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Effect of auxins on the asymbiotic seed germination of *Malaxis acuminata*- A medicinally important orchid

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Abstract: *Malaxis acuminata* D. Don (= *Microstylis wallichii* Lindl.), The Crimson Shield Orchid, is a widely distributed species found in Thailand, China, Burma and Indo-China. In India, it is available in temperate and subtropical Himalaya, Western Ghats, Nilgiri Hills and Andaman Islands. An important ingredient of '*Ashtavarga*' drugs used in the preparation of an ayurvedic tonic '*Chavyanprash,*' the species is marketed under the trade name '*Rshbhak*'. Immature seeds procured from green capsules (12 wap) of *Malaxis acuminata* were inoculated in Mitra medium for the assessment of their germination potential in media containing different concentrations of IAA, IBA and NAA. 55 and 64% seeds germinated in basal M medium in the absence and presence of AC, respectively. Healthy seedlings were obtained on basal M medium under light conditions in 33 weeks. When the medium was enriched with IAA, as many as 60% seeds germinated under light conditions only at lower concentration of 4.4 µM. However, IAA, in general, supported morphogenesis only till the protocorm stage. Pseudobulbs were formed only at 8.8 µM. IBA supported 45% seed germination at 8.8 µM concentration followed by leaf formation as well. NAA when supplied at 8.8 µM led to a marked increase in the percentage of seeds germinating 63.5%; eventually leading to the multiplication of pseudobulbs. AC was beneficial in rooting in basal M medium and enhancing the seed germination in basal M medium and IBA medium at 4.4 µM concentration.

Keywords: Activated charcoal, Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), *Malaxis acuminata*, Mitra medium, 1-Naphthalene acetic acid (NAA).

Abbreviations: AC: Activated charcoal; D: dark conditions; IAA: Indole-3-acetic acid; L: Light conditions; M: Mitra *et al*. (1976).

1. INTRODUCTION

Malaxis, a group of Adder's Mouth orchids, comprising more than 300 deciduous or evergreen species of terrestrial or rarely epiphytic orchids, is distributed in hot tropical and cold temperate regions, primarily in Asia and Oceania. *Malaxis acuminata* D. Don (= *Microstylis wallichii* Lindl.), The Crimson Shield Orchid, is a widely distributed species found in Thailand, China, Burma and Indo-China. In India, it is available in temperate and subtropical Himalaya (from Himachal Pradesh to Sikkim; 6000-7000 ft), Western Ghats, Nilgiri Hills and Andaman Islands. It is an erect, terrestrial herb, 30 cm high having stem with few sheaths at the base (Handa, 1986; Suyal *et al.,* 2020). The species forms an important ingredient of '*Ashtavarga*' drugs used in the preparation of an ayurvedic tonic '*Chavyanprash*' and is marketed under the trade name '*Rshbhak*' (Handa, 1986). '*Rshbhak*' provides strength, enhances sperm formation and makes a person free from diseases borne by *vat, pit, kaf* and tuberculosis (Khasim and Mohana Rao, 1999; Hegde and Ingalhalli, 1988). The dried pseudobulbs are important ingredients of century old ayurvedic drug 'Ashtavarga' and a polyherbal immune-booster nutraceutical 'Chyavanprash', known to restore vigour, vitality and youthfulness (Bose *et al*., 2017).

Rapid multiplication through tissue culture emerged as an important strategy to release their natural populations from profit-making collection pressures. Plant tissue culture could be one of the most suitable alternative tools to minimize the pressure on natural population of medicinal orchids and their sustainable utilization (Pant, 2013). Widespread pressure of commercial collections and the destruction of its habitat have eventually affected the size and frequencies of natural populations of *Malaxis acuminata* which even otherwise stand impaired due to poor fruit set and slow vegetative propagation. There have been only some reports on the *in vitro* propagation of the species (Deb and Temjensangba, 2006, Pathak *et al*., 2001; Deb and Arenmongla 2014), but they have been few and far-between. The present studies were, therefore, undertaken with a view to develop protocols for propagating medicinally important taxon *Malaxis acuminata in vitro* by using various explants and nutrient recipes.

