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In vitro plant regeneration through protocorm formation in orchid *Calanthe odora*

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Calanthe odora Griff. is a lithophyte terrestrial orchid found in the North West and the North Eastern part of India, viz. Sikkim, Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram and Nagaland. The species has been listed as endangered by the IUCN red list of threatened species (Barik *et al*. 2018). This orchid plant produces white colour flowers during May to July. Flower stem arises from previous years leafless pseudobulb. Propagation of the genus *Calanthe* by asymbiotic germination of seeds has been reported (Shin *et al*. 2011). *Calanthe* orchid is difficult to propagate due to very poor seed germination and very limited number of plants by pseudobulbs. Orchid seeds germinate and form small spherical tuber-like bodies referred to as protocorms. The protocorm has only one meristematic domain at the anterior end where new leaves and roots are formed sequentially. Protocorm-like bodies (PLBs) resemble protocorms structurally and are triggered from explants or calluses (Chugh *et al.* 2009). During the initiation of PLBs, callus cells from the explant form compact regions that are composed of meristemoids. Polarized growth starts from the surface cells of each compact pool of meristemoids (Lee *et al*. 2013). The first leaf is formed from the PLB and the root is usually produced at the base of the first leaf (Hong *et al*. 2008). Since there is no standard protocol available for commercial propagation of *Calanthe odora* so this research work was conducted to develop methodology for *in vitro* regeneration of plants from protocorms and PLBs by asymbiotic seed germination.

The present investigation was conducted in the Tissue Culture Laboratory of Departmentof Floriculture, Medicinal and Aromatic Plants, Faculty of Horticulture, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar, West Bengal and the experiments on hardening of plantlets were conducted at Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal during 2014–15. The four months mature capsules of *Calanthe odora* were collected from greenhouse and first washed in running tap water to remove any debris from their surfaces. Each capsule was rinsed thoroughly with Teepol for 2–3 minutes and then with sterile distilled water. The capsules were incised longitudinally and the seeds were scooped out into a Petri dish. Seeds were dipped in water for ten minutes and rinsed with three changes of sterile water. Then seeds were sterilized with 2.0% Bavistin (Carbendazim 50% w/w) for 10-15 minutes, 70% Ethanol (v/v) for 2 minutes and 0.01% Mercuric chloride for 1-2 minutes and rinsed thoroughly with sterile distilled water 4-5 times. Murashige and Skoog (1962) media were used supplemented with Kinetin (Kn) alone (0.5, 1.0, 1.5 and 2. mg/l) and in combination with α-Napthalene acetic acid (NAA) at the concentration on 1.0 mg/l (Table 1) for all the parameters studied. The jam bottles were placed in the culture room at 20–25°C under 16–24 h photoperiod conditions. Swollen embryos breaks from testa start to form protocorms after 2-3 weeks in culture and the culture will appear crowded. The well-developed protocorms and PLBs were subcultured transferring on MS media fortified with Kinetin (0.5, 1.0, 1.5 and 2.0 mg/l) alone and in combination with NAA (1.0 mg/l) and the different parameter were studied (Table 1). Young plantlets (5 to 7 cm in length) were primary hardened transplanting to plastic pots containing potting mixture vermicompost and vermiculite (1:1) and kept under day/night temperatures of 25/15 °C and 16-24 hour photoperiod for one week. The primary hardened *in vitro* raised plantlets were transferred to potting mix containing different ratio of garden soil, sand, vermicompost and vermiculite (Fig 1) and were maintained in polyhouse for 4 weeks.

The effects of the PGRs were analyzed through parameters, viz. seed germination, PLBs formation, development of protocorm and differentiation into shoots and roots after 5 weeks of culture. All the experiments were laid out under factorial experiment in CRD (Completely Randomized Design). The data were analyzed by Fisher's analysis of variance (ANOVA) technique and results were interpreted.

The data and pictorial evidences of this experiment have been presented in Table 1 and Fig 1 & 2. The initiation of asymbiotic seed germination and protocorm-

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Table 1 Effect of different growth regulators on seed germination and PLBs formation and development of protocorm to plantlets in *Calanthe odora*

Treatment combinations	Seed germination and PLBs formation		Development of protocorm to plantlets				
	Initiation of germination of seeds (weeks)	Capability of PLBs formation	Time for protocorm formation (weeks)	No. of shoots per culture	Length of shoot (cm)	No. of shoots with roots	No. of roots per plant
MS	12.33	very poor	16.67	21.33	0.97	14.67	1.67
MS + 0.5 mg/l Kn	8.33	slow growth	12.33	36.33	1.53	23.67	2.67
MS + 1.0 mg/l Kn	7.00	slow growth	10.67	43.33	2.20	32.33	2.67
MS + 1.5 mg/l Kn	6.67	slow growth	10.00	57.33	2.43	42.00	3.67
MS + 2.0 mg/l Kn	6.33	Greenish globular protocorms	9.67	58.00	2.60	46.33	4.00
MS + 0.5 mg/l Kn + 1.0 mg/l NAA	6.33	Greenish globular protocorms	9.33	60.33	2.43	52.00	5.67
MS + 1.0 mg/l Kn + 1.0 mg/l NAA	5.67	Greenish globular protocorms	8.33	62.67	3.07	59.00	6.33
MS + 1.5 mg/l Kn + 1.0 mg/l NAA	6.00	Greenish globular protocorms	8.67	70.67	4.13	59.33	5.67
MS + 2.0 mg/l Kn + 1.0 mg/l NAA	6.00	slow growth	9.00	58.33	3.97	52.67	5.33
SEm ±	0.33		0.29	1.95	0.12	2.62	0.50
CD	1.28		1.13	7.48	0.47	8.05	1.91

