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Micropropagation of Terrestrial Orchid *Eulophia graminea* Lindl. Available in the Chittagong Hill Tracts, Bangladesh

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Abstract

For *ex situ* conservation, *Eulophia graminea*, a terrestrial orchid, can be micro-propagated on MS medium supplemented with different concentrations and combinations of plant growth regulators (PGRs), auxins (IAA, IBA, NAA, Picloram) and cytokinins (BAP, Kn) to produce MSBs or PLBs from *in vitro* grown rhizome and leaf segments of germinated plantlets accordingly. In the case of rhizome segments, organogenesis occurred on MS medium supplemented with 3.0 mg/l BAP and 1.5 mg/l NAA producing the highest percentage of MSBs per segment. The average number of induced MSBs was 7.8 ± 0.58 and the rate was 89.33 ± 1.96 percent within the minimum required time of 4.42 ± 0.10 weeks. However, embryogenesis took place in the leaf segment and the MS medium supplemented with 3.0 mg/l BAP and 1.5 mg/l Picloram produced the prime percentage of greenish PLBs. The maximum rate was 78.67 ± 2.24 percent in the shortest amount of time, which was 5.36 ± 0.09 weeks. Following 30 days of mini shoot bud culture, the maximum elongation rate on liquid conditions was 4.23 ± 0.04 cm, whereas the maximum rate on semi-solid media was 4.17 ± 0.03 cm. MS supplemented with 2.0 mg/l BAP and 1.2 mg/l NAA was found to be best for semi-solid and liquid cultures for the growth and elongation of MSBs from rhizome segments. MS supplemented with 1.0 mg/l IBA and 1.0 mg/l NAA was best for root induction and development. The length of the roots was 3.76 ± 0.04 cm and the number of roots per seedling was 5.00 ± 0.28. After successive phases of acclimatization hardened seedlings were transferred to the outside condition and the survival rate was 74.29%.

Introduction

The Orchidaceae are one of the largest families of angiosperms and are considered the most attractive among all flowering plants. With 28,237 species, it is the most developed family among monocotyledons (Willis 2017). They are found all across the world mainly in the wet tropical area but are absent from polar and desert regions (Besi et al. 2023).

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However, due to taxonomic disagreements, the precise number of approved species is unknown, despite a more recent estimate from the Royal Botanic Garden of Kew listing 880 genera and about 22,000 species (Prakash and Pathak 2019). Because of their exquisitely beautiful and intricately complex blossoms, orchids are without a doubt the ornamental elite. The reason for this is that orchids are now a multibillion-dollar industry (De LC and Pathak 2015). Many orchids have evident therapeutic value in addition to their aesthetic value (Choudhary et al. 2023, Kumar et al. 2018, Prakash et al. 2018).

Orchids are renowned for their unusually formed, long-lasting and exquisitely attractive blossoms. Bangladesh is home to numerous native orchids that are appreciated primarily in the Sundarbans mangrove forest, Chittagong Hill Tracts (CHT), Chattogram, Cox's Bazar, greater Sylhet and Gazipur (Rahman et al. 2017). Orchid conservation is becoming a global problem due to the startling rate at which their native populations are declining. The creation of long-term conservation plans for orchids is greatly influenced by *in vitro* technologies (Bhattacharyya et al. 2015). Micropropagation is the most acceptable option for orchid conservation and propagation. This technique is an excellent method for obtaining a large number of disease-free plantlets (Bhowmik et al. 2024).

The terrestrial orchid *Eulophia graminea* Lindl. is native to tropical and subtropical regions of Asia (Pemberton et al. 2008); and status is Not Evaluated (NE) in Bangladesh (Huda 2008). Self-pollinated flowers yielded less fruit than out-pollinated blooms, despite the fact that *E. graminea* is an out-crossing, self-compatible plant that relies on pollinators (Karuppusamy 2007). Extracts from *E. graminea* tubers have been used as eardrops to relieve ear pain (Narkhede et al. 2016) and they are also thought to be a fantastic food for children and those recuperating from illness (Dressler 1993). Linalool and ferulic acid are the active chemical elements *of E. graminea* juice extract, which is used as a tonic and is beneficial in treating cough and paralysis (Singh 2022).

Developing a reliable, repeatable and efficient method for the bulk multiplication and micropropagation of the native terrestrial orchid *E. graminea* was the aim of the current study.