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2. MATERIALS AND METHODS

The plants of *Malaxis acuminata* were procured from Tara Devi Hills Shimla (Figs. 1, 3) which were maintained in the Orchid House, Botany Department, Panjab University, Chandigarh, for easy availability (Fig. 2). Glassware and instruments (Petri plates, containers, etc.) and surgical instruments (forceps, scalpels, spatulas, scissors, etc.) were treated with chromic acid solution (0.1%) for 24 h, scrubbed with neutral detergent ('Teepol'), washed in running tap water, and dried in an electric oven. Muslin wrapped absorbent cotton plugs were used to plug the test tubes and flasks. All the glassware and surgical instruments were steam sterilized in an autoclave for 40 min at a temperature of 121ºC and pressure of 1.1 kg cm^{-2}, and subsequently dried in an electric oven. Nutrient medium, namely M (Mitra *et al.*, 1976) was used for seed germination of *M. acuminata.* Growth Additives IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), and NAA (1-Naphthalene acetic acid) were added at 4.4, 6.6, and 8.8 µM concentration. The media were gelled with agar (0.85% for M medium); uniform heating and constant swirling ensured proper gelling of the medium.
Depending upon the nature of the experiment, required quantities of plant growth regulators (PGRs) (IAA, IBA, NAA) were incorporated into the medium. In some set of experiments, activated charcoal (AC) powder was used. Defined quantities of the medium, thus prepared, were dispensed into the culture vessels, which were plugged with muslin wrapped cotton plugs. These were then autoclaved for 20 min under pressure of 1.1 kg cm^{-2}. Subsequently the vessels were placed in an appropriate position (test tubes in slanting position) in order to allow the medium to gel. AC, whenever used, was dispersed well by swirling the vessels immediately after autoclaving when the medium was hot.

2.1 Explanting and surface sterilization

Seeds (from 12 weeks after pollination capsules in *Malaxis acuminata*) (Fig. 4) were used to source the explants for culture initiation *in vitro*. The explants were invariably surface sterilized prior to inoculation. They were scrubbed gently with detergent ('Teepol'; 0.5%) using a soft brush, and washed thoroughly under running tap water for 20 min. The explants were further processed under aseptic conditions in a 'Clean air' Laminar air-flow cabinet. The capsules were harvested from the 'Orchid House' which served as the source for seeds (12 wap). The capsules were dipped in 70% ethyl alcohol for 30 sec, flamed, and then sterilized for 10 min in $HgCl_2$ solution (0.1%) with 'Teepol' (1-2 drops) as wetting agent. These were then washed (3-4 times) with sterilized distilled water to remove all traces of $HgCl_2$. After $HgCl_2$ treatment, the capsules were treated with 0.05% 'Bavistin' (10-15 min) and 0.05% Streptomycin (10 min). Each step was followed by a thorough rinse with sterilized distilled water. The capsules were then split open with the help of a scalpel to scoop out the immature seeds.

2.2 Inoculations and Culture conditions

The inoculations were performed manually under aseptic conditions in a 'Clean air' Laminar air-flow cabinet using sterilized surgical instruments. The cultures were maintained at 25±2ºC temperature regime and kept under a 16 h photoperiod. Some sets of experiments were carried out under dark conditions as well, for the initial 12 weeks. The physical conditions were same for culture initiation, subculture, and re-culture experiments. The seeds of *M. acuminata* could not be counted and care was taken to inoculate a similar amount in each test tube.

2.3 Data recording

The observations were made at weekly intervals and the responses were recorded on the basis of visual observations and photographs were clicked on 35 mm SLR camera (Pentax, Japan) and close up lenses (Sonia, India) on Kodak GOLD 100 film. The various morphogenetic events studied during seed/embryo germination of *M. acuminata* included the swelling of embryos; the emergence of spherules through the apical and/or vertical slits in the seed coats; their development into protocorms; formation of leaf/root; formation of pseudobulbous shoots and their multiplication; and the formation of seedlings. The data are expressed as means ± standard deviation (SD) of mean and statistical analysis was performed with SPSS 14.0 for Windows Evaluation Version. Analysis of Variance (ANOVA) was performed followed by Scheffe's test for multiple comparisons. Difference with $P \leq 0.05$ was considered statistically significant.

