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Efficient micropropagation of the medicinal orchid *Pholidota imbricata* Hook. f. from pseudobulb and leaf explants in Bangladesh

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Abstract
Pholidota imbricata Hook. f. is a medicinally significant epiphytic orchid native to Bangladesh, currently facing population decline due to habitat destruction and over collection. This study establishes a reliable and efficient protocol for the mass propagation using pseudobulb and leaf explants derived from *in vitro* seedlings. The seeds were germinate *in vitro* on agar solidified four basal media *viz.* KC, MS, MVW and PM and produce a substantial number of plantlets, which were subsequently used for micropropagation studies. Murashige and Skoog (MS) medium supplemented with various concentrations of 6-Benzylaminopurine (BAP), Kinetin (Kn), α-Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) and Picloram were evaluated for shoot induction and proliferation. Direct organogenesis was observed from pseudobulb segments, with the highest frequency of multiple shoot buds (MSBs) formation (90.67% ± 1.96) recorded on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA. Conversely, leaf segments underwent somatic embryogenesis, producing protocorm-like bodies (PLBs) with a maximum induction rate of 74.67% ± 2.24 on the same hormonal combination. For shoot elongation, agar solidified MS medium fortified with 1.5 mg/l BAP and 0.9 mg/l NAA proved superior to liquid media, yielding the highest shoot length increase (2.72 ± 0.03 cm). Rooting was most effective on half strength MS medium supplemented with 1.0 mg/l IAA and 1.0 mg/l IBA, resulting in a mean root number of 4.33 ± 0.33 and root length of 3.85 ± 0.03 cm. Following acclimatization, 75% of the plantlets survived and were successfully established in the greenhouse. This protocol offers a viable strategy for the commercial propagation and *ex situ* conservation of this near threatened orchid species.

Keywords: *Pholidota imbricata*, micropropagation, explants, MSbs, Plbs

Introduction

Orchidaceae, one of the largest and most diverse families of flowering plants, comprises approximately 25,000-35,000 species [1]. Beyond their ornamental value, many orchids possess significant therapeutic properties used in traditional medicine systems globally [2]. In Bangladesh, recent ethnobotanical and pharmacological studies have highlighted the immense potential of indigenous orchids like *Acampe praemorsa* and *Acampe papillosa* for treating inflammation and neurological disorders, reinforcing the urgent need for their study and conservation [3-4].

Pholidota imbricata Hook. f., locally known as the "Rattlesnake Orchid" due to its imbricate floral arrangement, is an epiphytic species distributed across South and Southeast Asia, including Bangladesh, India and Thailand [5]. In traditional medicine, the leaves and roots of *P. imbricata* are applied externally to heal fractured bones, while the pseudobulbs are used to alleviate rheumatic pain and abdominal discomfort [6-7].

Despite its medicinal importance, *P. imbricata* is categorized as 'Near Threatened' in Bangladesh due to indiscriminate collection and deforestation. In the wild, orchid propagation is hindered by the lack of endosperm in seeds, necessitating a specific mycorrhizal association for germination [8]. Conventional vegetative propagation is slow and yields a limited number of propagules, which is insufficient for commercial exploitation or large-scale conservation [9]. Consequently, biotechnological interventions, specifically plant tissue culture, offer a powerful tool for the rapid mass multiplication of desired genotypes [10].

Micropropagation through protocorm-like bodies (PLBs) or direct shoot organogenesis from vegetative parts such as pseudobulbs, leaf and nodal explants has been successfully reported in various orchid genera, including *Dendrobium*, *Cymbidium* and *Coelogyne* [11-12]. However, protocols are often species-specific and the morphogenic response is heavily influenced by the type of explant and the synergistic interaction of PGRs [13] While seed germination studies exist, there is a scarcity of literature regarding efficient clonal propagation protocols for *P. imbricata* using vegetative explants in Bangladesh.

The present study aims to develop a reproducible and efficient micropropagation protocol for *P. imbricata* using *in vitro* derived pseudobulb and leaf explants. We investigated the effects of various cytokinins (BAP, Kn) and auxins (NAA, IAA, IBA, Picloram) on shoot multiplication, somatic embryogenesis, shoot elongation, and rooting to facilitate the mass production and conservation of this valuable medicinal orchid.

