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In vitro propagation of *Acampe papillosa* Lindl. (Orchidaceae) through direct somatic embryogenesis using leaf explants

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Abstract

A method has been developed for the regeneration of the Orchid cultivar *Acampe papillosa* Lindl. The present study reports a research carried out with different concentrations and combinations of auxins and cytokinins to activate *in vitro* propagation without an intermediate callus stage. Young leaf sections from *in vitro* plants were used as explants source. Protcorm like body was produced on these explants. The leaf explant was cultured on ½ inorganic MS Media containing 2 % sucrose which was solidified with 8 % agar and contained fractional combinations of auxin (NAA) and cytokinin (BAP) at concentrations of 0.5, 1, 2, 4 mgl^{-1} and 1, 2, 4, 8 mgl^{-1} respectively. The induction of PLBs was observed in the absence of any exogenous PGRs, but the addition of BAP with ½ inorganic MS improved the shoot growth significantly. Optimum growth was observed in the presence of 4 mgl^{-1} BAP, where 20 PLBs per explants was obtained. *In vitro* responding shoots were transferred to root induction medium consisting of ½ inorganic MS suplimented with cytokinin. Rooting was best observed in medium supplemented with 0.8 mgl^{-1}. The rooted plantlets were acclimatized and transferred to field conditions. The study of *in vitro* propagation of five month old undehisced green capsules of *Acampe papillusa* Lindl. through direct somatic embryogenesis using leaf explant was carried out in semisolid culture media. The experiment was conducted with plant growth regulators to propagate this orchid for commercial production due to its economic and aesthetic value.

INTRODUCTION

The word 'orchid' has originated from the Greek word 'Orchis which literally means 'Testicle'. 'Orchid' brings to one's mind some vision of unique or implausible form of flower, colour, beauty, fragrance etc. Like roses, lilies, and anthuriums, Orchids are also a particular group of flowering plants belonging to family Orchidaceae. The orchids are among the most advanced flowering plants, highly specialized in many ways. The family *Orchidaceae* is probably the largest among all angiosperms, with an estimated 25000 species [1-2]. Orchids are well known for the great diversity evaluated in their growth habits and life forms, in the contrivances of pollination and ecological adaptabilities. They have a worldwide distribution occurring in various ecological habitats. According to Atwood (1986) [1] more than 70% of orchid species are epiphytic that mostly inhabit in the tropics. Near about 163 genera and 1100 species have been found in India. Among them more than 300 species are endemic [3]. The major orchid-rich phytogeographical regions of India are North-eastern India, the Eastern and the Western Himalayas. As they bear attractive flowers, the orchids comprise a major group of horticultural crop. Apart from this ornamental value, a large number of species are also medicinally important [3,4]. The essence of vanilla, which is obtained from the seed capsule of the climbing orchid *Vanilla planifolia*, is also well known. Variations in floral morphology are often associated with the specialized and bizarre mechanisms of insect pollination. The relationship between orchids and their insects pollinators is not based on mutualism, and orchids are regarded as parasites on behaviour patterns of the pollinators [5].

In spite of their manifold utility, for a long time the cultivations of orchids for commercial purposes were considered very difficult, since most orchids show extremely slow rate of vegetative multiplication. The breakthrough in orchid propagation came many years ago, when it was first demonstrated that orchid seeds could be germinated in artificial culture conditions. However, this technology was not commercially utilized in many orchid-rich countries (especially of Asia) until recently. In Asia commercial propagation of orchids using *in vitro* culture methods began in the Gavinlertvatana and Prutpongse [6]. Therefore, for many years collection of plants from the natural habitats to meet the commercial demand was a common practice with many orchid traders.

The uncontrolled collections of plants from the wild, together with depletion of natural habitat have resulted in the extinction of many important species [7-8]. According to the recent figures of IUCN (International Union for Conservation of Nature and Natural Resources) Red List of Threatened plants, 1779 species of orchids are threatened with extinction [9]. However, it should be noted that this number is at a global scale, and numerous other species are threatened at national levels. In India 215 species have been declared as endangered and 14 nearly extinct. These include species like *Vanda coerulea*, *Renanthera imschootiana* and *Paphiopedilum wardii*. For preventing further damage, the government of India under the provision of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) have brought all orchid species under Appendix- II of Wild Life Protection Act [3]. These provisions make it obligatory on the part of the trader to propagate the orchids before trading with them.