Materials and Methods

For micropropagation, *Eulophia graminea* Lindl. plantlets were grown *in vitro*. The *in vitro* developed rhizome and leaf explants were cut into 0.5-1.0 cm pieces while being kept in a laminar air flow cabinet (HYSC, Korea). The cuttings were then put in a culture vessel with 0.8% (w/v) agar solidified MS based micropropagation media that had been enhanced with 25 distinct PGRs concentrations and combinations. Full strength MS semi-solid and liquid media supplemented with 25 different types of PGRs concentrations and combinations were used for elongation of mini plantlets after micropropagation. Agar was not used for liquid medium, although 0.8% (w/v) agar was utilized in solid media as well. Three types of auxins (IAA, IBA and NAA) and nineteen different types of 0.8% (w/v) agar solidified MS medium were used to encourage the growth of a strong root system.

i) The mean number of MSBs per explant was calculated using the following formula:

$$\text{Mean number of MSBs} = \frac{\text{Total number of shoot buds}}{\text{Number of explants inoculated}}$$

ii) The percent of explants giving rise to MSBs/PLBs was calculated using the following formula:

$$\%\text{ of explants producing MSBs/PLBs} = \frac{\text{Number of explants producing MSBs/PLBs}}{\text{Total number of explants cultured}} \times 100$$

iii) The average length of shoots/seedlings was calculated as follows:

$$\text{Average length of shoots/seedlings (cm)} = \frac{\text{Total length of shoot/seedlings}}{\text{Number of shoots/seedlings}}$$

iv) The mean number of roots per shoot/seedling was calculated using the following formula:

$$\text{Mean number of roots} = \frac{\text{Total number of roots}}{\text{Number of roots/seedlings}}$$

v) The average length of roots was calculated as follows:

$$\text{The average length of roots (cm)} = \frac{\text{Total length of roots}}{\text{Number of roots/seedlings}}$$

vi) The percent of seedlings survived in different plant species was calculated using the following formula:

$$\%\text{ of seedlings survived} = \frac{\text{Number of seedlings survived}}{\text{Total number of transplanted seedlings}} \times 100$$

Ninety-day-old plantlets with three to four leaves and healthy roots were selected for hardening. A method of progressive hardening was used in order to produce healthy plantlets. This process involved leaving cultured vessels open in the culture room for many hours and then exposing them to natural light for a day. Additionally, plantlets were washed twice with double distilled water to remove the adhering agar. Fungicide was sprayed on the roots and auxins were utilized to encourage *ex vitro* rooting in plants. The *E. graminea* seedlings were placed in plastic pots with a potting mixture of sand, activated charcoal, pit moss and sterilized soil in a 1 : 1 : 1 : 1 ratio. The pots were then placed in the green house (at 25-30°C temperature and 60-70% Relative Humidity). For two to three months, transplanted seedlings received frequent watering, which helped them establish and succeed.

Five to six replicates of each treatment were used in the three trials. Data on the morphogenic responses of explants under various settings were recorded using varying combinations of PGRs and basal medium strengths. Following the necessary number of days for culture, the data on various parameters were recorded. The entire design of the experiment was randomized and Microsoft Excel 2013 was used to prepare all of the

data. The SPSS software program was used to statistically evaluate the data (Gomez and Gomez 1984). At the 5% level of significance (P=0.05), DMRT performed an ANOVA and mean comparison.

Results and Discussion

Micropropagation or *in vitro* mass rapid clonal propagation of plants is currently being used worldwide. Modern orchid micropropagation began in 1949 when *Phalaenopsis* orchid was developed at Cornell University by *in vitro* culture (Rotor 1949). In the past, several scientists, Bhadra and Hossain in *Geodorum densiflorum* (2003), Bhadra and Bhowmik in *Arundina graminifolia* (2005), Kauth et al. in *Calopogon tuberosus* (2006), Bhattacharyya and Banerjee in *Geodorum densiflorum* (2020) have also used *in vitro* derived rhizome explants for micropropagation.

