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In vitro Micropropagation and Karyomorphological Characterization of Indian Sarsaparilla, *Hemidesmus indicus* (L.) R. Br.

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Key words: Anantamul, Callus induction, Indirect regeneration, Hardening, Karyotype analysis

Abstract

This study aimed to optimize *in vitro* regeneration techniques and cytological characterization for medicinally potential plant *Hemidesmus indicus*. The shoot apices and nodal segments of field grown plants directly differentiated into multiple shoots buds (MSBs) when cultured on MS medium supplemented with different combinations of PGR's. Shoot apices produced the highest number of MSBs (6.15 ± 0.14) per explant along with simultaneous development of green compact callus at the base when cultured on MS medium augmented with 2.0 mg/l BAP + 1.0 mg/l NAA. The synergistic effect of 1.0 mg/l BAP and 1.0 mg/NAA on MS medium produced the highest no. of MSBs (8.51 ± 0.16) per nodal explant. The leaf segments (91%) gave response to white friable calli in MS + 1.0 mg/1 2,4-D + 1.0 mg/l BAP. The calli further differentiated into MSBs showed indirect organogenesis. The elongated shoot buds were individually grown on rooting media. Maximum (8.17 ± 0.13) numbers of roots per micro shoots with the highest length (5.22 ± 0.03 cm) took place in ½MS + 1.0 mg/l IBA + 1.0 mg/l IAA medium. Well rooted plantlets were finally hardened to earthen pots with 88% survival rate. Comparative cytological analysis between *in vivo* and *in vitro* raised plants revealed 22 chromosomes in each somatic cell. The karyotypic formula for *in vivo* grown plants was $2L^{sm} + 6M^{sm} + 6M^{m} + 8S^{m}$ whereas that of the *in vitro* plants was $2L^{sm} + 4M^{sm} + 4M^{m} + 12S^{m}$.

Introduction

Hemidesmus indicus (L.) R. Br. (Synonym- *Periploca indica* L.) earlier placed in the family Asclepiadaceae, is now belongs to Apocynaceae following phylogenetic classifications. It is commonly known as anantamul (Indian sarsaparilla) is a slendar perennial, prostrate or slightly twining, rootstock woody available at Sal Forest of Dhaka-Tangail and Chittagong Hill Tracts. The roots of *H. indicus* used as a substitute for true sarsaparilla

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(*Smilax febrifuga*, family: Smilacaceae). It is valuable alterative, tonic, purgative, demulcent, diaphoretic, diuretic, anti-pyretic, aphrodisiac, antidiarrheal, and blood purifier. It is employed in nutritional disorders, loss of appetite, syphilis, leukoderma, itching, chronic rheumatism, gravel and other urinary diseases, leucorrhoea, fever, asthma, bronchitis, and skin diseases; useful in hemicranias, pain in the join and piles, juice is given to children to tonsilitis. Aqueous extract of root is bacteriostatic against *Mycobacterium leprae*. Fifty per cent ethanolic extract is reported as antiviral. The stem is diaphoretic, diuretic and laxative, lessens, inflammations; useful in leukoderma, paralysis, cough and asthma. The leaves are good for vomiting, colds, wounds and leukoderma (Asolkar et al. 1992).

The roots contain coumarins, hemidesmins I and II, sitosterols, hexatriacontane and an essential oil consisting principally of hydroxyl-methoxybenzaldehydes. The stem contains triterpene lactone, lupanone and its acetate derivative, lupeol, dehydrolupeol acetate, ketolupeneolide, hexadecanoic acid, 4-hydroxy-3-methoxybenzaldehyde. Leaves contains significant amount of rutin (Ghani, 2003). Various bioactive phytochemicals such as 2-hydroxy-4-methoxybenzoic acid (HMBA), 2-hydroxy-4-methoxybenzaldehyde (MBALD), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 3-hydroxy-4-methoxybenza ldehyde (isovanillin), lupeol acetate, hindicusine and di-*O*-acetylhindicusine and β-amyrin palmitate were reported from this plant (Mahalingam and Kannabiran 2009, Fimognari et al. 2011, Kundu et al. 2012, Ferruzzi et al. 2013, Zhao et al. 2013, Nair et al. 2014, Turrini et al. 2018).

In *Ayurveda*, the plant is used in the treatment of fever, stress, topical wound, bone-loss, low body weight and psoriasis (Deshpande et al. 2015). It is considered as one of the *Rasayana* plants of *Ayurveda*. *H. indicus* is used as a traditional remedy for dysentery and diarrhea, infections, skin disease, menorrhagia, post-partum recovery, nephritic complaints, diabetes, stomach-ache, gastrointestinal disorders, jaundice, fever, cough, headache, pain, inflammation, edema, mouth sores, toothache, gonorrhea, syphilis, impotence, snakebite, scorpion sting, leucoderma, psoriasis and rheumatism. It is also being used as body coolant, appetizer, as well as health and vitality promoter (Mohan et al. 2015). Few polyherbal formulations containing this plant have shown anti-ulcerogenic, cytotoxic and anti-tubercular activities (Nipanikar et al. 2017, Suryavanshi et al. 2019).

In spite of its auspicious application, overharvesting, rapid urbanization and industrialization have led to the depletion of plant resources, placing it at risk of extinction. The research work was conducted to develop a rapid and reproducible *in vitro* regeneration protocol of *H. indicus* for both conservation and medicinal utilization purposes. The present findings will be useful for further researches of this species.

Karyotype refers to the phenotypic appearance of the somatic chromosomes as opposed to their genetic makeup. Several researchers have carried at cytogenetic studies particularly chromosome number and morphology at mitotic division, chromosomal association and behavior during meiotic division in some of the medicinal plant species.

