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In Vitro Seed Germination, Seedlings and SPSs Development in a Terrestrial Medicinal Orchid *Eulophia Graminea* Lindl. of Bangladesh

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Abstract— *Full strength MS medium with PGRs combination gave the topmost response (86.67%) of seeds germination descended by PGRs supplemented PM (80.00%), VW (53.34%) and KC (46.67%) media. The lowest required time for initiation of seed germination was recorded on full strength PGRs fortified MS medium (10.37 ± 0.26 wks) followed by PM (12.37 ± 0.28 wks), VW (14.07 ± 0.42 wks) and KC (17.63 ± 0.35 wks) media. Half strength PGRs free KC, MS, PM and VW media did not show any response. The minimum time required for development of protocorms, differentiation of first leaf primordia, differentiation of first root primordia and development of seedlings was recorded on full strength PGRs fortified MS medium (14.57 ± 0.31 wks), (19.23 ± 0.30 wks), (25.60 ± 0.35 wks) and (32.77 ± 0.35 wks) accordingly. The liquid MS medium containing 2.0 mg/l BAP + 1.2 mg/l NAA showed best results in terms of SPSs development (94.67% response and 6.24 ± 0.14 wks time). Well-developed rooted seedlings were transferred to environment by successive phases of acclimatization.*

Keywords— *of Eulophia graminea, in vitro germination, PGRs, SPSs.*

I. INTRODUCTION

Orchids are regarded as nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine [1]. They are aesthetically, floriculturally, medicinally important and also regarded as ecological indicators [2, 3]. Orchidaceae is the most diverse family of flowering plants consisting of 30,000-35,000 species belonging to 600-800 genera [4]. Bangladesh is rich in orchids with 187 species [5].

Terrestrial herb orchid *Eulophia graminea* Lindl. found in dry partial shaded forests. Flowering time is March to April and status is Not Evaluated (NE). The species reigns in India, Sri Lanka, Nepal, Myanmar, China, Taiwan, Hong Kong and Malaysia. In Bangladesh, the species was collected from the Naikhongchhari, Bandarban district. The rhizomes are used as vermifuge [6]. This species is rarely distributed due to habitat destruction and mass collection of orchid hunters.

Mature green capsules were collected from Naikhongchhari, Bandarban, Bangladesh. In order to develop a mass propagation protocol for this species, the current study was created to assess the asymbiotic germination potential of seeds in vitro after the development of protocorms, differentiation of first leaf primordia, differentiation of first root primordia, development of seedlings and development of SPSs.

II. MATERIALS AND METHODS

2.1. Sterilization of capsules

Mature green capsules of *E. graminea* were collected and used as explants in the current study from Naikhongchhari, Bandarban, Bangladesh. The collected capsules were extensively cleansed with Teepol (0.01%), washed under running water for ten to fifteen minutes and then washed twice with sterile distilled water. The capsules were then cleaned with three times double distilled water after being dipped in 70% ethyl alcohol for 30 seconds. The capsules were then surface sterilized for 10 minutes with 0.1% (w/v) $HgCl_2$ and rinsed three times with double-distilled water. The seeds were then scooped out of the sterilized capsules lengthwise using a sterilized blade in an aseptic environment inside a laminar airflow cabinet.

2.2. Culture medium and culture condition

On 0.8% (w/v) agar solidified half strength without PGRs, full strength without PGRs and PGRs (0.5 mg/l BAP and 0.5 mg/l NAA) supplemented full strength KC [7], MS [8], PM [9] and VW [10] media, the germination potential of mature seeds was as sucrose was employed as a source of carbohydrates, with 3% (w/v) for MS and 2% (w/v) for KC, PM and VW medium. The pH of the medium was adjusted to 5.8 in MS and 5.4 in KC, PM and VW media using 0.1N NaOH or HCl and the medium was gelled using 0.8% (w/v) agar (Fluka, USA). About 50 ml of media was distributed into 100 ml of each culture vessel after the agar was dissolved by boiling the mixture in a water bath. The medium was then autoclaved (Hisense, South Korea) at 121 ^{0}C for 30 minutes at 15 lb/cm^2 pressure. The culture was kept at a temperature of 25°C and given 14 hours of 3500 lux light. The answers were recorded based on visual observations after the cultures were periodically (every 3-4 days). After six weeks, sub-culturing was continued in fresh medium.

