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In vitro Germination and Mass Multiplication of an Endangered Medicinal Orchid *Bulbophyllum crassipes* Hook. f. from Bangladesh

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Key words: Bulbphyllum crassipes, Medicinal, Protocorms, MSBs, Endangered orchids

Abstract

An efficient procedure for mass multiplication of an epiphytic medicinal orchid from Bangladesh *Bulbophyllum crassipes* was established by *in vitro* seed culture. Seeds obtained from immature pods of *B. crassipes* were cultured on four different types of nutrient media *viz.* MS, PM, modified VW and B5. The maximum percentage of seed germination (90.75 ± 0.61) was recorded on MS where various plant growth regulators (PGRs) were used either individually or in combination for secondary protocorm development and multiple shoot buds (MSBs) induction. Secondary protocorm were developed from primary protocorm on MS with BAP, Kn and NAA. The highest number of secondary protocorms (21.07 ± 0.12) were obtain from primary protocorm in MS with 1.0 mg/l Kn and 0.5 mg/l NAA. Here 1.0 mg/l BAP and 0.5 mg/l NAA showed the best performance on plant height (3.64 ± 0.07 cm). Maximum multiple shoot buds (8.40 ± 0.26) per single shoot were developed on MS with 1.0 mg/l BAP and 0.5 mg/l NAA. For root induction single shoots were sub-cultured on ½ MS supplemented with NAA, IAA and IBA. Maximum root induction (6.50 ± 0.29) and its growth (4.11 ± 0.08 cm) was observed on ½ MS supplemented with 1.0 mg/l IAA. The well rooted plantlets were acclimatized and successfully transferred to pot containing coconut husk, brick pieces and charcoal at a ratio of 2:1:1 for further growth and development.

Introduction

Among the angiosperms, orchids are the largest and most diverse group of plants and it is mainly cultivated as ornamental plants. It's prized as cut flowers due to their exotic appearance and long-lasting flowers, many of them are also used for herbal medicine, food and other cultural purposes (Arditti 1967, Khasim and Rao 1999). Because

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of habitat destruction and indiscriminate collection many orchid species are threatened (Pant 2013). It is well understood that orchids are widely used in traditional Chinese medicine and ancient Indian system of medicine called Ayurveda (Singh and Duggal 2009, Pant 2013). However, many orchids have apparent medicinal and glycosidal importance and the possibility of their role in herbal medicine is often overlooked (Hossain 2011).

Orchids have been widely used in the indigenous medical system for more than 3000 years and are rich in alkaloids and other phytochemical components. The leaves, pseudobulbs, and flowers of orchids are highly significant in ethnobotany and are applied externally and internally by indigenous people to treat a variety of illnesses (Priya and Krishnaveni 2005). Different types of orchids are reported in Bangladesh, but they are most prevalent in Chattogram, the Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur, and Sunarbans (Rashid et al. 2017, Rahman et al. 2017).

The causes of local orchid extinction include habitat damage, deforestation, climate change, and indiscriminate collecting by enthusiasts and traders (Huda and Jahan 2019, Bhattacharjee and Islam 2014). However, it is necessary to take measures for the conservation and propagation of local orchids. The genus *Bulbophyllum* plays an integral significance in many civilizations, serving as a sacred, protective, ornamental, cosmetic, and therapeutic plant. *Bulbphyllum crassipes* belonging to the genus *Bulbophyllum* is an epiphytic medicinal orchid of Bangladesh. Pseudobulb of *B. crassipes* is eaten raw to treat stomach problems, acidity, constipation, dysentery and as tonic (Bhinija et al. 2021).

Many tribal groups living in India consume this orchid as a source of nutritional supplements (Mudalkar and Marandi 2023). *Bulbophyllum crassipes* is one of the most threatened species in India and also in the sub-continent (Barik et al. 2018). Vegetative propagation of orchid species is common but the developmental process is very slow and expensive (Bhattacharya and Banerjee 2020). The symbiotic seed germination method of orchid was first developed by Knudson (1922). Seeds of *Bulbphyllum crassipes* are minute and non-endospermic and its vegetative propagation takes a long time. Due to slow and difficult conventional propagation, mass propagation through *in vitro* culture is a must needed and useful technique for *B. crassipes*. In view of this, the present investigation was undertaken for the development of an efficient *in vitro* protocol for asymbiotic seed germination, protocorm development and mass propagation of *B. crassipes* using suitable plant growth regulators for its conservation and commercial cultivation purposes in Bangladesh.