The detailed information on the chromosome characteristics of *in vivo* and *in vitro* grown medicinal plants is very limited. Karyomorphological studies of some medicinal plant species, however, carried out at times by previous researchers such as Huziwara 1962, Sreeranjini and Thoppil 2005, Zhou et al. 2009, Rohami et al. 2010, Barandozi and Akbari 2013, Ramesh 2015, Haque et al. 2016, Roy and Mukhopadhyay 2017, Foysol et al. 2019, Dehery et al. 2020, Majumder et al. 2021, Dehery and Das 2022, Panda et al. 2023, Anjum et al. 2024 and Sirin 2025.

Information on karyomorphological studies of *H. indicus* in natural and *in vitro* grown plants is very scanty. Chromosome characteristics of the selected medicinal plant species was studied by characterizing their mitotic metaphase chromosomes based on centromeric type (CT), relative length (RL), centromeric index (CI), karyotypic similarity or Total Form Percent (TF%) and finally on the basis of karyotypic grouping as proposed by Stebbins (1958). In plant classification, breeding and genetic researches, information about chromosome karyotypes can be beneficial. It also utilized for identifying species and analysis of hybrid populations. The size and shape of chromosome can suggest the evolutionary relationship between various plant species.

Materials and Methods

The shoot apices, nodal and leaf segments from juvenile twigs of field grown plants of *H. indicus* were collected and thoroughly washed under running tap water. The materials were then separated into small pieces, surface sterilized with 5% savlon and liquid soap for 5-10 min with constant shaking. Then the explants were washed with distilled water 3-4 times for complete removal of detergent and taken under laminar airflow cabinet then transferred to 500 ml sterilized beaker. After rinsing with 70% ethanol for less than 60 sec, they were immersed in 0.1 ml $HgCl_2$ for 2 min and washed with sterile distilled water 4-5 times and then disinfected explants were cut into small pieces (0.5-1.0 cm) with a sterilized surgical blade and then inoculated onto the culture media. MS medium containing 8% (w/v) agar supplemented with various concentrations and combinations of cytokinins (BAP and Kn) and auxins (NAA and IAA) were used for organogenesis. In order to induce differentiation, the regenerated plantlets were subcultured on medium supplemented with various concentration and combination of auxins (NAA and IAA) and cytokinins (BAP and Kn) and these were then cultured on elongation media. After that induced shoot buds were carefully removed from the medium and further washed with double distilled water appropriately to avoid any trace of medium on roots. *In vitro* regenerated shoot buds (3-4 cm long) were transferred onto the half-strength MS medium supplemented with the auxins (NAA, IBA and IAA) for induction of root. The pH of the medium was adjusted to 5.8 prior to the addition of agar before autoclaving at 121°C for 30 min under a pressure of 15 psi. All the culture vessels with inoculated explants were then taken to the culture room for inoculation. The culture room was maintained at a regular cycle of 16 hours photoperiod with a light intensity of 2000-3000 lux. The temperature of culture room was maintained at 25°C ± 2°C. After proper root formation, the rooted plantlets with well-developed foliage were gently washed to remove residual agar attached to the roots and rinsed with running tap water for complete removal of medium; the well-developed plantlets with roots were placed to pots containing a mixture of soil and compost (1 : 1). After gradual acclimatization, the pots with *in vitro* developed plantlets were finally transferred to the outside natural environment. The percentage of survived plants was recorded after two months. Each treatment was repeated five times. The graphs and mean values of parameters were compared by analysis of variance using SPSS (Ver. 26) software.

The roots of *in vivo* and *in vitro* grown plants were collected when they become 1-1.5 cm in size at 9.30 to 10.30 a.m. First growing healthy roots were collected and pretreated with saturated solution of Para-dichlorobenzene (PDB) for 3 hrs at room temperature. The roots were deep into 1 : 3 acetoalcohol for 24 hrs then preserved in 70% (v/v) alcohol for further studies. The pretreated roots were hydrolyzed with 2-3 ml 1 N HCl for 10 sec at 60°C. After gradually washing with distilled water, roots were deep into 2% (w/v) aqueous solution of iron alum for 5-10 min. Then the roots were further washed with distilled water for 3-4 times. Roots were stained in 0.5% (w/v) aqueous solution of hematoxylin for 15-20 min and then squashed in a drop of 0.2% (w/v) acetocarmine. After that the hydrolyzed root tips were soaked using a filter paper and placed on a glass slide. After that the meristematic region of roots was cut with a fine blade. A clean cover glass was placed on the material then the prepared slides were then examined under an Optika Vision Pro Microscope at a magnification of 1000X using oil immersion.

In terms of characterization of chromosomes and determination of karyotype asymmetry the following parameters were considered: (i) shortest (s) and, (ii) longest (l) chromosome length, (iii) arm ratio (l/s), (iv) total length of chromosomes (CL), (v) proportion of chromosomes with arm ratio more than 2 : 1, (vi) Centromeric index (CI= length of short arm/total length of chromosome X 100), (vii) Total form percent (TF%) (Huziwara 1962), (viii) Qualitative classification for determination of asymmetry according to Stebbins (1958), and (ix) karyotypic formula. Classification of chromosomes was done on the basis of the nomenclature of Levan (1964). Several karyomorphological parameters were estimate by KaryoType software (Karyotype XY).

Results and Discussion

The shoot apices and nodal segments of *H. indicus* directly differentiated into multiple shoot buds when cultured on MS medium supplemented with a broad spectrum of PGR's combinations. A significant variation was found by the effect of various concentrations of BAP and Kn singly or in combination of BAP with IAA, NAA for formation of MSBs from shoot apices and nodal explants. The percentage of nodal explants showing proliferation varied from 46 to 96%. Maximum number of nodal segments (96%) produced multiple shoot buds when cultured on MS medium fortified with 1.0 mg/l BAP + 1.0 mg/NAA (Table 1). The average number of MSBs (8.51 ± 0.16) per explant was also highest in this medium combination. On the other hand, shoot

Table 1. Effects of different concentrations and combinations of PGRs on shoot buds induction from shoot apices and nodal segments of *H. indicus*, when cultured on MS medium.