Conventional methods of orchid propagation are extremely slow and the number of propagules produced by these methods is low. Tissue culture techniques provide a solution for producing large number of propagules within a limited period of time. According to Moral (1974) [10] more than four million plantlets can be obtained in a year from a single explants. Among the explants most commonly used are shoot tips and auxiliary buds [11] although stem, leaf, root and infloresence can also be used [12].

Also, a wide range of recipe has been used in the *in vitro* culture of orchids [13]. In a study carried out by we *et al* on shoot tip culture of oriental orchids,[14] it was observed that several factors influence shoot formation and *in vitro* multiple shoot production in orchids. When growing the orchids in our laboratory, we found that the plantlets of *Acampe papillosa* do not grow fast enough in the commonly used Vacin & Went [15] and Knudson C (KdC) medium [16] and do not produce roots which are strong enough to withstand the acclimatization conditions. As literature for the commercial propagation of *Acampe papillosa* specifically are scarce, due to obvious reasons, a study was conducted to compare the efficiency of different nutrient media, growth regulators and medium supplements for the *in vitro* propagation of orchid *Acampe papillosa* suitable for commercial exploitation. The experiment was conducted with plant growth regulators to propagate this orchid for commercial production due to its economic and aesthetic value. Here we report a reproducible protocol for high frequency plant regeneration via PLB bodies from *in vitro* grown leaf samples that could be suitable for commercial exploitation.

MATERIALS AND METHODS

Surface sterilisation of capsule

The study was conducted with five-month old undehisced green capsules of *Acampe papillosa* Lindl. The pods were washed under running tap water (30 min) followed by treatment with 7.5 % (v/v) Lizol (Reckitt Benckiser, India) for 30 min. The pods were dipped in Bavestin (1 g/100 ml of distilled water) and then which was rinsed 56 times using autoclaved tap water. These were surface-disinfected by treating with 0.1 % (w/v) mercuric chloride (8 min) followed by rinsing with autoclaved distilled water (56 changes) and soaked with tissue paper. The pods were then cut and seeds were removed from them and also the seeds were inoculated into different nutrient media

In vitro seed germination

After opening the capsule aseptically with a scalpel, the seeds were taken out and sown on the surface of semisolid culture media in Erlenmeyer flask (250 ml). The seeds were scoped and transferred on different nutrient media such as ½ strength inorganic nutrients of [17], ½ MS0, MS0, Hyponex, OKF_1, Knudson C [16] supplemented with 0.1 % (w/v) peptone and 2 % (w/v)sucrose to study their germination percentage. The pH of the culture media was adjusted to 5.2 the inoculated culture vessels were transferred to the culture room

Clonal propagation using *in vitro* leaf tissue

The experiment was performed with juvenile leaves of *A. papillosa*, collected from 6 month old seedlings. Since the seedlings were raised aseptically under *in vitro* conditions; there was no need for surface sterilisation of the plant material. The excised leaves, 8-10 mm in length, were inoculated on the surface of culture medium, 1per replicate tube (15mm). There were 5 replicate culture tubes per treatment, each containing 10ml culture medium. The culture medium consisted of half strength inorganic nutrient of [17], supplemented with 0.1 % (w/v) peptone and 2 % (w/v) sucrose. The plant growth regulator (PGR) treatments comprised of BAP (0, 1, 2 and 4 mgl^{-1}) and NAA (0, 0.5, 1 and 2 mgl^{-1}). The pH of culture media was adjusted to 5.2 prior to autoclaving. After addition of 0.9 % (w/v) agar the media were autoclave at 1.02 $kgcm^{-2}$ for 20 min.

Maintenance of culture

The cultures were maintained at 25± 2°C under 10-h photoperiod provided white fluorescent lamps of 37.5 µmol $m^{-2}sec^{-1}$ irradiance. Data of different morphogenetic responses were collected after one month intervals up to six months of culture.