For micropropagation of *E. graminea* rhizome and leaf segments of *in vitro* grown plantlets were cultured on MS medium supplemented with different concentrations of BAP and Kn singly and in combination with IAA or NAA or Pic. The results are presented in Table 1 and Figs 1a-b. Rhizome segments produce MSBs in all the media combinations but higher concentrations of BAP with higher concentrations of NAA or IAA were more effective for induction of MSBs. The effectiveness of a medium was determined by counting the number of shoot buds that each explant generated. The maximum number of explants (89.33%) produced MSBs in the medium containing 3.0 mg/l BAP + 1.5 mg/l NAA with the highest number of MSBs per explant. This PGRs combination also took the lowest time (4.42 ± 0.10 wks) for induction of MSBs. Whereas the minimum number of explant (13.33%) produced MSBs with the lowest number of MSBs in the medium containing 1.0 mg/l Kn alone. The effect of BAP with NAA was almost similar to that of BAP with IAA. There is no significant difference ($P < 0.05$) between the media containing 3.0 mg/l BAP + 1.5 mg/l NAA and 3.0 mg/l BAP + 1.5 mg/l IAA. Whereas required time for MSBs development showed significant variation ($P < 0.05$) and was lowest compared to all treatment. Previous research on the orchids *Geodorum densiflorum* (Bhadra and Hossain 2003) and *Calopogon tuberosus* (Kauth et al. 2006) has shown similar results. BAP was the most successful in promoting the growth of shoot buds in *Vanda spathulata* (Lekshmi and Decruse 2018), *Dendrobium bensoniae* (Riva et al. 2016) and *Cymbidium gigantean* (Hossain et al. 2010).

The leaf segments proliferated and induced Protocorm Like Bodies (PLBs) in maximum media combinations except 1.0 mg/l BAP/Kn or 2.0 mg/l Kn containing MS medium and MS0 (Control). The maximum response (78.67%) was found in the MS medium having 3.0 mg/l BAP + 1.5 mg/l Pic. Minimum time (5.36 ± 0.09 wks) was required for induction of green PLBs in this PGRs combination. Here the effect of BAP with Pic and BAP with IAA were also similar in terms of PLBs induction. The PGRs combinations 3.0 mg/l BAP + 1.5 mg/l Pic and 3.0 mg/l BAP + 1.5 mg/l IAA did not show significant differences ($P < 0.05$) for induction of PLBs. Individual treatment of BAP (2.0

Table 1. Development of MSBs/PLBs from *in vitro* raised rhizome and leaf explants of *E. graminea* on agar solidified MS medium with various concentrations and combinations of PGRs.

