J. Orchid Soc. India, 37: 59-67, 2023 *ISSN 0971-5371*

IN VITRO SEED GERMINATION, SEEDLING AND SPSs DEVELOPMENT IN *HABENARIA DIGITATA* LINDL. ON DIFFERENT GROWTH ADDITIVES AND PGRs SUPPLEMENTED MS MEDIUM

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Abstract

In the present investigation, seeds procured from green capsules of *Habenaria digitata* Lindl. were cultured on 0.8% (w/v) agar solidified MS medium supplemented with eight different growth additives namely, activated charcoal (AC), banana powder (BP), peptone (P), vanilla powder (VP), amaranth juice (AJ), coconut water (CW), pineapple juice (PJ), and tomato juice (TJ). Maximum percentage (93.34%) of seed germination was found on MS medium fortified with 1 gl^{-1} activated charcoal, 2 gl^{-1} peptone, and 20% coconut water. The lowest time required for initiation of seed germination (7.21±0.34 wks), induction of protocorms (10.29±0.49 wks), and development of seedlings (25.79±0.60 wks) was achieved on MS medium supplemented with 1 gl^{-1} AC. After 30 days of culture, the maximum increase in length (3.91±0.04 cm) and number of roots (4.17±0.31) was observed on MS medium supplemented with IBA (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}). The highest (96.00%) greenish shoot primodia like structures (SPSs) were developed within the lowest time (6.22±0.15 wks) in liquid MS medium supplemented with KN (1.0 mgl^{-1}) and NAA (0.6 mgl^{-1}). The healthy seedlings with well-developed roots were transferred to natural environment following successive phases of acclimatization.

Introduction

THE ORCHIDS, belonging to the family Orchidaceae, are renowned for their beauty, colour combinations, flower shapes, and are amongst the most significant decorative plants. These plants occupy a wide range of habitats and exhibit highly specialized pollination mechanisms and morphological, structural, and physiological characteristics (Lal *et al.,* 2021; Prakash and Pathak, 2020a,b, 2022). These plants comprise the second largest family of flowering plants and have around 29,481 species (WFO, 2023) across over 705 genera (POWO, 2023). In Bangladesh, orchids are fairly common and like in other tropical areas, the majority of them are epiphytes, representing 188 species under 72 genera, of these, 117 species under 41 genera are epiphytic and 71 species under 31 genera are terrestrial (Huda *et al.,* 2020; Rahman *et al.,* 2017). Orchids are expensive and the orchid industry in the world is well established and has further scope for enlargement. A well-known family in ethnomedicine, the Orchidaceae has been used in folk medicine in many different regions (Pathak *et al.,* 2010; Vibha *et al.,* 2019). For centuries, people have valued orchids for their therapeutic benefits and as a source of medications (Prakash and Pathak, 2019; Tsering *et al.,* 2017). The Orchidaceae family is distinguished by tiny dusty seeds with an ellipsoidal to spherical embryos encased in thin, translucent fusiform testa. Some researchers have shown a connection between orchid seed size and plant habit (Clifford and Smith, 1969; Rasmussen, 1995; Swamy *et al*., 2004; Verma *et al*., 2013; Vij *et al*., 1992) and the seeds of epiphytic orchids are often smaller than those of terrestrial orchids.

Habenaria Willd. is a genus of terrestrial orchids that are widespread throughout the tropical, subtropical, and temperate regions (Batista *et al.,* 2013), with centres of diversity in Brazil, Southern and Central Africa, East Asia (Kurzweil and Weber, 1992), and a large number of them are found in the Indian region (Choudhury *et al.,* 2011). In order to meet the high demand of the pharmaceutical industry, raw materials are currently taken from the wild at a high rate. This had a significant negative impact on the availability of this plant in its native environment, and the species has been classified as rare in the Himalayan region (Chinmay *et al*., 2009). As *Habenaria digitata* contains substances that are both analgesic and anti-inflammatory (Mahnashi *et al*., 2021), its populations are declining in its natural habitat due to higher economic importance. Hence, this species needs urgent conservation measures. Some attempts have been made earlier to propagate and conserve a few orchid species from diverse habits and habitats using *in vitro* asymbiotic seed germination technique (Anuprabha and Pathak, 2019; Bhowmik and Rahman, 2020, 2022a; Kaur *et al.,* 2017; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al*., 2016, 2017, 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021). Hence, the current study was designed to evaluate the effectiveness of additives on asymbiotic germination potential of seeds, differentiations, and seedlings development *in vitro*. Induction of strong and stout

Received: April 18, 2023; Accepted: June 22, 2023

rooting system and SPSs development were established for mass propagation protocol for this species.