Materials and Methods

For this study mature plants of *Bulbphyllum crassipes* were collected from Sylhet, Bangladesh. The plants were maintained in the Orchid shade house of Institute of Biological Sciences (IBSc), University of Rajshahi. The flowering period of this plant is in the month of October and the fruiting period is in the month of December - March.

Immature fresh capsules were retrieved and the seeds were cultured on medium in aseptic condition. The collected capsules were cleaned by washing under running tap water and later soaked them in aqueous solution of detergent for 10-15 min. The capsules were surface sterilized by dipping them with 0.2% $HgCl_2$ solution for 5.0 min and washed them 4-5 times by sterile double distilled water in a laminar air flow cabinet. With a sterile surgical blade, the green capsules were dissected longitudinally and the seeds were cultured.

Four types of media *viz*. MS, PM (phytomax™), modified VW (Vacin and Went 1949) and B5 (Gamborg et al. 1968) were used for seed germination and protocorm development. MS was supplemented with 3% sucrose while PM, B_5 and modified VW were amended with 2% sucrose. The pH of all media (PM = 5.6, VW, B_5 and MS = 5.8) was adjusted before adding agar and autoclaving.

The cultured vessels were kept in the growth chamber at 25 ± 2°C under photoperiod of 16 hrs light and 8 hrs dark cycle. Seed germination data were recorded after 30 days of inoculation. Percentage of germination was calculated by using the following formula and was repeated thrice.

$$\text{Percentage (\%) of seed germination} = \frac{\text{Number of seed swelling of the embryo}}{\text{Total number of seeds}} \times 100$$

Once the spherules (irregular shaped cell mass) were formed, observation were recorded at an interval of one week to trace protocorm development. Later protocorm were taken out aseptically from culture vessels and transferred into fresh culture vessels containing the same germination medium. *In vitro* grown sixty days old protocorm of *B. crassipes* were used as explants in this experiment. These *in vitro* grown protocorms were cultured on MS medium with various concentrations and combinations of plant growth regulators for the production of secondary protocorm and its sub-sequent development. Different concentrations of BAP (0.5-2.0 mg/l), Kn (0.5-2.0 mg/l) and NAA (0.5, 1.0 and 2.0) mg/l) were used in this study. In this case MS basal medium was considered as control.

To evaluate elongation of plantlets, they were transferred to MS medium supplemented with different concentrations of PGRs either single or in combination. The PGRs used in this study were BAP (0.5-2.0 mg/l), Kn (0.5-2.0 mg/l) and NAA (0.25-1.0 mg/l). For multiple shoot bud induction, the shoot segments were transferred to MS medium with above mentioned PGRs of same concentration and combination.

Shoot without roots were cultured on half strength MS medium with auxins for root induction. Different concentrations (0.5-2.0 mg/l) of NAA, IAA and IBA were used alone for the assessment of root development. The well rooted mature plantlets were hardened successfully in a pot containing coconut husk, brick pieces and charcoal at a ratio of 2:1:1 and kept in the orchid shade house of IBSc, University of Rajshahi. Data about seed germination percentage, production of secondary protocorm, plant height, development of shoot and root were recorded. Data were also recorded on the basis of production of

several protocorm from one primary protocorm and their viability to developed plantlets after thirty days of culture. Mean values of 30 culture vessels were taken for each treatment and all experiment were repeated thrice. For this study 10 explants were taken for each time.

Five replicates were taken per medium for seed germination. To investigate the main effect of media, PGRs and their interaction on plant multiplication of *B. crassipes*, data were assigned to analysis of variance, and the difference of means were separated by the Duncan's Multiple Range test (DMRT) with significance at the 5% level. When required the results were expressed as the means ± SE.

Results and Discussion

For seed germination and protocorm development all four media were used where no PGRs was added. Seeds were collected from immature pod (Fig. 1a). Immature seeds were selected because for many orchid species, immature seeds offer higher germination rates than mature seeds (Rasmussen 1995, Yamazaki and Miyoshi 2006).