PGRs Concentration (mg/l)					Rhizome Segments (Mean ± SE)			Leaf Segments (Mean ± SE)		
BAP	Kn	NAA	IAA	Pic	% of induced MSBs/ segment	Required time (weeks) for development of MSBs	No. of MSBs produced/ segment	% of induced PLBs/ segment	Required time (weeks) for development of PLBs	Nature of PLBs (Colour)
1.0	-	-	-	-	26.67 ± 1.72^{cd}	6.32 ± 0.09^{ijk}	3.0±0.71^{abcd}	0.00±0.00^{a}	0.00 ± 0.00^{a}	-
2.0	-	-	-	-	38.67 ± 2.24^{ef}	5.74 ± 0.15^{gh}	4.4±0.51^{cdefghij}	26.67±1.72^{de}	7.08 ± 0.10^{i}	G
3.0	-	-	-	-	65.33 ± 1.44^{ij}	5.26 ± 0.09^{cde}	6.0±0.71^{hijklmno}	33.33±1.72^{ef}	6.44 ± 0.09^{fgh}	WG
-	1.0	-	-	-	13.33 ± 1.72^{ab}	6.52 ± 0.07^{kl}	2.2 ± 0.37^{ab}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-
-	2.0	-	-	-	20.00 ± 1.72^{bc}	6.44 ± 0.09^{jkl}	2.4 ± 0.51^{abc}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-
-	3.0	-	-	-	40.00 ± 1.72^{ef}	5.74 ± 0.15^{gh}	4.2 ± 0.73^{bcdefghi}	13.33 ± 1.72^{bc}	7.32 ± 0.10^{i}	G
1.0	-	0.5	-	-	57.33 ± 1.96^{hi}	5.36 ± 0.09^{cdef}	5.6 ± 0.68^{ghijklmn}	38.67 ± 2.24^{fg}	6.44±0.09^{fgh}	YG
2.0	-	1.0	-	-	77.33 ± 1.96^{kl}	4.66 ± 0.16^{a}	7.2 ± 0.58^{mno}	46.67 ± 1.72^{gh}	6.12±0.14^{ef}	G
3.0	**-**	**1.5**	**-**	**-**	**89.33 ± 1.96^{m}**	**4.42 ± 0.10^{a}**	**7.8 ± 0.58^{o}**	58.67 ± 2.24^{ij}	5.62±0.14^{bc}	G
1.0	-	-	0.5	-	52.00 ± 2.24^{gh}	5.46 ± 0.09^{defg}	5.4 ± 0.51^{fghijklmn}	38.67 ± 2.24^{fg}	6.34 ± 0.07^{efg}	YG
2.0	-	-	1.0	-	70.67 ± 1.96^{jk}	5.06 ± 0.10^{bc}	6.8 ± 0.80^{klmno}	57.33 ± 1.96^{ij}	5.74 ± 0.15^{cd}	WG
3.0	**-**	**-**	**1.5**	**-**	**84.00 ± 1.96^{lm}**	**4.54 ± 0.12^{a}**	**7.4 ± 0.51^{no}**	**70.67 ± 1.96^{kl}**	**5.42 ± 0.10^{bc}**	**G**
1.0	-	-	-	0.5	46.67 ± 1.72^{fg}	5.66 ± 0.16^{fg}	4.8 ± 0.58^{defghijk}	53.33 ± 1.72^{hi}	6.10 ± 0.14^{ef}	YG
2.0	-	-	-	1.0	65.33 ± 2.24^{ij}	5.18 ± 0.08^{cd}	6.2 ± 0.58^{ijklmno}	65.33 ± 2.24^{jk}	5.58 ± 0.12^{bc}	G
3.0	**-**	**-**	**-**	**1.5**	77.33 ± 1.96^{kl}	4.76 ± 0.16^{ab}	7.0 ± 0.71^{lmno}	**78.67 ± 2.24^{l}**	**5.36 ± 0.09^{b}**	**G**
-	1.0	0.5	-	-	33.33 ± 1.72^{de}	6.22 ± 0.09^{ijk}	3.4 ± 0.51^{abcdef}	6.67 ± 0.00^{ab}	7.42 ± 0.10^{i}	WG
-	2.0	1.0	-	-	58.67 ± 2.24^{hi}	5.36 ± 0.09^{cdef}	5.8 ± 0.58^{ghijklmno}	26.67 ± 1.72^{de}	6.74 ± 0.13^{h}	G
-	3.0	1.5	-	-	72.00 ± 2.24^{jk}	5.18 ± 0.08^{cd}	6.4 ± 0.75^{jklmno}	46.67 ± 1.72^{gh}	6.62 ± 0.14^{gh}	YG
-	1.0	-	0.5	-	26.67 ± 1.72^{cd}	6.22 ± 0.09^{ijk}	3.2 ± 0.58^{abcde}	20.00 ± 1.72^{cd}	7.24 ± 0.14^{i}	WG
-	2.0	-	1.0	-	38.67 ± 2.24^{ef}	6.02 ± 0.13^{hi}	4.0 ± 0.71^{bcdefgh}	33.33 ± 1.72^{ef}	6.62 ± 0.14^{gh}	G
-	3.0	-	1.5	-	52.00 ± 2.24^{gh}	5.46 ± 0.09^{defg}	5.2 ± 0.58^{efghijklm}	52.00 ± 2.24^{hi}	6.00 ± 0.13^{de}	WG
-	1.0	-	-	0.5	20.00 ± 1.72^{bc}	6.44 ± 0.11^{jkl}	2.6 ± 0.51^{abc}	20.00 ± 1.72^{cd}	6.12 ± 0.15^{ef}	G
-	2.0	-	-	1.0	33.33 ± 2.11^{de}	6.12 ± 0.12^{ij}	3.8 ± 0.58^{bcdefg}	34.67 ± 2.24^{ef}	6.52 ± 0.15^{gh}	WG
-	3.0	-	-	1.5	46.67 ± 1.72^{fg}	5.58 ± 0.12^{efg}	5.0 ± 0.71^{defghijkl}	65.33 ± 1.44j^{k}	5.64 ± 0.12^{bc}	YG
MS0 (Control)					**6.67 ± 1.22^{a}**	**6.72 ± 0.16^{l}**	**1.6 ± 0.40^{a}**	**0.00 ± 0.00^{a}**	**0.00 ± 0.00^{a}**	**-**

