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In vitro seed germination and seedlings development of a terrestrial orchid, *Habenaria digitata* Lindl of Bangladesh

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Abstract
Habenaria digitata Lindl. is a indigenous terrestrial orchid, infrequently distributed in subtropical mountains and rainforests area of Jaflong, Sylhet, Bangladesh. The seeds of this orchid can be germinated asymbiotically *in vitro* for rapid propagation. In this study, half and full strength KC, MS, PM and VW basal media with or without PGRs combination showed various responses. Amongst four basal media used, MS medium with PGRs combination gave the maximum percentage response (86.87%) followed by PGRs supplemented PM (80.00%), VW (53.34%) and KC (46.67%) media. Minimum percentage of seed germination was observed on full strength PGRs free KC (26.67%) medium. Half strength PGRs free KC and VW media did not show any responses for germination of this orchid species. Minimum time needed for initiation of germination in full strength PGRs supplemented MS (8.07 ± 0.13^{b} weeks) medium, whereas, maximum time required (15.13 ± 0.22^{i}) in PGRs free full strength KC medium.

Keywords: *habenaria digitata*; PGRS; PLBS; terrestrial orchid

1. Introduction
Orchids are highly evolved, fascinating group of angiosperm known for their aesthetic beauty and great floral architecture [1]. With amazing ornamentation, brilliant color combinations and extended blooming period, they acquire highest admiration from scientists, horticulturists and industrialists [2-3]. Orchids are very sensitive and high specificity about pollution free environment of their niches and need much care for protection of the habitat for their existence. Heavy shrinkage of forest areas has created increasing threat of survival of entire orchid group. However, destruction of habitats and many other anthropogenic pressures, some species of orchids have vanished from the nature [4-5].

The genus *Habenaria* comprises eight species are reported in Bangladesh [6]. *Habenaria digitata* is a terrestrial herb species flowered at August to November [7] and inflorescence of this orchid looks very attractive, so it has good horticultural value [8].

In nature, orchid seeds do not germinate easily, these germinate if infected by a suitable mycorrhizal fungus [9-10]. Multiplication of orchids using tissue culture methods have been preferred, as there exists great opportunity in improving the quality and increasing the numbers of plantlets through mass propagation of several important orchids, hybrids or a new variety within a short time period [11-14]. In the present study, rapid and efficient method for mass propagation through asymbiotic seed germination with or without PGRs supplemented half and full strength four basal media is used for *Habenaria digitata*. During the last few years, tissue culture technique has been extensively exploited for the large scale propagation as well as *ex situ* conservation [15-16]. However, no work has been done of this species in Bangladesh. Therefore, in the present study was undertaken to develop effective *in vitro* germination and seedlings development protocol for the conservation of *Habenaria digitata* orchid.

2. Materials and Methods
2.1. Source and Collection of Materials
Mature seeds from undehisced capsules of *Habenaria digitata* were collected from subtropical mountains and rainforests area of Jaflong, Sylhet, Bangladesh. Capsules were collected in two times during the month of December and January.

2.2. Sterilization of Capsules
The collected capsules were washed with running tap water for 10 min to wash away the dust and other external particles from the green capsules. The capsules were transferred to laminar air flow cabinet; where they were surface sterilized with 3% (w/v) sodium hypochlorite (NaOCl) solution for 10 min followed by rinsing with sterile distilled water, subsequently they were treated with 70% ethanol (v/v) for 30 sec and washed with double distilled water. Then, the capsules were dipped in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 min followed by three times rinsing with sterile distilled water.

2.3. Culture medium and incubation
In the present investigation, half strength, full strength and plant growth regulators (PGRs) *viz.* BAP (0.5 mg/l) and NAA (0.5 mg/l) supplemented media of KC [17], MS [18], PM [19] and VW [20] were used for *in vitro* seed germination and seedling development. Basal medium were fortified with 30g/l sucrose for MS (Murashige and Skoog) and 20g/l sucrose for KC (Knudson c), PM (Phytamax), VW (Vacin and Went) and with or without different plant growth regulators (PGRs) like BAP & NAA (Table 1). Agar (0.8% w/v) was used as a gelling agent for all tested media. pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, PM and VW by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 50 ml of media was dispensed into 100 ml each culture vessel and autoclaved at 121 ^{0}C for 20 minutes at 15 lb/cm^2 pressure.