In vitro germination of immature seeds (12 wap) of Malaxis acuminata on M medium and its various combinations with growth additives, IAA, IBA, NAA under light and dark conditions

Growth adjunc T	L/D	In vitro germination(%)	Time taken for formation of (weeks)		Time taken for development of (weeks)						Remarks
			Spherule	Protocorm	Chlorophyll	First leaf	Pseudobulb	First root	Seedling	Pseudobulb multiplication	

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M	L D	$55\pm0.82^{X,t}$ 40 ± 1.63	$10.88\pm0.25^{O,P,Q,o}$ 8.75 ± 1.26	$13.25\pm0.5^{Q,R,S,o,p}$ 10 ± 0.82	$15.13\pm0.63^{P,Q,R,p}$ 11.75 ± 1.26	$22.75\pm1.56^{R,q}$ 22.13 ± 0.63	$20\pm0.82^{S,q}$ 22.38 ± 0.48	- -	- -	$23.13\pm0.63^{R,p,q}$ 24.25 ± 1.5	Healthy pseudobulbous shoots Pseudobulbous shoots
M*	L D	64.25 ± 0.96 20 ± 1.63	10.88 ± 1.03 9.53 ± 0.41	12.5 ± 0.58 11.88 ± 0.63	14.75 ± 0.96 15.5 ± 1.73	26.38 ± 0.48 26.25 ± 0.87	25.63 ± 0.95 26.75 ± 1.04	30.13 ± 0.86 -	33 ± 0.82 -	28.13 ± 0.63 31.75 ± 0.5	Healthy seedlings (33 weeks) Leaf formed
M+IAA$_{4.4}$	L D	$60.25\pm1.71^{W,r}$ 39.75 ± 1.71	$9.25\pm0.5^{T,o,p,q}$ 11.88 ± 0.85	$10.38\pm0.48^{T,p,q}$ 13.13 ± 0.63	$11.38\pm0.48^{P,Q,o}$ 16 ± 1.41	- -	$-^{O,p}$ -	- -	- -	$-^{O,p}$ -	Protocorms failed to develop Protocorms failed to develop
M*+IAA$_{4.4}$	L D	80 ± 0.82 58 ± 1.63	10.25 ± 0.87 11.88 ± 0.63	11.25 ± 0.87 13.13 ± 0.63	12.5 ± 1.0 16.13 ± 0.63	- -	- -	- -	- -	- -	Chlorophyllous protocorms failed to differentiate further Chlorophyllous protocorms
M+IAA$_{6.6}$	L D	14.5 ± 2.52^{q} 65.25 ± 1.71	13.75 ± 0.5^{r} 12 ± 0.41	$14.88\pm0.63^{r,s}$ 13.13 ± 0.63	- 15 ± 0.41^{r}	- -	$-^{o}$ -	- -	- -	$-^{p,q}$ -	Achlorophyllous protocorms Chlorophyll developed
M*+IAA$_{6.6}$	L D	5 ± 0.82 30 ± 1.63	17.88 ± 0.63 13.13 ± 0.63	- 15.5 ± 1.0	- -	- -	- -	- -	- -	- -	Spherules did not develop further Achlorophyllous protocorms
M+IAA$_{8.8}$	L D	$15\pm1.63^{o,p}$ 5 ± 0.82	$7.75\pm0.5^{p,q}$ 13 ± 0.82	$8.63\pm0.48^{q,r}$ -	10 ± 0.82^{q} -	- -	12.25 ± 0.29^{q} -	- -	- -	16.25 ± 0.5^{o} -	Pseudobulb multiplication Spherule development arrested
M*+IAA$_{8.8}$	L D	25.5 ± 1.73 10 ± 0.82	12.5 ± 0.58 12.38 ± 0.95	19.75 ± 0.5 13.63 ± 1.11	- -	- -	- -	- -	- -	- -	Achlorophyllous protocorms Achlorophyllous protocorms
M+IBA$_{4.4}$	L D	$44\pm1.63^{T,r}$ 24.75 ± 1.26	$6.38\pm0.48^{O,P,o,p,q}$ 9.75 ± 1.26	$8.25\pm0.5^{O,P,p,q}$ 10.75 ± 1.26	$9.38\pm0.48^{O,o}$ 12.25 ± 0.5	- -	$10.63\pm0.75^{P,p}$ 15.75 ± 0.29	- -	- -	$14.75\pm0.5^{O,P,p}$ 19 ± 0.82	Pseudobulbous shoots Pseudobulbous shoots
M*+IBA$_{4.4}$	L D	29.5 ± 1.73 20 ± 0.82	10 ± 0.41 10.88 ± 0.63	11 ± 0.41 11.88 ± 0.63	13.38 ± 0.48 -	- -	- -	- -	- -	- -	Chlorophyllous protocorms failed to develop Achlorophyllous protocorms

