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In vitro micropropagation of *Heliotropium indicum* Linn.: An important medicinal Herb

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Abstract
A rapid *in vitro* multiple shoot regeneration protocol has been developed for *Heliotropium indicum* Linn., an important medicinal herb. Apical buds and nodal segments of field grown plants were used as explants and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) namely IAA, NAA, BAP and Kn. Maximum number of shoots per explant (6.40±0.08 shoots/shoot apex and 7.16±0.50 shoots/nodal explant) was induced after 30 days of culture in the MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l NAA. The highest elongation of shoot buds (6.01± 0.13) took place on MS medium fortified with 1.5 mg/l BAP + 0.5 mg/l NAA. In terms of rooting, well developed shoots were excised and implanted individually in rooting medium containing half strength MS fortified with 1.0 mg/l IBA. Regenerated plantlets were hardened, acclimatized and successfully established in field condition. About 85% plantlets survived under field conditions.

Keywords: *Heliotropium indicum*, explant, organogenesis, micropropagation

1. Introduction
Heliotropium indicum Linn. is commonly known as "*Hathisura*" in Bengali due to the shape of its inflorescence and belongs to the family Boraginaceae. This medicinal herb is found in the tropical and temperate regions of the world including Bangladesh. This plant is characterized by the presence of deep green leaves with borage or rough surface, white flowers arranged on the curved inflorescence axis, which appears like an elephant trunk. This plant is highly valued in the folklore medicine and is believed to be used in treating Malaria, abdominal Pain, fever, dermatitis, venereal diseases, insect bites, menstrual disorders, uriticaria and sore throat [1]. Decoction of different part is used for the treatment of kidney stone [2]. The leaf paste is applied externally to cure rheumatism and skin infections [3]. The tribals use the leaf paste over fresh cut and wounds and claim for its promising activity [4]. This plant is potential in wound healing and also used for anti-tumor and anti-leukemic activities due to the presence of some secondary metabolites [5-6].
Wound healing capability of extract of *H. indicum* has been reported by Dodehe [7]. Decoction of leaves and young shoots is used for the treatment of ringworm, gonorrhoea, pharyngitis and tonsillitis [8]. Roots are used for the treatment of night blindness [9]. Due to the above properties and their prevalence in the wild habitat, the species has been brutally exploited by the agents of Vaidya or the labourers of the Pharma companies.
For the conservation of germplasm of such important medicinal plants which are considered to be rare or threatened, different strategies are being followed. Changes in the life style, conversion of forest cover into urban and industrial development, release of polluted water in the open fields, brutal collection of medicine plants from their wild population all have perturbed the natural habitat leading to gradual extinction of several species in general and the medicinal plants in particular. Conservation through seeds or cutting is not applicable in all the species and plant tissue culture techniques can be used as a suitable alterative. Tissue culture technique is now being utilized for the mass production and germplasm conservation of different endangered and threatened plant species including valuable medicinal plants such as *Aegle marmelos* [10], *Hybanthus enneaspermus* [11], *Aloe vera* [12], *Tectona grandis* [13], *Holostemma ada-kodien* [14], *Sauropus androgynous* [15], *Boerhaavia diffusa* [16], *Rauvolfia tetraphylla* [17], and *Boerhavia diffusa* [18]. The present study was therefore undertaken to develop a protocol for mass scale clonal propagation of this important medicinal herb through *in vitro* culture.

2. Materials and Methods

2.1 Plant Materials

Healthy apical buds and nodal explants of *Heliotropium indicum* Linn. Were collected from wild grown mature plants in the University campus of Chittagong, Chittagong, Bangladesh. Both explants were washed thoroughly under running tap water for 30 minutes followed by a treatment with liquid detergent for 10 minutes and then dipping in 5% (v/v) savlon solution for 10 minutes. They were then washed several times with distilled water and then surface sterilized with 70% alcohol for 60 sec and 0.1% (W/V) mercuric chloride solution ($HgCl_2$) for 6-10 minutes. Explants were then washed with sterile distilled water (3-4 washes, 5 min each), to remove even the trace of the chemical from the explants. The surface sterilized explants were cut into small pieces (0.5-1.0cm) with a sterilized surgical blade and then inoculated onto culture media.

2.2 Culture Medium and Culture Condition

Three types explants were inoculated into Murashige and Skoog (MS) medium supplemented with plant growth regulators (auxins and cytokinins) in different concentrations, such as α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5–3.0 mg/l) and Benzyladenine (BA; 0.1–1.0 mg/l); 3 % (*w*/*v*) of sucrose (Himedia) as a carbon source; 0.8 % of agar (Himedia) as a gelling agent; and the pH of the medium was adjusted to 5.8 before autoclaving at 121 °C under a pressure of 1.1kg/cm^2 for 30 minutes. All cultures were incubated at 25 ± 2 °C under a regular cycle 14 hours light and 10 hours of dark.