2.3. SPSs development

For the development of shoot primordia like structures (SPSs); *in vitro* grown seed originated seedlings were grown on twenty five types of different PGRs supplemented MS solid

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and liquid media. In all of the species, huge quantities of SPSs were produced on some of the culture vessels at the basal zone of the seedlings.

2.4. Transplantation

The succeeding rounds of acclimation eventually toughened healthy seedlings with three to four leaves and two to three roots. The seedlings were transplanted to a pot containing a combination (sterilized soil, sand, activated charcoal and pit moss in a ratio of 1:1:1:1) after being thoroughly rinsed in sterile distilled water to remove agar.

2.5. Computation and presentation of data

The data on different parameters from different experiments were recorded after the required days of culture. The parameters are:

2.5.1. Percent of (%) of culture vessel germinate

The percent of culture vessel germinated in different strength and conditions of four basal media was calculated using the following formula.

$$\% \text{ of culture vessel germinate} = \frac{\text{Number of cultured vessels germinated}}{\text{Total number of cultured vessels}} \times 100$$

2.5.2. Frequency (%) of SPSs development

The percent of cultured vessel developed SPSs at the base of seedlings in *E. graminea* were calculated using the following formula.

$$\% \text{ of cultured vessel developed SPSs} = \frac{\text{Number of cultured vessels developed SPSs}}{\text{Total number of cultured vessels}} \times 100$$

2.5.3. Percent of seedlings survived

The percent of seedlings that survived was calculated using the following formula.

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

2.5.4. Percent of seedlings survived

The percent of seedlings survived in different plant species was calculated using the following formula.

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

2.6. Statistical analysis

Three separate experiments were carried out, each with a different number of repetitions for each treatment and the findings were given as means with standard error (mean ± SE). Microsoft Excel 2013 was used to determine the standard deviation (SD). The significant differences were identified using Duncan's Multiple Range Test [11] at a 5% level of significance (P = 0.05) after the data were subjected to an analysis of variance (ANOVA). The IBM SPSS (Statistical Product and Service Solutions) Statistics software was used to analyze the data.

III. Results and Discussions

Among KC, MS, PM and VW based twelve types of germination media used, full strength MS medium with PGRs combination gave the topmost response (86.67%) of seeds germination in *E. graminea* descended by PGRs supplemented PM (80.00%), VW (53.34%) and KC (46.67%) media (Table - 1; Figs. 1 - 6). Poor percentage of seed germination (33.34%) was observed on full strength PGRs free KC medium. The lowest required time for initiation of seed germination was recorded on full strength PGRs fortified MS medium (10.37 ± 0.26 wks) followed by PM (12.37 ± 0.28 wks), VW (14.07 ± 0.42 wks) and KC (17.63 ± 0.35 wks) media. PGRs free full strength KC medium needed the utmost time (19.10±0.32 wks) for seeds germination. Half strength PGRs free KC, MS, PM and VW media did not show any response for germination of *E. graminea*. After inoculation of seeds, the minimum required time was found on full strength PGRs fortified MS medium for the development of protocorms (14.57 ± 0.31 wks), differentiation of first leaf primordia (19.23 ± 0.30 wks), differentiation of first root primordia (25.60 ± 0.35 wks) and development of seedlings (32.77 ± 0.35 wks) subsequently PM medium (17.33 ± 0.40; 23.57 ± 0.37; 30.80 ± 0.38; 37.30 ± 0.30 wks accordingly). Full strength KC medium without PGRs required maximum time for the development of protocorms (24.43 ± 0.39 wks), differentiation of first leaf primordia (31.20 ± 0.30 wks), differentiation of first root primordia (38.60 ± 0.25 wks) and development of seedlings (45.70 ± 0.34 wks).