All cultures were maintained at 25±2 ^{0}C under 350-500 lux illumination for 14h photoperiod using white fluorescent tubes and 10h dark.

2.4. Seed culture and Sub-culturing

Surface sterilized mature green capsules were kept on sterilized petri dish containing sterilized filter paper for drying. They were cut longitudinally with the help of sharp sterilized surgical blade. The mature seeds were scooped out with the help of sterilized spatula and transferred to and spread over the surface of different strength of KC, MS, PM and VW media supplemented with or without combination of NAA and BAP. Sub-culturing was carried out every 4-6 weeks into fresh medium till the protocorms grew and formed complete seedlings. The entire experiment was performed in aseptic condition under laminar air flow hood to prevent contamination.

2.5. Hardening and Transplantation

For hardening, 90 days old plantlets with good rooting and 3-4 leaf conditions were selected. A gradual system of hardening was taken place in order to grow healthy plantlets. In this process, cultured vessels were kept open in the culture room for several hours, and then it was exposed to natural light for a day. Further, plantlets were washed by double distilled water to remove the adhering agar. Plants were treated with auxins to induce *ex vitro* rooting and roots were treated with fungicide. Then the seedlings of *H. digitata* were transferred to plastic pots containing a potting mixture of sterilized Soil, Sand, Activated Charcoal and Pit Moss, Vermicompost at a ratio of 1: 1: 1: 1 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

2.6. Computation and presentation of Data

The experiments were conducted thrice using 15 replicates per treatment. Different strength of basal media and PGRs combinations were considered to record data on morphogenic responses of explant under different conditions. The data on different parameters were recorded after required days of culture.

2.7. Statistical analysis

Experiment was set up as a randomized complete design and all graphs were prepared with using Microsoft Excel 2013. The data were statistically analyzed, using SPSS software package. ANOVA and mean comparison were carried out by DMRT at 5% level of significance (P=0.05).

3. Results and Discussion

The seeds of *Habenaria digitata* were aseptically grown on 0.8% (w/v) agar solidified half strength, full strength and PGRs (0.5 mg/l BAP and 0.5 mg/l NAA) supplemented full strength KC, MS, PM and VW media for *in vitro* germination and seedling development of indigenous terrestrial orchid *Habenaria digitata* (Table 1). Amongst four basal media used, MS medium with PGRs combination gave the maximum percentage response (86.87%, Fig. 1a) followed by PGRs supplemented PM (80.00%, Fig. 1b), VW (53.34%) and KC (46.67%) media. Minimum percentage of seed germination was observed on full strength PGRs free KC (26.67%) medium. Half strength PGRs free KC and VW media did not show any responses for germination of this orchid species; whereas, seeds can be germinate and produce protocorm, seedlings development in half strength MS and PM media. Minimum time required for initiation of germination in full strength PGRs supplemented MS (8.07 ± 0.13^b weeks) medium, followed by (10.23 ± 0.25^d weeks) PM, (12.30 ± 0.24^f weeks) VW and (13.77 ± 0.15^g weeks) KC media. PGRs free full strength KC medium needed maximum time (15.13 ± 0.22^i weeks) for seeds germination.

First visible yellowish green spherule like protocorms were develop after seed inoculation in PGRs supplemented full strength MS (12.57 ± 0.20^b weeks) medium followed by PM (14.33 ± 0.25^c weeks), VW (15.17 ± 0.22^d weeks) and KC (18.33 ± 0.22^g weeks) media respectively. Differentiation of first leaf primodia was shown on after 17 weeks in PGRs fortified MS (17.23 ± 0.23^b weeks) subsequently PM (19.27 ± 0.22^c weeks), VW (19.60 ± 0.35^c weeks, Fig. 1c) and KC (24.56 ± 0.32^f weeks, Fig. 1d) media. Whereas, first root primodia was exposed on PGRs supplemented MS (23.87 ± 0.25^b weeks) medium followed by PM (25.33 ± 0.27^c weeks, Fig. 1e), VW (26.60 ± 0.33^d weeks) and KC (30.73 ± 0.28^g weeks) media. Within 29 weeks of culture, complete seedlings were first showing on PGRs fortified MS (29.60 ± 0.37^b weeks, Fig. 1f) medium followed by PM (30.57 ± 0.30^c weeks), VW (32.53 ± 0.25^d weeks) and KC (38.63 ± 0.31^h weeks) media.

