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Full Length Research Paper

In vitro plant regeneration of *Esmeralda clarkei* Rchb.f. via protocorm explant

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***Esmeralda clarkei* is an epiphytic native orchid species of Nepal growing under medium amount of light with fragrant flowers. Reliable protocols for *in vitro* plant regeneration of *E. clarkei* via protocorm explants were developed. Protocorms obtained from *in vitro* germinated seeds cultured on Murashige and Skoog's (MS) medium supplemented with 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) individually and in combinations responded positively by induction of multiple shoots. The cultures were maintained at 25 ± 2°C under a 16/8h light/dark cycle photoperiod provided by fluorescent lamps (Philips, India). BAP increased from 0.5 to 2.0 mg/l individually induced the maximum number of shoots (11 to 14 shoots per treatment) which is followed by combinations of BAP (0.5, 1.0 and 1.5mg/l) and NAA (0.5mg/l) which induced 7 to 8 shoots per treatment. The highest number of roots (3 roots per treatment) was observed in NAA (0.5 and 1.0 mg/l) supplemented medium among the other tested medium.**

Key words: 6-Benzylaminopurine, *Esmeralda clarkei*, *in vitro*, α-naphthalene acetic acid, protocorm.

INTRODUCTION

Esmeralda clarkei Rchb.f., an epiphytic monopodial orchid native to Nepal (Pearce and Cribb, 2002), grows on *Rhododendron* sp. in cool to intermediate climatic conditions under medium amount of light. Plants blooms from fall to winter with three to four fragrant flowers (Ghimire, 2008). It has high commercial value in horticulture and is faced with extensive over-collection and habitat destruction pressures. As a consequence, its populations are depleted and the species is now restricted to very narrow pockets in its natural habitats. Commercially threatened species require immediate conservation measures. *E. clarkei* has a low rate of multiplication under natural/greenhouse conditions, and like other monopodial orchids, survival of mother plant is not conducive to a shoot tip/meristem based micropropagation system. It is thus necessary to devise a rapid and efficient micropropagation protocol for obtaining true-to-type regenerants without detriment to the survival of mother plant/donor plant and saving its populations from getting rarer in nature (Kapai et al., 2010). Methods for rapid multiplication of orchids are essential to meet the commercial demand. *In vitro* propagation is a valuable tool for orchids in this context. Previous work on *in vitro* culture include propagation from explants such as *Cymbidium* shoot tips (Morel, 1960), *Dendrobium moschatum* stem (Kanjilal et al., 1999) ad *Cymbidium ensifolium* rhizomes (Chang and Chang, 2000).

A significant number of identical clones can be raised from a single protocorm and shoot tip explants through direct or callus mediated organogenesis (Arditti, 1977). Because of lack of protocol, it is necessary to develop the reliable procedure for its micropropagation. In the present

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Abbreviations: ANOVA, Analysis of variance; **BAP,** 6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **NAA,** α-naphthalene acetic acid; **SD,** standard deviation; **SPSS,** Statistical Package for Social Sciences.

paper, we reported for the first time, an efficient multiplication method through protocorm of *E. clarkei* to produce plantlets. This protocol is simple, reproducible and suitable for mass propagation.

MATERIALS AND METHODS

Plants of *E. clarkei* were collected from their natural habitat, Gaurishankar Conservation Area, Dolakha (Alt. 1330 m), Nepal and planted in the botanical garden of Central Department of Botany, Tribhuvan University, Kathmandu. Green capsule of this plant were harvested and brought to the laboratory for *in vitro* germination.

Establishment of culture by *in vitro* seed germination

The harvested immature green capsule was rinsed with detergent in running water for 1 to 2 h to remove the adhering soil particles. The capsule was surface sterilized with 70% ethanol for 2 min and then with 1% sodium hypochlorite solution for 10 min. The capsule was rinsed with sterile distilled water and allowed to air dry. Green capsule was dissected longitudinally with sterile surgical blade. The immature seeds scooped out of the sterilized capsule were germinated in the solidified full strength MS medium (Murashige and Skoog, 1962).

The medium was solidified with Agar-agar (Qualigens, India) at a concentration of 0.8% (w/v). The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 min at 15 psi, 121°C. The cultures were maintained at 25 ± 2°C under a 16/8 h light/dark cycle photoperiod provided by cool white fluorescent lamps (Philips, India). Protocorms developed from germination of seed were used as explant for further investigation.

In vitro plant regeneration from protocorm culture

The experiment was performed with the protocorms obtained from *in vitro* germination of seeds. Single protocorm was inoculated on solidified full strength MS medium with different concentrations of BAP (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) and NAA (0.0 and 0.5 mg/l) individually and in combination with NAA (0.5 mg/l). The cultures were maintained in the culture condition as mentioned above. After 120 days of culture, morphogenetic response of the treatments was evaluated in terms of frequency of shoot development from the protocorms.