Hardening

The well-developed seedling (3-4 cm height) were removed from culture vessel and thoroughly washed with tap water to remove adhering medium completely without causing damage to the roots. Then the plantlets were treated with the fungicide solution (Bavestin) at 4 % concentration and transferred to perforated plastic pots. Plastic pots were filled with a mixture of uniform, small charcoal pieces and brick pieces (1:1). After a thorough wash of the pots and the potting media in water and treatment with 0.2 % diethane M 45 fungicide, the seedling were transplanted. The roots of the seedling were closely touching the surface of the charcoal pieces. Care was also taken so that 1-2 roots of the seedling passed through the space in between charcoal pieces. The potted plants were kept under a green house (25 % light) and mist irrigated. After two weeks both misting and foliar application of NPK mixture (Vijay complex 17:17:17) were followed. The application of the later was done twice a week. Observations on the establishment of the seedlings were recorded at bi-weekly intervals.

Data analysis

Data on present response and the number of shoots per explant were determined at monthly intervals over a period of six months following culture initiation. Data were analyzed with ANOVA for a completely randomized design (CRD). Duncan's new multiple range test (DMRT) [18] was used to separate the means to determine significant effects.

RESULTS

Seed germination

The seeds taken from the green pods were first sown in the ½ inorganic MS medium (Table 1) containing various concentrations of two organic nutrients, namely peptone and sucrose, and then in ½ MS0, MS0, Hyponex, OKF_1, Knudson C medium supplemented with 0.1 % (w/v) peptone and 2 % (w/v) sucrose to study their germination percentage.

Germination of seeds started after three weeks of culture and was considered to have occurred when the embryo emerged from the ruptured seed testa. Swelling of the embryos were first noticed within 10 days. Highly significant variation among different media had been observed in required days to seed its germination (Table 2). Maximum days (56 days) to seed germination was recorded by Knudson C medium while the minimum period (28 days) was required by ½ MS0 medium.

The highest seed germination (90.32 %) was observed in ½ inorganic MS medium supplemented with peptone 0.1 % (W/V) and Sucrose 2 % (W/V), followed by ½ MS0 (83.12 %), MS0 (75.78 %) and Hyponex medium (71.65 %), while Knudson C, demonstrated poor performance (49.23 %).

Survival of leaf explants

Survival of leaf explants was 100 % in the PGR-free control (Table 3). Necrosis of leaf explants in the presence of NAA took place after two months of culture (Table 3). However, the application of BAP along with NAA completely prevented the NAA promoted leaf necrosis.

Table 1. Effect of ½ inorganic MS with peptone and different concentration of sucrose on seed germination and required days to seed germination of *Acampe papillosa*

Media	Explant	Peptone % (W/V)	Sucrose % (W/V)	PH	Days required to seed germination	Culture duration (in days)	Seed germination frequency (%)
½ inorganic MS	Seeds	0.1	0	5.2	45	70	41.43 ± 2.76^{f}
½ inorganic MS	Seeds	0.1	1	5.2	32	70	75.31 ± 2.65^{b}
½ inorganic MS	Seeds	0.1	2	5.2	25	70	90.32 ± 2.65^{a}
½ inorganic MS	Seeds	0.1	3	5.2	36	70	70.87 ± 2.76^{c}
½ inorganic MS	Seeds	0.1	4	5.2	39	70	67.56 ± 2.23^{d}
½ inorganic MS	Seeds	0.1	5	5.2	42	70	64.39 ± 1.99^{e}

Data looped from a total of three separate experiments each comprising of ten replicate conical flask containing 1-4 in vitro leaf explants per conical flask. Mean values within column with different superscript alphabets are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Table 2. Effect of different media on seed germination and required days to seed germination of *Acampe papillosa*

Type of Media	Explant	Peptone % (W/V)	Sucrose % (W/V)	PH	Culture duration (in days)	Days required to seed germination	Seed germination frequency (%)
MS0	Seeds	0.1	2	5.2	70	39	75.78 ± 2.80^{d}
½ MS0	Seeds	0.1	2	5.2	70	28	83.12 ± 1.81^{b}
Hyponex	Seeds	0.1	2	5.2	70	47	71.65 ± 1.99^{c}
OKF_1	Seeds	0.1	2	5.2	70	51	54.29 ± 3.71^{e}
Knudson C	Seeds	0.1	2	5.2	70	56	49.23 ± 2.65^{f}