Multiple Shoot Buds (MSBs); Protocorm Like Bodies (PLBs); '-' Indicate no response; Greenish PLBs (G), Yellowish Green PLBs (YG), Whitish Green PLBs (WG); Values represent mean ± SE of each experiment consist of five replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

and 3.0 mg/l) and Kn (3.0 mg/l) gave positive response that showed significant variation ($P < 0.05$) for induction of PLBs and required time for PLBs development. Higher concentration of Kn (3.0 mg/l) and BAP treatment significantly higher ($P < 0.05$) compared

to lower concentrations (1.0-2.0 mg/l). Development of PLBs is very important for *in vitro* micropropagation of orchids (Islam et al. 2015). In *Oncidium flexuosum* (Mayer et al. 2010), *Coelogyne Cristata* (Naing et al. 2011), *Phalaenopsis gigantea* (Niknejad et al. 2011), *Vanilla planifolia* (Tan et al. 2011), *Coelogyne flaccida* (Kalyan and Sil 2015), *Vanda* sp (Budisantoso et al. 2017), *Dendrobium chrysotoxum* (Bhowmik and Rahman 2020), *Rhynchostylis retusa* (Kumari and Pathak 2021) and *Satyrium nepalense* (Vasundhra et al. 2021) developed PLBs *via*. embryogenesis from the leaf explants to promote of these plant regeneration.

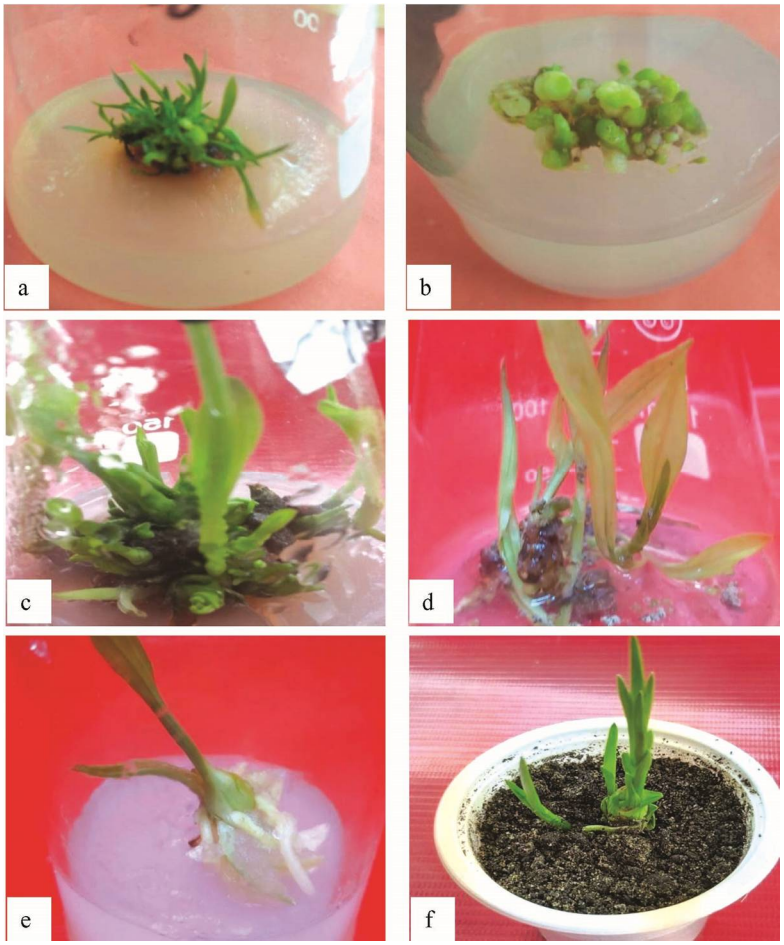

Fig. 1. Various phases of *E. graminea* seedling development and *in vitro* micropropagation: (a) Development of MSBs in *E. graminea* sprouted from *in vitro* raised rhizome segment on agar solidified MS + 3.0 mg/l BAP + 1.5 mg/l NAA, (b) Induction of PLBs in *E. graminea* created from *in vitro* derived leaf segment on agar solidified MS + 3.0 mg/l BAP + 1.5 mg/l Pic, (c) MBSs raised plantlets of *E. graminea* undergoing elongation on agar solidified MS + 2.0 mg/l BAP + 1.2 mg/l NAA, (d) MBSs derived plantlets of *E. graminea* undergoing elongation in liquid MS + 2.0 mg/l Kn + 1.2 mg/l NAA, (e) Development of root system in MSBs derived plantlet of *E. graminea* on MS + 1.0 mg/l IBA + 1.0 mg/l NAA and (f) *In vitro* developed *E. graminea* seedlings growing in pot outside of the culture room.

Table 2. Elongation of MSBs derived plantlets of *E. graminea* on 0.8% (w/v) agar solidified and liquid MS medium with different concentrations and combinations of PGRs.