Seed germination and protocorm development were influence by the types of medium and they responded in different ways and at different times. The highest response (90.75 ± 0.61%) of seed germination was found on MS medium with lowest required time than PM (79.90 ± 1.36%), MVW (70.65 ± 1.17%) and B_5 (47.65 ± 0.98%). It was observed that within 4-5 weeks the germination was first evident by swelling and emergence of the embryo from testa.

The undifferentiated embryos formed an irregular shaped cell mass as spherules. Within 1-2 weeks these spherules turned green and form round shape (Fig. 1b) protocorms. It became visible with vegetative apex after 6 weeks of culture initiation (Fig. 1c). Later they transformed into seedling with the development of 1-2 leaf primordia (Fig. 1d) followed by the development of young plantlets (Fig. 1e, Table 1). Further sub-culture was done at a lower density on the same culture media (Fig. 1f). Within two weeks these plantlets with 1-2 leaves were ready to use for further study (Fig. 1g) medium being used as control.

Sixty days old primary protocorms were sub-cultured on MS with different type of auxin and cytokinin to assess their effectiveness on multiplication and generation of secondary protocorm. PGRs with different concentrations of BAP (0.5-2.0 mg/l) and Kn (0.5-2.0 mg/l) were used singly. On the other hand, different concentrations of BAP (0.5-2.0) mg/l, Kn (0.5-2.0) mg/l and NAA (0.5, 1.0 and 2.0) mg/l were used in combination. MS basal after 3 weeks of culture initiation secondary protocorm were started to produce directly instead of shoot formation. The maximum number of protocorm (21.07 ± 0.12) were observed after 28 days of culture on MS with 1.0 mg/l Kn and 0.5 mg/l NAA followed by 2.0 mg/l Kn and 1.0 mg/l NAA (Table 2). Combined effect of Kn and NAA was more effective over other PGRs on production of secondary protocorm of *B. crassipes*. Within 4-5 weeks maximum protocorms were formed vegetative apex (Fig. 2a) followed

by the development of leaf primordia (Fig. 2b) and later converted successfully into healthy plantlets (Figs 2c-d). Minimum number of protocorm were developed on MS with

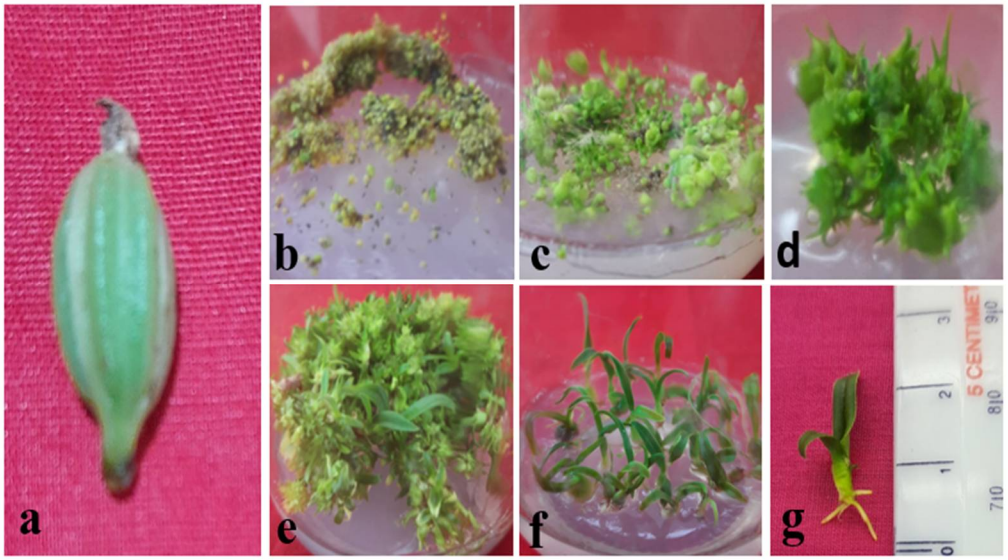

Fig. 1 (a-g). *In vitro* seed germination stages of *Bulbophyllum crassipes*. (a) immature seed pods; (b) germinated seeds (after 4 weeks); (c) round shaped of primary protocorm with shoot apex; (d) seedlings with leaf primordia; (e-f) young plantlets and (g) a complete plantlet with roots.