Data looped from a total of three separate experiments each comprising of ten replicate conical flask containing 1-4 in vitro leaf explants per conical flask. Mean values within column with different superscript alphabets are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Formation of PLB and Plant regeneration from leaf explants

Formation of protocorm like body (PLB) as small globular structure with ½ inorganic MS, was noted after three weeks of culture (Table 3). After 3 to 4 weeks PLBs were induced on each explant. The PLBs which were developed in to shoots with 0.79 - 2.32 cm high after 8 weeks of culture. Germination of PLBs & formation of shoot primordia took place in the initial PLB induction medium. Protocorm formation was done by using ½ inorganic MS medium with four different concentration of BAP (1.0, 2.0, 4.0 and 8.0 mgl^{-1}) and NAA(0.5, 1.0, 2.0 and 4.0 mgl^{-1}). Both individual and combined effects of BAP, NAA and BAP + NAA were analyzed (Table 1). Interestingly, two different concentrations of NAA (1.0 and 4.0) the explants were survived well but no PLB was formed. Highest protocorm like bodies was produced on the medium which containing 4 mgl^{-1} BAP, followed by 1.0 mgl^{-1} BAP and 8.0 mgl^{-1} BAP in the present MS medium. Only lowest number of protocorm like bodies was produced on the medium contained 0.5 mgl^{-1} NAA. The same principle was followed to quantify the combined effects of BAP and NAA. Highest no of protocorm like bodies was produced by the medium which contained 2.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA (Table 3). Lowest number of protocrms was produced by the medium containing 8.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA.

Root formation was noted following transfer of the PLBs to the NAA containing ½ inorganic MS medium (Fig.1c). Number of roots per plantlets were highest in ½ inorganic MS medium supplemented with peptone 0.1 % (W/V), Sucrose 2 % (W/V) and NAA (0.1 mgl^{-1}) while the same rooting medium supplemented with NAA(0.8 mgl^{-1}) produced the lowest number of roots.

Plantlet regeneration and acclimatisation

When the PLBs were sub-cultured into MS medium supplemented with 4.0 mg $BAPl^{-1}$, $30gl^{-1}$ sucrose and $2gl^{-1}$ Phytagel, they grew in size. After two weeks leaves were seen protruding from the PLBs (Fig. 1a and Fig. 1b). Upon sub-culturing into fresh medium, plantlets with well-formed leaves and roots were obtained (Fig 1c). These were potted into baskets containing wood charcoal and coco peat separately acclimatized under the conditions mentioned in the materials and methods section to obtain normal plants. Hardened plantlets showed significantly high survival rate (84 %) after seven months.

DISCUSSION

Tissue culture production of ornamental plants in general and orchids in particular forms the foundation for the entire horticultural industry. Orchids, the doyen among ornamental plants, are one of the few flowering floras of commercial value to be propagated *in vitro*, both through seed and tissue culture.

Table 3. Effects of BAP & NAA on leaf culture of Acampe papillosa after 1st, 2nd, 3rd and 4th month of cultures