Materials and Methods

Plant Material and Sterilization

Green capsules of *Pholidota imbricata* were collected from Naikhongchhari, Bandarban (Chittagong Hill Tracts). The capsules were washed under running tap water, treated with a detergent solution and surface sterilized with 0.2% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. This was followed by a one-minute rinse in 70% ethanol and three-four times washes with sterile double distilled water.

Media Preparation for Seed Germination

Four basal media (MS [14], PM [15], MVW [16] and KC [17]) were prepared to test germination efficiency and seedlings development. All media were gelled with 0.8% agar (Fluka) and the pH was adjusted (5.8 for MS; 5.4 for PM, MVW; 5.0 for KC) prior to autoclaving at 121°C for 20 minutes. Cultures were incubated in a growth chamber maintained at 25 ± 2°C under a 14-hour photoperiod provided by cool white fluorescent tubes (approx. 2000–3000 lux).

Explant Preparation
Healthy 5-6 cm tall *in vitro* seedlings derived from seed cultures served as the source of explants. Pseudobulbs were cut transversely into 1.0-1.5 cm segments and leaves were cut into 0.5-1.0 cm segments for use as explants in micropropagation experiments.

Culture Media for Micropropagation, Elongation and Rooting
The basal medium consisted of MS salts and PGRs supplemented medium with 3% (w/v) sucrose. For micropropagation and shoot proliferation, the medium was fortified with various concentrations and combinations of BAP, Kn, NAA, IAA and Picloram. For the elongation study, both solid (0.8% agar) and liquid (agar-free) MS media supplemented with PGRs were evaluated. For rooting experiments, half strength MS with auxins (IAA, IBA, NAA) and MS0 (control) were used.

Experimental Design
Regeneration of Plantlets
Seeds were collected from green capsules and cultured on four basal media MS [14], PM [15], MVW [16] and KC [17] medium for *in vitro* seedlings development.

Shoot induction: Pseudobulb and leaf segments were cultured on MS medium supplemented with different combinations of PGRs (Table 1). Data on the percentage of explants responding, days to induction and number of shoots/PLBs per explant were recorded after 4-6 weeks.

Elongation: Multiple shoot buds (MSBs) were sub-cultured onto different MS elongation media (solid vs. liquid) containing combinations of BAP, Kn and auxins. Shoot length increment was record after 30 days of culture.

Rooting: Elongated shoots (2-3 cm) were transfer to different half strength MS with PGRs and MS0 (control) rooting media containing IAA, IBA and NAA alone or in combination. Root number and length were record after 30 days of inoculation.

Acclimatization
Well rooted seedlings were removed from culture vessels, washed to remove agar and treated with a mild fungicide. They were transplanted into small pots containing a mixture of moist coconut coir, saw dust and coal. The plantlets were kept in a green house with controlled humidity and watered regularly. Survival percentage was recorded after 2-3 months.

Statistical Analysis
All experiments were set up in a completely randomized design (CRD) with five replicates per treatment. Data were analyzed using One-Way Analysis of Variance (ANOVA). Mean values were presented as Mean ± Standard Error (SE).

Results
Seed germination and plantlets development
The seeds of *Pholidota imbricata* an epiphytic indigenous orchid was aseptically germinated on four basal media *viz*. MS [14], PM [15], MVW [16] and KC [17] and finally produce large number of plantlets. Among the four basal media MS was superior (80.00%, Figure 1) responses than PM (73.34%, Figure 2), MVW (53.34%, Figure 3) and KC (46.67%, Figure 4) respectively.

Micropropagation from pseudobulb and leaf explants
The morphogenic response of *P. imbricata* explants varied significantly depending on the explant type and hormonal combination (Table 1). Pseudobulb segments exhibited direct organogenesis, producing multiple shoot buds (MSBs) without an intervening callus phase. In contrast, leaf segments underwent indirect morphogenesis, producing Protocorm-Like Bodies (PLBs).
The highest frequency of shoot induction (90.67% ± 1.96) was observed on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA (Figure 5). This combination also yielded the maximum number of shoots per explant (6.4 ± 0.51) with the minimum time required for induction (5.38 ± 0.10 weeks). A combination of 2.0 mg/l BAP and 1.0 mg/l IAA also showed favorable results (84.00% response). Media containing Kinetin generally produced lower responses compared to BAP-supplemented media.
Leaf segments proliferated to form greenish PLBs. The maximum induction frequency (74.67% ± 2.24) was recorded on MS medium fortified with 2.0 mg/l BAP and 1.0 mg/l NAA, taking approximately 5.66 ± 0.10 weeks (Figure 6). The combination of 2.0 mg/l BAP + 1.0 mg/l Picloram also supported PLB formation (58.67%) but was less effective than the BAP + NAA combination. Control media (MS0) showed negligible response for both explant types.