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M+I $BA_{6.6}$	L D	30 ± 1.63^{q} 5.25 ± 2.06	7.38 ± 0.48^{r} 10 ± 0.82	$8.38\pm0.48^{r,s}$ 11.63 ± 0.75	9.38 ± 0.48^{r} -	- -	11.5 ± 0.41^{o} -	- -	- -	$16.5\pm0.41^{p,q}$ -	Pseudobulb multiplication Protocorms did not develop further
M*+I $BA_{6.6}$	L D	20 ± 0.82 0	10.75 ± 0.96 -	11.75 ± 0.96 -	13 ± 1.41 -	- -	- -	- -	- -	- -	Protocorms did not differentiate further No germination
M+I $BA_{8.8}$	L D	$45\pm1.63^{o,p}$ 12.25 ± 2.06	$7\pm0.41^{p,q}$ 11.25 ± 0.87	$8.13\pm0.63^{q,r}$ 12.13 ± 0.63	10.25 ± 0.5^{q} 14.13 ± 0.25	$21.75\pm0.65^{C,Q,b,p}$ -	11.38 ± 0.48^{q} 20.25 ± 0.5	- -	- -	15.38 ± 0.48^{o} 23.13 ± 0.63	First leaf formed Pseudobulb multiplication
M*+I $BA_{8.8}$	L D	15 ± 1.63 10 ± 0.82	10.38 ± 0.48 11.13 ± 0.85	11.5 ± 0.71 12.25 ± 1.04	- -	- -	- -	- -	- -	- -	Achlorophyllous protocorms Achlorophyllous protocorms
M+N $AA_{4.4}$	L D	$46\pm2.45^{V,r}$ 5 ± 0.82	$8\pm0.82^{O,P,o,p,q}$ 13.25 ± 1.26	$9\pm0.82^{O,p,q}$ -	$10\pm0.82^{O,o}$ -	- -	$11.13\pm0.63^{Q,p}$ -	- -	- -	$15.88\pm0.63^{O,P,p}$ -	Pseudobulbous shoots Spherule development arrested
M*+ NAA $_{4.4}$	L D	40.25 ± 2.06 26 ± 1.63	9.38 ± 0.48 7.88 ± 0.25	10.38 ± 0.48 9 ± 0.41	12.13 ± 0.48 10.38 ± 0.48	- -	14.38 ± 0.48 14 ± 0.41	- -	- -	19.25 ± 0.5 18.13 ± 0.63	Pseudobulbous shoots Pseudobulbous shoots
M+N $AA_{6.6}$	L D	4.75 ± 1.26^{q} 47 ± 1.63	12 ± 1.63^{r} 6.63 ± 0.48	- $8.63\pm0.48^{r,s}$	- 9.75 ± 0.5^{r}	- -	- 11.88 ± 0.25^{o}	- 28.38 ± 1.11	- -	- $17.88\pm0.63^{p,q}$	Spherule development arrested Root formed
M*+ NAA $_{6.6}$	L D	45 ± 0.82 30 ± 2.45	10.25 ± 0.87 12 ± 0.82	11.25 ± 0.87 13.13 ± 1.03	12.13 ± 0.63 14.38 ± 1.11	- -	17.63 ± 0.48 -	- -	- -	20.5 ± 0.58 -	Pseudobulb multiplication Chlorophyllous protocorms
M+N $AA_{8.8}$	L D	$63.5\pm1.92^{o,p}$ 20 ± 1.63	$7.13\pm0.63^{p,q}$ 6 ± 0.41	$9\pm0.82^{q,r}$ 7.88 ± 0.25	10.13 ± 0.63^{q} 11 ± 0.41	- -	11.13 ± 0.63^{q} 12.88 ± 0.63	- -	- -	16.88 ± 0.63^{o} 18.63 ± 0.48	Pseudobulb multiplication Pseudobulbous shoots
M*+ NAA $_{8.8}$	L D	30.25 ± 2.87 10 ± 0.82	11.88 ± 0.25 10 ± 1.41	13 ± 0.41 11.63 ± 0.48	19.88 ± 0.63 -	- -	29.63 ± 0.48 -	- -	- -	- -	Pseudobulbs formed Achlorophyllous protocorms

