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In vitro Mass Propagation of a Ground Orchid - *Phaius tancarvilleae* (L'Her.) Blume through Shoot Tip Culture

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Abstract

High frequency direct shoot proliferation was induced from the shoot tip explants derived from the *in vitro* grown seedlings of a critically endangered and horticulturally important ground orchid *Phaius tancarvilleae* (L'Her) Blume. Shoot tip explants cultured on solidified MS with alone or combination of various concentrations of NAA and BAP produced shoots and multiple shoots. The maximum number of healthy shoots was observed on MS with BAP (1.0 mg/l) with an average of 13.3 shoots per culture in 20 weeks; where shoot multiplication was initiated after 4 weeks of culture. Regenerated shoots rooted on MS with various concentrations of NAA, IAA, IBA. MS with NAA (0.5 mg/l) was the most appropriate condition for rooting. The well developed *in vitro* rooted plantlets were hardened successfully in the potting mixture containing cocopeat and sphagnum moss in the ratio of 2 : 1.

Introduction

Phaius is the small genus of Orchidaceae comprises approximately 30 species of often large and spectacular, principally terrestrial orchids. The distribution range of *Phaius* is largest one, extending from East Africa and Madagascar, throughout tropical Asia and Indonesia to the Himalayas. In Nepal, two species of *Phaius* have been reported i.e. *Phaius flavus* and *P. tancarvilleae* (Press et al. 2000). *Phaius tancarvilleae* (L'Her.) Blume is one of the critically endangered and floriculturally important terrestrial orchids of Nepal, found in a damp places near stream side and prefers partial shade. It is commonly known as "Tall grass orchid" and is reported in central and eastern part of the country at an elevation of 400 - 1200 m (Raskoti 2009). Frequently numerous flowers usually open successively, so that the plants remain bloom for a long time (Fig. 1). Apart from the floricultural value, it has medicinal properties as well. The dried tubers of it are commonly used as tonic in traditional healing system by various ethnic groups. Habitat destruction, illegal and indiscriminate collection by orchid enthusiast is some of

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the threats facing by this species causing to largely decline its population from its natural habitat. Thus being categorized as endangered species under Environment Protection and Biodiversity Conser-vation Act (Briggs and Leigh 1996).

Orchid requires a combination of multiplicity of factor for continued reproduction in nature. The propagation of this species through sexual means is a very slow process as its seeds lack endosperm and need fungal stimulant for germination in nature; the fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (Arditti et al. 1982). In nature, only 2 - 5% of seeds germinate (Rao 1997) even if they do so, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. This difficulty in natural population drives the many species of orchid to extinction. It is therefore important to take initiative for the mass propagation of this orchid and establish in nature. We are applying tissue culture technique as a potential alternative method for mass scale propagation and conservation of rare, endangered and threatened orchid.

Shoot tip culture is an efficient system for the production of large number of plantlets in a short period of time. Knudson (1946) first developed the asymbiotic germination of orchid seeds while Morel (1960) revolutionized developing protocol for *in vitro* micropropagation by shoot tip culture. Since then, commercial orchids are predominantly produced by tissue culture and this technique is used routinely in many countries for mass scale production of orchid seedlings. Establishment of a reliable cloning methodology for *P. tancarvilleae* is important in terms of enabling the rapid propagation and production of a large number of high quality plants. We have already reported the *in vitro* propagation of *P. tancarvilleae* by immature seed culture (Pant et al. 2011). The present investigation is undertaken to develop an efficient protocol for *in vitro* micropropagation of this commercially threatened species through shoot tip culture and to introduce this species in natural habitat.

Materials and Methods

Shoot tips of *Phaius tancarvilleae* (L'Her.) Blume were used from 20-week-old *in vitro* grown seedlings. This experiment was assessed on MS with or without different concentrations and combinations of various growth regulators to compare and investigate the effect of hormone concentration on shoot multiplication and rooting of *P. tancarvilleae*. The medium was supplemented with 3% sucrose as carbon source and solidified with 0.8% agar. The pH was adjusted to 5.8 before autoclaving. The medium was autoclaved at 15 psi and 121ºC for 20 min.