Material and Methods

Sterilization of Capsules

In the present study, green capsules of *H. digitata* were collected from Jaflong, Sylhet, Bangladesh and used as explants. The collected capsules were thoroughly cleaned with Teepol (0.01%), washed under running water for 10 min, and then washed twice with sterile distilled water. After being submerged in 70% ethyl alcohol for 30 sec, the capsules were cleaned three times with double-distilled water. After being surface sterilized for 10 min with 0.1% (w/v) $HgCl_2$, the capsules underwent three rinses with double-distilled water. The seeds were then extracted from the sterilized capsules

Fig. 1A-I. *In vitro* seed germination, seedling and SPSs development in *Habenaria digitata* on different growth additives and PGRs supplemented MS medium: A, Seed germination on agar solidified MS medium with 20% CW; B, Seed germination on agar solidified MS medium with 2.0gl^{-1} P; C, Shoots on MS medium with 1.5 gl^{-1} AC; D, Shoots on MS medium with 4.0 gl^{-1} BP; E, Development of seedlings on MS medium with 1.0 gl^{-1} AC; F, Plantlets developed on MS medium with 8.0 gl^{-1} VP; G, Induction of strong and stout root system in seedlings on MS + 1.0 mgl^{-1} IBA + 1.0 mgl^{-1}NAA; H, Development of SPSs at the base of the shoots in liquid MS + 1.0 mgl^{-1}KN + 0.6 mgl^{-1}NAA; I, Development of SPSs at the base of the shoots on agar solidified MS + 1.0 mgl^{-1} KN + 0.6 mgl^{-1}NAA.