PGRs (mg/l)				Solid media		Liquid media	
BAP	Kn	NAA	IAA	Initial length (cm) of plantlets after micro-propagation (Mean ± SE)	Increased in length (cm) of plantlets within 30d of culture (Mean± SE)	Initial length (cm) of plantlets after micropropagation (Mean ± SE)	Increased in length (cm) of plantlets within 30d of culture (Mean ± SE)
0.5	-	-	-	1.33 ± 0.02^{a}	3.34 ± 0.03^{f}	1.32 ± 0.03^{abc}	3.37 ± 0.04^{fg}
1.0	-	-	-	1.36 ± 0.03^{a}	3.43 ± 0.03^{fgh}	1.34 ± 0.02^{abc}	3.48 ± 0.04^{ghi}
1.5	-	-	-	1.34 ± 0.02^{a}	3.51 ± 0.02^{hi}	1.33 ± 0.02^{abc}	3.68 ± 0.04^{ikl}
2.0	-	-	-	1.31 ± 0.03^{a}	3.64 ± 0.03^{jk}	1.31 ± 0.02^{abc}	3.77 ± 0.03^{lmn}
-	0.5	-	-	1.34 ± 0.03^{a}	2.88 ± 0.03^{b}	1.36 ± 0.02^{abc}	3.03 ± 0.03^{b}
-	1.0	-	-	1.35 ± 0.02^{a}	2.95 ± 0.03^{ab}	1.33 ± 0.03^{abc}	3.08 ± 0.04^{bc}
-	1.5	-	-	1.33 ± 0.03^{a}	3.02 ± 0.04^{bc}	1.30 ± 0.02^{ab}	3.14 ± 0.04^{cd}
-	2.0	-	-	1.32 ± 0.03^{a}	3.15 ± 0.03^{de}	1.34 ± 0.02^{bc}	3.20 ± 0.04^{de}
0.5	-	0.3	-	1.36 ± 0.03^{a}	3.56 ± 0.03^{ij}	1.35 ± 0.02^{abc}	3.64 ± 0.04^{ijk}
1.0	-	0.6	-	1.32 ± 0.02^{a}	3.73 ± 0.03^{klm}	1.36 ± 0.02^{abc}	3.82 ± 0.03^{mno}
1.5	**-**	**0.9**	**-**	**1.33 ± 0.02^{a}**	**4.03 ± 0.03^{p}**	1.30 ± 0.02^{abc}	4.01 ± 0.04^{qr}
2.0	**-**	**1.2**	**-**	**1.35 ± 0.03^{a}**	**4.17 ± 0.03^{q}**	**1.34 ± 0.03^{abc}**	**4.23 ± 0.04^{s}**
0.5	-	-	0.3	1.30 ± 0.02^{a}	3.38 ± 0.04^{fg}	1.35 ± 0.02^{abc}	3.53 ± 0.04^{hi}
1.0	-	-	0.6	1.36 ± 0.03^{a}	3.66 ± 0.08^{jkl}	1.34 ± 0.02^{c}	3.72 ± 0.04^{klm}
1.5	-	-	0.9	1.34 ± 0.02^{a}	3.82 ± 0.03^{mn}	1.31 ± 0.02^{abc}	3.88 ± 0.04^{nop}
2.0	-	-	1.2	1.35 ± 0.03^{a}	3.92 ± 0.03^{no}	1.32 ± 0.02^{abc}	3.92 ± 0.04^{opq}
-	0.5	0.3	-	1.33 ± 0.03^{a}	4.28 ± 0.04^{r}	1.34 ± 0.03^{abc}	3.42 ± 0.03^{gh}
-	1.0	0.6	-	1.31 ± 0.02^{a}	3.68 ± 0.03^{kl}	1.36 ± 0.03^{abc}	3.76 ± 0.03^{lm}
-	1.5	0.9	-	1.30 ± 0.02^{a}	3.88 ± 0.03^{no}	1.32 ± 0.02^{ab}	3.97 ± 0.04^{pqr}
-	**2.0**	**1.2**	**-**	1.34 ± 0.03^{a}	3.97 ± 0.04^{op}	**1.33 ± 0.03^{abc}**	**4.07 ± 0.05^{r}**
-	0.5	-	0.3	1.34 ± 0.03^{a}	3.08 ± 0.04^{cd}	1.33 ± 0.03^{abc}	3.25 ± 0.03^{de}
-	1.0	-	0.6	1.37 ± 0.03^{a}	3.21 ± 0.03^{e}	1.36 ± 0.02^{abc}	3.31 ± 0.03^{ef}
-	1.5	-	0.9	1.33 ± 0.02^{a}	3.47 ± 0.04^{ghi}	1.32 ± 0.02^{a}	3.58 ± 0.03^{ij}
-	2.0	-	1.2	1.35 ± 0.03^{a}	3.76 ± 0.03^{lm}	1.34 ± 0.03^{abc}	3.71 ± 0.04^{klm}
MS0 (Control)				**1.31 ± 0.02^{a}**	**2.72 ± 0.03^{a}**	**1.33 ± 0.02^{a}**	**2.87 ± 0.03^{a}**

Values represent mean ± SE of each experiment consist of five replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to Duncan's Multiple Range Test.