2.3 Shoot buds initiation and multiplication

The surface sterilized apical and nodal explants were cultured on MS basal medium as well as the same supplemented with various concentrations of cytokinins (BAP and Kn: 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) either alone or in combinations with auxins (IAA, IBA and NAA: 0.5 and 1.0 mg/l) for shoot inductions and multiplications. Sub culturing was done on the same media for further response at an interval of 10-15days and culture vessels were maintained in the culture room in the same light and temperature conditions

2.4 Rooting of Shoots

Experiments of adventitious root formation on the shoots proliferated *in vitro* were conducted only after having sufficient amount of shoot cultures. Different rooting experiments were carried out with half strength MS medium with or without growth regulators to determine the suitable media composition, optimum growth requirements. After 10-20 days, the proliferated multiple shoots were separated and individual shoots were placed in rooting media. *In vitro* raised elongated shoots (3-4cm) were individually transferred to half strength MS medium fortified with various concentrations of auxins (IAA, IBA and NAA) for rooting.

2.5 Acclimatization and transfer of *in vitro* raised plantlets to soil

For acclimatization, the platelets with well-developed roots were removed from culture vessels, washed in sterile distilled water to remove the remnants of agar and platelets were planted separately onto poly cup filled with potting mixture of soil and compost (1:1). Plantlets were maintained at 30±2°C temperature and 80-85% relative humidity under bright sunlight. Plantlets were frequently watered and gradually exposed to the natural environment.

2.6 Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explants and number of roots per shoot. Means and standard deviations were calculated for each treatment. The data means ± SD of at least three different experiments were represented.

3. Results and Discussion

Shoot apex and leaf segments from field grown plants of *Acalypha indica* L. were aseptically inoculated on MS basal medium fortified with various concentrations of auxin such as IAA and NAA in combinations with BAP and Kn (Table-1).When both explants were cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators, a differential response with regards to shoot induction was observed (Figure1-A&B) after 2 weeks of incubation (Table-1). The present study exemplifies a positive modification of multiple shootbud induction efficacy on MS with combination of auxin (IAA, NAA) and cytokinins (BAP, Kn) was producing maximum number of shoots and longer shoot length. The highest percentage of shoots proliferation was found in MS medium supplemented with 1.0 mg/l BAP and 1.0mg/l NAA. The highest shoot bud proliferation was observed in nodal explant than shoot apex explant (Figure 1-A&B) within 30 days of culture (7.16±0.50 shoots, 6.40±0.08 shoots, respectively). The maximum shoot length (6.01± 0.13) was observed in MS medium fortified with 1.5 mg/l BAP and 0.5 mg/l NAA. (Figure1-C, D & F) within two weeks of incubation. Both for apical bud and nodal explant, BAP was found to be more efficient than Kn with respect to shoot initiation and proliferation of shoots (Table-1). The activity of BAP compared to other cytokinins is also reported in many such plants i.e., *Helicteres isora* [19], *Plumbago rosea* [20], *Picrorhiza Kurroa* [21], Strawberry [22], *Plumbago zeylanica* [23] and *Toddalia asiatica* [24].

Table 1: Effect of growth regulators in MS basal medium on shoot proliferation from shoot buds and nodal explants of *Heliotropium indicum*.

Growth regulators (mg/l)				Apical bud		Nodal segment	
BAP	Kn	NAA	IAA	Shoot buds initiation (%)	Average* no. of shoot buds / explant	Shoot buds initiation (%)	Average* no. of shoot buds/ explant
1.0	-	-	-	25	1.26±0.11	31	1.59±0.01
2.0	-	-	-	33	2.20±0.15	40	3.20±0.16
3.0	-	-	-	20	1.06±0.01	22	1.37±0.14
-	1.0	-	-	21	1.10±0.04	27	1.44±0.20
-	2.0	-	-	12	1.01±0.09	20	1.20±0.13
1.0	-	0.5	-	65	5.17±0.31	86	5.54±0.35
1.0	-	1.0	-	80	6.40±0.08	97	7.16±0.50
1.5	-	1.0	-	70	6.06±0.42	82	6.70±0.31

2.0	-	0.5	-	61	5.12±0.63	75	6.20±0.22
1.0	-	-	0.5	65	5.00±0.02	71	5.36±0.19
1.5	-	-	1.0	60	4.56±0.30	66	5.17±0.20
2.0	-	-	1.0	55	4.41±0.05	62	4.92±0.05
-	1.0	0.5	-	57	4.33±0.21	60	4.70±0.31
-	1.5	1.0	-	48	3.81±0.17	56	4.46±0.20
-	2.0	1.0	-	40	3.16±0.12	50	3.58±0.33
-	1.0	-	0.5	38	3.12±0.08	45	3.36±0.12
-	1.5	-	1.0	31	3.00±0.01	39	3.10±0.21
-	2.0	-	1.0	26	2.41±0.06	30	3.07±0.30

