IN VITRO SEED GERMINATION AND SEEDLING DEVLOPMENT OF PHAIUS *TANCARVILLEAE* (L' HER.) BLUME.

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Abstract*: In vitro* seed germination and seedling development of *Phaius tancarvilleae* (L'Her.) Blume. was carried out on 0.8%(w/v) agar solidified MS Medium (Murashige and Skoog, 1962) without hormones or supplemented with different concentration and combination of Naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP). MS medium supplemented with 0.5 mg/l of BAP was the most ideal condition for early seed germination, protocorm formation and development of seedlings. Germination started after 7 weeks of culture and complete seedlings were obtained after 24 weeks of culture. This protocol might be helpful for mass propagation of orchids by asymbiotic seed germination.

Keywords: Orchid; *Invitro;* Protocorm; Media.

INTRODUCTION

Nepal with its unique geographical position and climate offers an excellent growing condition for orchids. As a result about 388 species under 102 genera has been reported so far. Out of these, 12 species are endemic to Nepal (Raskoti, B.B., 2009). Most of them are epiphytic some are terrestrial. Seeds of terrestrial orchid are more difficult to grow than epiphytic orchid because they have hard seed coat and require more nutritions for *in vitro* germination. The species selected for present investigation has beautiful long lasting flower belonging to the genus *Phaius* (*Phaius* refers to dusky). In Nepal, two species of *Phaius* have been reported i.e. *P. flavus* and *P. tancarvilleae* (Press *et al*., 2000). Frequently numerous flowers usually open successively, so that the plants remain blooming for a long period.

Phaius tancarvilleae (L' Her.) Blume. occurs as a terrestrial herb in the subtropical and temperate zones of east Nepal at 1300-2100 m altitude. It blooms in the month of March-May. It is one of the commercially potential orchids of Nepal having horticultural as well as medicinal value. Tuber of this species is used as a tonic (Pant *et al*., 2007). Illegal collection for trade and consumption and loss of habitat has decreased the population of this species at an alarming rate in natural habitats. So to conserve this species and to meet its commercial demand, *in vitro* seed germination has been utilized to produce large quantities of uniform seedlings.

Orchid seeds are very small, dusts like and are produced in large number, often over a million seeds per capsule. In nature, germination rate is very slow and takes a long time because they require mycorrhizal fungi for germination and seedling development as they lack their own food reserves i.e. endosperm. Tissue culture has become the standard method of propagation for the conservation of orchids. Germinating seeds *in vitro* is a breakthrough in orchid multiplication (Fay, 1996). *In vitro* asymbiotic germination is possible only by tissue culture method because it substitutes the action of fungus with a nutrient medium. Germination and development take place much quicker *in vitro*, since there is a controlled environment, and there is no competition with fungi and bacteria. Therefore, a systematic research on propagation technique especially the tissue culture should be promoted for commercially valuable orchids.

The present study is carried out on *in vitro* seed germination and seedling development of *P. tancarvilleae* (L' Her.) Blume.

MATERIALS AND METHODS

The materials used for the present investigation were the immature capsule of *P. tancarvilleae* (L' Her.) Blume. obtained from the cultivated plants.

Surface Sterilization of capsule

The immature healthy capsules of the orchid were dipped in water containing few drops of teepol solution. Then, they were subjected to running tap water for 30-40 minutes and rinsed with distilled water. The orchid pod was then wrapped with cotton, soaked in 70% ethyl alcohol for 1 minute and surface sterilized in 1% sodium hypochlorite solution for 10 minutes. Finally they were rinsed with sterile water for 3-5 times.

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Media

MS medium was used as the basal medium (BM) alone and supplemented with different concentrations and combinations of NAA and BAP (as given in the Table 1.) for the inoculation of seeds. Medium was adjusted to pH 5.8 with 0.1N NaOH or HCl before autoclaving and solidified with 0.8% w/v Difco Bacto Agar. Medium was heated upto boiling to melt the agar. When the solution became transparent, about 20 ml medium per culture tube was dispensed into sterile culture tubes and each tube was sealed with aluminium foil. The culture tubes containing medium were autoclaved at 121^{i}C and pressure of 15 lb/sq inch for 20 minutes. After cooling down, the tubes were taken out and kept in slanting position in the culture room.