Full strength with or without PGRs supplemented with different media gave significant difference ($P<0.05$) for the development of seedlings except PGRs fortified full strength KC and PGRs free full strength VW media. For differentiation of first leaf primordia with or without PGRs supplemented full strength of different media showed significant difference ($P<0.05$) while PGRs free full strength PM, VW and PGRs fortified full strength KC media illustrate insignificant variation ($P<0.05$). Different strengths of media with or without PGRs combinations showed significant differences ($P<0.05$) in the initiation of seed germination, development of protocorms and differentiation of first root primordia. But there are no significant differences ($P<0.05$) between PGRs free full strength KC and PGRs supplemented full strength VW; full strength PGRs free MS and full strength PGRs supplemented VW media for initiation of seed germination. Again, full strength PGRs free PM and VW; full strength PGRs free MS and full strength PGRs supplemented VW media showed insignificant differences ($P<0.05$) for the development of protocorms.

In the current investigation, there were four different basal media, each of which had a unique chemical makeup. While PM, VW and KC medium have relatively modest amounts of macro and micro nutrients, with or without vitamins, respectively, MS medium is significantly enriched in both macro and micro nutrients as well as a variety of vitamins [12-13]. Because KC medium lacks vitamins, the highest percentage of seed germination was found under various circumstances of MS, PM and VW medium. Numerous

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researches describe how vitamins boost seedling growth and germination in a variety of orchid species. *Cymbidium elegans* and *Coelogyne punctulata* seeds were observed to germinate more readily and develop into seedlings when different vitamins were added to the medium [13-14]. According to Mariat [15], vitamin B improved *Cattleya* seedlings germination and differentiation. The most effective nutrients for producing *Cattleya* hybrids were biotin, nicotinic acid and thiamine. Pyridoxine was demonstrated to be essential for the production of chlorophyll in another study and it was found that combining biotin with nicotinic acid improved *Orchis laxiflora* seed germination [16].

Due to their extremely low concentrations of macro and micronutrients, the current exploration found that half strengths of the KC, MS, PM and VW medium did not aid in the seed germination to plantlets formation. All of the full strength basal media produced respectable results, but the time required for germination and seedling development was much longer than the medium supplemented with PGRs. Auxins like NAA, IAA and IBA are known to cause the production of roots in *in vitro* culture, whereas cytokinins like BAP, Picloram, and Kinetin are known to induce the creation of axillary and adventitious shoots [17-18]. PGR, like BAP, was reported to increase the germination frequency of *Cymbidium pendulum*, *Erythrodes humilis*, *Habenaria macroceratitis*, *Cypripedium candidum* and protocorm multiplication in *Erythrodes humilis* [18-20]. *Habenaria digitata* [13], *Aerides ringens* [21], *Cymbidium elegans* [22], *C. iridiodes* [23], *Dendrobium thyrsiflorum* [24], *Phaius tancarvilleae* [25] and *Vanda tessellata* [26] have also reported the synergistic action of cytokinin and auxin in germination and plantlet development as shown in the current study.

From the aforementioned findings, it was concluded that MS medium, as opposed to PM, VW and KC media, was more favorable for earlier germination, a highest number of protocorms production and seedlings development. Similar findings showing MS medium to be the most suited medium above other nutrient media were reported for *Habenaria digitata* [13], *Cleisostoma racemifefum* [27], *Coelogyne suaveolens* [28], *Malaxix khasiana* [29] and *Vanda coerulea* [30].

The liquid MS medium containing 2.0 mg/l BAP + 1.2 mg/l NAA showed best results in terms of SPSs development (94.67% response and 6.24 ± 0.14 wks time) in *E. graminea.* In agar solidified MS medium containing same PGRs gave utmost results for SPSs development (89.33% response and 7.08 ± 0.12 wks). The colour of SPSs was greenish in both the cases. Liquid MS medium fortified with 2.0 mg/l Kn and 1.2 mg/l NAA gave the almost similar results (90.67%; 6.46 ± 0.09 wks). The lowest results of SPSs development were observed in both agar solidified (9.33%; 8.72 ± 0.14 wks) and liquid (12.00%; 8.76 ± 0.14 wks) MS medium supplemented with 0.5 mg/l Kn (Table 2 and Figures 7 to 8). Fewer hairy yellowish green SPSs were present, with the majority being hairy greenish. Similar conclusions were reached with *Calanthe densiflora* [31], *Dendrobium aphyllum* [32] and *Geodorum densiflorum* [33].