Shoot Elongation
To enhance the growth rate, MSBs were sub-cultured onto various elongation media compared to the efficacy of solid (agar-gelled) versus liquid MS media supplemented with PGRs (Table 2).
The highest increase of shoot elongation (2.72 ± 0.03 cm) was achieved on solid MS medium supplemented with 1.5 mg/l BAP and 0.9 mg/l NAA (Figure 7) followed by solid MS with 1.0 mg/l BAP + 0.6 mg/l NAA (2.61 ± 0.04 cm). In comparison, liquid media showed slightly lower efficacy, with the maximum elongation (2.37 ± 0.04 cm) observed in liquid MS with 1.5 mg/l BAP and 0.9 mg/l NAA (Figure 8). Overall, solid media proved superior to liquid media for the elongation of *P. imbricata*.

Rooting
Elongated shoots (2-3 cm) were transferred to rooting media and the results indicated that the inclusion of auxins significantly improved root quality and number compared to the control. The highest root length increase (3.85 ± 0.03 cm, Graph 1) and the maximum number of roots per seedling (4.33 ± 0.33, Graph 2) were observed on half strength MS medium supplemented with a synergistic combination of 1.0 mg/l IAA and 1.0 mg/l IBA (Figure 9). Individual applications of auxins were effective but less so than the combined treatment.

Acclimatization
The *in vitro* raised seedlings with well-developed root systems were exposing to a gradual hardening process. After transferring to pots containing coconut coir, sawdust and coal, the seedlings showed a survival rate of 75%. The surviving plants exhibited resumption of growth and formation of new leaves after 2-3 months in the greenhouse conditions.

Discussion
The establishment of an efficient micropropagation protocol depends heavily on the selection of explants and the precise

manipulation of PGRs. In the present study, *in vitro* derived pseudobulb segments of *P. imbricata* demonstrated a high capacity for direct organogenesis. This aligns with findings in other orchid species, such as *Dendrobium palpebrae* [11] and *Coelogyne stricta*, where pseudobulbs serve as excellent storage organs capable of regenerating shoots due to the presence of axillary buds.

The synergistic effect of cytokinins (BAP) and auxins (NAA) was evident in both shoot multiplication and PLBs induction. We observed that media supplemented with BAP and NAA yielded significantly higher results than those with Kinetin or single PGR. BAP is often reported as the most effective cytokinin for orchid tissue culture, known for breaking apical dominance and stimulating multiple shoot formation [13, 18]. The specific combination of 2.0 mg/l BAP and 1.0 mg/l NAA provided the optimal balance for maximizing shoot proliferation (90.67%) from pseudobulbs and PLBs formation (74.67%) from leaves. This is consistent with results reported by Reddy *et al.* [19] in *Dendrobium nobile* and Gladies *et al.* [20] in *Pholidota pallida*.

Crucially, our findings are corroborating by very recent studies on indigenous orchids of Bangladesh. For instance, Bhowmik and Rahman [10] reported that a combination of 3.0 mg/l BAP and 1.5 mg/l NAA was most effective for *Eulophia graminea* and Bhowmik and Rahman [11] found a similar synergistic requirement (2.0 mg/l BAP + 1.0 mg/l NAA) for *Dendrobium crepidatum*. The similarity in hormonal requirements suggests a conserved physiological response among these epiphytic and terrestrial orchids in the region.

Leaf explants followed a pathway of somatic embryogenesis, forming PLBs. This is a desirable trait for mass propagation as PLBs can proliferate rapidly and produce large numbers of plantlets. The success of leaf culture in *P. imbricata* suggests that highly differentiated tissues can revert to meristematic activity under appropriate hormonal stimuli, a phenomenon supported by studies on *Aerides multiflora* [21] and *Eria lasiopetala*, where MSBs were successfully induced from protocorms [22].

For shoot elongation, solid media proved superior to liquid media in this specific study, contrasting with some reports where liquid culture enhances nutrient uptake [23]. However, this finding aligns with the recent work on *Dendrobium crepidatum*, where solid media also performed competitively for shoot quality [24]. The structural support provided by agar may have prevented vitrification, a common issue in liquid culture, allowing for healthier shoot development in *P. imbricata*.