Inoculation of seeds

The surface sterilized orchid capsule was then transferred into the petridish and dissected longitudinally by using sterile scalpel. Immature orchid seeds in cluster were inoculated on the surface of MS medium alone and combination with NAA and BAP. Sterile forceps were used to spread seeds on agar medium. The cultures were incubated at 25^{i}C (±2 ^{i}C) temperature under the photoperiod of 16 hours. Cultures were subculture into fresh media once every 8 weeks.

RESULTS AND DISCUSSIONS

The seed germination rate of *P. tancarvilleae* (L' Her.) Blume. in hormone free MS medium and MS medium supplemented with various concentrations of NAA and BAP was found to be variable (Table 1). About 85% of the seeds were germinated and began to form protocorms. Though MS basal medium favored seed germination, the rate of seed germination was less in comparison to MS medium supplemented with hormone. MS basal medium took 10 weeks to start the germination (Fig. 1). MS medium supplemented with BAP (0.5 mg/l) was found to be the most appropriate condition for fastest germination of these seeds which was started after 7 weeks of culture (Fig. 2). This concentration was also effective for the development of protocorms, leaf and root primordial as compared to other. The first primordial leaf and root were developed after 12 weeks (Fig. 3) and 18 weeks of culture respectively on this medium. Hence media with low concentration of BAP was found to be more effective for seed germination and subsequent development of this terrestrial orchid. It was followed by MS + BAP (1mg/l) + NAA (0.5 mg/l). In this medium, germination was started after 8 weeks of culture.

Protocorms undergoes further differentiation on MS medium alone and MS medium supplemented with different concentration and combination of growth regulator. MS medium supplemented with BAP (0.5mg/l) favored the development of complete seedlings directly from seeds after 24 weeks of culture. This result was also followed by MS+BAP (1mg/l) +NAA (0.5mg/l) (Fig. 4), MS+BAP (1mg/l) and MS+NAA (0.5 mg/l). While in hormone free MS medium, the seeds could not undergo further differentiation because immature seeds require more regulated conditions of hormones especially in terrestrial orchids. Seeds were also not undergoing further differentiation on MS + BAP (0.5mg/l) + NAA (0.5mg/l) apart from hairy protocorms even after 30 weeks of culture (Fig. 5). This is due to the capacity to form seedling increase with BAP which is treated alone or media modified with strong concentration of BAP and weak NAA combination. Incorporation of same concentration of BAP and NAA suppressed the seedling formation (Pierik 1987). The protocorms formed in different media were chlorophyllous and globular. Seedlings were changed to fusiform or cylindrical in appearance.

In the present study, MS medium alone and MS medium supplemented with different concentrations of hormones were found to be efficient for the germination of immature seeds upto the development of protocorm. MS + BAP (0.5mg/l) was found to be the most suitable culture condition for immature seed germination where germination was observed after 7 weeks of culture, protocorms were developed after 9 weeks of culture and complete seedlings were obtained after

Table 1: Effects of growth regulators in MS Medium on seed germination and seedling growth of *Phaius tancarvilleae* (L'Her.) Blume

Medium	Plant Growth Regulators	Concentration of Hormones (mg/l)	Time taken in weeks					Remarks
			Initiation of Germination	Development of Protocorm	Differentiation of			
					1st Leaf Primordia	1st Root Primordia	Seedling	
MS	BM	-	15	19	-	-	-	Germination favored
"	BAP	0.5	7	9	12	18	24	Germination and seedling development favored
"	BAP	1	12	15	20	28	35	Germination and seedling development favored
"	NAA	0.5	13	18	23	32	38	Germination and seedling development favored
"	BAP + NAA	0.5 + 0.5	13	17	24	39	-	Germination favored
"	BAP + NAA	1 + 0.5	8	11	14	19	29	Germination and seedling development favored

Culture conditions: MS medium, 25±2 ºc, 40 weeks of culture, 16 hrs photoperiod, 4 replicates were used in each combination.