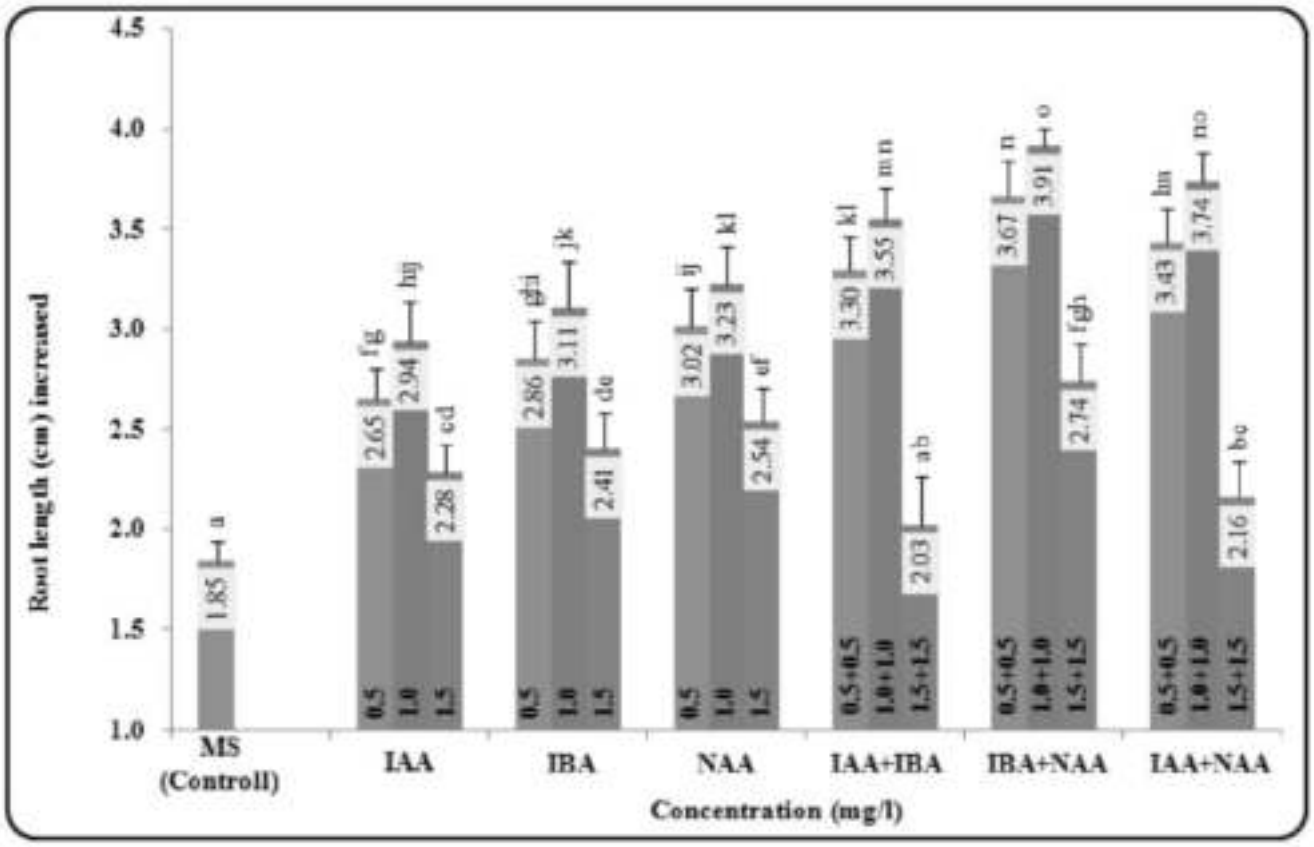

Fig. 2. Increased root length (cm) of seed originated plantlets of *H. digitata* in auxin supplemented MS medium after 30 days of culture. Values represent Mean±SE of each experiment consist of six replicates. Mean values of each bar of a graph followed by different letters at the upper position of SE bar are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Test.

using a sterilized blade aseptically, inside a laminar airflow cabinet.

In Vitro Seed Germination

Habenaria digitata was cultured on 0.8% (w/v) agar solidified MS (Murashige and Skoog, 1962) medium fortified with 3% (w/v) sucrose and eight different types of growth additives namely, activated charcoal (AC), banana powder (BP), peptone (P), vanilla powder (VP), amaranth juice (AJ), coconut water (CW), pineapple juice (PJ), and tomato juice (TJ). Three concentrations of each additive were used for rapid germination of this orchid species (Table 1).

In Vitro Rooting

MS medium supplemented with different combinations and concentrations of plant growth regulators (PGRs; IAA, IBA, and NAA) singly or in various combination was used for the development of strong and stout rooting system.

Transplantation

Healthy seedlings with three to four leaves and two to three roots were finally hardened by the subsequent acclimatization cycles. The seedlings were properly rinsed in sterile distilled water to remove agar before being transplanted into a pot filled with potting mixture (sterilized soil, sand, activated charcoal, and pit moss in a ratio of 1:1:1:1).

SPSs Development

In vitro grown seedlings were cultivated on twenty five different combinations of PGRs (BAP, KN, NAA, and IAA) enriched MS solid and liquid media in order to develop shoot primordia like structures (SPSs) (Table 2).

Computation and Presentation of Data

The data on various growth parameters was recorded. The parameters were:

i) Per cent (%) of seed germination

The following formula was used to calculate the % of seeds that germinated on MS medium:

$$\% \text{ of seeds germinated} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

ii) Number of roots per plantlet

The number of roots per plantlet was recorded individually and the mean number of roots per plantlet was calculated using the following formula:

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

iii) Length of roots per plantlet

The length of roots per plantlet was measured in centimeter (cm) and the average length of roots was calculated as follows:

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

iv) Frequency (%) of SPSs development

The following formula was used to calculate the % of SPSs formed at the base of seedlings:

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

v) Per cent of seedlings survived

The formula below was used to calculate the % of seedlings that survived in external environment:

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

Statistical Analysis

The results were presented as means with standard error (Mean±SE) from three separate trials. The standard deviation (SD) was calculated using Microsoft Excel (2013) software. The data was subjected to an analysis of variance (ANOVA) and the significant differences were found using Duncan's Multiple Range Test (Gomez and Gomez, 1984) at a 5% level of significance ($P<0.05$). The data was analyzed using the IBM, SPSS (Statistical Product and Service Solutions) Statistics software program.