With or without PGRs supplemented full strength KC, MS, PM and VW media was found to be the most suitable culture condition for mature seed germination of *H. digitata* up to seedling development. Here also noted that PGRs supplemented media took lesser time for germination (Fig. 2), protocorm proliferation (Fig. 3), first leaf and root primodia differentiation and seedlings development (Fig. 4) than full strength of PGRs free medium. Therefore the present study showed that full strength KC, MS, PM and VW media supplemented with PGRs was the best and followed by without PGRs free full and half strength KC, MS, PM and VW media respectively.

Mature seeds of *H. digitata* undertake diverse developmental stages throughout germination. Germination of orchid seeds is unlike from other seeds. Orchid seeds are twisted in large numbers inside a pod. The seeds are very little and contain undifferentiated embryos and without endosperm. In few orchids self-pollination is not feasible and some are possible like *Vanda*, which have to wait for 4-6 months for pod development [21]. In the present research, mature green pods were taken for *in vitro* culture. Due to non-endospermic character of seed, the germination in environment is a exclusive incident and requires specific mycorrhizal fungal infection. Germination is an important factor for *in vitro* culture development. Cells of mature seeds first turned into smooth walled globular structures called spherules after eight weeks of seed culture due to the enlargement of embryos. This indicates the first observable sign of germination of cultured seeds. Light yellowish cultured seeds first changed into light green and finally to green in color throughout development of protocorms. Protocorms develop further to give go up to shoot and root without undergoing callus development in *H. digitata*. All the testing conditions showed seed germination but full strength MS media supplemented with BAP (0.5mg/l) and NAA (0.5mg/l) was the most effective condition for enhancing seed germination and seedling development.

Four different basal media working in present study were

different from one another in their chemical composition. MS medium is highly enriched with macro and micro nutrients with diverse vitamins whereas PM, VW and KC medium limited comparatively low amount of macro and micro nutriments and with or without vitamins [22] respectively. Due to this, maximum percentage of seed germination was observed in different conditions of MS, PM and VW medium rather than Knudson medium as they lack vitamins. There are numerous reports explaining perfection of germination and seedling growth and improvement by vitamins on different orchids. Addition of various vitamins into the medium was reported to be promoting for seeds germination and seedlings development of *Cymbidium elegans* and *Coelogyne punctulata* [23]. Mariat [24] reported that vitamin B enhanced for germination and differentiation in *Cattleya* seedlings. He showed that thiamine, nicotinic acid and biotin were most efficient in *Cattleya* hybrids production. In other study reported that, Pyridoxine was shown to be vital for chlorophyll synthesis and combination of nicotinic acid and biotin favored for better germination of *Orchis laxiflora* seeds [25]. In present exploration, half strength of KC and VW medium were not helpful for plantlet formation as they have very low amount of macro and micro nutrients. All of the basal media of full strength gave acceptable result but the time taken for germination and seedling development was quite longer than PGRs supplemented medium. In *in vitro* culture, cytokinins like BAP, Picloram and Kinetin are generally known to induce both axillary and adventitious shoots formation and auxins like NAA, IAA and IBA for root induction [26-27]. PGR like BAP was known to increase the germination frequency of *Habenaria macroceratitis*; *Cypripedium candidum*; *Erythrodes humilis* [27-29]; protocorm multiplication and shoot formation in *Erythrodes humilis* and *Cymbidium pendulum* [29-30]. The synergistic effect of cytokinin and auxin in germination and plantlet development as found in present study has also been reported in *Aerides ringens* [31], *Cymbidium elegans* [32], *C. iridiodes* [33], *Dendrobium thyrsiflorum* [34], *Phaius tancarvilleae* [35] and *Vanda tessellata* [36]. In present investigation, protocorms were globular and chlorophyllous in all testing conditions of MS and PM medium; whereas, in KC and VW medium, they were light yellowish in color.