L: Light conditions; D: Dark conditions; *: Medium containing AC; Figures mentioned as subscripts indicate the concentrations of the growth adjuncts used. o-z are means for groups in homogenous subsets are displayed using Scheffe's Post hoc tests. Similar superscripts denote similar subsets. Post hoc tests are not performed for AC/nAC and Light/Dark because there are fewer than three groups or error term has zero degrees of freedom. #: Original observation quoted.

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3. RESULTS AND DISCUSSION

In basal M medium, 55±0.82% seeds germinated in 10.88±0.25 weeks. However, under dark conditions, the germination percentage was reduced to 40±1.63%, though the time taken for spherule formation was reduced to 8.75±1.26 weeks. The spherules turned green and developed into protocorms (Fig. 5, 6) in 13.25±0.5 weeks; the differentiation of leaf primordial occurred in 22.75±1.56 weeks. Though the pseudobulbous shoots with 2-3 juvenile leaves were obtained (Fig. 7), rhizogenesis failed to occur. Pseudobulbs obtained thereafter. Additional use of AC enhanced the percentage of germinating seeds to 64.25±0.96%; complete healthy seedlings with 2-3 leaves and 1-2 roots (Fig. 8) were obtained in 33±0.82 weeks. Addition of AC, however, did not prove beneficial under dark conditions as far as germinating percentage and rhizogenesis was concerned; though it induced leaf formation and pseudobulb multiplication.

IAA fortified medium: Equipment of the medium with IAA at 4.4 µM enhanced seed germination (60.25±1.71%) in 9.25±0.5 weeks; although the development was arrested at protocorm stage. Incubation under dark conditions, however, led to a decline in the germination percentage. Addition of AC to the medium was functional in escalating the percentage of germination to 80±0.82%. However, the growth and development of the germinating entities was detained at chlorophyllous protocorm stage. Dark conditions reduced the per cent of seeds germinating. When the medium was supplemented with 6.6 µM concentration of IAA, the germination percentage was reduced to 14.5±2.52% and protocorms failed to develop chlorophyll. Under dark incubation, however, 65.25±1.71% seeds germinated and chlorophyllous protocorms were obtained in 15±0.41 weeks. Additional use of AC proved deleterious. Fortification of the medium with 8.8 µM concentration of IAA supported only 15±1.63% seed germination in 7.75±0.5 weeks; though pseudobulb multiplication was observed. Accessory use of AC sustained 25.5±1.73% germination; though only till the protocorm stage.

IBA supplemented medium When IBA was added to the medium at 4.4 µM concentration, germination was supported in 44±1.63% seeds in 6.38±0.48 weeks and pseudobulbs multiplied as early as 14.75±0.5 weeks. However, incubating the cultures under dark conditions reduced the germination percentage and delayed the onset of spherule formation and subsequent morphogenetic changes. Pseudobulbs obtained under both light and dark conditions multiplied. Protocorms failed to differentiate leaf and root primordial. Addition of AC reduced the per cent of seed germination. IBA when added at 6.6 µM concentration, assisted in germination of 30±1.63% seeds in 7.38±0.48 weeks and pseudobulb multiplication occurred; whereas dark conditions drastically reduced the percentage. On the addition of IBA at 8.8 µM concentration, seed germination slightly increased to 45±1.63% in 7±0.41 weeks. Leaves formed in 21.75±0.65 weeks and pseudobulbs multiplied in 15.38±0.48 weeks. Additional use of AC in the medium was also ineffective irrespective of the photoperiod conditions; the entities failed to develop beyond the achlorophyllous protocorm stage.