PGRs concentration in MS medium (mg/l)						Shoot apices			Nodal segments		
						% of explants gave response	Average* no. of MSBs per explant (Mean ± SE)	Green compact callus growth	% of explants gave response	Average* no. of MSBs per explant (Mean ± SE)	Average* length of shoot (cm) (Mean ± SE)
BAP	Kn	BAP+NAA	BAP+IAA	Kn+NAA	Kn+IAA						
0.5	-	-	-	-	-	55	3.23 ± 0.06^{efh}	-	75	5.65 ± 0.11^{ac}	1.86 ± 0.17^{cd}
1.0	-	-	-	-	-	61	4.12 ± 0.03^{eg}	-	63	7.15 ± 0.17^{dfh}	2.35 ± 0.22^{af}
2.0	-	-	-	-	-	66	5.15 ± 0.07^{af}	+	76	3.80 ± 0.12^{deg}	3.50 ± 0.19^{ab}
3.0	-	-	-	-	-	52	3.08 ± 0.20^{cd}	+	62	6.77 ± 0.16^{adf}	3.90 ± 0.12^{abc}
-	0.5	-	-	-	-	65	2.33 ± 0.06^{agh}	++	65	4.67 ± 0.13^{ad}	3.29 ± 0.10^{bc}
-	1.0	-	-	-	-	63	4.17 ± 0.01^{bc}	++	73	5.15 ± 0.14^{dgh}	2.24 ± 0.11^{ac}
-	2.0	-	-	-	-	56	3.08 ± 0.13^{dh}	++	78	3.82 ± 0.10^{egh}	1.75 ± 0.03^{ab}
-	3.0	-	-	-	-	72	3.11 ± 0.01^{ah}	+	64	6.47 ± 0.19^{acf}	3.77 ± 0.15^{bd}
-	-	1.0 + 0.5	-	-	-	59	3.65 ± 0.15^{afg}	-	79	5.36 ± 0.08^{bg}	2.38 ± 0.15^{ch}
-	-	1.0 + 1.0	-	-	-	74	3.07 ± 0.02^{ad}	-	96	8.51 ± 0.16^{bdh}	4.78 ± 0.13^{ad}
-	-	2.0 + 0.5	-	-	-	76	4.13 ± 0.31^{bdf}	-	86	8.27 ± 0.01^{abf}	3.66 ± 0.16^{ac}
-	-	2.0 + 1.0	-	-	-	87	6.15 ± 0.14^{abd}	++	77	6.01 ± 0.15^{acd}	1.11 ± 0.05^{cd}
-	-	3.0 + 0.5	-	-	-	82	5.39 ± 0.12^{adf}	+++	72	7.24 ± 0.07^{cdf}	3.11 ± 0.01^{bc}
-	-	3.0 + 1.0	-	-	-	78	3.10 ± 0.23^{cf}	+	68	4.10 ± 0.09^{ade}	2.48 ± 0.14^{acd}
-	-	-	1.0 + 0.5	-	-	57	3.11 ± 0.02^{e}	-	67	4.22 ± 0.11^{bfg}	1.83 ± 0.21^{cd}
-	-	-	1.0 + 1.0	-	-	69	2.17 ± 0.05^{eh}	-	59	5.76 ± 0.12^{cfh}	3.28 ± 0.20^{bc}
-	-	-	2.0 + 0.5	-	-	67	4.35 ± 0.04^{dh}	-	63	4.23 ± 0.02^{dh}	4.03 ± 0.16^{ad}
-	-	-	2.0 + 1.0	-	-	70	5.16 ± 0.11^{bcg}	++	55	3.32 ± 0.03^{ac}	3.93 ± 0.21^{ac}
-	-	-	3.0 + 0.5	-	-	50	1.05 ± 0.01^{fh}	-	60	2.51 ± 0.10^{ach}	4.23 ± 0.06^{cd}
-	-	-	3.0 + 1.0	-	-	62	1.22 ± 0.04^{dgh}	+	52	2.11 ± 0.04^{acg}	3.50 ± 0.11^{ad}
-	-	-	-	1.0 + 0.5	-	75	2.93 ± 0.03^{cfh}	-	70	3.16 ± 0.07^{bdg}	2.22 ± 0.13^{b}
-	-	-	-	1.0 + 1.0	-	54	4.01 ± 0.07^{abd}	-	66	4.51 ± 0.04^{abd}	2.72 ± 0.02^{ac}
-	-	-	-	2.0 + 0.5	-	66	4.12 ± 0.04^{cfg}	-	56	2.27 ± 0.01^{acf}	3.02 ± 0.13^{d}
-	-	-	-	2.0 + 1.0	-	61	4.86 ± 0.12^{af}	++	46	1.25 ± 0.07^{ad}	4.86 ± 0.08^{c}
-	-	-	-	3.0 + 0.5	-	52	3.36 ± 0.07^{bcd}	+++	62	2.24 ± 0.17^{cdg}	3.04 ± 0.17^{ae}
-	-	-	-	3.0 + 1.0	-	43	2.11 ± 0.01^{bcf}	+	67	3.10 ± 0.08^{acd}	2.76 ± 0.13^{b}
-	-	-	-	-	1.0 + 0.5	80	6.12 ± 0.04^{adfh}	++	79	4.21 ± 0.11^{bfg}	3.51 ± 0.22^{bd}
-	-	-	-	-	1.0 + 1.0	76	6.04 ± 0.16^{bd}	+	82	6.76 ± 0.15^{cfh}	2.04 ± 0.06^{bc}
-	-	-	-	-	2.0 + 0.5	77	4.38 ± 0.03^{b}	-	85	7.12 ± 0.13^{adh}	1.78 ± 0.13^{a}
-	-	-	-	-	2.0 + 1.0	73	5.03 ± 0.11^{ach}	+	74	6.32 ± 0.03^{ac}	2.29 ± 0.22^{d}
-	-	-	-	-	3.0 + 0.5	60	5.43 ± 0.05^{bg}	-	71	5.51 ± 0.22^{ace}	2.56 ± 0.15^{bc}
-	-	-	-	-	3.0 + 1.0	62	3.93 ± 0.02^{bd}	+	62	4.11 ± 0.03^{cd}	2.20 ± 0.07^{ab}