Rooting of regenerated shoots

For the root induction experiments, the multiple shoot obtained from the above experiment were used. Single un-rooted shoot (3 to 4 cm in length) were cultured in MS medium supplemented with different concentration of NAA, IAA and IBA (0.5, 1.0, 1.5 and 2.0 mg/l). The cultures were incubated for 3 months under the conditions described for the previous experiment.

Experimental design and statistical analysis

All the experiments were conducted with a minimum of six replicates per treatment and the experiments were repeated once. The results are expressed as mean ± SD of two experiments. Data of shoot induction from protocorm were subjected to analysis of variance (ANOVA) and means were compared by Duncan's multiple range test at $P<0.05$ using SPSS ver. 11.5 (SPSS Inc., USA). Data of root induction were analyzed by paired sample t-test.

RESULTS AND DISCUSSION

The regeneration competence of the protocorm explants seems to be markedly influenced by different growth regulators in nutrient media (Vajrabhaya, 1978). In the present investigation, regeneration capacity of protocorm explants was tested for *E. clarkei*. Protocorms obtained from *in vitro* cultures of seeds, used as explants responded readily on MS medium supplemented with or without growth regulators of various concentrations. Addition of various concentrations of BAP and NAA was essential for the formation of plantlets from protocorm explants indicated by significant variations in the morphogenetic characters of plantlets (Table 1). Among the different combinations tried in this study, BAP (0.5, 1.0 and 1.5 mg/l) combination with NAA (0.5 mg/l) considerably induced many shoots (7 to 8 shoots per treatment) from single protocorm explants without any root differentiation (Figure 1).

This result was analogous to multiple shoot production from protocorm of *Dendrobium* (Sheela et al., 2004; Yin and Hong, 2009), *Aerides crispum* (Murthy, 2005), *Cleisostoma racemiferum* (Deb and Temjensangba, 2006) and protocorm proliferated into plantlets of *Vanda helvola* (David et al., 2008). The MS medium supplemented with combination of BAP (2.0 mg/l) and NAA (0.5 mg/l) had least number of shoot production as compared to other combination tried. Protocorm explants showed variably increase in the shoots number (11 to 14 shoots per treatment) as the concentration of BAP increased from 0.5 to 2.0 mg/l (Table 1 and Figure 2) as compared to BAP and NAA together. The result agrees with the results for *Dendrobium chrysotoxum* and *Dendrobium* crosses (Nagaraju et al., 2004), *Dendrobium* (Hsia et al., 2005), *Coelogyne stricta* (Basker and Narmatha, 2006) and *Cymbidium aloifolium* (Nongdam and Chongtham, 2011). MS medium without any growth regulators also gives 7 shoots per treatment from protocorm explants (Figure 3) which is similar to that of *Dendrobium* (Sheela et al., 2006). Shoot length showed significant variation in all the media. The longest shoot length (3.5 to 3.6 cm) was observed on protocorms cultured with 0.5 mg/l of NAA.

The well developed un-rooted shoot was removed from the culture tubes and transferred to the rooting media supplemented with various concentrations of NAA, IAA and IBA. All media positively responded to development of roots. In this study, 90% of the shoots rooted well after been transferred to these media (Table 2). The highest number of roots occurred on shoot cultured on medium supplemented with NAA (0.5 and 1.0 mg/l) with an average of 3 roots per shoot (Figure 4). In this condition, the rooting started after 3 weeks of culture. The concentration of NAA increased from 1.5 to 2.0 mg/l and different concentrations of IAA and IBA showed poor induction of root in the cultured shoot. This may be due to the presence of enhanced level of NAA. This result agree

Table 1. *In vitro* plant regeneration from protocorm explants cultured on Murashige and Skoog (MS) media with different concentrations of 6-benzyl aminopurine (BAP) and naphthalene acetic acid (NAA).

Medium	NAA (mg/l)	BA (mg/l)	Mean no. of shoots (±SE)	Mean length of shoot (cm) (±SE)	Mean no. of roots (±SE)	Mean length of root (cm) (±SE)
1	0	0	7.00±3.69^{b}	3.55±0.55^{a}	0.75±0.47^{b}	0.50±0.28^{b}
2	0.5	0	7.50±2.72^{b}	3.67±0.56^{a}	3.00±0.40^{a}	2.37±0.23^{a}
3	0	0.5	14.00±2.48^{a}	2.87±0.23^{a}	0.0^{c}	0.0^{c}
4	0	1.0	13.75±1.25^{a}	2.75±0.32^{b}	0.0^{c}	0.0^{c}
5	0	1.5	11.50±3.88^{a}	2.25±0.32^{b}	0.0^{c}	0.0^{c}
6	0	2.0	14.00±2.34^{a}	2.37±0.23^{b}	0.0^{c}	0.0^{c}
7	0.5	0.5	7.50±1.89^{b}	1.75±0.32^{bc}	0.0^{c}	0.0^{c}
8	0.5	1.0	7.25±1.70^{b}	2.50±0.20^{b}	0.0^{c}	0.0^{c}
9	0.5	1.5	8.75±1.93^{b}	2.50±0.35^{b}	0.0^{c}	0.0^{c}
10	0.5	2.0	4.75±2.42^{c}	3.12±0.23^{a}	0.0^{c}	0.0^{c}
Chi square			13.53	14.97	32.54	32.77
P			0.14	0.09	0.0	0.0

Culture conditions: 25 ± 2°C, 120 days of culture, 16 h photoperiod, 6 replicates were used in each combination. The values with same superscript are not significantly different at P≤0.05.