For improving mini shoot bud elongation, 0.8% (w/v) agar solidified and liquid MS media were made with various concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Picloram). The increase in length of the shoot system during 30 days of culture was used to assess the effectiveness of a medium in promoting shoot elongation. The elongation of several shoot buds produced tiny plantlets (Table 2 and

Figs 1c-d). The maximum elongation of MSBs raised plantlets (4.17 ± 0.03cm) was achieved on agar solidified MS medium supplemented with 2.0 mg/l BAP + 1.2 mg/l NAA. Agar solidified MS medium fortified with 1.5 mg/l BAP + 0.9 mg/l NAA gave almost same result in MSBs derived plantlets (4.03 ± 0.03 cm). But in liquid condition, the highest increased elongation of MSBs derived plantlets (4.23 ± 0.04 cm) was recorded in MS medium fortified with 2.0 mg/l BAP + 1.2 mg/l NAA followed by MS + 2.0 mg/l Kn + 1.2 mg/l NAA (4.07 ± 0.05 cm). The lowest results of elongation were recorded in MSBs derived plantlets (2.88 ± 0.03 cm) after 30d of culture on agar solidified MS medium supplemented with 0.5 mg/l Kn. From the data table it is clear that, elongation of MSBs raised planlets was better in liquid medium than solid medium. In *E. graminea*, liquid medium was better in respect of PGRs the BAP and NAA combinations were better for elongation of MSBs derived plantlets.

Combined treatments of BAP and NAA in all concentrations were showed significant variation ($P < 0.05$) for the elongation of MSBs derived shoot buds. Lower concentrations of combined used of BAP and IAA or Kn and NAA were proved significant differences ($P < 0.05$). At elongation stage, higher concentrations of combined used of Kn and IAA showed the significant variation ($P < 0.05$). Statistically at higher concentration (2.0 mg/l) of BAP exposed significant difference ($P < 0.05$) except in liquid culture for the elongation of MSBs derived shoot buds. In liquid condition every individual concentrations of Kn were showed insignificant variation ($P < 0.05$). Liquid medium probably facilitated more uptakes of nutrients because of more surface exposure of cultured plantlets and thereby contributing to the better and prolific growth of orchid plantlets. Combine effect of auxin and cytokinin have been shown by several researchers to have positive effects on the height increase of plantlets of certain orchids, including *Cymbidium finlaysonianum* (Islam et al. 2015) and *Dendrobium crepidatum* (Dhungana et al. 2022). The MS medium supplemented with 1.5 mg/1 BAP and 0.5 mg/1 NAA showed the highest shoot bud elongation in *Vanda tessellata* (Bhattacharjee and Islam 2014). It is clear that a liquid medium, as opposed to solidified conditions, promoted shoot bud elongation more successfully and MS medium was found to be more effective for elongating the shoot bud (Bhadra and Hossain 2003, Sunitibala and Kishor 2009, Julkiflee et al. 2014, Bhattacharjee and Islam 2015).

The roots of young seedlings cannot develop adequately in the elongation media. For the purpose of promoting the development of a strong and stout rooting system, full strength MS0 (control) and eighteen distinct PGRs (IAA, IBA and NAA) supplemented MS medium were utilized (Figs 1e, 2 and 3). The length and number of roots that emerged from each seedling within 30 days of culture were used to measure the effectiveness of the rooting medium. The maximum increase in length, as well as the number of roots of *E. graminea* induced in MSBs raised plantlets (3.76 ± 0.04 cm, 5.00 ± 0.37 no) were achieved in MS medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA. MS medium fortified with 0.5 mg/l IBA + 0.5 mg/l NAA gave almost same result in MSBs derived (3.61 ± 0.06 cm, 4.83 ± 0.40 no) seedlings. The lowest findings of increased length

and number of roots were recorded on agar solidified MS medium supplemented with 1.5 mg/l IBA in MSBs derived (2.08 ± 0.07 cm, 2.83 ± 0.31 no) seedlings.