After 60 DAI SPSs development significantly higher ($P<0.05$) in higher concentration of PGRs treatments. After 60 days of inoculation both solid and liquid culture of MS medium with different concentrations and combinations of PGRs treatments showed the insignificant variation ($P<0.05$). Percentage of highest response and required minimum time for SPSs development after 60 DAI illustrate insignificant variation ($P<0.05$) in agar solidified MS medium with 2.0 mg/l BAP + 1.2 mg/l NAA and 1.5 mg/l BAP + 0.9 mg/l NAA whereas, in liquid medium the insignificant variation ($P<0.05$) was observed in 2.0 mg/l BAP + 1.2 mg/l NAA and 2.0 mg/l Kn + 1.2 mg/l NAA combinations.

E. graminea seedlings that were healthy underwent gradual hardening through subsequent phases of adjustment. The seedlings were transplanted to a pot containing a mixture (sterilized soil, sand, activated charcoal and pit moss) at the ratio of 1:1:1:1 in a humidity chamber after being properly cleaned with sterile distilled water to remove agar. The development of new roots and leaves was regarded as an indication that the seedlings had successfully acclimated. The survival rate of seedlings that received frequent irrigation and nutritional solution spraying was 72.38%.

A fruitful attempt was performed in the current work to assess the *in vitro* seed germination and subsequent differentiation of *E. graminea* on four media, KC, MS, PM and VW. Seed originated seedlings derived SPSs were used in plant tissue culture for mass scale propagation of this orchid species. This procedure could be helpful in determining the ideal state for this priceless orchid species' mass reproduction and *ex situ* conservation.

IV. Conclusions

Based on the results of the current study, MS medium is preferred over PM, VW and KC media for the *in vitro* seed germination, protocorm development, differentiation of first leaf primordia, differentiation of first root primordia and development of seedlings in *E. graminea*. Additionally, PGRs fortified all basal media highest responded from germination to seedlings development. Highest percentage of SPSs development was more effective in liquid MS medium supplemented with high concentrations of BAP and NAA treatment than solidify culture.

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Table 1: Effect of different strength of KC, MS, PM and VW media with or without PGRs on *in vitro* seed germination, differentiation and seedlings development of *Eulophia graminea* Lindl.

Medium	Strength of medium	Time taken in weeks					% of culture vessel germinated	Remarks
		Initiation of germination (Mean ± SE)	Development of protocorms (Mean ± SE)	Differentiation of 1st leaf primordia (Mean ± SE)	Differentiation of 1st root primordia (Mean ± SE)	Development of seedlings (Mean ± SE)		
KC	Half without PGRs	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-
	Full without PGRs	**19.10 ± 0.32^{g}**	**24.43 ± 0.39^{g}**	**31.20 ± 0.30^{g}**	**38.60 ± 0.25^{g}**	**45.70 ± 0.34^{h}**	**33.34**	**+**
	Full with PGRs	17.63 ± 0.35^{f}	22.37 ± 0.31^{e}	29.53 ± 0.37^{f}	36.17 ± 0.40^{ef}	43.23 ± 0.36^{g}	46.67	+
MS	Half without PGRs	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-
	Full without PGRs	13.40 ± 0.30^{d}	19.30 ± 0.32^{d}	25.40 ± 0.27^{d}	32.77 ± 0.33^{d}	38.20 ± 0.26^{d}	66.67	++
	Full with PGRs	**10.37 ± 0.26^{b}**	**14.57 ± 0.31^{b}**	**19.23 ± 0.30^{b}**	**25.60 ± 0.35^{b}**	**32.77 ± 0.35^{b}**	**86.67**	**+++**
PM	Half without PGRs	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-
	Full without PGRs	16.13 ± 0.32^{e}	23.20 ± 0.27^{f}	29.70 ± 0.34^{f}	35.63 ± 0.33^{e}	41.57 ± 0.37^{f}	53.34	++
	Full with PGRs	**12.37 ± 0.28^{c}**	**17.33 ± 0.40^{c}**	**23.57 ± 0.37^{c}**	**30.80 ± 0.38^{c}**	**37.30 ± 0.30^{c}**	**80.00**	**+++**
VW	Half without PGRs	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-
	Full without PGRs	17.50 ± 0.39^{f}	23.20 ± 0.24^{f}	30.20 ± 0.29^{f}	36.50 ± 0.37^{f}	43.13 ± 0.37^{g}	40.00	+
	Full with PGRs	14.07 ± 0.42^{d}	19.67 ± 0.32^{d}	26.37 ± 0.33^{e}	33.07 ± 0.28^{d}	40.73 ± 0.36^{e}	53.34	++