Treatment (mg/L)		1st month			2nd month			3rd month			4th month		
BAP	NAA	Frequency of explants survival (%)	Frequency of PLB formation (%)	Mean number of PLBs per explant	Frequency of explants survival (%)	Frequency of PLB formation (%)	Mean number of PLBs per explant	Frequency of explants survival (%)	Frequency of PLB formation (%)	Mean number of PLBs per explant	Frequency of explants survival (%)	Frequency of PLB formation (%)	Mean number of PLBs per explant
1	0	100a	60^{b}	1.2 ± 0.58^{a}	100^{a}	100^{a}	8.4 ± 3.66^{a}	100^{a}	100^{a}	11.4 ± 4.46^{c}	100a	100a	19.6 ± 6.00^{a}
2	0	100^{a}	60^{b}	1.2 ± 0.44^{a}	100^{a}	60^{b}	4.8 ± 2.05^{b}	100^{a}	60^{b}	8.4 ± 3.47^{d}	100a	60b	14.8 ± 6.20^{b}
4	0	100^{a}	80^{a}	1.4 ± 0.39^{a}	100^{a}	100^{a}	9.6 ± 2.65^{a}	100^{a}	100^{a}	14.0 ± 3.69^{a}	100a	100a	20.0 ± 5.88^{a}
8	0	100^{a}	60^{b}	0.6 ± 0.24^{c}	100^{a}	100^{a}	8.2 ± 2.39^{a}	100^{a}	100^{a}	13.6 ± 3.58^{b}	100a	100a	19.2 ± 5.12^{a}
0	0.5	100^{a}	00	0.0 ± 0.00	100^{a}	20^{d}	0.2 ± 0.19^{e}	40^{b}	40^{c}	0.4 ± 0.44^{h}	40b	40c	0.4 ± 0.24^{g}
0	1	100^{a}	00	0.0 ± 0.00	40^{b}	00	0.0 ± 0.00	0	00	0.0 ± 0.00	00	00	0.0 ± 0.00
0	2	100^{a}	20^{d}	0.2 ± 0.19^{d}	20^{c}	20^{d}	0.4 ± 0.39^{d}	20^{c}	20^{d}	1.2 ± 0.24^{g}	20c	20d	1.6 ± 1.59^{f}
0	4	100^{a}	00	0.0 ± 0.00	40^{b}	00	0.0 ± 0.00	20^{c}	00	0.0 ± 0.00	20c	00	0.0 ± 0.00
1	1	100^{a}	20^{d}	0.6 ± 0.59^{c}	100^{a}	20^{d}	2.8 ± 2.79^{c}	100^{a}	20^{d}	4.0 ± 3.99^{e}	100a	20d	5.6 ± 5.59^{d}
2	1	100^{a}	60^{b}	1.0 ± 0.54^{b}	100^{a}	60^{b}	5.6 ± 3.30^{b}	100^{a}	60^{b}	7.6 ± 4.06^{d}	100a	60b	7.6 ± 4.97^{c}
4	1	100^{a}	40^{c}	0.4 ± 0.24^{d}	100^{a}	40^{c}	1.6 ± 0.93^{c}	100^{a}	40^{c}	2.8 ± 1.73^{f}	100a	40c	5.2 ± 3.31^{d}
8	1	100^{a}	0	0.0 ± 0.00	100^{a}	40^{c}	0.4 ± 0.24^{d}	100^{a}	40^{c}	1.4 ± 0.67^{g}	100a	40c	2.2 ± 1.31^{c}
0	0	100^{a}	0	0.0 ± 0.00	100^{a}	20^{d}	0.4 ± 0.39^{d}	100^{a}	40^{c}	1.0 ± 0.77^{g}	100a	40c	1.4 ± 0.97^{f}

Data looped from a total of three separate experiments each comprising of ten replicate conical flask containing 1-4 in vitro leaf explants per conical flask. Mean values within column with different superscript alphabets are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Table 4. Root induction in 1st and 2nd month of culture of *Acampe papillosa*

Treatment		1st month		2nd month	
NAA	Frequency of rooting	Mean No. of roots	Root length(mm)	Mean No. of roots	Root length(mm)
0.1 mgl^{-1}	100%	4^{a}	4^{a}	4.6^{a}	13^{a}
0.2mgl^{-1}	100%	3.6^{b}	3^{b}	3.8^{b}	7^{b}
0.4mgl^{-1}	100%	3.4^{c}	2^{c}	3.6^{c}	4^{c}
0.8mgl^{-1}	100%	1.4^{d}	1^{d}	1.6^{d}	2^{d}
Control	0%	0	0	0	0

Data looped from a total of three separate experiments each comprising of ten replicate conical flask containing 1-4 in vitro leaf explants per conical flask. Mean values within column with different superscript alphabets are significantly different ($p \leq 0.05$; Duncan's New Multiple Range Test)