Table 1. Comparative effect of four culture media on seed germination and protocorm development of *B. crassipes*.

Culture media	Time required (weeks)		Percentage (%) of seed germination (Mean ± SE)
	Spherule formation	Protocorm formation	
MS	4 -5	6 -7	90.75 ± 0.61[a]
PM	6 -7	8 -9	79.90 ± 1.36[b]
Modified VW	10-11	12-13	70.65 ± 1.17[c]
B_5	11-12	13-14	47.65 ± 0.98d[d]

MS = Murashige and Skoog (1962) PM = Phytamax™ (Sigma, USA), B_5 = Gamborg et al. (1968), Vacin and Went (1949) and MVW- Modified Vacin and Went medium.

2.0 mg/l BAP but the lowest number of protocorm was obtained from MS basal medium which was used as control (Table 2). *In vitro* grown young plantlets derived from protocorms were transferred to MS medium supplemented with different types of cytokinin and auxin to assess their efficacy on elongation of plant. Later elongated shoot segments were sub-cultured on fresh MS medium with same concentration and

combination of PGRs which was used either single or in combination to observed shoot induction. The lowest elongation (1.20 ± 0.04 cm) was found when it was cultured on MS with 2.0 mg/l Kn. MS medium with BAP and NAA was more effective for elongation of plant than other PGRs used in this study (Fig. 2e).The highest shoot bud induction (8.40 ± 0.26) was recorded on MS with 1.0 mg/l BAP and 0.5 mg/l NAA and lowest was recorded when MS supplemented with 2.0 mg/l Kn (Fig. 2f).

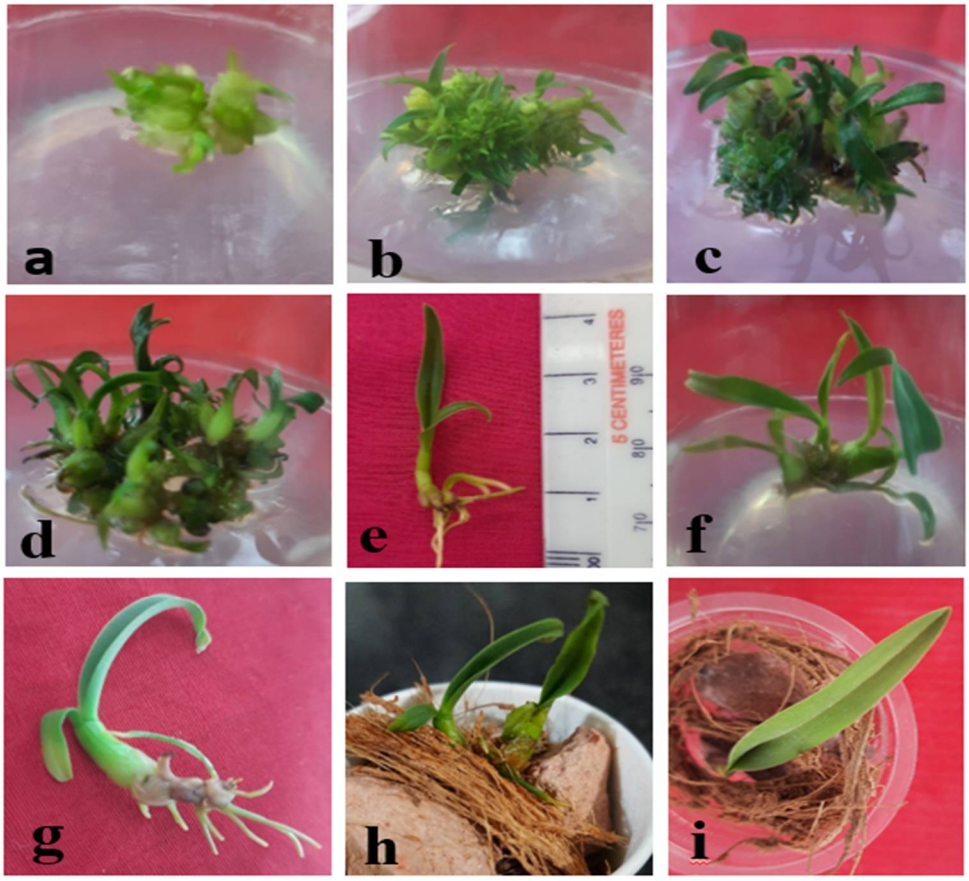

Fig. 2 (a-i). Production of secondary protocorm and its subsequent regeneration of *B. crassipes*. (a) secondary protocorm developed from single protocorms with vegetative apex; (b) leaf primordia formation; (c-d) healthy plantlets, (e) elongated plantlets; (f) MSBs induction; (g) well rooted plant (h) plants transferred to pot after acclimatization and (i) plants grown in pot.