Rooting is a critical step for successful acclimatization. A combination of IAA and IBA on half strength MS medium was most effective. Reduced salt strength (half MS) is often preferred for rooting in epiphytes to mimic their natural low nutrient environment [25]. The combination of two auxins likely provided a complementary effect, enhancing both root initiation and elongation, which is crucial for the uptake of water and nutrients during the transition to *ex vitro* conditions.

Conclusion

This study establishes a complete and reproducible protocol for the micropropagation of *Pholidota imbricata* Hook. f. The optimum pathway involves the culture of pseudobulb or leaf segments on MS medium with 2.0 mg/l BAP + 1.0 mg/l NAA for multiplication, followed by elongation on solid MS + 1.5 mg/l BAP + 0.9 mg/l NAA, and rooting on 1/2 MS + 1.0 mg/l IAA + 1.0 mg/l IBA. With a 75% survival rate during acclimatization, this protocol can be effectively utilized for large-scale commercial production and conservation of this near-threatened medicinal orchid.

Fig 1: *In vitro* seed germination on agar solidified basal MS medium

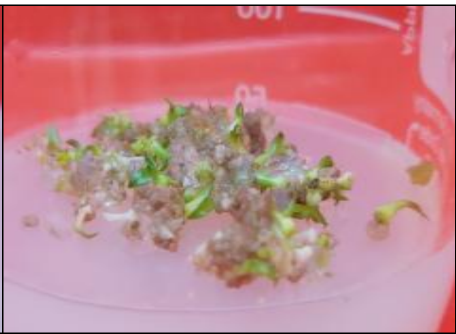


Fig 2: *In vitro* seed germination on agar solidified basal PM medium

Fig 3: *In vitro* seed germination on agar solidified basal MVW medium

Fig 4: *In vitro* seed germination on *agar* solidified basal KC medium

Fig 5: Development of MSBs raised from pseudobulb segment on MS + 2.0 mg/l BAP + 1.0 mg/l NAA

Fig 6: Development of PLBs develop *from* leaf segment on MS + 2.0 mg/l BAP + 1.0 mg/l NAA

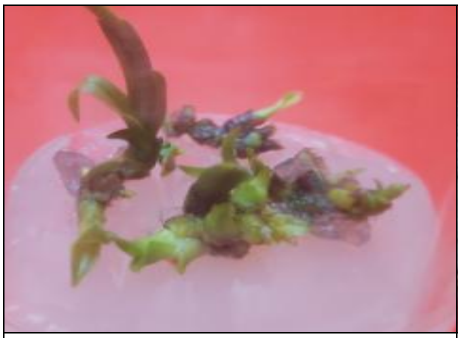


Fig 7: Shoot buds' elongation on agar solidified MS + 1.5 mg/l BAP + 0.9 mg/l NAA

Fig 8: Shoot buds' elongation in liquid MS + 1.5 mg/l BAP + 0.9 mg/l NAA

Fig 9: Induction of rooting on MS + 1.0 *mg*/l IAA + 1.0 mg/l IBA

Table 1: Development of MBSs/PLBs from *in vitro* raised pseudobulb and leaf explants of *P. imbricata* on agar solidified MS medium with different kinds of PGRs