Results and Discussion

Additional presence of all additives excepting for CW demonstrated better seed germination responses at the moderate level than at the lower or higher levels. The highest percentage of seed germination (93.34%) was observed on MS medium supplemented with 1 gl^{-1} activated charcoal, 2 gl^{-1} peptone, and 20% coconut water. This was followed by 86.67 % response in MS medium fortified with 1.5 gl^{-1} activated charcoal, 1 gl^{-1} peptone, 10 ml^{-1} amaranth juice, 15% coconut water, and 15 ml^{-1} pineapple juice. MS medium supplemented with 1 gl^{-1} AC, enhanced the initiation of seed germination (7.21±0.34 wks), induction of protocorms (10.29±0.49 wks), and development of seedlings (25.79±0.60 wks) (Table 1; Fig. 1C). MS medium supplemented with 2 gl^{-1} P provided almost similar results. For the onset of germination, induction of protocorms, and development of seedlings, a lower concentration of AC (0.5 gl^{-1}) exhibited significantly different variation ($P<0.05$) than a medium concentration (1.0 gl^{-1}). Furthermore, for initiation of seed germination, induction of protocorms, and the development of seedlings at a higher (3.0 gl^{-1}) P concentration demonstrated significant differences ($P<0.05$) from the medium concentration (2.0 gl^{-1}). For the development of protocorms, the concentrations of the VP and AJ additives (lower and medium) varied significantly ($P<0.05$). When 10% and 20% of CW was added to MS medium, the differences for protocorm induction and seedling growth were significant ($P<0.05$). For the induction of protocorms and the growth of seedlings, higher concentrations of BP and TJ exhibited significant differences ($P<0.05$) than moderate values. Finally, it was established that the optimal medium for initiating seed germination, inducing protocorms, and developing seedlings was 1.0 gl^{-1} AC supplemented MS medium. Earlier, the

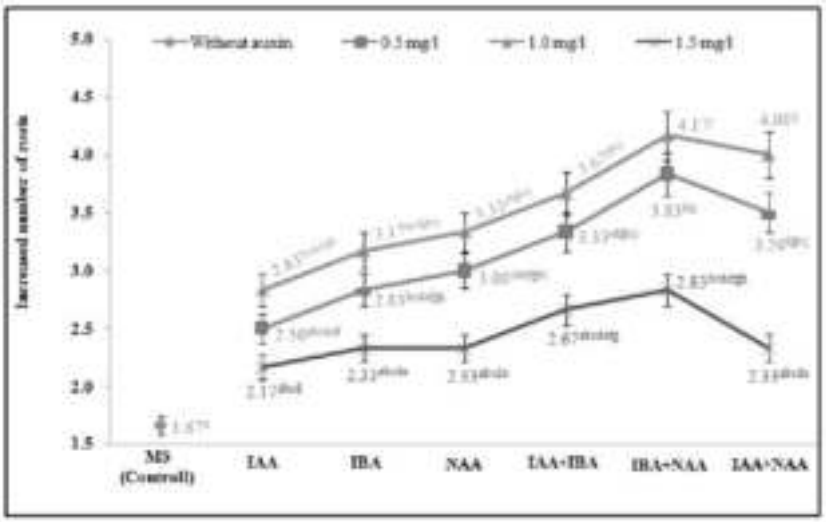

Fig. 3. Increased root number of seed originated plantlets of *H. digitata* in auxin supplemented MS medium after 30 days of culture. Values represent Mean±SE of each experiment consist of six replicates. Mean values of each point of a graph followed by different superscript letters are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Test.