**Note. ***Values are the mean of three replicates each with 15 explants

Table 2: Effect of different plant growth regulators in MS medium on elongation of multiple shoot buds of *Heliotropium indicum.*

PGRs combination(mg/l)	Average* initial length (cm) of individual shoot bud (mean ± SE)	Average* length(cm) of multiple shoot bud after 30days of culture (mean ± SE)
BAP+ NAA 0.5 + 1.0	0.80 ± 0.01	2.77 ± 0.02
1.0 + 0.5	1.30 ± 0.13	4. 00± 0.19
1.5 + 0.5	2.06 ± 0.11	6.01± 0.13
1.5+ 1.0	1.19 ± 0.01	4.45 ± 0.05
2.0 + 1.0	0.94 ± 0.15	4.01 ± 0.12
BAP + IAA 0.5 + 1.0	0.75 ± 0.30	2.58 ± 0.01
1.0 + 0.5	1.01± 0.01	3.00 ± 0.13
1.5 + 1.0	2.11 ± 0.10	4.02 ± 0.10
2.0 + 1.0	1.23 ± 0.17	3.15 ± 0.01
Kn + NAA 0.5 +1.0	0.56 ± 0.02	2.15 ± 0.10
1.0 + 0.5	0.96 ± 0.13	3.87 ± 0.20
1.5 + 1.0	1.80± 0.05	4.68 ± 0.11
2.0 + 1.0	0.91 ± 0.01	3.41 ± 0.10
Kn + IAA 0.5+1.0	0.56 ± 0.01	2.72 ± 0.05

Note.* Values are the mean of three replicates each with 15 explants

In order to get complete plantlets *in vitro* grown elongated individual shoot buds were separated and transferred to rooting media. Half strength MS medium fortified with different concentration of auxins (NAA, IAA & IBA) was used for rooting experiment. Data were recorded after 3 weeks of inoculation (Table-3). The highest frequency of rooting (97%), mean number of roots per shoot (14.05±0.32) and mean root length (7.10±0.33cm) was obtained (FIgure1-F&G) on half strength MS medium supplemented with IBA (1.0 mg/l). Half strength MS fortified with IBA also exhibited the highest *in vitro* rooting in *Holostemma ada-kodien* [26], *Mentha piperita* [27], *Aegle marmelos* [28], *Psoralea corylifolia* [29], *Rhinacanthus nasutus* [30] and *Emblica officinales* [31].

Table 3: Effect of auxins on root induction from *in vitro* raised micro shoots of *H. indicum* in half strength MS medium after 3 weeks of culture.

Concentration of PGRs mg/l			Rooting %	Mean* no. of roots per shoot bud(±SE)	Mean*length of roots (cm) (±SE)
IAA	IBA	NAA			
0.5	-	-	45	5.12±0.13	4.25±0.17
1.0	-	-	55	5.60±0.10	4.82±0.12
1.5	-	-	67	6.28±0.07	5.19±0.23
2.0	-	-	50	4.03±0.16	3.51±0.20
-	0.5	-	82	10.30±0.11	6.52±0.31
-	1.0	-	97	14.05±0.32	7.10±0.33
-	1.5	-	75	9.01±0.02	6.21±0.40
-	2.0	-	66	6.31±0.27	5.45±0.26
-	-	0.5	30	5. 01±0.30	5.29±0.15
-	-	1.0	41	4.32±0.03	4.72±0.21
-	-	1.5	33	4.12±0.24	4.51±0.13
-	-	2.0	21	3.02±0.23	4.10±0.01

Note.*Values are the mean of three replicates each with 15 explants

The regenerated plants with healthy, delicate roots were hardened under controlled environmental conditions in the poly cups containing a mixture of soil: compost in the ratio 1:1.The plantlets were reared under semi-controlled temperature (30±2^{O}C) and light (2000 lux) in a chamber with 80 percent humidity. During this period of acclimation shoots elongated, leaves expanded and turned deep green and healthier (Figure1-H). After three weeks, plants were transferred to an open place and gradually acclimated to outdoor conditions, where 85 percent plants were survived.