Several researchers using various plant parts as an explant and culture media, introduced tissue culture methods for *in vitro* regeneration of orchids. Germination of orchid seeds is not like germination of other seeds. The longevity of the orchid seeds is highly variable. Some lose their viability in nine months [19] while some others in two months or less [20]. Orchid seeds are produced in huge number within a capsule. The seeds are extremely small, contain an undifferentiated embryo composed of 80-100 cells without any functional endosperm. In certain orchids, self-pollination is not possible, and even if possible as in the case of *Vanda*, one has to wait for 4-6 months for pod development [21] Green pod cultures as against mature/dehisced pod culture, is

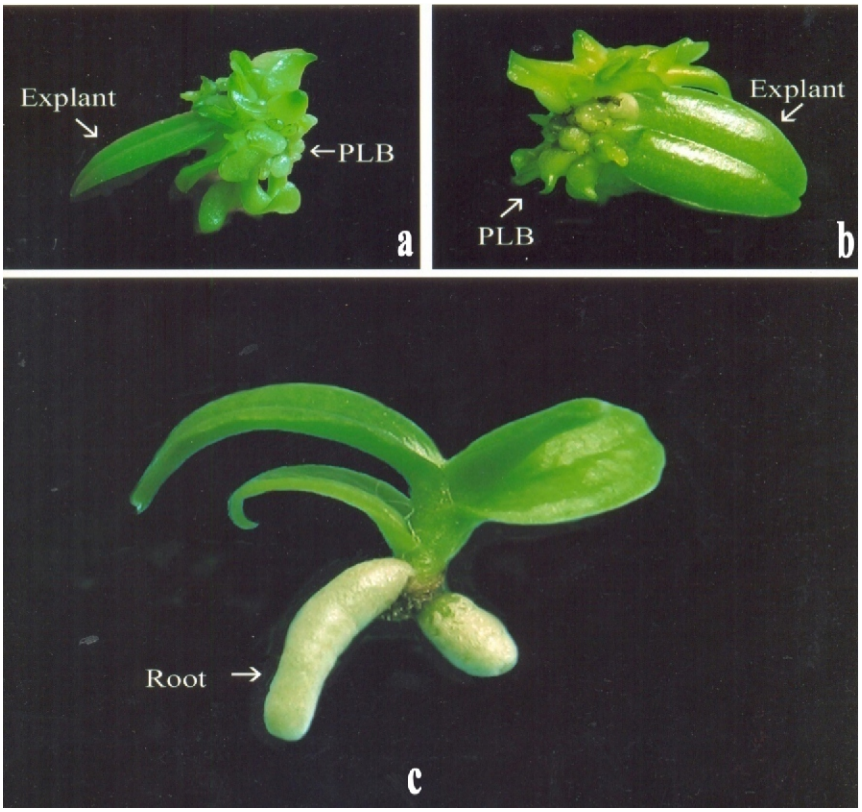

Fig. 1. In vitro regeneration of Acampe papillosa plant from leaf explants.

a & b. PLB formation from basal cut-end of leaf explant, c. Rooting of PLB-derived plantlets

desirable to save time and to avoid contamination.In this study, Minimum (28 days) to seed germination was recorded by ½ MS0 medium. Minimum days required for seed germination of *Dendrobium* sp. in MS medium is 51 and 55 days respectively demonstrated by [22-23]. On the contrary, in the present investigation, the seed germination of *Acampe papillosa* sp. was best on the ½ inorganic MS medium supplemented with peptone 0.1 % (W/V) and Sucrose 2 % (W/V). Due to the non-endospermic nature of the seed, its germination *in vivo* is a unique phenomenon and requires fungal association. Germination is much more successful *in vitro. S*everal authors have suggested different nutrient solution suitable at different stages of growth for various species [24-32]. There was a highly significant variation in present seed germination among different media (Table 1 and Table 2). Knudson C supplemented with peptone 0.1 % (W/V) and Sucrose 2 % (W/V) usually have very less beneficial effect on orchid seed germination.

The highest seed germination (90.32 %) was observed in ½ inorganic MS medium supplemented with peptone and sucrose followed by ½ MS0, MS0 and Hyponex medium, while Knudson C, demonstrated poor performance. This result is in agreement with an experiment conducted by Ismat (1982) [33] with *Dendrobium pierardii* on MS and Hyponex media. More or less similar results (% of seed germination was 81 and 74 in MS and Hyponex media respectively) was found by Hoque (1993) [23] in case of *Dendrobium formusum*.