Table 1. Effect of different additives in MS medium on asymbiotic seed germination, protocorms induction, and seedlings development, in *Habenaria digitata*.

Additives	Concentration	% of seeds responded	Time taken in wks (Mean±SE)		
			Initiation of seed germination	Induction of protocorms	Development of seedlings
AC(gl^{-1})	0.5	80.00	10.93 ± 0.25^{bcdefg}	17.14 ± 0.52^{bcdef}	35.71 ± 0.64^{cdef}
	1.0	93.34	9.71 ± 0.26^{ab}	15.57 ± 0.52^{ab}	33.14 ± 0.75^{ab}
	1.5	86.67	11.57 ± 0.41^{efgh}	16.07 ± 0.48^{abcd}	34.64 ± 0.58^{bcd}
BP(gl^{-1})	2.0	73.34	10.14 ± 0.34^{abcd}	16.86 ± 0.57^{abcdef}	35.71 ± 0.59^{cdef}
	4.0	80.00	9.86 ± 0.45^{ab}	16.07 ± 0.48^{abcd}	34.21 ± 0.52^{bc}
	6.0	73.34	11.93 ± 0.41^{fgh}	17.57 ± 0.60^{defg}	36.64 ± 0.51^{defg}
P(gl^{-1})	1.0	86.67	11.64 ± 0.42^{efgh}	16.71 ± 0.58^{abcde}	32.93 ± 0.66^{ab}
	2.0	93.34	9.36 ± 0.34^{a}	15.21 ± 0.57^{a}	31.57 ± 0.57^{a}
	3.0	80.00	10.86 ± 0.45^{bcdefg}	17.21 ± 0.57^{bcdef}	34.21 ± 0.52^{bc}
VP(gl^{-1})	4.0	73.34	11.36 ± 0.42^{cdefgh}	18.14 ± 0.57^{efg}	36.86 ± 0.60^{efg}
	8.0	86.67	10.07 ± 0.38^{abc}	16.58 ± 0.47^{abcde}	34.86 ± 0.71^{bcde}
	12.0	80.00	10.79 ± 0.42^{bcdefg}	17.36 ± 0.52^{cdef}	34.79 ± 0.75^{bcd}
AJ(ml^{-1})	5.0	73.34	11.57 ± 0.40^{efgh}	18.36 ± 0.51^{efg}	37.79 ± 0.57^{g}
	10.0	80.00	10.14 ± 0.39^{abcd}	16.86 ± 0.50^{abcdef}	35.57 ± 0.69^{cdef}
	15.0	73.34	10.93 ± 0.37^{bcdefg}	17.64 ± 0.56^{defg}	36.36 ± 0.71^{defg}
CW(%)	10.0	80.00	11.29 ± 0.29^{cdefgh}	17.79 ± 0.58^{defg}	32.93 ± 0.66^{ab}
	15.0	86.67	10.79 ± 0.42^{bcdefg}	16.29 ± 0.50^{abcd}	31.50 ± 0.56^{a}
	20.0	93.34	9.86 ± 0.50^{ab}	15.79 ± 0.54^{abc}	34.29 ± 0.52^{bc}
PJ(ml^{-1})	10.0	73.34	11.21 ± 0.42^{cdefgh}	17.71 ± 0.57^{defg}	36.57 ± 0.54^{defg}
	15.0	86.67	10.50 ± 0.41^{abcde}	16.93 ± 0.48^{abcdef}	35.79 ± 0.60^{cdef}
	20.0	80.00	12.07 ± 0.41^{gh}	18.57 ± 0.49^{fg}	37.50 ± 0.53^{fg}
TJ(ml/l)	5.0	73.34	11.43 ± 0.44^{defgh}	17.57 ± 0.47^{defg}	36.50 ± 0.57^{defg}
	10.0	80.00	10.64 ± 0.37^{abcdef}	17.71 ± 0.50^{defg}	35.64 ± 0.64^{cdef}
	15.0	73.34	12.29 ± 0.42^{h}	18.36 ± 0.51^{efg}	37.43 ± 0.49^{fg}
	MS0 (Control)	66.67	13.50 ± 0.33^{i}	19.29 ± 0.43^{g}	38.21 ± 0.42^{g}