NAA fortified medium Presence of NAA (4.4 µM) in the medium reduced (46±2.45%) seed germination; the chlorophyllous protocorms were obtained in 10±0.82 weeks. Pseudobulb multiplication was also induced. Dark conditions were, conversely, inhibitory. Medium fortified with AC reduced the germination percentage and morphogenesis occurred till the pseudobulb multiplication stage. Dark incubation, however, led to a reduction of germination percentage to almost half although the onset of germination was advanced (7.88±0.25 weeks). Increase in the concentration of NAA to 6.6 µM was, nonetheless, restraining. On the other hand, incubation of the cultures under dark conditions, led to the germination of 47±1.63% seeds. It induced root formation in 28.38±1.11 weeks in addition to supporting multiplication of pseudobulbs. Supplying the medium with AC remarkably enhanced seed germination. An enhancement in the concentration of NAA to 8.8 µM led to a marked increase in the percentage of seeds germinating (63.5±1.92%); eventually leading to the multiplication of pseudobulbs in 16.88±0.63 weeks. Additional supply of AC to the medium reduced the germination and delayed its onset, irrespective of the photoperiod conditions.

3.1 Discussion

Orchid seeds are known to contain very low amounts of auxins (cf. Arditti and Ernst, 1984). Their auxin requirements for germination in nature are augmented by the fungal partner in mycorrhizal association (Hayes, 1969). Novak *et al.* (2014) reviewed the involvement of auxins in orchid germination, PLB formation, and somatic embryogenesis. Presently, the effect of IAA, IBA and NAA was assessed separately. Auxins often promote orchid seed germination, protocorm conversion, and seedling development (Kang *et al.*, 2020).

When the medium was enriched with IAA, as many as 60% seeds germinated under light conditions only at lower concentration of 4.4 µM. However, IAA, in general, supported morphogenesis only till the protocorm stage. Pseudobulbs were formed only at 8.8 µM. In disagreement with the above consequences, Manrique *et al.* (2005), however, reported impaired germination frequency in *Comparettia falcata* in IAA supplemented medium. In contrast to