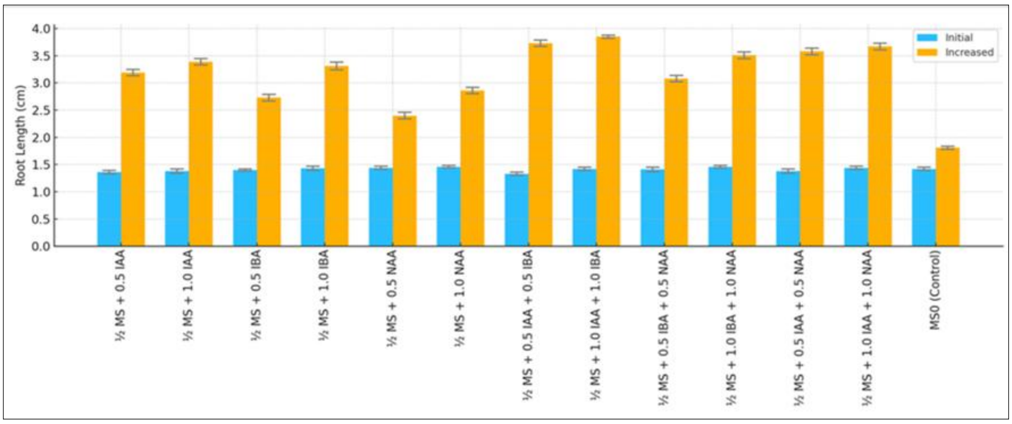
Sl. No.	PGRs Concentration (mg/l)					Pseudobulb Segment (Mean ± SE)			Leaf Segment (Mean ± SE)		
	BAP	Kn	NAA	IAA	Pic	% of induced MBSs/ segment	Time required (week) for development of MSBs	No. of MBSs produced/ segment	% of induced PLBs/ segment	Time required (week) for development of PLBs	Nature of PLBs (Colour)
1.	1.0	-	-	-	-	26.67±1.72	7.26±0.09	2.4±0.51	0.00±0.00	0.00±0.00	-
2.	2.0	-	-	-	-	38.67±2.24	6.72±0.16	2.0±0.45	6.67±0.00	7.76±0.12	G
3.	-	1.0	-	-	-	20.00±1.72	7.34±0.08	1.8±0.37	0.00±0.00	0.00±0.00	-
4.	-	2.0	-	-	-	26.67±2.72	7.26±0.09	2.2±0.37	0.00±0.00	0.00±0.00	-
5.	1.0	-	0.5	-	-	77.33±1.96	5.64±0.12	5.8±0.58	52.00±2.24	6.24±0.06	YG
6.	2.0	-	1.0	-	-	90.67±1.96	5.38±0.10	6.4±0.51	74.67±2.24	5.66±0.10	G
7.	1.0	-	-	0.5	-	70.67±1.96	6.08±0.10	5.2±0.58	46.67±1.72	6.52±0.07	WG
8.	2.0	-	-	1.0	-	84.00±1.96	5.56±0.12	6.0±0.71	57.33±1.96	6.08±0.10	G
9.	1.0	-	-	-	0.5	65.33±1.44	6.12±0.12	5.0±0.71	53.33±1.72	6.32±0.07	YG
10.	2.0	-	-	-	1.0	77.33±1.96	5.72±0.14	5.6±0.51	58.67±2.24	6.10±0.11	G
11.	-	1.0	0.5	-	-	46.67±1.72	6.66±0.16	3.8±0.58	20.00±1.72	7.44±0.08	G
12.	-	2.0	1.0	-	-	57.33±1.96	6.34±0.09	4.6±0.51	33.33±1.72	7.08±0.10	YG
13.	-	1.0	-	0.5	-	45.33±1.44	6.68±0.16	3.6±0.40	20.00±1.72	7.56±0.07	WG
14.	-	2.0	-	1.0	-	57.33±1.96	6.34±0.09	4.4±0.51	26.67±1.72	7.24±0.07	G
15.	-	1.0	-	-	0.5	45.33±1.44	6.74±0.15	3.4±0.51	13.33±1.22	7.64±0.12	G
16.	-	2.0	-	-	1.0	52.00±2.24	6.56±0.07	4.0±0.71	25.33±1.89	7.36±0.07	WG
17.	MS0 (Control)					6.67±0.00	7.62±0.11	1.2±0.20	0.00±0.00	0.00±0.00	-

Multiple Shoot Buds (MBSs); 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.

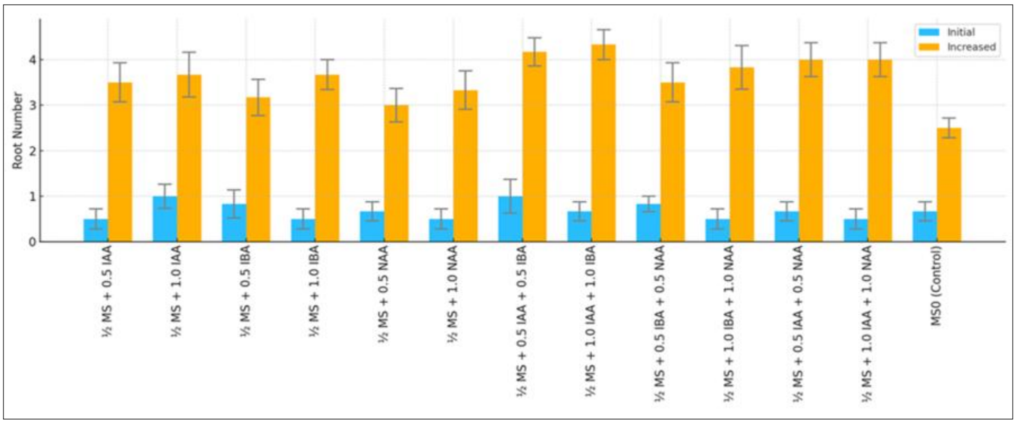
Table 2: Elongation of pseudobulb raised MSBs of *P. imbricata* on agar solidified and liquid MS medium with different kinds of PGRs