The number of protocorms was few on Knudson medium and highest in MS medium but size of protocorms was superior on Knudson medium. Low amount of macro and micro elements of Knudson medium could have been effective for enlargement of protocorms due to nutritional stress but not for increasing their number. Production of larger size of protocorms indicates that culture seeds require sufficient amount of nutrients. Thus, the nutrient system for orchid culture is specific species to species and no particular culture medium is generally relevant for all the orchid species. From above result, it was accomplished that MS medium enriched with high concentration of nutritional compounds was suitable for earlier germination, large number of protocorm formation and seedling development rather than PM, VW and KC media. Similar findings were reported in *Cleisostoma racemifefum* [37], *Coelogyne suaveolens* [38], *Malaxix khasiana* [39] and *Vanda coerulea* [40] where MS medium was shown to be the most suitable medium over other nutrient media. In the present study, a flourishing effort was made to evaluate the *in vitro* seed germination and their consequent differentiation of *H. digitata* on four media *viz.* KC, MS, PM and VW. This protocol might be useful for choice of best state for mass propagation and *ex situ* conservation of this precious orchid species.

The *in vitro* derived plantlets were acclimatized at room temperature. The seedlings of *H. digitata* were transferred to pots containing soil, sand, pit moss, saw dust and charcoal. Rooted plantlets were able to grow into normal plantlets in *ex vitro* condition after a short period of acclimatization and transplanted seedlings were watered regularly for about 2-3 months. .

4. Conclusion

Full strength PGRs supplemented MS medium was found superior than KC, PM and VW media respectively. PGRs supplemented full strength all basal media were higher responses than PGRs free full and half strength media for promoting germination of this orchid seeds. *Ex situ* conservation by tissue culture technique of this species is highly recommended. Mass propagation using seed culture can be started in commercial scale to conserve this species in their natural habitat.

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Table 1: Effect of different strength of KC, MS, PM and VW media with plant growth regulators on *in vitro* seed germination, differentiation and seedling development of *Habenaria digitata Lindl.*

Medium	Culture condition	Time taken in weeks					% of culture vessel germinated	Remarks
		Initiation of Germination (Mean ± SE)	Development of protocorm (Mean ± SE)	Differentiation of 1st leaf primodia (Mean ± SE)	Differentiation of 1st root primodia (Mean ± SE)	Development of seedling (Mean ± SE)		
KC	*	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}	-	-
	**	15.13 ± 0.22^{i}	21.20 ± 0.30^{i}	27.17 ± 0.30^{h}	34.23 ± 0.27^{i}	42.20 ± 0.35^{i}	26.67	+
	***	13.77 ± 0.15^{g}	18.33 ± 0.22^{g}	24.56 ± 0.32^{f}	30.73 ± 0.28^{g}	38.63 ± 0.31^{h}	46.67	+
MS	*	11.33 ± 0.21^{e}	16.24 ± 0.21^{e}	22.60 ± 0.30^{e}	29.17 ± 0.24^{f}	37.20 ± 0.28^{g}	40.00	+
	**	9.57 ± 0.18^{c}	14.50 ± 0.20^{c}	19.47 ± 0.33^{c}	25.07 ± 0.28^{c}	32.03 ± 0.29^{d}	53.34	++
	***	8.07 ± 0.13^{b}	12.57 ± 0.20^{b}	17.23 ± 0.23^{b}	23.87 ± 0.25^{b}	29.60 ± 0.37^{b}	86.77	+++
PM	*	15.03 ± 0.20^{i}	20.23 ± 0.18^{h}	26.17 ± 0.33^{g}	32.23 ± 0.30^{h}	39.20 ± 0.22^{h}	33.34	+
	**	12.10 ± 0.28^{f}	16.07 ± 0.25^{e}	21.77 ± 0.25^{d}	28.20 ± 0.24^{e}	34.27 ± 0.25^{e}	46.67	+
	***	10.23 ± 0.25^{d}	14.33 ± 0.25^{c}	19.27 ± 0.22^{c}	25.33 ± 0.27^{c}	30.57 ± 0.30^{c}	80.00	+++