MSBs = Multiple shoot buds. *Values are the means of five replicates of 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.
- No response, + Poor growth, + + Moderate growth, + + + Good growth.

apices (87%) gave response for induction of multiple shoot buds along with simultaneous development of green compact callus (Fig. 5A) at the base when cultured on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA with the average number of MSBs (6.15 ± 0.14) per explant. Whereas, the minimum percentage (43%) of multiple shoot buds induction on MS medium containing 3.0 mg/l Kn + 1.0 mg/l NAA with lowest number of MSBs (2.11 ± 0.01) per explant. Good growth of green compact callus at the base of shoot apices was reported when cultured in MS medium supplemented with 3.0 mg/l BAP or Kn and 0.5 mg/l NAA. Both explants underwent direct organogenesis giving rise to multiple shoot buds (MSBs) but the nodal explants were better than shoot apices. Relatively higher amount of cytokinin in combination with lower amount of auxin promoted direct organogenesis. The synergetic effect of BAP in combination with NAA on induction of MSBs has been noted in other medicinal plant species such as *Boerhaavia diffusa* (Roy 2008), *Artemisia annua* (Gopinath et al. 2014), *Ocimum sanctum* (Jamal et al. 2016) and *Aloe vera* (Das and Bora 2018).

The leaf segments produced white friable callus (Fig. 5B) on different PGRs supplemented media. The maximum number of leaf segments (91%) produced callus tissue within 12-18 days when cultured in MS medium containing 1.0 mg/1 2, 4-D + 1.0 mg/l BAP (Fig. 1). In this combination notable amount of callus tissue was induced. Similar proliferation of callus tissue was also recorded in other medicinal plants including *Abrus precatorius* (Biswas et al. 2007), *Barleria prionitis* (Shukla et al. 2011), *Asteracantha longifolia* (Kumar and Nandi 2015) and *Viola serpens* (Jha et al. 2020). It is pertinent to mention here that the leaf explants did not induce any callus tissue when cultured on MS medium containing Kn individually. It was reported that the combination of 2, 4-D with BAP was better than the combination of 2, 4-D with Kn in terms of frequency amount of callus induction.

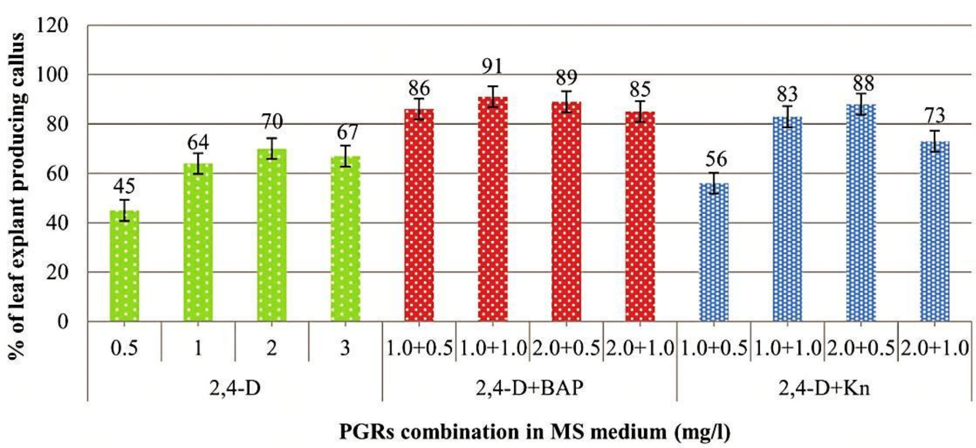

Fig. 1 Effects of 2, 4-D individually and in combination with BAP and Kn in MS medium on callus induction in leaf segments of *H. indicus.* Vertical bar indicates standard error.

In order to induce tissue differentiation in leaf segments derived white friable callus as well as shoot apices derived green compact callus, a broad spectrum of PGRs supplemented MS medium was used. Maximum number of callus (93%) gave response in MS medium containing 1.0 mg/l BAP + 1.0 mg/l NAA producing the highest number of MSBs (6.15 ± 0.11) within 16-22 days of culture (Fig. 2 and Fig. 5C). There are some reports on indirect organogenesis in other medicinal plant species such as *Withania somnifera* (Rani et al. 2003), *Balanites roxburghii* (Shivamurthy et al. 2004), *Plumbago indica* (Bhadra et al. 2004), *Vitex negundo* (Jawahar et al. 2008), *Abutilon indicum* (Rout et al. 2009), *Rauvolfia serpentina* (Singh et al. 2009), *Scoparia dulcis* (Majumder et al. 2011), *Alternanthera versicolor* (Preetha et al. 2013) and *Coccinia grandis* (Kashem and Rahman 2018).