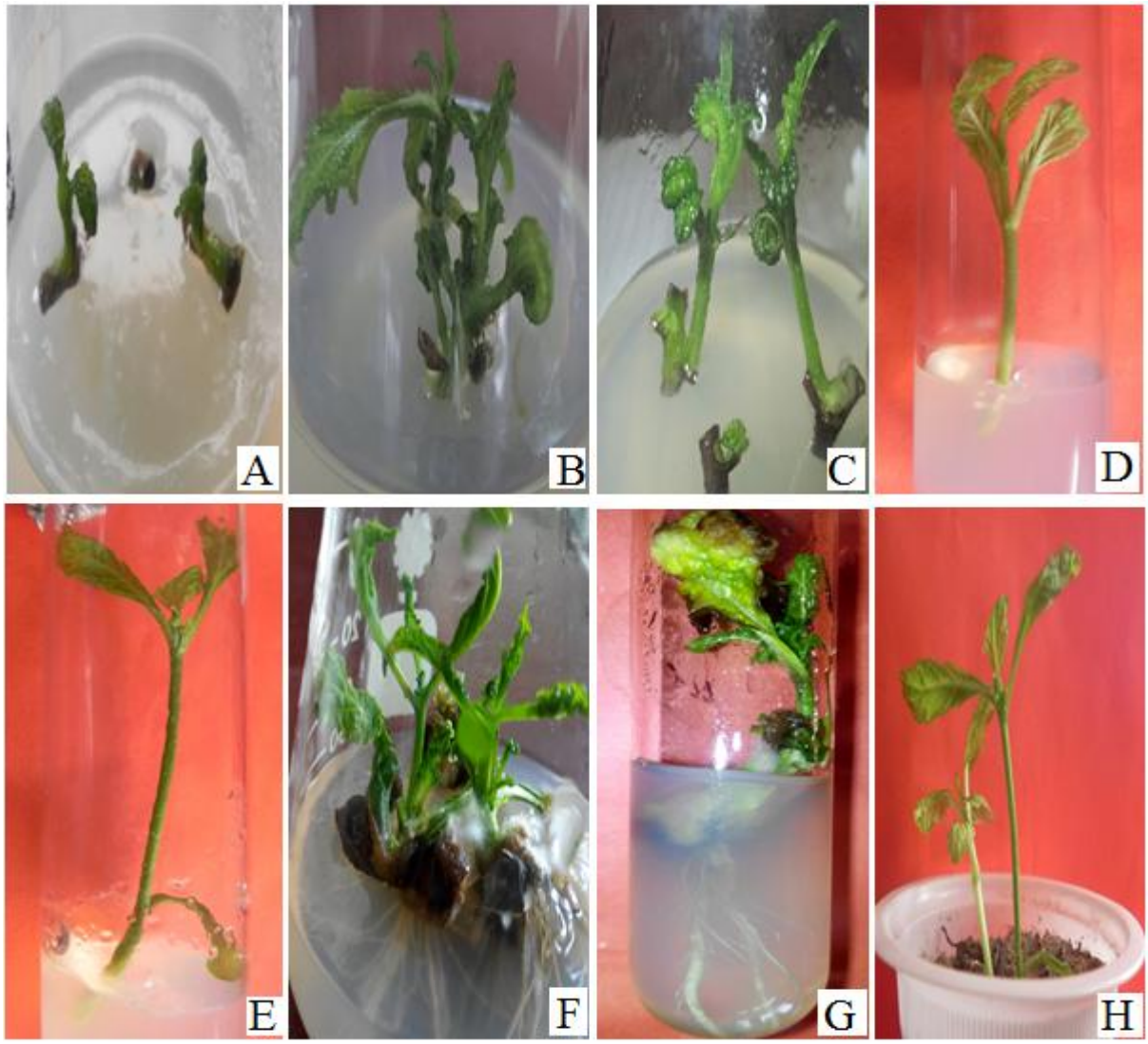

Fig 1: *In vitro* regeneration and plant establishment of *H. indicum* (A) Induction shoots after 2 weeks of culture; (B) Development and multiplication of shoot buds after 3 weeks of culture;(C, D & E) Different steps of elongation of micro shoots; (F-G) Rooting of *in vitro* regenerated shoots in half strength MS+1.0 IBA (H) Transplantation of plantlets into plastic pots.

4. Conclusion

In conclusion, the present investigation reports an efficient and reproducible regeneration protocol for mass propagation of *H. indicum*. MS basal medium containing 1.0 mg/l BAP + 1.0 mg/l NAA gave the best response of shoot induction from nodal segments. Maximum elongation of shoot buds took place on MS supplemented with BAP at 1.5 mg/l in combination with 5.0 mg/l NAA. Half strength MS medium fortified with 1.0 mg/l IBA was found to be the best treatment for root formation in *H. indicum*. The plantlets obtained survived and grew normally in the outside environment. The protocol developed for seedlings of *H. indicum* can be used reliably for propagation in a commercial scale and *ex situ* conservation of this valuable medicinal plant species.

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