AC, Activated Charcoal; BP, Banana Powder; P, Peptone; VP, Vanilla Powder; AJ, Amaranth Juice; CW, Coconut Water; PJ, Pineapple Juice; and TJ, Tomato Juice. Values represent mean±SE of each experiment consist of fifteen replicates. Mean values followed by different superscript letters within a column are significantly different at $p<0.05$ according to DMRT.

highest percentage of seed germination was observed on culture medium supplemented with 20% CW in *Smithsonia maculata* (Decruse and Gangaprasad, 2018). Coconut water has been shown to improve seed germination in *Acampe papillosa* (Piri *et al*., 2013), *Cypripedium macranthos* (Huh *et al*., 2016), *Dendrobium parishii* (Buyun *et al*., 2004), *Paphiopedilum wardii* (Zeng *et al*., 2012), and *Rhynchostylis retusa* (Thomas and Michael, 2007). Peptone has been shown to improve seed germination in *Aerides ringens* (Srivastava *et al*., 2015), *Dendrobium lasianthera* (Utami *et al*., 2017), *Epidendrum ibaguense* (Hossain, 2008), and *Spathoglottis plicata* (Hossain and Dey, 2013). For improving the germination of seeds, amino acids, amides, and vitamin contents of peptone are thought to be important (Oliva and Arditti, 1984). The seeds cultured on nutrient medium fortified with pineapple juice (PJ) showed the highest germination rate in *Orchis simia* (Fatahi *et al*., 2022). Activated charcoal (AC) was also found to be effective in seed germination of *Epidendrum ibaguense* (Hossain, 2008). Adsorption of unknown morphogenetically active or toxic substances (Klein and Bopp, 1971) and adsorption of inhibitory phenolics and carboxylic compounds manufactured by tissues (Fridborg *et al*., 1978) were two of the potential benefits of charcoal in culture media. Tomato juice (TJ) was proved to be effective in the germination of orchid seeds in *Geodorum densiflorum* (Muthukrishnan *et al*., 2013) and *Vanda helvola* (David *et al.,* 2015)

Table 2. Development of Shoot Primodia like Structures (SPSs) in *H. digitata* on agar solidified and liquid MS media with PGRs.