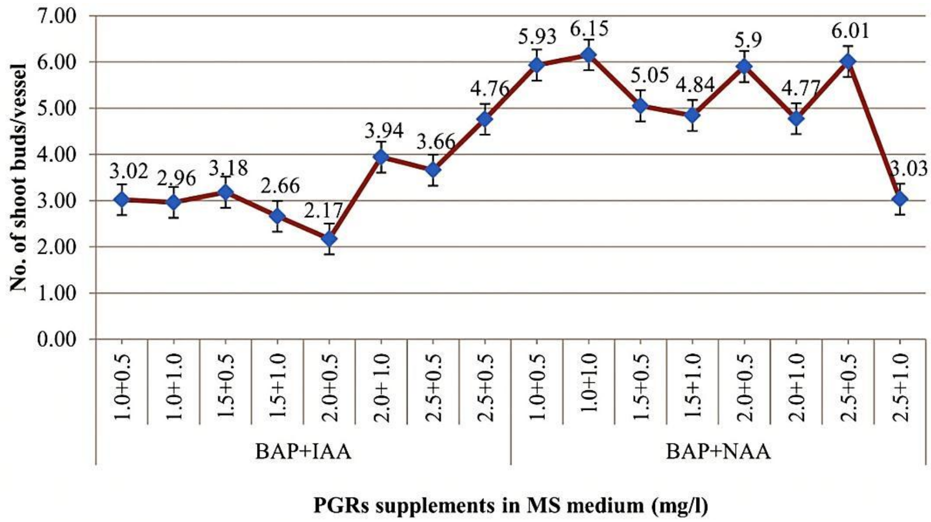

Fig. 2. Effects of BAP in different concentrations and combinations with IAA or NAA in MS medium on induction of MSBs in induced callus of *H. indicus*. Vertical bar indicates standard error.

In order to enhance rapid elongation, the multiple shoot buds of *H. indicus* that developed directly and indirectly were further cultured on elongation media. MS medium supplemented with different concentrations and combinations of PGRs were used for this purpose. MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l IAA was proved best for elongation of this species. The highest elongation (3.82 ± 0.28 cm) of individual shoot bud after 30 days of culture was recorded in this medium composition (Table 2 and Fig. 5D). In the box plot graph, the highest median value for shoot length elongation was observed in MS media having BAP + NAA followed by MS media with BAP + IAA. Median values for shoot length elongation for MS media supplemented with BAP in combination with NAA or IAA was found to be almost identical (Fig. 3) whereas, a significantly lower median was reported for MS media with Kn + IAA. The effect of BAP in combination with NAA has been noted in many other medicinal plant species viz. *Plumbago indica* L. (Bhadra et al. 2004), *Gynura procumbens* (Keng et al. 2009), *Aloe vera* (Das and Rout 2018), *Withania somnifera* (Goswami et al. 2022), *Leucas biflora* (Paul and Biswas 2024), *Spilanthes acmella* (Sana and Rani 2025) and *Actinidia deliciosa* (Uddin et al. 2025).

Table 2. Effects of different concentrations and combinations of plant growth regulators (PGRs) on the elongation of directly and indirectly produced shoot buds of *H. indicus* when cultured on MS medium.

PGRs supplement in MS medium (mg/l)				Average* initial length (cm) of individual shoot bud (mean ± SE)	Average* length(cm) of individual shoot buds after 30d of culture (mean ± SE)	Average* increase in length(cm) of individual shoot buds after 30d of culture (mean ± SE)
BAP + NAA	BAP + IAA	Kn + NAA	Kn +IAA			
1.0 + 0.5	-	-	-	1.62 ± 0.17^{cdh}	3.87 ± 0.16^{cg}	2.25 ± 0.16^{c}
1.0 + 1.0	-	-	-	1.46 ± 0.13^{abd}	3.91 ± 0.15^{ceg}	2.45 ± 0.25^{a}
2.0 + 0.5	-	-	-	1.33 ± 0.11^{cdh}	4.53 ± 0.19^{cd}	3.20 ± 0.34^{c}
2.0 + 1.0	-	-	-	1.53 ± 0.08^{ceh}	3.91 ± 0.21^{dh}	2.38 ± 0.21^{d}
-	1.0 + 0.5	-	-	1.25 ± 0.04^{bcd}	3.76 ± 0.20^{abh}	2.51 ± 0.26^{c}
-	1.0 + 1.0	-	-	1.61 ± 0.15^{abe}	3.59 ± 0.17^{bcf}	1.98 ± 0.40^{b}
-	2.0 + 0.5	-	-	1.33 ± 0.08^{dc}	2.94 ± 0.12^{abd}	1.61 ± 0.32^{a}
-	2.0 + 1.0	-	-	1.90 ± 0.02^{ah}	5.72 ± 0.08^{abcf}	3.82 ± 0.28^{c}
-	-	1.0 + 0.5	-	2.00 ± 0.05^{abf}	2.65 ± 0.06^{ab}	0.65 ± 0.11^{c}
-	-	1.0 + 1.0	-	1.20 ± 0.09^{acd}	3.77 ± 0.10^{f}	2.57 ± 0.16^{c}
-	-	2.0 + 0.5	-	1.64 ± 0.13^{d}	3.76 ± 0.09^{cgh}	2.12 ± 0.30^{d}
-	-	2.0 + 1.0	-	1.38 ± 0.10^{fh}	2.55 ± 0.16^{abe}	1.17 ± 0.31^{b}
-	-	-	1.0 + 0.5	1.69 ± 0.18^{cfh}	3.47 ± 0.09^{bcg}	1.78 ± 0.14^{c}
-	-	-	1.0 + 1.0	1.53 ± 0.03^{abe}	2.75 ± 0.12^{ch}	1.22 ± 0.10^{c}
-	-	-	2.0 + 0.5	1.41 ± 0.06^{cgh}	3.32 ± 0.19^{ac}	1.91 ± 0.02^{d}
-	-	-	2.0 + 1.0	1.72 ± 0.04^{cdf}	3.07 ± 0.20^{adf}	1.35 ± 0.30^{b}

d = days. *Values are the means of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

To get complete plantlets, the elongated shoot buds were individually grown on rooting media with the idea that initiation and proliferation of root formation took place in stress nutrient condition or auxin supplemented media. Accordingly, half-strength MS medium without or with auxin (IBA, IAA and NAA) individually or in combinations were used for root initiation. Of the different kinds of media combinations, the average numbers of roots per micro shoots were 8.17 ± 0.13 (Fig. 4a) and the average length of roots after 30 days of culture was 5.22 ± 0.03 cm (Fig. 4b) took place in half-strength MS supplemented with 1.0 mg/l IBA + 1.0 mg/l IAA (Fig. 5E) indicating that combination of both low nutrient and auxin supplementation promoted initiation of roots in micro shoots of *H. indicus*. Half-strength MS medium with combination of two auxins was found effective for rooting of many other medicinal plants such as *Ficus religiosa* (Hassan et al. 2009), *Mentha piperata* (Manik et al. 2012), *Bacopa monnieri* (Pandiyan and Selvaraj 2012), *Caralluma diffusa* (Prabu et al. 2013) and *Clausena heptaphylla* (Majumder and Rahman 2016).