The production of orchid seedling from seed involves sequential phases of germination, protocorm formation and seedling development. In the present investigation same sequence of plant development was observed when the selected orchid leaf of *Acampe papillosa* Lindl. spp was grown on the medium for plantlet formation. From the results of different fractional combinations of auxin (NAA) and cytokinin (BAP) treatments it is evident that the application of NAA is deleterious for the survival of leaf explants. The necrosis of leaf explants in the presence of NAA took place after two months of culture. However, the application of BAP along with NAA completely prevented the NAA promoted leaf necrosis. Previously a similar role of exogenous cytokine in preventing leaf necrosis was reported in *Doritaenopsis* [33].

In orchid the germinated embryo is called a protocorm. After emergence from the seed coat the protocorms were initially white without any visible shoot apex, but soon they turned green and subsequently developed tiny leaf primordia at the apex and clusters of rhizoids from the basal region. Later the protocorms showed leaf expansion, followed by root initiation at the protocorm base to form complete seedlings. Such developmental stages are common to most orchid species [34]. *In vitro* plant regeneration via PLB is a common phenomenon in the members of orchidaceae. For a long time the exact nature of PLBs wasn't known. However recent histological studies have shown that PLBs are actually somatic embryos & always originate from a single cell [33, 35-36]. Protocorm formation (after 3 to 4 weeks) was done by using ½ inorganic MS medium with BAP and NAA. In the present study both individual and combined effects of BAP, NAA and BAP + NAA stimulated shoot and root growth in *Acampe papillosa* sp. as also reported in *Epidendrum nocturunum* [37] and growth of cymbidium seedlings.

Root formation was prominent following transfer of the PLBs to the NAA containing ½ inorganic MS medium. Number of roots

per plantlets was highest in ½ inorganic MS medium supplemented with peptone, Sucrose and NAA while the same rooting medium supplemented with NAA produced the lowest number of roots. Generally, auxins stimulate root formation and cytokinins enhance shoot development and cell division. The positive effect of cytokinins (e.g., benzyladenine or kinetin) on protocorm development was reported by [38] and the negative effect of kinetin was obsedved by Rasmussen HN [38]. In our experiment, orchid seedlings had significantly higher growth in the presence of auxin and cytokinin combinations. The growth differences in response to auxins and cytokinins do not suggest strong species-specificity in the species studied. Our study showed that treatment of mature seeds with 7.2 % calcium hypochlorite, peptone or auxin (IAA, NAA), and the addition of cytokinin (zeatin, BA) to the culture medium substantially improved germination and seedling growth in *Dactylorhiza incarnata* subsp. *serotina*, *Dactylorhiza maculata* subsp. *maculata* and *Liparis loeselii*. When the PLBs were sub-cultured into MS medium supplemented with BAP, sucrose and Phytagel, they grew in size. Plantlets with well-formed leaves and roots were obtained after two weeks from the PLBs.

CONCLUSION

The present investigation revealed that the ½ inorganic MS medium supplemented with certain concentrations of inorganic nutrients influenced seed germination and ½ inorganic MS medium supplemented with certain concentrations of inorganic nutrients and certain concentration of growth regulators influenced production of protocorm like bodies, shoot multiplication and root initiation. The leaf explants of *Acampe papillosa* have high regenerative potential. Plant regeneration took place through direct PLB formation. Since, the morphogenetic pathway does not involve any intermediary callus phase; the possibility of the occurrence of somaclonal variation is low. Although induction of PLBs could occur in the absence of any exogenous PGRs, addition of BAP improved the yield considerably. The *in vitro* raised seedlings were successfully established in the potting medium. Further growth and development of seedlings and molecular characterisation for soma clonal variation will be observed in further. This opens up the route for *in vitro* clonal mass multiplication of this commercially important species. Therefore, the present method of micro propagation could be used successfully to propagate this important orchid successfully for commercial production. From the above results, it may be suggested that the ½ inorganic MS medium was better for *in vitro* seed germination and clonal propagation from leaf of *Acampe papillosa*.

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