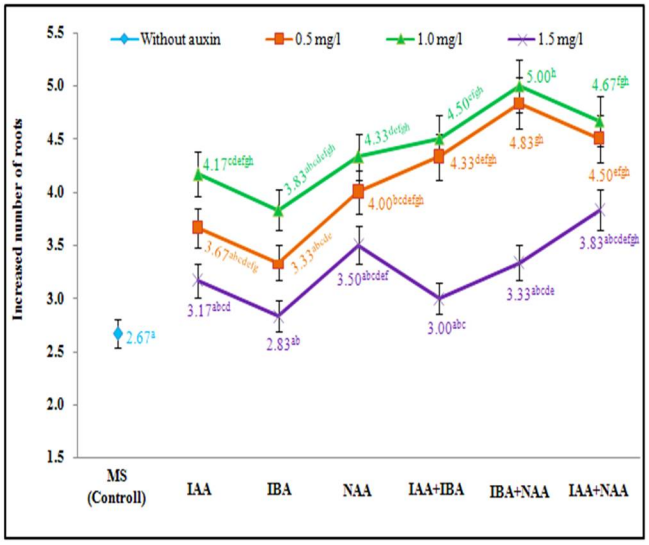

Fig. 2. Increased root number of MSBs derived plantlets of *E. graminea* in auxin supplemented MS medium after 30d of culture. Values represent mean ± SE of each experiment consist of six replicates. Mean values of each point of a figure followed by different superscript letters are significantly different at p ≤0.05 according to Duncan's Multiple Range Test.

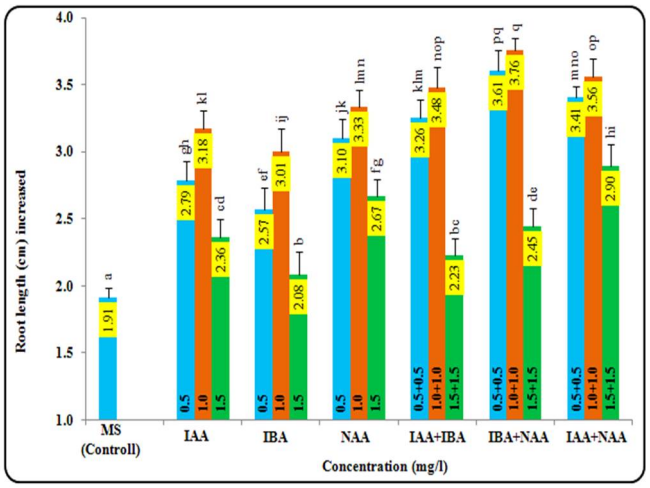

Fig. 3. Increased root length (cm) of MSBs derived plantlets of *E. graminea* in auxin supplemented MS medium after 30d of culture. Values represent mean ± SE of each experiment consist of six replicates. Mean values of each bar of a figure followed by different letters at the upper position of SE bar are significantly different at p ≤0.05 according to Duncan's Multiple Range Test.

Increase in length and number of roots in both seed originated and MSBs derived plantlets gave the insignificant variation (P <0.05) was recorded in 1.0 mg/l IBA + 1.0 mg/l NAA and 0.5 mg/l IBA + 0.5 mg/l IAA treatments. In seed derived and MSBs raised plantlets, individual and combined treatment of different PGRs *i.e.* IAA, IBA, NAA with different concentrations proved that moderate concentration is significantly lower (P <0.05) in higher or lower concentrations of PGRs treatment. After 30 days of culture in rooting media both seed originated and MSBs derived plantlets illustrate the insignificant differences (P <0.05) in different concentrations and combinations of PGRs treatments. The beneficial effect of NAA on root induction was also reported by Pant and Shrestha (2011) and Thokchom et al. (2017) in *Phaius tancarvilleae*. IBA was effective for best rooting in *Dendrobium* orchid (Aktari et al. 2007), *Coelogyne cristata* (Pant et al. 2008), *Cymbidium iridioides* (Pant and Swar 2011), *Ilex khasiana* (Dang et al. 2011), *Cymbidium finlaysonianum* (Islam et al. 2015) and *Cymbidium aloifolium* (Paul et al. 2019).

The mature plants were transferred from the growth room to the outdoor environment after a number of acclimatization phases (Fig. 1f). Of the seedlings developed *in vitro*, 74.29% lived and continued to grow in the greenhouse's pots. In the end, they were set up in the Botanical Garden's Orchidarium at Chittagong University in Chattogram, Bangladesh.

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