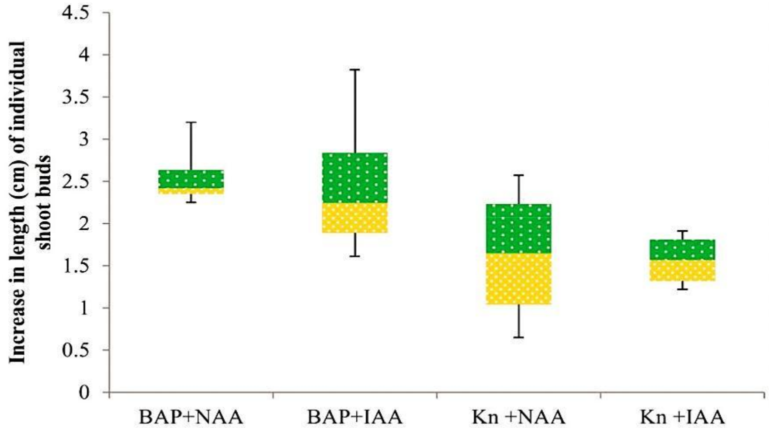

Fig. 3. Box plots representing the effect of different PGRs combinations in MS medium on increase in length (cm) of directly and indirectly produced individual shoot buds of *H. indicus*.

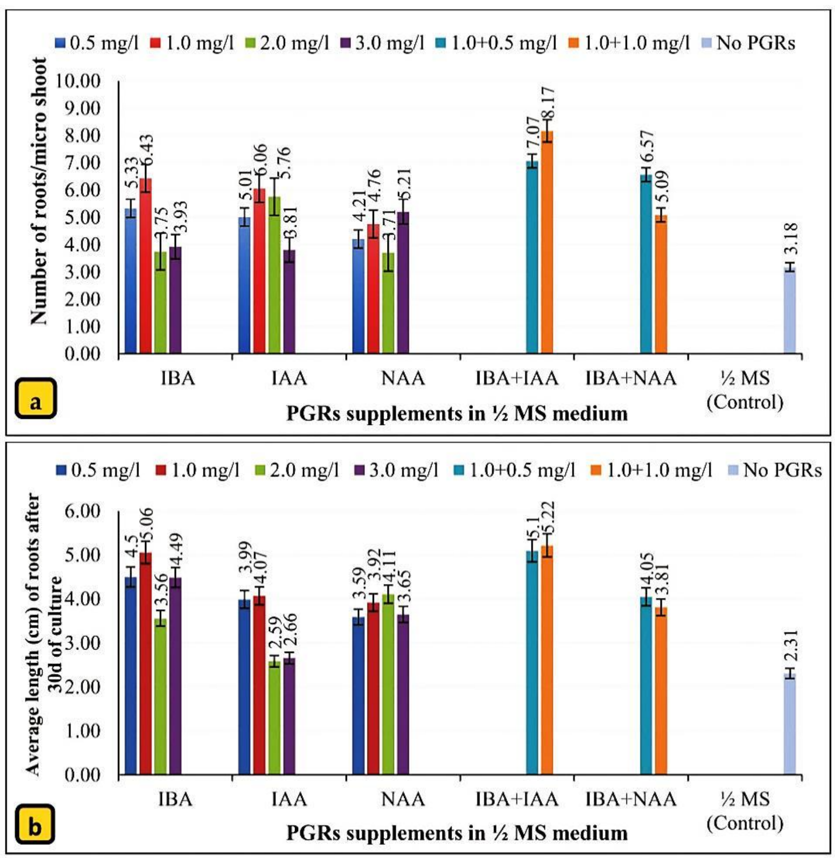

Fig. 4(a-b). Effects of ½MS medium with different concentrations and combinations of auxins on the development of roots in elongated shoot buds of *H. indicus*: (a) number of roots/micro shoot, and (b) average length (cm) of roots after 30d of culture. Vertical bar indicates standard error.

After establishment of root system in elongated shoot buds the complete plantlets were transferred to earthen pots and acclimatized to natural environment. About 88% of the transferred plantlets finally survived and established in outside environment showing normal growth (Fig. 5F). Verma and Vashistha (2016) recorded almost 80% survival rate in their experiment in this species. Thus the protocol developed for micropropagation of *H. indicus* is quite efficient and can be followed for rapid propagation of this species in mass scale.

