and sand (3:1) and acclimatized in a mist house. The surviving rates of *R. gigantea* were more than 95%.

DISCUSSION

Orchids efficient regeneration could be obtained by using a few kinds of explants: leaf (Temjensangba, 2005; Chen and Chang, 2000a, 2004; Decruse et al., 2003), flower-stalk (Chen and Chang, 2000b), node (Gangaprasad et al., 2000; Martin et al., 2005; Geetha and Shetty, 2000), or inflorescence tip (Geetha and Shetty, 2000; Ravindra et al., 2004; Malabadi et al., 2005; Lam-Chan and Lee, 1996) and mature or immature seeds (Nagayoshi et al., 1996; Lin and Hsieh, 2002; Hirano et al., 2004; Thammasiri, 2000; Kitsaki et al., 2004). The calli or protocorm-like bodies (PLBs) from seeds, protocorms, or shoot tips were not only good explants for propagation, but also usable materials for genetic transformation (Liau et al., 2003; Men et al., 2003). In this report, calli and PLBs of the white flower *R. gigantea* mutant were efficiently induced from the seed-derived early stage protocorms on medium supplemented with 2.0 mg l^{-1} BA and 1.0 mg l^{-1} NAA. PLBs can be maintained and proliferated by transferring to the same fresh medium every 2 months. These PLBs were easily germinated on hormone-free MS medium, rooted on MS medium contain 100 g l^{-1} banana and may be used as target explants for transformation.

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