MS medium without PGRs provide poor height of plantlets and MSBs induction than PGRs supplemented medium. BAP and NAA were proved superior over other PGRs used for MSBs induction (Table 3). It was certain that in case of shoot development of *B. crassipes* cytokinin in addition with auxin provided better result than single cytokinin. Well rooted plantlets were acclimatized in pots containing potting mixture (Figs 2g-h).

Table 2. Effects of PGRs on production of secondary protocorm of *B. crassipes.*

PGRs (mg/l)			No. of secondary protocorms (Mean ± SE)	Time required (weeks)
BAP	Kn	NAA		
0.5	-	-	6.10 ± 0.29^{c}	
1.0	-	-	7.50 ± 0.15^{b}	6-8
1.5	-	-	8.00 ± 0.26^{a}	
2.0	-	-	5.03 ± 0.09^{d}	
-	0.5	-	7.80 ± 0.12^{c}	
-	1.0	-	9.90 ± 0.31^{a}	
-	1.5	-	9.00 ± 0.35^{b}	6-8
-	2.0	-	6.87 ± 0.23^{d}	
0.5	-	0.5	9.50 ± 0.29^{e}	
1.0	-	0.5	14.13 ± 0.27^{a}	
0.5	-	1.0	10.30 ± 0.15^{d}	
2.0	-	1.0	12.83 ± 0.20^{b}	5-6
1.0	-	2.0	11.00 ± 0.35^{c}	
1.0	-	1.0	8.27 ± 0.18^{f}	
2.0	-	2.0	7.00 ± 0.12^{g}	
-	0.5	0.5	16.43 ± 0.30^{c}	
-	1.0	0.5	21.07 ± 0.12^{a}	
-	0.5	1.0	12.50 ± 0.15^{e}	
-	2.0	1.0	18.00 ± 0.23^{b}	4-5
-	1.0	2.0	10.77 ± 0.24^{f}	
-	1.0	1.0	15.00 ± 0.31^{d}	
-	2.0	2.0	9.30 ± 0.17^{g}	
Control (MS0)			3.20 ± 0.12	7-9

Values represent mean ± SE (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD) Test ($p < 0.05$).

After one week they were shifted under natural condition at orchid shed house of Institute of Biological Sciences, University of Rajshahi (Fig. 2i). For the assessment of root development elongated shoots were sub-cultured on ½ MS with different concentrations of auxins. Here maximum number of roots (6.50 ± 0.29) per shoot was developed when it was cultured on ½ MS supplemented with 1.0 mg/l IAA. The highest length of root (4.11 ± 0.08 cm) was also observed on same medium. Significant difference on root formation and its elongation at different concentrations and combinations of auxins are shown in Figs 3a-b. Data was recorded after 30 days of culture. In case of auxin supplemented

media IBA was less effective for root formation and elongation. But the lowest root induction was observed on basal medium used as control.

Table 3. Effects of MS with different concentrations and combinations of PGRs on plant elongation and multiple shoot buds (MSBs) induction after 30 days of culture initiation in *B. crassipes*.