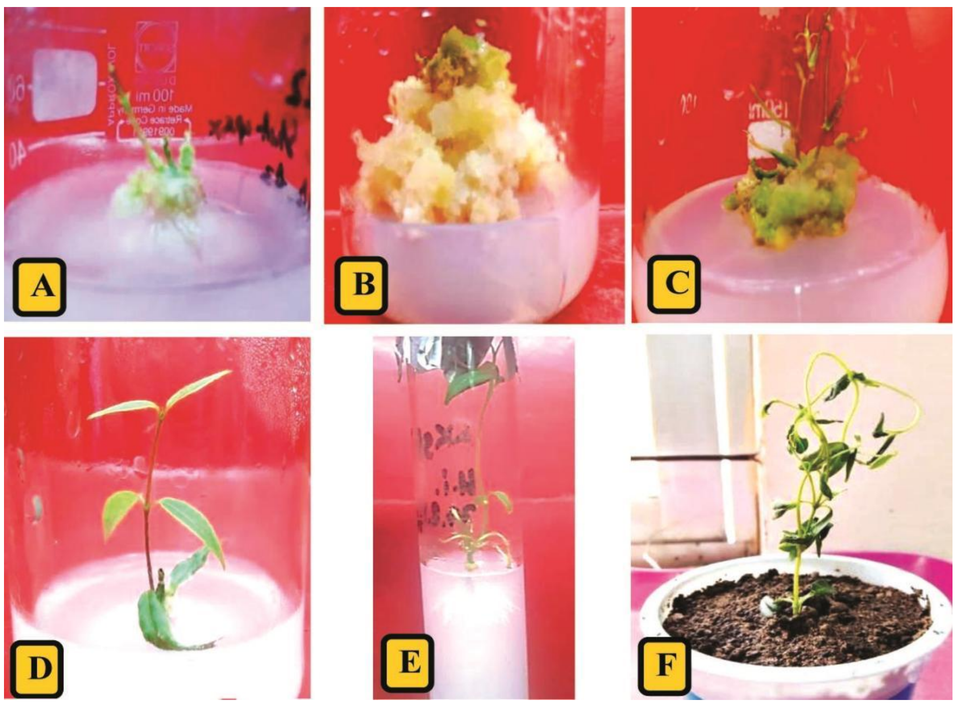

Fig. 5(A-F). Induction and development of MSBs in *Hemidesmus indicus* through direct organogenesis: (A) initiation of MSBs formation with simultaneous development of callus from 10 days culture of shoot apices, (B) callus induction from leaf segment in MS + 2.0 mg/1 BAP + 1.0 mg/l NAA, (C) differentiation of callus tissue into MSBs in MS + 1.0 mg/l BAP + 1.0 mg/l NAA, (D) elongation of shoot buds in MS + 2.0 mg/l BAP + 1.0 mg/l IAA after 30 days, (E) induction of roots in half-strength MS medium supplemented with 1.0 mg/l IBA + 0.5 mg/l IAA, and (F) *In vitro* developed complete plantlets after transplantation in plastic pot.

Cytological study of *H. indicus* indicated that the chromosome number remain same (2n= 22) in *in vivo* and *in vitro* grown plants. The length of individual chromosome varied from 2.17 to 5.69 μm with a median value of around 3.70 μm in mother plant and in *in vitro* grown plant the length ranged from 2.00 to 5.76 μm (Table 3) with a median value of 3.65 μm (Fig. 6a). The range of relative length for *in vivo* plant was 5.40-14.15% with a median value 9.20% and that of *in vitro* raised plant was 4.87-14.09% with a median value of 8.89% (Fig. 6b). In the genomic set of mother plant there were 4 submetacentric and 7 metacentric chromosomes (4sm + 7m) and *in vitro* plant had 3 submetacentric and 8 metacentric chromosomes (3sm + 8m). The numbers of metacentric chromosomes were found to be higher in the *in vitro* plant when compared with that of *in vivo* plant. The 4sm + 7m centromeric type as noted in mother plants can be considered as more primitive in comparison to 3sm + 8m type that was recorded in micropropagated plants. No telocentric or acrocentric chromosomes were present in any of the plants. In case of both plants, none of the chromosomes had secondary constrictions. Similar type of observation was noted in some other plants such as *Tylophora indica* (Samaddar et al. 2012), *Allium hookeri* (Toijam et al. 2013) and *Gloriosa superba* (Biswas et al. 2014).

Table 3 Comparison of chromosome characters between *in vivo* and *in vitro* grown plants of *H. indicus*.

Chromosome characters		*In vivo* raised plant	*In vitro* raised plant
Somatic chromosome number		2n=22	2n=22
Total haploid chromatin length (TCL) (µm)		40.20	41.03
Range of individual chromosome length (µm)		2.17 – 5.69	2.00 – 5.76
Total length of haploid complement	Long arm (µm)	22.93	23.14
	Short arm (µm)	17.27	17.89
Range of relative length (RL%)		5.40 – 14.15	4.87 – 14.09
Centromeric Index (CI)		34.99 – 50.00	33.85 – 50.00
Ratio of longest/smallest chromosome		2.47 : 1	2.88 : 1
The total form percentage (TF%)		42.96	43.60
Karyotypic formula	n=11	$1L^{sm}+3M^{sm}+3M^{m}+4S^{m}$	$1L^{sm}+2M^{sm}+2M^{m}+6S^{m}$
	2n=22	$2L^{sm}+6M^{sm}+6M^{m}+8S^{m}$	$2L^{sm}+4M^{sm}+4M^{m}+12S^{m}$
Karyotype class		1B	1B