VW	*	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]	-	-
	**	14.47 ± 0.27[h]	17.37 ± 0.25[f]	23.30 ± 0.33[e]	29.37 ± 0.31[f]	35.13 ± 0.30[f]	33.34	+
	***	12.30 ± 0.24[f]	15.17 ± 0.22[d]	19.60 ± 0.35[c]	26.60 ± 0.33[d]	32.53 ± 0.25[d]	53.34	++

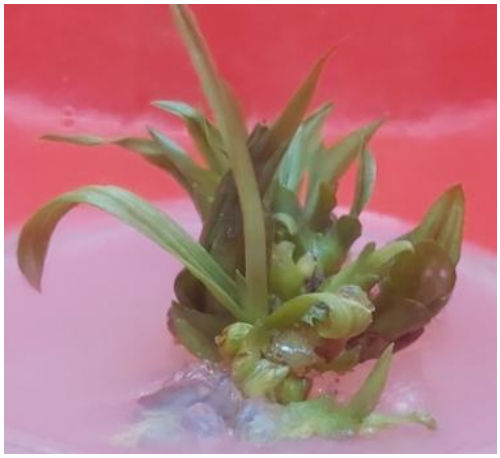
*Half strength without PGRs, **Full strength without PGRs, *** Full strength with PGRs (0.5mg/l BAP + 0.5mg/l NAA); '-' Indicate no response, + = Minimum germination (0% ≤ + ≤ 49%), ++ = Medium germination (50% ≤ ++ ≤ 74%), +++ = Maximum germination (75% ≤ +++ ≤ 100%). Values represent mean ± SE of each 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.

a. Germination of *Habenaria digitata* seeds on full strength PGRs supplemented agar solidified MS medium.

b. Germination of *Habenaria digitata* seeds on full strength PGRs supplemented agar solidified PM medium.

c. Germinated PLB's *Habenaria digitata* turned into small shoots on full strength VW medium.

d. Germinated PLB's *Habenaria digitata* turned into small shoots on full strength of KC medium.



e. Development of seedlings and small root of *Habenaria digitata* on full strength PM medium.

f. Plantlets of *Habenaria digitata* developed on MS + 0.5 mg/l BAP + 0.5 mg/l NAA.

Fig. 1: Different stages of *in vitro* seed germination and seedling development of *Habenaria digitata.*

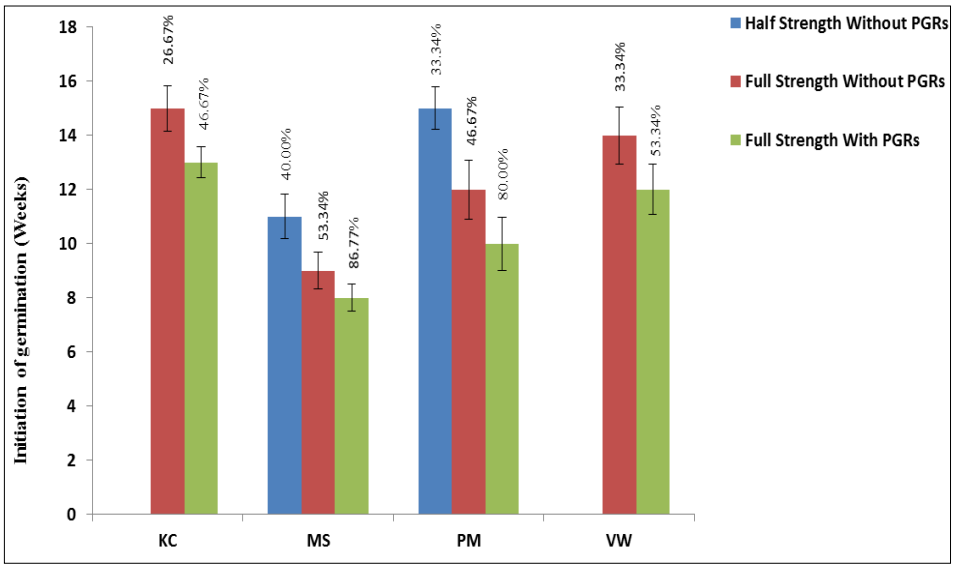


Fig 2: Initiation of germination pattern of Habenaria digitata *Lindl*.