PGRs (0.5mg/l BAP + 0.5mg/l NAA); + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each experiment consist of 15 replicates. Mean values followed by different superscript letters within a column are significantly different at p = 0.05 according to DMRT.

Table 2 : Development of Shoot Primodia like Structures (SPSs) in *E. graminea* on agar solidified and liquid MS medium with different kinds of PGRs.

Sl. No.	PGRs Concentration (mg/l)				Solid media			Liquid media		
	BAP	Kn	NAA	IAA	% of response (Mean ± SE)	Required time (weeks) for development of SPSs (Mean ± SE)	Colour of SPSs	% of response (Mean ± SE)	Required time (weeks) for development of SPSs (Mean ± SE)	Colour of SPSs
1.	0.5	-	-	-	32.00±2.55^{efgh}	8.26±0.09^{ghi}	GW	30.67±1.96^{efg}	8.12±0.12^{jk}	WG
2.	1.0	-	-	-	38.67±2.83^{ghi}	8.12±0.12^{fg}	GY	38.67±2.24^{ghi}	8.00±0.15^{ij}	YG
3.	1.5	-	-	-	42.67±1.96^{hij}	8.18±0.08^{gh}	YG	46.67±2.72^{ijk}	7.66±0.12^{hi}	G
4.	2.0	-	-	-	64.00±1.96^{mnop}	7.58±0.12^{bcd}	WG	65.33±2.55^{mno}	7.22±0.15^{efg}	GY
5.	-	0.5	-	-	9.33±0.94^{ab}	8.72±0.14^{j}	G	12.00±1.44^{ab}	8.76±0.14^{n}	GW
6.	-	1.0	-	-	13.33±1.22^{abc}	8.68±0.16^{j}	YG	14.67±1.44^{abc}	8.62±0.11^{mn}	G
7.	-	1.5	-	-	16.00±1.54^{bc}	8.62±0.12^{ij}	YG	17.33±0.94^{bcd}	8.52±0.15^{lmn}	GW
8.	-	2.0	-	-	21.33±1.44^{cde}	8.42±0.12^{ghij}	GY	21.33±2.24^{bcde}	8.42±0.07^{klmn}	GY
9.	0.5	-	0.3	-	50.67±1.96^{jkl}	8.06±0.12^{efg}	G	53.33±2.72^{jkl}	7.46±0.09^{fgh}	YG
10.	1.0	-	0.6	-	68.00±2.55^{nopq}	7.52±0.15^{bcd}	G	72.00±2.24^{nop}	7.02±0.13^{cde}	G
11.	**1.5**	**-**	**0.9**	**-**	**85.33±2.24st**	**7.26±0.09^{ab}**	**WG**	86.67±2.72^{qrs}	6.56±0.13^{ab}	YG
12.	**2.0**	**-**	**1.2**	**-**	**89.33±1.96^{t}**	**7.08±0.12^{a}**	**G**	**94.67±1.44^{s}**	**6.24±0.14^{a}**	**G**
13.	0.5	-	-	0.3	33.33±2.72^{fgh}	8.20±0.14^{gh}	G	42.67±1.96^{hij}	7.72±0.14^{hi}	G
14.	1.0	-	-	0.6	53.33±2.72^{jklm}	7.76±0.16^{def}	GY	57.33±1.96^{klm}	7.38±0.10^{efgh}	GW
15.	1.5	-	-	0.9	70.67±2.31^{opqr}	7.44±0.11^{abcd}	GW	76.00±2.61^{opq}	6.74±0.13^{bc}	GY
16.	2.0	-	-	1.2	78.67±2.24^{qrst}	7.36±0.09^{abc}	GW	84.00±1.96^{qrs}	6.62±0.11^{b}	G
17.	-	0.5	0.3	-	28.00±2.55^{defg}	8.34±0.11^{ghij}	G	34.67±2.24^{fgh}	8.14±0.12^{jkl}	GY
18.	-	1.0	0.6	-	57.33±1.96^{klmn}	7.72±0.14^{cde}	G	61.33±1.44^{lmn}	7.36±0.09^{efgh}	G
19.	-	1.5	0.9	-	74.67±2.24^{pqrs}	7.42±0.10^{abcd}	GW	80.00±2.72^{pqr}	6.78±0.14^{bcd}	YG
20.	**-**	**2.0**	**1.2**	**-**	81.33±2.24^{rst}	7.34±0.11^{abc}	G	**90.67±1.96^{rs}**	**6.46±0.09^{ab}**	**WG**