Table 2. Induction of root on shoot cultured on MS media with different concentrations of NAA, IAA and IBA.

PGRs used	Concentrations (mg/l)	Mean number of roots (±SE)	Mean length of root (cm) (±SE)
NAA	0.5	3.0±0.40	2.12±0.12
	1.0	3.0±0.57	2.93±0.47
	1.5	1.25±0.25	2.12±0.23
	2.0	2.67±0.33	1.67±0.44
IAA	0.5	2.0±0.40	1.25±0.14
	1.0	2.33±0.33	1.67±0.16
	1.5	2.0±0.40	1.5±0.20
	2.0	2.0±0.57	1.33±0.33
IBA	0.5	2.25±0.25	1.75±0.32
	1.0	1.67±0.33	1.66±0.16
	1.5	2.25±0.47	1.25±0.14
	2.0	1.33±0.33	1.0±0.0

Culture conditions: 25 ± 2°C, 90 days of culture, 16 h photoperiod, 6 replicates were used in each combination.
PGRs, Plant growth regulators; NAA, naphthalene acetic acid; IAA, indole-3-acetic acid; BAP, 6-benzyl aminopurine.

with previous findings of rooting of *Micropera pallida* (Bhadra and Hossain, 2004), *Coelogyne stricta* (Basker and Narmatha, 2006), *Dendrobium strongylanthum* (Kong et al., 2007) and *Vanda tessellata* (Rahman et al., 2009).

Conclusion

The regeneration potential of plantlets greatly depend on the growth regulators in the medium, and the presence of growth regulators proved significant in increasing the development of the plantlets. The ability of protocorms of orchids to regenerate multiple shoot suggests that *in vitro* culture can be successfully employed for rapid multiplication by suitably adjusting the nutrient environment.

Large number of shoots was induced when increasing concentration of BAP from 0.5 to 2.0 mg/l followed by BAP (0.5, 1.0 and 1.5 mg/l) combination with
NAA (0.5 mg/l) supplemented medium. Large number of roots was induced in shoot on medium supplemented with NAA increased from 0.5 to 1.0 mg/l. The protocol we established for plant regeneration from protocorm explants of *E. clarkei* is feasible and may be adopted as

Figure 1. Multiplication of protocorm on 0.5 mg/l NAA and 1.5 mg/l BAP.

Figure 3. Formation of multiple shoot from protocorm explants cultured on MS medium.

Figure 2. Induction of multiple shoots from protocorm explants cultured on 1.0 mg/l BAP.

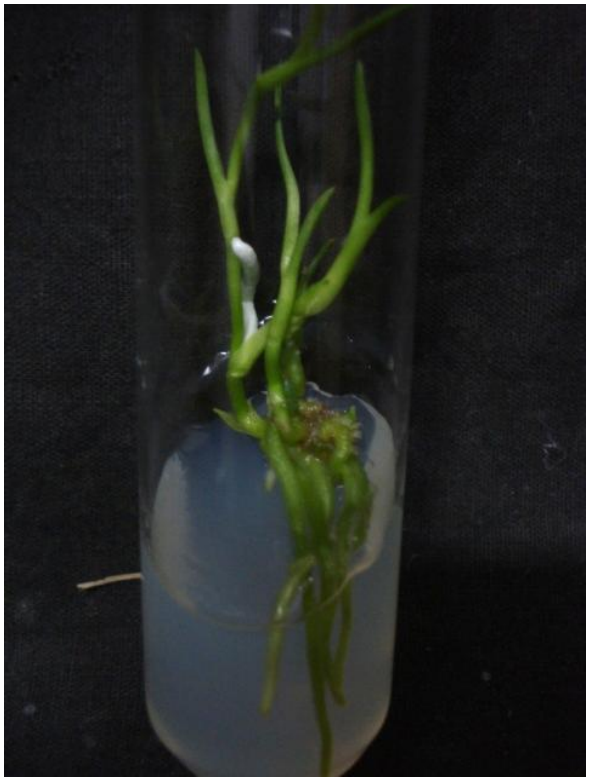

Figure 4. Induction of root on shoot cultured on 0.5mg/l NAA.

an effective propagation method for this species.

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