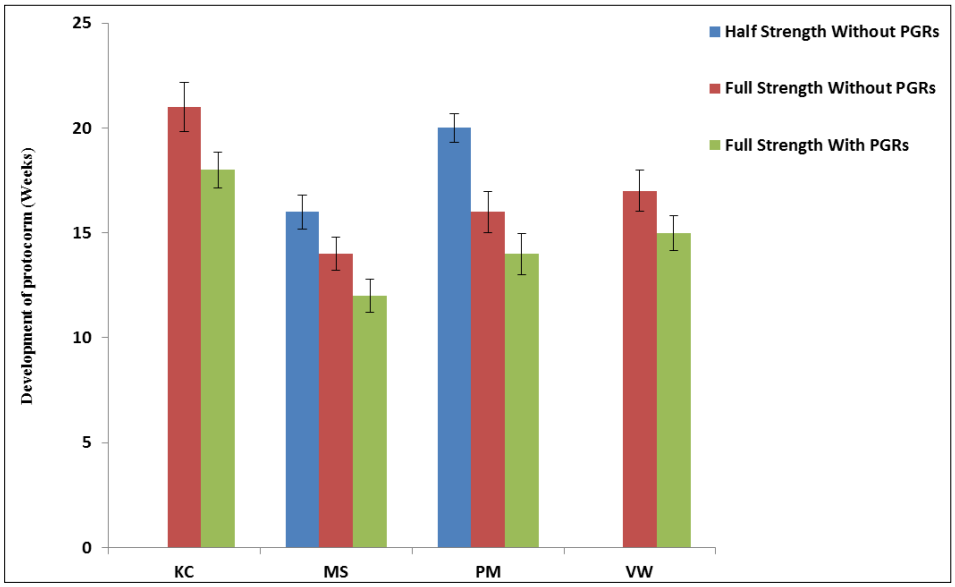
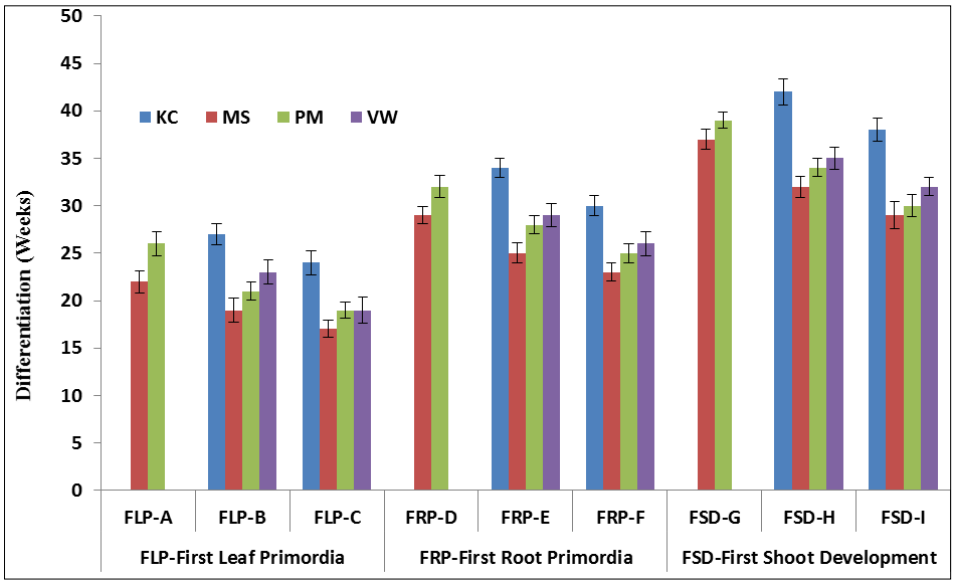


Fig 3: Development of protocorm pattern of *Habenaria digitata Lindl*.



A+D+G=Half Strength without PGRs; B+E+H=Full Strength without PGRs; C+F+I= Full Strength With PGRs

Fig 4: Differentiation pattern of *Habenaria digitata Lindl*.

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