Sl. N.	PGR Conc. (mg/l)				Solid media (Mean± SE)			Liquid media (Mean± SE)		
	BAP	Kn	NAA	IAA	Initial length (cm) of MSB	Length (cm) of seedlings after 30d of culture	Increased in length (cm) within 30d of culture	Initial length (cm) of MSB	Length (cm) of seedlings after 30d of culture	Increased in length (cm) within 30d of culture
1.	1.0	-	-	-	1.38±0.02	3.72±0.04	2.33±0.03	1.38±0.02	3.37±0.05	1.99±0.03
2.	1.5	-	-	-	1.35±0.02	3.76±0.05	2.41±0.03	1.35±0.03	3.42±0.04	2.07±0.04
3.	2.0	-	-	-	1.31±0.02	3.52±0.05	2.21±0.04	1.28±0.02	3.15±0.04	1.87±0.04
4.	-	1.0	-	-	1.38±0.02	3.41±0.04	2.03±0.03	1.27±0.02	2.95±0.05	1.68±0.03
5.	-	1.5	-	-	1.32±0.02	3.40±0.03	2.08±0.03	1.34±0.03	3.05±0.06	1.71±0.03
6.	-	2.0	-	-	1.34±0.02	3.34±0.03	2.00±0.03	1.39±0.02	3.05±0.04	1.66±0.04
7.	1.0	-	0.6	-	1.36±0.03	3.98±0.06	2.61±0.04	1.32±0.01	3.60±0.04	2.28±0.03
8.	1.5	-	0.9	-	1.41±0.02	4.13±0.02	2.72±0.03	1.34±0.02	3.70±0.05	2.37±0.04
9.	2.0	-	1.2	-	1.35±0.02	3.73±0.03	2.38±0.03	1.38±0.02	3.49±0.02	2.11±0.03
10.	1.0	-	-	0.6	1.37±0.03	3.90±0.06	2.52±0.04	1.33±0.02	3.52±0.04	2.19±0.03
11.	1.5	-	-	0.9	1.39±0.02	3.95±0.04	2.57±0.04	1.26±0.02	3.50±0.03	2.24±0.04
12.	2.0	-	-	1.2	1.33±0.02	3.70±0.02	2.36±0.03	1.29±0.03	3.33±0.03	2.04±0.04
13.	-	1.0	0.6	-	1.43±0.02	3.92±0.02	2.49±0.03	1.32±0.02	3.48±0.04	2.16±0.03
14.	-	1.5	0.9	-	1.32±0.02	3.85±0.04	2.54±0.04	1.31±0.03	3.52±0.03	2.21±0.03
15.	-	2.0	1.2	-	1.41±0.02	3.71±0.04	2.30±0.03	1.27±0.02	3.28±0.04	2.01±0.03
16.	-	1.0	-	0.6	1.43±0.02	3.70±0.04	2.27±0.03	1.39±0.02	3.34±0.05	1.96±0.04
17.	-	1.5	-	0.9	1.42±0.02	3.86±0.05	2.44±0.03	1.28±0.02	3.41±0.02	2.14±0.03
18.	-	2.0	-	1.2	1.36±0.02	3.52±0.03	2.16±0.04	1.37±0.02	3.20±0.05	1.84±0.04
19.	MS0 (Control)				1.34±0.03	3.15±0.04	1.81±0.02	1.35±0.02	2.82±0.04	1.48±0.03

Values represent mean ± SE of each experiment consist of five replicates



Graph 1: Comparison of initial and increased root length (cm) by PGR concentration (mg/l)



Graph 2: Comparison of initial and increased root number by PGR concentration (mg/l)

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Conflicts of interest

The authors declare that they have no conflicts of interest related to the publication.

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