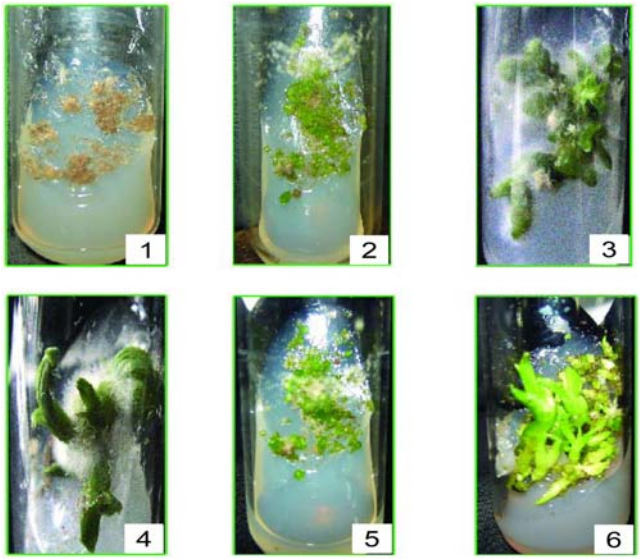

Fig (1-6): *In vitro* Seed germination of *Phaius tancarvilleae* (L' Her.) Blume : 1. Hormone free MS media after 10 weeks of culture; 2. MS media with BAP (0.5 mgl') after 7 weeks of culture; 3. MS media with BAP (0.5 mgl') after 12 weeks of culture showing PLBs and hairy shoot primordial; 4. MS + BAP (1mg/l)+ NAA (0.5 mg/l) medium after 29 weeks of culture showing hairy shoot primordial 5. MS + BAP (0.5mg/l) + NAA (0.5 mg/l) medium after 30 weeks of culture showing PLBs; 6. MS + BAP (0.5 mg/l) medium after 24 weeks of culture showing protocorm like bodies and shoots.

24 weeks of culture (Fig. 6). This was found on the basis of time taken for germination, high amount of protocorms formation and further differentiation into seedling. In MS basal medium, germination was observed after 15 weeks of culture and protocorms were seen after 19 weeks of culture. Further differentiation into seedlings was ceased. This result showed that hormone supplemented media was found to be more effective for seed germination than hormone free medium. Arditti *et al.*, (1981) reported that the improvement in the nutritional status of the basal medium with additives like vitamins, aminoacids and hormones promote seed germination in many orchids (especially terrestrial species).

Further increase in the concentration of BAP to 1mg/l, took more time for seed germination and protocorm formation. This result was supported by Nagarju *et al.*, (2003) who reported that proliferated protocorms were developed in *Cattleya* and *Cymbidium* on medium supplemented with BAP (0.5mg/l). Similar result has been obtained by De Pauw *et al.*, (1995) on seed germination of *Cypripedium candidum* where 0.8 mg/l BA enhanced faster germination, multiple protocorm formation increased with cytokinin.

Use of BAP and NAA in combination also favored germination and further differentiation into seedling. In present study, MS+BAP (1mg/l)+NAA (0.5mg/l) was also found to be another suitable culture condition after MS+BAP (0.5mg/l) where germination was observed after 8 weeks of culture and complete seedling was obtained after 29 weeks of culture. This is in conformity with the findings of Vij *et al.*, (2004) who reported that a combination containing BAP (1.5 mg/l) and NAA (1.0 mg/l) proved best for accelerated development of PLBs in *Cymbidium*.

It was found that in *P. tancarvilleae* (L' Her.) Blume., MS medium supplemented with BAP showed stimulatory response rather than inhibitory effect. MS supplemented with BAP (0.5mg/l) was the most effective medium for early germination, higher number of protocorm formation and their further differentiation. The use of BAP (1mg/l) + NAA (0.5 mg/l) also favored seed germination and seedling formation.

CONCLUSIONS

P. tancarvilleae (L'Her.) Blume. is a robust terrestrial orchid species listed as endangered under the Environment Protection and Biodiversity Conservation Act. 1999. Habitat destruction, illegal and indiscriminate collection by orchid enthusiast is some of the threats facing to this species. Thus, from both commercial and conservation point of view, it is important to develop quick methods of propagation of this species. *In vitro* techniques of micropropagation are the method that can meet both commercial and conservational demand. There is no information regarding the *in vitro* seed germination of *P. tancarvilleae* to our knowledge so far. This investigation may provide alternative for *in vitro* germination of such horticulturally and medicinally important orchid species.

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