PGRs (mg/l)			Plant height (cm)			No. of shoots per single shoots Mean ± SE
BAP	Kn	NAA	Initial length Mean ± SE	Final length Mean ± SE	Increased length Mean ± SE	
0.5	-	-	2.24 ± 0.03	4.24 ± 0.05	2.00 ± 0.09^{b}	4.00 ± 0.23^{c}
1.0	-	-	2.26 ± 0.04	4.78 ± 0.06	2.52 ± 0.03^{a}	5.50 ± 0.25^{a}
1.5	-	-	2.28 ± 0.02	4.10 ± 0.09	1.82 ± 0.07^{c}	4.80 ± 0.15^{b}
2.0	-	-	2.26 ± 0.01	3.64 ± 0.03	1.38 ± 0.05^{d}	3.20 ± 0.12^{d}
-	0.5	-	2.24 ± 0.02	4.14 ± 0.05	1.90 ± 0.05^{b}	3.50 ± 0.17^{c}
-	1.0	-	2.23 ± 0.03	4.52 ± 0.04	2.29 ± 0.03^{a}	4.90 ± 0.15^{a}
-	1.5	-	2.28 ± 0.04	4.00 ± 0.06	1.72 ± 0.02^{c}	4.10 ± 0.06^{b}
-	2.0	-	2.25 ± 0.03	3.45 ± 0.04	1.20 ± 0.04^{d}	3.00 ± 0.21^{d}
0.5	-	0.25	2.23 ± 0.02	4.69 ± 0.04	2.46 ± 0.04^{c}	6.30 ± 0.31^{c}
1.0	-	0.50	2.26 ± 0.02	5.90 ± 0.05	3.64 ± 0.07^{a}	8.40 ± 0.26^{a}
1.5	-	0.75	2.21 ± 0.03	5.19 ± 0.03	2.98 ± 0.08^{b}	7.27 ± 0.12^{b}
2.0	-	1.0	2.21 ± 0.02	4.14 ± 0.05	1.93 ± 0.05^{d}	5.90 ± 0.17^{d}
-	0.5	0.25	2.23 ± 0.01	4.38 ± 0.04	2.15 ± 0.04^{c}	6.00 ± 0.06^{c}
-	1.0	0.50	2.25 ± 0.03	5.25 ± 0.07	3.00 ± 0.05^{a}	7.83 ± 0.09^{a}
-	1.5	0.75	2.20 ± 0.01	4.86 ± 0.04	2.66 ± 0.06^{b}	6.60 ± 0.12^{b}
-	2.0	1.0	2.24 ± 0.02	3.88 ± 0.05	1.64 ± 0.03^{d}	5.13 ± 0.03^{d}
0.5	0.5	-	2.25 ± 0.02	3.86 ± 0.04	1.61 ± 0.02^{c}	4.50 ± 0.26^{c}
1.0	0.5	-	2.30 ± 0.04	4.83 ± 0.04	2.53 ± 0.05^{a}	5.40 ± 0.23^{a}
0.5	1.0	-	2.28 ± 0.04	4.58 ± 0.04	2.30 ± 0.04^{b}	5.00 ± 0.25^{b}
1.0	1.0	-	2.24 ± 0.01	3.64 ± 0.05	1.40 ± 0.04^{d}	3.80 ± 0.12^{d}
MSO (Control)			2.26 ± 0.01	3.16 ± 0.05	0.90 ± 0.03	2.80 ± 0.058

PGRs = Plant growth regulators, values represent mean ± SE (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD) at $p < 0.05$ level.

In the present study, for *in vitro* seed germination of *B. crassipes* four types of basal media were used. Among the media, MS medium proved to be the best one where 90.75% seeds were germinated. Here protocorm formation was also recorded highest on MS medium. The effectiveness of MS medium on seed germination of different orchid species was reported by several researchers on several orchid species such as *Cymbidium*

mastersii, Rynchostylis resuta, Bulbophyllum auricomum (Mohanty et al. 2012, Bhattacharjee and Islam 2015, Aung et al. 2022). In this study MS supplemented with 1.0 mg/l Kn and 0.5 mg/l NAA showed the highest rate of secondary protocorm developed from primary protocorms. Kn and NAA also proved to be the best for secondary protocorm development of *Vanda stangeana* (Pebam et al. 2016). Different research works showed that, nutrient requirements and PGRs varies from species to species for the production of secondary protocorm. According to the findings of some researchers BAP and NAA was best for secondary protocorm development (Mohanty et al. 2012, Hossain 2014, Bhattacharjee and Islam 2015). Kang et al. (2020) reported that ½ MS medium with TDZ was best for secondary protocorm formation. On the contrary, Hossain et al. (2010) reported that, maximum number of secondary protocorm developed from *Cymbidium giganteum* on PM medium supplemented with BAP and NAA.