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the above results, Devi *et al*. (2006) reported IAA to favour PLB multiplication in *Vanda amesiana*; while root and leaf formation was favoured only at 0.1 and 2.0 mg l^{-1}; however, it proved ineffective (0.5 mg l^{-1}) and detrimental to growth (1.0 mg l^{-1}) at lower concentrations in consonance with the current investigations. In the present study, IAA was not found to have any positive impact on the seed germination percentage and differentiation of protocorms occurred at higher concentrations of 6.6 and 8.8 µM. However, Talukdar (2001) and Sharma (2009) reported stimulatory effect of IAA (10.0 mg l^{-1}) on seedling growth and protocorm differentiation and leaf formation in *Dendrobium aphyllum* and *Habenaria commelinifolia* and *Malaxis acuminata*, respectively. Arenmongla and Deb (2012) reported the beneficial effect of NAA in enhancing the seed germination of *Malaxis acuminata* till 85%. Nongdam and Tikendra (2014) reported best seed germination percentage in *Dendrobium chrysotoxum* in Mitra medium containing 0.4% AC and 2 mg/l BAP and 2 mg/l IAA. In contrast to the present studies, IAA was the most influential among auxins tested in promoting rooting and shooting and prominent shoot development and maximum root formation were noticed in medium supplemented with 2.0 mg/L IAA in *Dendrobium thyrsiflorum* (Tikendra et al., 2018).
The presence of IBA in the medium was unable to match up to the germination percentage shown by the basal medium in the current study. However, leaf formation and pseudobulb development occurred at a higher concentration of 8.8 µM. Similarly, IBA supplemented medium supported leaf formation in *Vanda amesiana* (Devi *et al*., 2006). In contrast to the current results, IBA supplemented medium supported cent per cent germination in *Bulbophyllum careyanum* and *Dendrobium chrysotoxum*, promoted protocorm multiplication in *D. chrysotoxum*, and favoured luxuriant seedling growth in *B. careyanum* and *D. chrysotoxum* (Kher, 1999); and seed germination in *Cymbidium lowianum* and healthy seedlings were obtained in 32 weeks in M medium (Nongdam *et al*., 2006). IBA improved the germination frequency but remained ineffective for subsequent morphogenetic events in the germinating entities in *Habenaria commelinifolia* (Sharma, 2009).
During the investigations at hand, NAA at 8.8 µM showed germination percentage of 63.5% which was comparable to basal M medium in the presence of AC. NAA at 6.6 µM was beneficial in supporting 47% seed germination, pseudobulb formation and rooting. However, contrary to our results, *Vanda tessellata* seeds formed roots in the medium containing IAA (1.0 mg l^{-1}) (Bhadra *et al*., 2005). NAA promoted seedling growth in *V. amesiana* but its effect on PLB induction was dose specific (Devi *et al*., 2006). In resemblance with the present study, Devi *et al*. (2006) stated that additional use of NAA favoured PLB multiplication of *V. amesiana* but their effect on leaf and root formation varied with the quality and concentration of the PGRs. In the current study, NAA was more effective at lower concentration, though an odd result was shown by NAA at 8.8 µM under light conditions (63% germination). In a similar way, Pant and Gurung (2005) reported that lower concentration of NAA was more useful than its respective higher concentration in inducing seed germination of *A. odorata*. Tang *et al*. (2005) also found NAA (0.5 mg l^{-1}) to produce highest number of roots and root length in *Dendrobium candidum*. However, stimulatory effect on protocorm differentiation and seedling formation was reported in NAA supplemented medium (5.0 and 10.0 mg l^{-1}) in *D. aphyllum* (Talukdar, 2001). NAA reduced the germination frequency but enabled earlier leaf differentiation in *Dactylorhiza hatageria* (Sharma, 2009). In the present study, root formation was stimulated by NAA (6.6 µM) enriched medium. Presence of NAA supported efficient seed germination and rapid protocorm development in *Cymbidium lowianum* in addition to proving best for producing higher number of roots and rapid root initiation (Nongdam *et al*., 2006). The present studies showed that NAA at 6.6 µM concentration supported rooting in *Malaxis acuminata*; while IAA and IBA proved ineffective in root formation. Presence of elevated level of IBA or NAA with low cytokinin content in medium generated more *in vitro* rooting, though IBA was found to be more effective in rooting induction as compared to NAA (Nongdam and Tikendra, 2014). The conversion of protocorms into seedlings was maximized by the supplementation of optimal levels of IBA (2 µM) or NAA (1 µM) in *Gastrochilus masturan*. Nevertheless, increasing the optimal level of IBA or NAA could decrease the frequency of seedling conversion. The maximum seedling conversion rate was 92.3% for protocorms grown on ½ MS (with vitamins) medium containing 1 µM NAA (Kang et al., 2020).

CONCLUSIONS

The present study revealed the prospects of developing regeneration protocols for rapid *in vitro* propagation of *Malaxis acuminata*. The asymbiotic seed germination of *M. acuminata* can be achieved by bypassing the fungal requirement *in vitro*. The basal Mitra medium was the best for seedling development, protocorm multiplication, pseudobulb formation and seedling formation. Basal Mitra medium also resulted in highest and earliest seed germination. AC promoted root formation and healthy seedling formation in Mitra medium. IAA when used at 4.4 µM concentration was useful for 60% seed germination and additional use of AC led to an increase in germination upto 80%. However, protocorms failed to develop further. IBA supported 45% seed germination at 8.8 µM concentration followed by leaf formation as well. NAA when supplied at 8.8 µM led to a marked increase in the percentage of seeds germinating 63.5%; eventually leading to the multiplication of pseudobulbs. A lower concentration of NAA (6.6 µM) supported root formation as

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well. AC was beneficial in rooting in basal M medium and enhancing the seed germination in basal M medium and IBA medium at 4.4 µM concentration.