The shoot tips (0.3 - 0.5 mm) were cut aseptically with the help of surgical blade and inoculated single shoot tip on MS with alone or combination of various concentrations of BAP (0.5 - 2.0 mg/l) and NAA (0.5 mg/l). After inoculation, the cultures were kept in control room and exposed to artificial light (fluorescent light) with a light/dark cycle of 16/8 hrs at 25 ± 2ºC. The observation was made at regular intervals of one week up to the 20 weeks and recorded their result.

For the induction of root, the regenerated multiple shoots of *P. tancarvilleae* were excised and a single shoot cultured on MS containing various concentrations of IAA, IBA and NAA (0.5 - 1.0 mg/l). The culture tubes were maintained in the culture room under the same condition as used for shoot multiplication. The observation was made at regular intervals of one week up to the 20 weeks of culture and recorded the root number and their length.

After the formation of complete rooted plantlets they were subjected to *ex vitro* hardening. The plantlets were removed from the culture tubes and were washed thoroughly with sterile double distilled water to remove any traces of the medium. They were then treated with 0.1% (w/v) Bavistin (fungicide) and again washed with sterile water. The rooted plantlets were planted in the potting mixture containing cocopeat and Sphagnum moss (2 : 1). The plants were covered with plastic bags for 30 days and maintained under humidity. Plants became acclimated to a reduced relative humidity by gradually opening the plastic cover and after 50 days they were completely uncovered and hardened to greenhouse conditions.

Significance of treatment effects on shoot multiplication and rooting of shoot tip were analyzed using one way ANOVA, $p \leq 0.05$ and comparison between mean values of treatments were made by Tuckey HSD test. All statistical analyses were performed using R development core team.

Results and Discussion

Multiple shoot and shoots were developed without any intervening callus and PLB formation on entire tested MS basal and MS with alone or various concentrations and combinations of BAP and NAA. Maximum number of shoots was observed after 20 weeks of culture in all the combinations. Multiplication of shoots together with shoot growth and root induction was observed. However there were quite differences between and within media in terms of number of shoot and their length**.** The highest number of multiple shoots (an average value of 13.3 shoots per explants) were obtained in MS with 1.0 mg/l BAP with an average 4.0 cm length of shoots (Fig. 2a). The shoot multiplication was initiated after 4 weeks of primary culture. As the concentration of BAP from 0.5 - 2.0 mg/l

considerably increased the multiple shoot production however increase in concentration of BAP to 2 mg/l decreased the multiplication rate. BAP influences shoot proliferation by stimulating quick cell divisions to induce large number of multiple shoots (Roy and Banerjee 2002, Ronzhina, 2003). Application of BAP at appropriate concentration effectively breaks apical dominance and induce lateral buds. Less quantity of BAP is required for shoot multiplication and even if more BAP is added, there is no significant increase in number of multiple shoots (Pierik 1997). Less number of roots was developed in media containing various concentrations of BAP. However, in the media supplemented with NAA root induction was increased (Fig. 2b). Maximum multiplication in MS + 1.0 mg/l BAP was followed by MS + 0.5 mg/l BAP (7.8 shoots/explants). It was followed by MS + 0.5 mg/l NAA (7.0 shoots/explants) and MS + 1.0 mg/l BAP + 0.5 mg/l NAA (5.5 shoots/explants). Least multiplication (1.45 shoots/explant) as well as least shoot growth (1.41 cm) was observed in MS + 2 mg/l BAP +0.5 mg/l NAA. Maximum length of shoot (6.33 cm) was observed in MS + 1 mg/l BAP + 0.5 mg/l NAA. Rooted multiple shoots were obtained on entire tested media (Table 1). In the present investigation, MS alone was not effective for induction of multiple shoots. Similar result was obtained in *Dendrobium* species (Yasugi et al. 1994). This revealed that the addition of plant growth regulators in nutrient medium

Table 1. Effect of BAP and NAA on growth and proliferation of shoot through shoot tip explants of *Phaius tancarvilleae*.