like bodies formation was significantly affected by different concentration of Kinetin alone and in combinations with NAA as a supplement of MS medium (Table 1A). The asymbiotic seed germination was also achieved by Shekarriz *et al.* (2014) in *Phalaenopsis* orchids. The significantly lowest mean time taken for seed germination (5.67 weeks) exhibited in MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l NAA (Table 1). The MS medium fortified with 0.5 mg/l Kinetin alone took maximum weeks (8.33) for initiation of seed germination from immature seed asymbiotically. Godo *et al.* (2010) reported similar result on the germination of terrestrial orchids in culture media. Cytokinins might have stimulated the germination of seeds by eliminating the inhibitory effects of abscisic acid on germination. Protocorm-like bodies formation was observed on all MS medium supplemented with Kinetin alone and in combinations with NAA. On the basis of overall morphology and visual growth the formation of PLBs were recorded (Table 1A). In lower concentration of Kinetin upto 1.5 mg/l the PLBs formation took place but the growth is very slow while in combination with 1.0 mg/l NAA greenish globular PLB formation was observed (Table 1A, Fig 2). Similar results on seed germination and PLB formation with growth regulators was achieved by Hrahsel and Thangjam (2015) in *Vanda coerulea.*

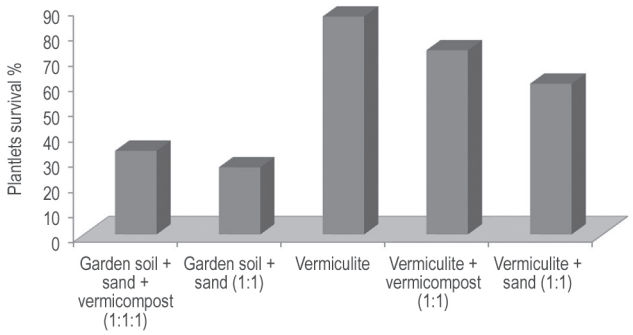

Fig 1 Effect of different hardening media on percent survival of plantlets of *Calanthe odora.*

The MS media supplemented with Kinetin alone and in combination with NAA affected protocorm multiplication significantly (Table 1B). The best respond of protocorm multiplication and development to become plantlet was found on MS media supplemented with 1.0 mg/l Kinetin in combination with 1.0 mg/l NAA (Table 1B). The results are in agreement with the findings of Shin *et al*. (2011) in *Calanthe sp.* and of Zeng *et al.* (2012) in *Paphiopedilum wardii.* The minimum time (8.33 weeks) for protocorm formation was observed on MS media supplemented with 1.0 mg/l Kinetin in combination with 1.0 mg/l NAA (Table 1B). As the concentration of Kinetin was increased, time required for protocorm formation was found to be decreased. Protocorm and PLBs formation derived from seed was also reported by Shin *et al*. (2011), Shekarriz *et al.* (2014). The maximum number (70.67) of shoots per culture were observed after 14 weeks of inoculation on MS medium supplemented with 1.5 mg/l Kinetin and 1.0 mg/l NAA which was significantly superior over other treatments (Table 1B). The maximum length of shoot (4.13 cm) was observed on MS medium supplemented with 1.5 mg/l Kinetin and 1.0 mg/l NAA wherein minimum (1.53 cm) was on MS media supplemented with 0.5 mg/l Kinetin alone. The number of rooted shoots was found to be increased with concentration of growth regulator is increased and