Tapash Kumar Bhowmik and Md. Mahbubur Rahman, "In Vitro Seed Germination, Seedlings and SPSs Development in a Terrestrial Medicinal Orchid Eulophia Graminea Lindl. of Bangladesh," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 5, Issue 6, pp. 27-33, 2022.

International Research Journal of Pharmacy and Medical Sciences
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21.	-	0.5	-	0.3	18.67 ± 1.44^{bcd}	8.52 ± 0.07^{hij}	GW	25.33 ± 2.24^{cdef}	8.34 ± 0.11^{jklm}	G
22.	-	1.0	-	0.6	24.00 ± 1.96^{cdef}	8.44 ± 0.11^{ghij}	GY	28.00 ± 2.24^{defg}	8.26 ± 0.09^{jklm}	GW
23.	-	1.5	-	0.9	46.67 ± 2.72^{ijk}	8.08 ± 0.12^{efg}	G	49.33 ± 2.61^{ijk}	7.56 ± 0.13^{gh}	G
24.	-	2.0	-	1.2	61.33 ± 2.55^{lmno}	7.66 ± 0.16^{cd}	WG	68.00 ± 2.24^{mno}	7.12 ± 0.12^{def}	GW
25.	**MS0 (Control)**				**4.00 ± 0.94^{a}**	**9.28 ± 0.10^{k}**	**GW**	**4.00 ± 1.54^{a}**	**9.22 ± 0.13^{o}**	**GY**

Greenish (G), Greenish Yellow (GY), Greenish White (GW), Yellowish Green (YG), Whitish Green (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.

Fig. 1: Germination of *E. graminea* seeds on full strength KC medium.

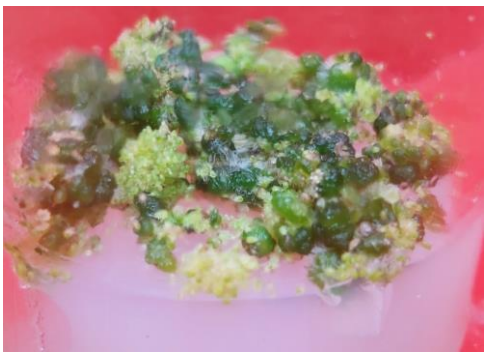

Fig. 2: Germination of *E. graminea* seeds on full strength PM medium.

Fig. 3: Germinated PLBs turned into small shoots on full strength VW medium.

Fig. 4: Germinated PLBs turned into small shoots on full strength MS medium.

Fig. 5: Development of plantlets of *E. graminea* on full strength MS medium.

Fig. 6: Plantlets of *E. graminea* developed on PM + 0.5 mg/l BAP + 0.5 mg/l NAA.

Tapash Kumar Bhowmik and Md. Mahbubur Rahman, "In Vitro Seed Germination, Seedlings and SPSs Development in a Terrestrial Medicinal Orchid Eulophia Graminea Lindl. of Bangladesh," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 5, Issue 6, pp. 27-33, 2022.

Fig. 7: Development of SPSs at the base of the shoots in liquid MS + 2.0 mg/l BAP + 1.2 mg/l NAA.

Fig. 8: Development of SPSs at the base of the shoots on agar solidified MS + 2.0 mg/l BAP + 1.2 mg/l NAA.

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