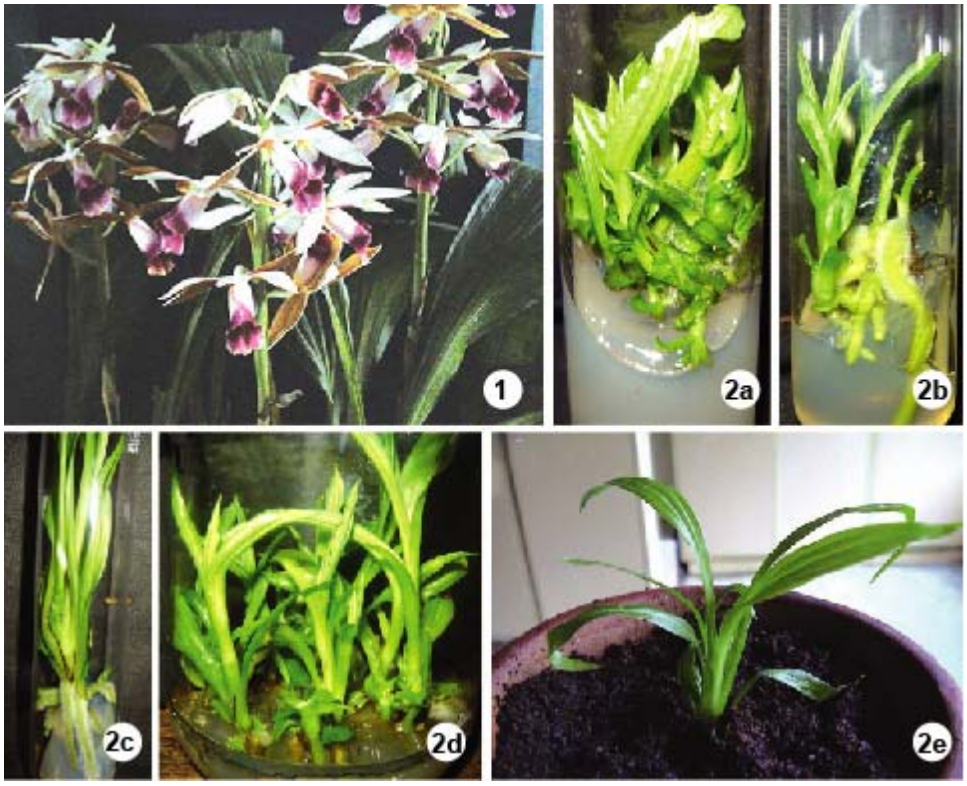
Media	Growth hormone	Conc. of hormones (mg/l)	Mean No. of shoots (± S.E)	Mean length of shoot (cm) (± S.E.)	Mean No. of roots (± S.E.)
MS	BM	0	1.0 ± 0.16	1.45 ± 0.17	1.16 ± 0.15
″	BAP	0.5	7.8 ± 2.4	4.9 ± 0.38	0.5 ± 0.31
″	BAP	1.0	13.3 ± 1.92	4.0 ± 0.66	1.33 ± 0.30
″	BAP	2.0	1.80 ± 0.68	2.26 ± 0.24	0.33 ± 0.19
″	NAA	0.5	7.0 ± 2.34	5.16 ± 0.54	4.25 ± 0.45
″	BAP + NAA	0.5 + 0.5	1.83 ± 0.28	3.66 ± 0.30	1.0 ± 0.23
″	BAP + NAA	1.0 + 0.5	5.5 ± 1.77	6.33 ± 0.56	1.41 ± 0.17
″	BAP + NAA	2.0 + 0.5	1.45 ± 0.42	1.41 ± 0.17	0.66 ± 0.28