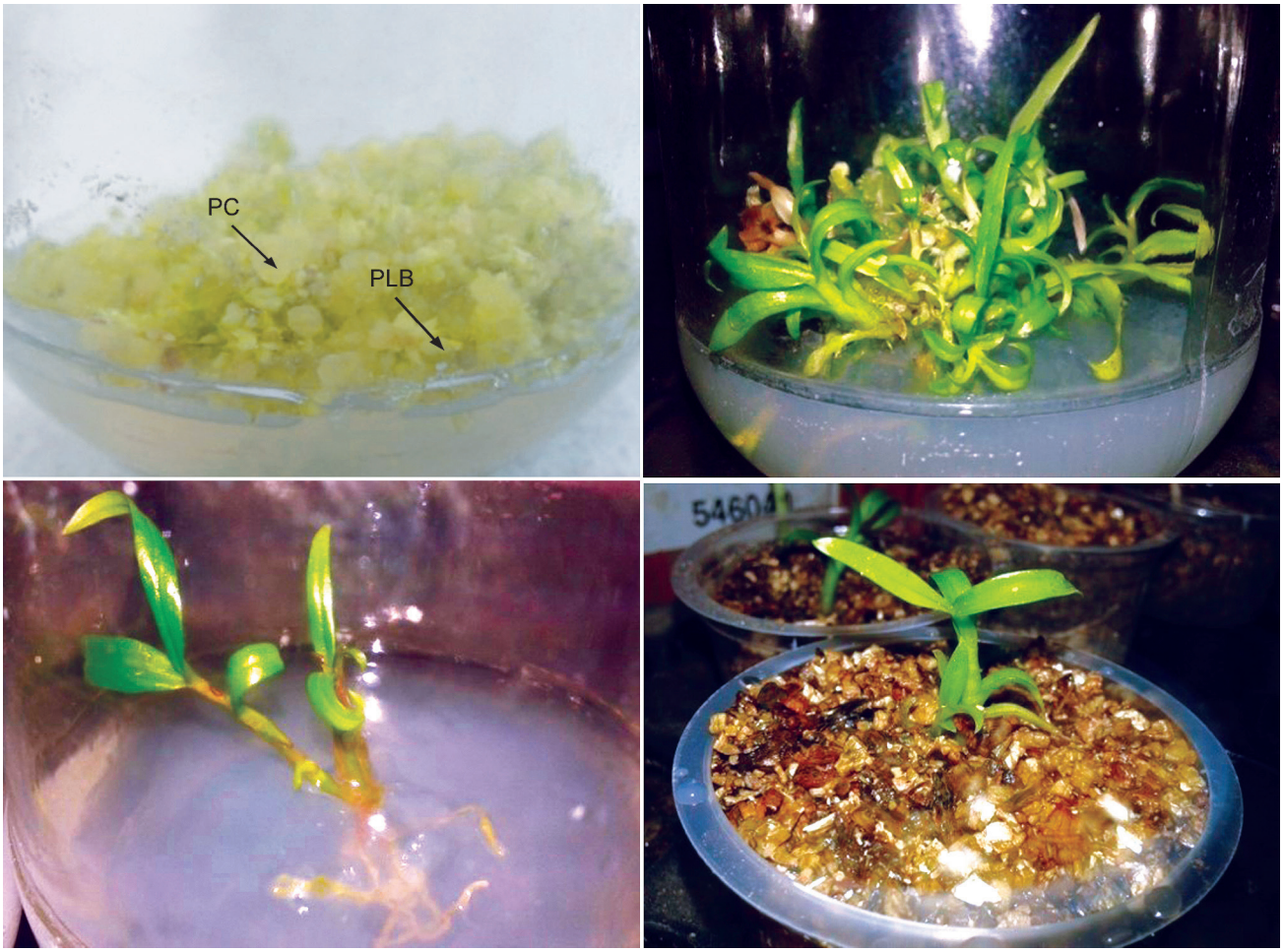

Fig 2 A. Emergence of protocorm and PLBs from asymbiotic germination of immature seeds; B. PLBs with emergence of leaves and shoots; C. Development of healthy shoots with roots; D. Hardening of plantlets in vermiculite medium

maximum (59.33) observed on MS fortified medium with 1.5 mg/l Kinetin and 1.0 mg/l NAA (Table 1B & Fig 2). The number of roots per plant was maximum (6.33) on MS fortified medium with 1.0 mg/l Kinetin and 1.0 mg/l NAA. The number of roots was found to be decreased with lowering the concentration of growth regulator in MS media. The maximum percent survival (86.7) of plantlets was recorded when plants were hardened on vermiculite media in polyhouse as were observed after four weeks of transfer to hardening media (Fig 1).

The present investigation indicated that *in vitro* seed germination of *Calanthe odora* and their subsequent regeneration from protocorm and PLBs can be achieved successfully with use of plant growth regulators. The standardized protocol of MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l NAA for germination and formation of protocorm-like bodies from immature seed followed by development on MS media fortified with 1.5 mg/l Kinetin and 1.0 mg/l NAA can be utilized for *in vitro* multiplication of *Calanthe odora*.

SUMMARY

The regeneration of plants through formation of protocorm and protocorm-like bodies was achieved by *in vitro* asymbiotic immature seed culture of *Calanthe odora.* This is the first report on *in vitro* regeneration of plantlets of *Calanthe odora* Griff., the IUCN red listed threatened species. The immature seeds as explants were most regenerative than mature seeds. The MS media fortified with different concentrations of Kinetin alone or in combination with NAA were tested to sort out as the best regeneration media. From the experimental results the best medium was sorted as MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l NAA for germination and formation of protocorm-like bodies from immature seed explants followed by transfer to MS media fortified with 1.5 mg/l Kinetin and 1.0 mg/l NAA for development of protocorm to plantlets. The plantlets after hardening in *in vitro* were transferred to polyhouse where 86.7% survival rate was recorded on vermiculite potting media.

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