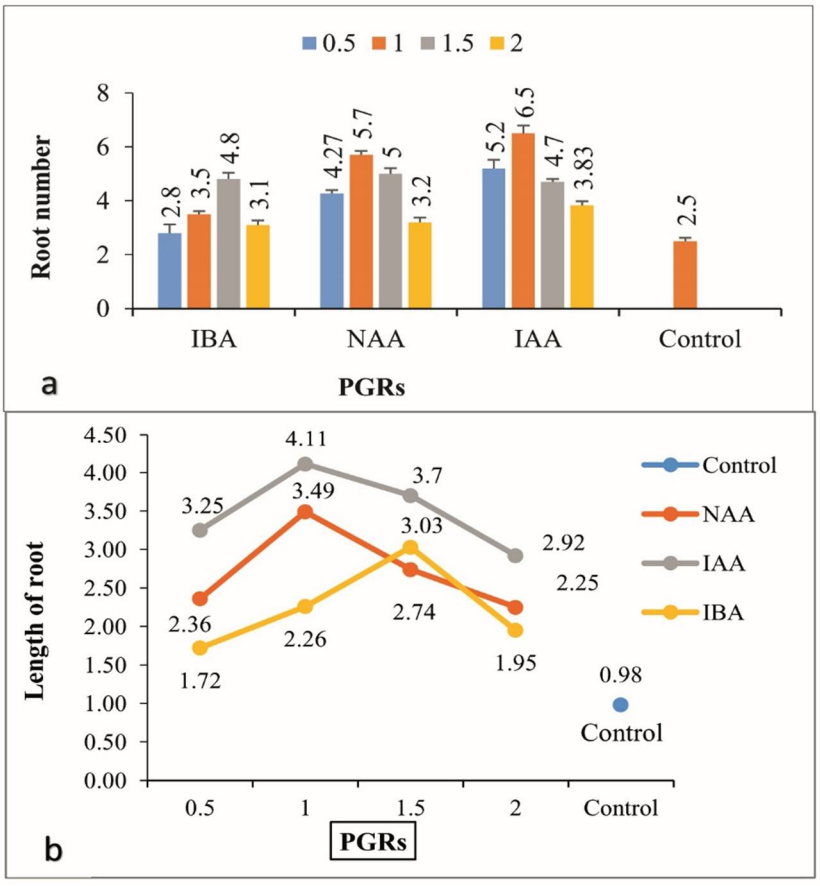

Fig. 3 (a-b). Effect of ½ MS with different auxins on root development of *B. crassipes*. (a) numbers of roots and (b) length of roots.

For the investigation of plant height development, young healthy seedlings were sub-cultured on MS with several types of PGRs with different concentrations either single or in combination. Highest rate of elongation was observed when sub-cultured on MS medium with 1.0 mg/l BAP and 0.5 mg/l NAA.

The same concentration of BAP combined with NAA was also provided the best result on multiple shoot induction. The positive effect of BAP combined with NAA on shoot development of orchid were reported by several researchers. The enhanced performance of PGRs on the plant height elongation of *Orchis catasetum*, number of leaves of *Orchis catasetum*, *Doritaenopsis* and multiple shoot induction of *Vanda tesselata, Dendrobium palpebrae* was reported by several researchers (Baker et al. 2014, Chowdhury et al. 2003, Rahman et al. 2009, Bhowmik and Rahman 2020).

In case of root formation and its elongation, IAA was the most effective than other auxins used in this study. It was observed that the highest number of roots were developed when it was cultured on ½ MS with 1.0 mg/l IAA. The media with same concentration of IAA was also showed better result for the elongation of root. Several researchers found the positive effect of IAA on root development of orchid (Pant and Gurung 2005, Sunitibala and Kishor 2009, Castillo-Pérez et al. 2022). For acclimatization the well rooted plantlets were transferred from culture vessels to pots with coconut husk, brick pieces and charcoal at a ratio of 2:1:1.

The present study is a complete protocol for the *in vitro* micropropagation of *Bulbophyllum crassipes* through seed culture. MS medium was best for both seed germination and protocorm development. Combined effect of PGRs was more effective for secondary protocorm development, induction of MSBs and plant height development than single uses. IAA was effective for root development. This micropropagation technique can be used for mass scale production of *B. crassipes*.

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