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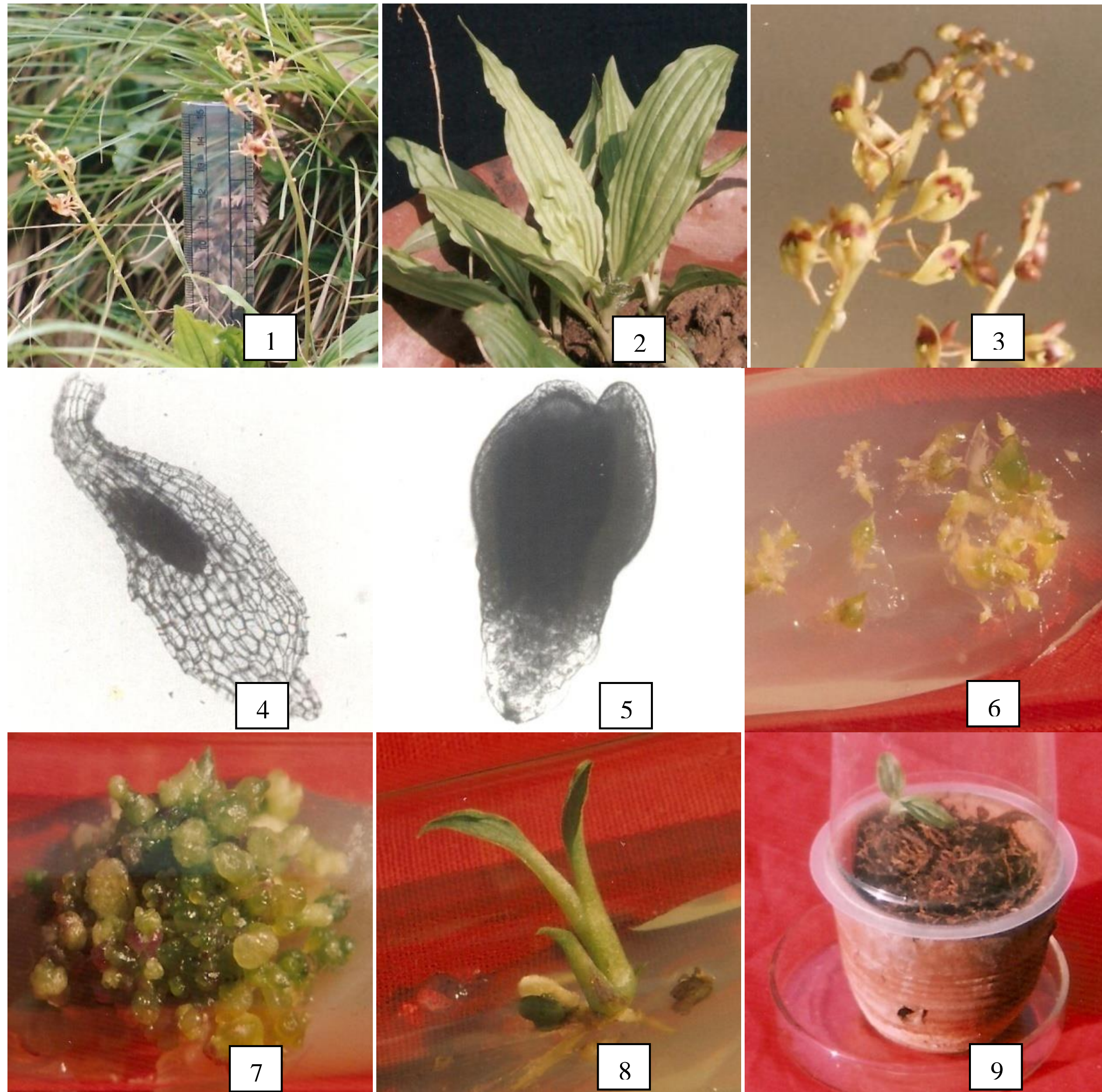

Fig 1- 9: 1, *Malaxis acuminata* in natural habitat in Tara Devi hills Shimla; 2, the plant in Orchid House; 3, inflorescence in natural habitat; 4, seed at the time of inoculation; 5, a protocorm; 6, protocorm formation; 7, pseudobulb multiplication; 8, a seeding; 9, lab to land transfer.

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