PGRs Concentration (mgl^{-1})				Solid medium			Liquid medium		
BAP	KN	NAA	IAA	% of response (Mean±SE)	Required time (wks) for development of SPSs (Mean±SE)	Colour of SPSs	% of response (Mean±SE)	Required time for development of SPSs (Mean±SE)	Colour of SPSs
0.5	-	-	-	14.67±1.44^{abc}	8.64±0.14^{j}	G	13.33±1.22^{abc}	8.66±0.13^{kl}	G
1.0	-	-	-	22.67±1.96^{bcde}	8.52±0.07^{hij}	GW	18.67±1.44^{bcd}	8.44±0.08^{ijkl}	GW
1.5	-	-	-	18.67±1.44^{bcd}	8.66±0.13^{j}	G	16.00±1.96^{bc}	8.56±0.13^{jkl}	WG
2.0	-	-	-	10.67±1.96^{ab}	8.72±0.14^{j}	WG	12.00±1.44^{ab}	8.74±0.13^{l}	G
-	0.5	-	-	36.00±1.96^{fgh}	8.34±0.09^{ghij}	G	32.00±2.24^{efg}	8.12±0.14^{hi}	YG
-	1.0	-	-	66.67±2.72^{mno}	7.62±0.13^{bcd}	YG	64.00±1.96^{mno}	7.24±0.16^{de}	GY
-	1.5	-	-	44.00±1.96^{hij}	8.16±0.14^{fgh}	GY	44.00±1.96^{hij}	7.66±0.16^{fg}	G
-	2.0	-	-	30.67±1.96^{defg}	8.44±0.11^{ghij}	G	24.00±1.96^{cde}	8.32±0.12^{ijk}	WG
0.5	-	0.3	-	52.00±2.24^{jkl}	8.16±0.12^{fgh}	G	53.33±2.72^{jkl}	7.58±0.12^{efg}	GW
1.0	-	0.6	-	76.00±1.96^{opqr}	7.52±0.14^{bcd}	GW	84.00±1.96^{rst}	6.64±0.12^{b}	G
1.5	-	0.9	-	69.33±1.96^{mnop}	7.68±0.15^{bcd}	GY	72.00±1.44^{opq}	7.12±0.12^{cd}	YG
2.0	-	1.2	-	26.67±2.72^{cdef}	8.44±0.11^{ghij}	GY	28.00±2.24^{def}	8.26±0.09^{ij}	GW
0.5	-	-	0.3	48.00±2.24^{ijk}	8.12±0.12^{fgh}	YG	48.00±2.24^{ijk}	7.64±0.12^{fg}	GY
1.0	-	-	0.6	73.33±2.72^{nopq}	7.54±0.12^{bcd}	G	74.67±2.24^{pqr}	7.08±0.10^{cd}	G
1.5	-	-	0.9	62.67±2.61^{lmn}	7.78±0.16^{def}	YG	68.00±1.44^{nop}	7.12±0.12^{cd}	GY
2.0	-	-	1.2	18.67±1.44^{bcd}	8.58±0.14^{ij}	WG	21.33±2.24^{bcd}	8.34±0.10^{ijk}	G
-	0.5	0.3	-	58.67±2.24^{klm}	7.74±0.13^{cde}	G	61.33±2.24^{lmn}	7.32±0.12^{def}	WG
-	1.0	0.6	-	90.67±1.96^{s}	7.12±0.12^{a}	G	96.00±1.54^{u}	6.22±0.15^{a}	G
-	1.5	0.9	-	86.67±2.72^{rs}	7.34±0.11^{ab}	GW	92.00±1.44^{tu}	6.46±0.09^{ab}	YG
-	2.0	1.2	-	40.00±2.72^{ghi}	8.20±0.14^{ghi}	GW	38.67±2.24^{ghi}	7.78±0.16^{gh}	GY
-	0.5	-	0.3	54.67±2.55^{jkl}	8.08±0.10^{efg}	G	57.33±2.31^{klm}	7.44±0.09^{defg}	GW
-	1.0	-	0.6	82.67±1.96^{qrs}	7.42±0.10^{abcd}	WG	88.00±2.24^{stu}	6.58±0.12^{ab}	G
-	1.5	-	0.9	80.00±2.72^{pqrs}	7.36±0.11^{abc}	GW	80.00±2.72^{qrs}	6.74±0.15^{bc}	GW
-	2.0	-	1.2	33.33±2.72^{efgh}	8.34±0.09^{ghij}	GY	36.00±1.96^{fgh}	8.06±0.12^{hi}	G
MS0 (Control)				4.00±1.54^{a}	9.36±0.09^{k}	G	4.00±0.94^{a}	9.34±0.11^{m}	GW

G, Greenish; GY, Greenish Yellow; GW, Greenish White; YG, Yellowish Green; WG, Whitish Green; 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.

After 30 days of culture, the topmost increase in length and number of roots developed (3.91±0.04 cm; 4.17±0.31 number) was observed on MS medium fortified with 1.0 mgl^{-1} IBA and 1.0 mgl^{-1} NAA (Figs. 1G, 2, 3). MS medium with 1.0 mgl^{-1} IAA and 1.0 mgl^{-1} NAA (3.74±0.06 cm; 4.00±0.37 number) gave almost similar results. Individual and combined treatment of different PGRs *i.e.* IAA, IBA, NAA with different concentrations showed that length increase in seed derived plantlets in moderate concentration is significantly lower ($P<0.05$) in higher or lower concentrations of PGRs treatment. After 30 days of culture in rooting media, seedlings illustrated the insignificant differences ($P<0.05$) in different concentrations and combinations of PGRs treatments. Seedlings gave the insignificant variation ($P<0.05$) for