Culture conditions: MS medium, 25 ± 2ºC, 20 weeks of primary culture, 6 replicates were used in each combination.

might be essential for further growth, development and proliferation of shoot tip explants. In this experiment, among the entire tested media MS with 1.0 mg/l BAP was found most effective for the shoot multiplication which indicates that MS alone with BAP might be suitable for the shoot proliferation. Similar result was also supported by our previous work and of several researchers in different

orchid species *Dendrobium densiflorum* (Luo et al. 2006), *Geodorum densiflorum* (Bhadra and Hossain 2003), *Cymbidium* and *Cattleya* (Nagarju et al. 2004).

Among the different combinations tested in this study, BAP (1.0 mg/l) and NAA (0.5 mg/l) were also found to be effective for the shoot multiplication as well as growth of shoot. The result showed that combination of BAP and NAA is also suitable for the shoot multiplication. The previous work of several researchers also showed that the high concentration of BAP and low concentration of NAA was favorable for the induction of multiple shoots. Similar



Figs 1 - 2: 1. Flower of *P. tancarvilleae*. 2. Micropropagation of *P. tancarvilleae* from shoot tip explants. (a) Multiple shoot regeneration on MS with BAP 1.0 mg/l. After 12 weeks of primary culture. (b) Multiple shoot with roots regenerated on MS with BAP 1.0 mg/l + NAA 0.5 mg/l. (c) Rooting of excised shoots on Ms with NAA 0.5 mg/l. (d) After 12 weeks of primary culture. (e) *In vitro* developed plantlets ready for acclimatization. (f) Hardened plantlet on potting mixture.

results were obtained in different orchid species *viz. Dendrobium* orchid (Talukdar et al. 2003), *Dendrobium transparens* (Sunitibala and Kishor 2009) and *Aerides odorata* (Pant and Gurung 2005). Hence, the regeneration potential of explants is markedly influenced by their physiological status and chemical

stimulus present in the nutrient pool. The quality, quantity and nature of growth regulators have foremost effect on the regeneration capacity of the shoot tip.

Well developed microshoots were transferred into MS with different concentrations of IAA, IBA and NAA for rooting. Auxin application to microshoots is said to intensify the number of adventitious roots by increasing the level of endogenous contents of enzymes. They are considered to have an increased effect on cell division, elongation and differentiation (Husen and Pal 2007). Han et al. (2009) revealed that auxins induce the sprouting of shoot buds which then stimulates the growth substances present in the roots for their growth and elongation. All tested auxins positively responded on development of roots after four weeks of culture. Though Liu et al. (2002) described as IBA is physiologically a more active auxin than NAA and IAA in promoting the root initiation as it acts as a precursor for endogenous IAA, in the present study highest numbers of roots were observed on MS with NAA 0.5 mg/l with an average 4.33 roots per explants and their average length of 3.0 cm among the all auxins tested. In this culture condition rooting started after two weeks of culture and the roots were thick, fleshy and hairy (Fig. 2c). Higher concentration of all auxins inhibited rooting. This result agrees with the previous finding of rooting of *Coelogyne stricta* (Bhaskar and Bai 2006) and also supported by the findings of Rout (2006) as quoted that NAA promotes the number of roots by enhancing cell division in the root primordia.

The *in vitro* well developed rooted plantlets of *Phaius tancarvilleae* were successfully hardened on potting mixture containing cocopeat and Sphagnum moss (2 : 1). Nearly 70% of plantlets survived were recorded (Fig. 1e). This work suggests that the mixture of cocopeat and moss will be favorable for the acclimatization of terrestrial orchids as in *Phaius tancarvilleae.*

This simple and efficient procedure for regenerating a large number of plantlets from shoot tip cultures of *Phaius tancarvilleae* could be used for large-scale propagation and *ex situ* conservation of this threatened orchid species.

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