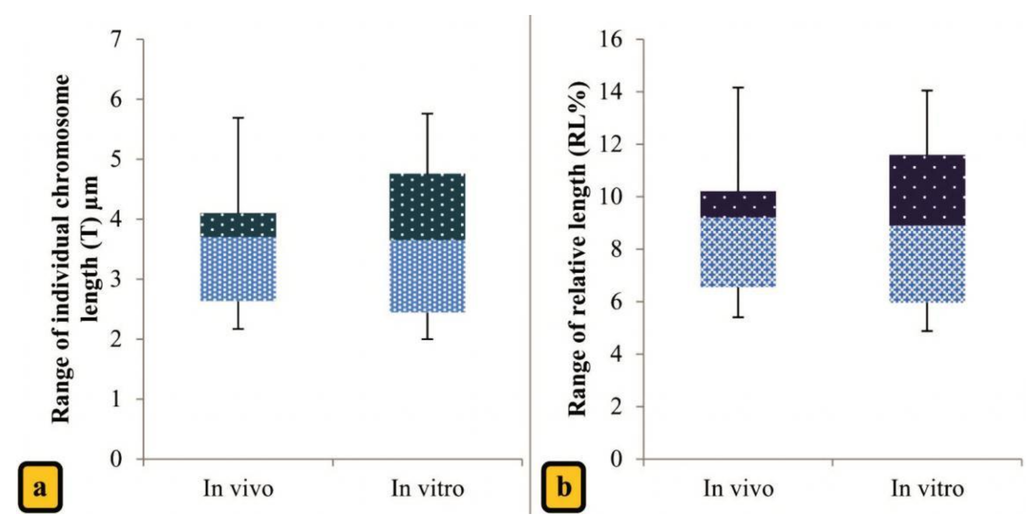

Fig. 6(a-b). Box plots showing the comparative studies between *in vivo* and *in vitro* grown *H. indicus* on: (a) total length of individual chromosome (µm), and (b) range of relative length (%).

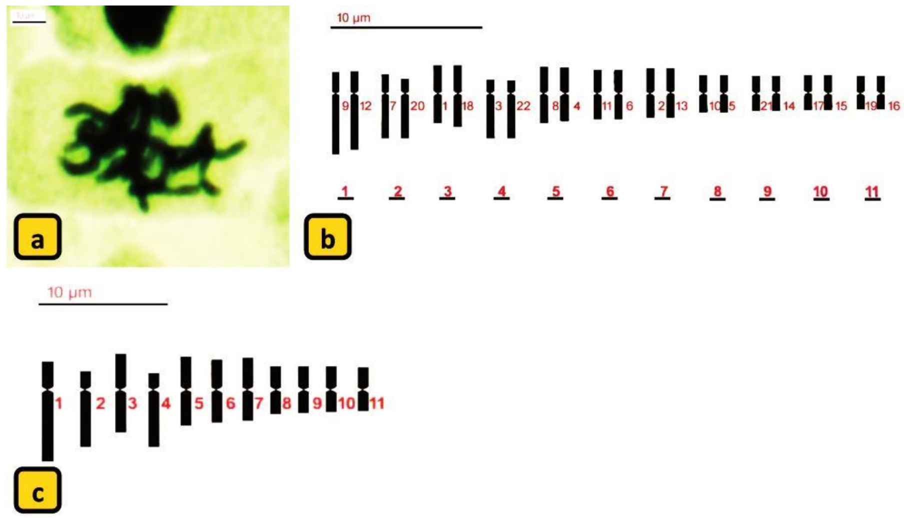

Fig. 7. (a) Microscopic photograph of somatic metaphase chromosomes of mother plant of *H. indicus*, (b-c) Ideogram of mother plant, Scale bar = 10 µm, (b) Diploid complement, 2n = 22, and (c) Haploid complement, n = 11.

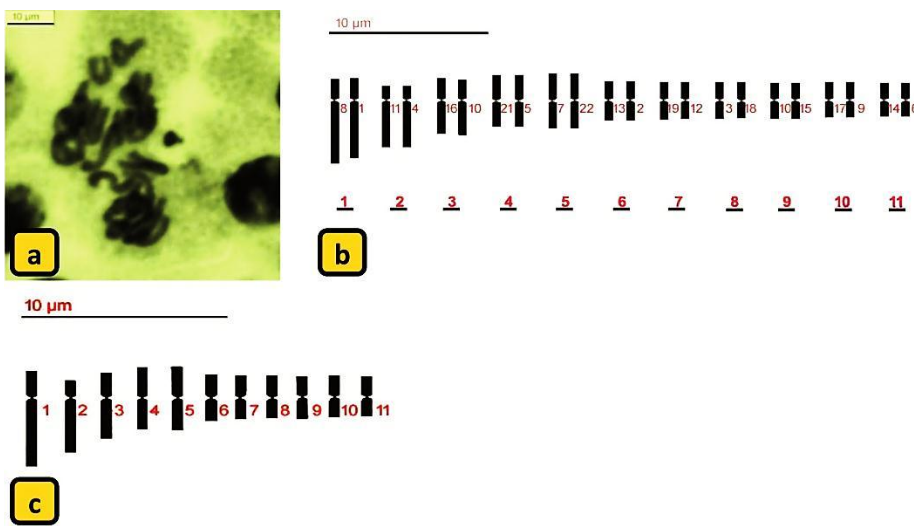

Fig. 8. (a) Microscopic photograph of somatic metaphase chromosomes of *in vitro* grown plant of *H. indicus*, (b-c) Ideogram of *in vitro* grown plant, Scale bar = 10 µm, (b) Diploid complement, 2n = 22, and (c) Haploid complement, n = 11.

Karyotypic characteristics of *in vivo* plants could be represented by $2n = 22 = 2L^{sm} + 6M^{sm} + 6M^{m} + 8S^{m}$ (Fig. 7) whereas, karyotypic formula for *in vitro* plant was $2n = 22 = 2L^{sm} + 4M^{sm} + 4M^{m} + 12S^{m}$ (Fig. 8). However, Samaddar et al. (2012) recorded 2 m.st + 12 M + 6 sm + 2 m for *Tylophora indica* (Burm. f.) Merrill. Toijam et al. (2013) reported karyotype formula: $m_2 + sm_{13} + st_7 + t_0 = 2n = 22$ for *A. hookeri*. Karyotypes were classified following

Stebbins (1958), while both plants had 1B type of karyotype. However, Biswas et al. (2014) noted 1A type of karyotype for two life forms of *Gloriosa superba* having diploid with 2n=22. TF% of mother and *in vitro* raised plant was 42.96% and 43.60%, respectively. Variation in TF% was not prominent in case of both plants. The present findings confirm the ploidy stability of all regenerated plants.

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