increase in length and number of roots in 1.0 mgl^{-1} IBA + 1.0 mgl^{-1} NAA and 1.0 mgl^{-1} IAA + 1.0 mgl^{-1} NAA treatments. IAA was proved to be the most effective in inducing roots in *Acampe praemorsa* (Nayak *et al*., 1997), *Cymbidium* (Barman *et al*., 2001), and *Ipsea malabarica* (Gangaprasad *et al*., 1999). NAA was found to be the most efficient for rooting in *Cymbidium* (Banerjee and Mandal, 1999). On the other hand, Pant and Swar (2011) indicated that IBA was the most beneficial for inducing rooting in *Cymbidium iridioides*.

For hardening, cultured vessels were kept open in the culture room for several hrs, and then these were 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 were transferred to plastic pots containing a potting mixture of sterilized small bricks: coconut husk: sawdust: activated charcoal (1:1:1:1 ratio) and kept in the green house (at 25-30°C and RH 60-70 %). For about 2-3 months, transplanted seedlings were watered regularly where the seedlings established and grew well.

In liquid MS medium supplemented with 1.0 mgl^{-1} KN and 0.6 mgl^{-1} NAA, the highest (96.00%) greenish SPSs were generated in the shortest amount of time (6.22±0.15 wks). Almost identical results (92.00%; 6.46±0.09 wks) were obtained with liquid MS medium enriched with 1.5 mgl^{-1} KN and 0.9 mgl^{-1} NAA. The fastest rate of greenish SPSs (90.67%) on agar solidified medium was attained on MS medium with 1.0 mgl^{-1} KN and 0.6 mgl^{-1} NAA, followed by MS + 1.5 mgl^{-1} KN + 0.9 mgl^{-1} NAA (86.67%; 7.34±0.11 wks). The lowest outcomes of SPSs development were seen on MS medium supplemented with 2.0 mgl^{-1} BAP and both agar solidified (10.67%; 8.72±0.14 wks) and liquid (12.00%; 8.74±0.13 wks) media. The specific findings of the SPSs development of *H. digitata* were presented in Table 2 and Fig. 1H-I. Both solid and liquid MS media cultures with various PGRs treatments and concentrations showed negligible modification after 60 days of inoculation ($P<0.05$). For lower concentrations of PGRs (1.0 mgl^{-1} and 0.6 mgl^{-1}) treatments, the production of SPSs was substantially higher ($P<0.05$) after 60 days of inoculation. The percentage of the maximum response and the least amount of time needed for SPSs development after 60 days of inoculation showed that there is no statistically significant difference ($P<0.05$) between the agar solidified liquid MS medium with 1.5 mgl^{-1} KN + 0.9 mgl^{-1} KN + 0.6 mgl^{-1} NAA combinations. In terms of SPSs development, the liquid MS medium containing 2.0 mgl^{-1} BAP + 1.2 mgl^{-1} NAA yielded the highest results (94.67 % response) in *Calanthe densiflora* (Bhowmik and Rahman, 2017); *Dendrobium aphyllum* (Bhadra *et al*., 2002); *Eulophia graminea* (Bhowmik and Rahman, 2022b), and *Geodorum densiflorum* (Bhadra and Hossain, 2003).

Conclusion

According to the findings of the present study, MS medium supplemented with various growth additives were preferred over basal MS medium for *in vitro* seed germination, differentiation, and seedlings development in *Habenaria digitata*. Moderate concentrations of AC, P or CW in MS medium showed the best performance for the seed germination to seedling development in *H. digitata*. For individual and combined treatment of different PGRs with different concentrations showed that increased length and number of roots were achieved in seed originated plantlets. In comparison to solidified culture, liquid MS medium supplemented with high concentrations of KN and NAA treatment was more effective at promoting the highest quantities of SPSs formation.

Acknowledgements

Our sincere gratitude is extended to the Prime Minister Higher Education Fund, Government of the People's Republic of Bangladesh, Prime Minister's Office, Dhaka, Bangladesh for providing the financial support to carry out the present research work. The Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Chittagong, Bangladesh, provided the required resources and logistical assistance for the research, for which the authors are grateful.

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