



Optimization of *in vitro* regeneration in *Hibiscus subdariffa* L. – An important medicinal plant

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ABSTRACT

In vitro propagation methods using nodal segments of young tissues of *Hibiscus subdariffa* plant have been established. The nodal shoot segments responded better than apical shoot tip explants. Murashige and Skoog (MS) semi-solid medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) induced 4.2 ± 0.24 shoots per node with 2.40 ± 0.18 cm in length after four weeks of inoculation. About 92% explants responded on this medium combination. The shoots were further multiplied (8.2 ± 1.37 shoots with 5.9 ± 1.05 cm length) by repeated subculture of mother explants and freshly induced shoot clumps on MS medium with the reduced concentrations of BAP and Kinetin (0.5 mg/L each) with additives within 4 weeks. Half strength MS medium augmented with 2.0 mg/L indole-3 butyric acid (IBA) was found most favorable for root induction with 98% response. On this medium 7.5 ± 0.61 roots with 4.8 ± 0.23 cm length of roots were recorded. The regenerated plantlets were successfully hardened and field transferred.

Keywords: *Hibiscus subdariffa*; plantlet regeneration; MS medium; growth regulators

1. INTRODUCTION

Hibiscus sabdariffa (Roselle) belongs to the family Malvaceae, it is native to India and Malaysia and distributed in tropical world such as Central America, India, Africa, Australia, Florida and Philippines (Gautam, 2004; Leung and Foster, 1996). *H. sabdariffa* is an erect annual herb commonly known as Bissap, Mesta and Karkadeh. It is widely cultivated for its seeds, petals and leaves (Dlazieli, 1973). It is reported as an aromatic, astringent, aphrodisiac, cholagogue, demulcent, digestive, purgative, refrigerant, sedative, stomachic and resolvent (Lin et al., 2007; Manandhar, 2002).

H. sabdariffa is used as a cooling herb and used in various medicinal purposes. It has equally gained significance in traditional as well as modern system of medicines. It is used as a folk remedy in the treatment of abscesses, bilious conditions, cancer, cough, debility, dyspepsia, fever, hangover, heart ailments, hypertension and neurosis (Vilasinee et al., 2005; Tom et al., 2013). Its decoction is useful to treat high blood pressure and cough. The petals used in the treatment of damaged liver, hypertension, leukemia and fever (Ross, 2003; Akanbi et al., 2009; Jonadet et al., 1990). Fresh calyx is rich in citric acid and pectins, used as flavoring agent in cakes, jellies, soups, sauces, pickles, puddings etc. Petals are used as natural antioxidants, natural colorants, and an ingredient of functional foods (Mahadevan et al., 2009; Lepengue et al., 2009).

Phytochemical investigations of *H. subdariffa* revealed the presence of various bioactive compounds like rhamnogalacturans, riboflavin, β -carotene, sabdaretin, resin, hibiscin, cholesterol, ergosterol, saponins, essential amino acids, alkaloids, anthocyanins, flavonoids, steroids and tannins (Pacome et al., 2014; Mohamed et al., 2007; Rao, 1996). This plant is also explored for its antioxidant (Usoh et al., 2005; Azevedo et al., 2010), anti-inflammatory (Dafallah and Mustafa, 1996), antihypertensive (Onyenekwe et al., 1999), cardioprotective (Jonadet et al., 1990), hepatoprotective (Wang et al., 2000), anticancer (Chang et al., 2005), antihyperlipidemic (Carvajal et al., 2005) and antibacterial activities (Garcia et al., 2006).

Recently, medicinal and food crops gained attention of tissue culture technologies which offer viable and reproducible tools for mass propagation, genetic manipulation and germplasm conservation. For successful genetic transformation in crop improvement research, development of effective micropropagation protocol is considered to be pre-requisite (Fan et al., 2011). Therefore, the present study aimed to develop *in vitro* regeneration methods from somatic tissues of *Hibiscus subdariffa* L.

2. MATERIALS AND METHODS

2. 1. Plant material and sterilization

The young and healthy plants were collected from the farmers belongs to east-coast of the south India and maintained in greenhouse after conducting the field surveys. Plant specimens were identified by The French Institute, Puducherry. The actively growing and disease free shoots were collected from the greenhouse grown plants. Apical shoot tips and nodal shoot segments with 2-3 nodes were used as explants. The explants were initially washed with running tap water.

Special care was taken in sterilizing the young explants because the stem has spongy and soft tissues. The explants were treated with 0.1% (w/v) Bavistin (systemic fungicide, BASF India Ltd.) for 5 min and HgCl₂ (disinfectant, HiMedia, India) for 3-5 min, and rinsed with autoclaved double distilled water for 6-8 times under laminar air flow chamber.

2. 2. Culture medium and culture conditions

The explants were cultured horizontally and vertically on MS basal medium (Murashige and Skoog 1962) supplemented with 30 g/L (w/v) sucrose, 8.0 g/L agar, growth hormones and additives (50 mg/L ascorbic acid, 25 mg/L each of citric acid, L-arginin and adenine sulphate). The culture medium was dispensed into culture tubes, flasks and bottles and capped with non-absorbent cotton plugs as well as polycarbonate caps. The cultures were incubated in the culture room at 25 ±2 °C and 50-70% Relative Humidity (RH) under a photoperiod of 12 hrs at 40-50 μmol m⁻²s⁻¹ Spectral Flux Photon Density (SFPD) light intensity provided by cool white florescent tubes (Philips Ltd, India).

2. 3. Induction of shoots from different explants

The explants were cultured on MS basal medium supplemented with different types and concentrations of growth regulators. To optimize the appropriate cytokinin for culture induction apical shoot buds and nodal explants were cultured on MS medium enriched with different concentrations of BAP and Kin ranging from 0.5 to 3.0 mg/L. The cultures were further multiplied by repeated transfer of mother explants and subculturing of *in vitro* produced shoots on fresh medium. The cultures were incubated in culture room at 25 ±2 °C temperature and 40-45 μmol m⁻²s⁻¹ SFPD light intensity for 12 h/d.

2. 4. *In vitro* shoot multiplication

The *in vitro* regenerated shoots were continuously transferred to MS media with optimized concentration of auxins and cytokinins. The shoot amplification was done by continuous subculture of mother explants along with regenerated shoots. The MS medium supplemented with various concentrations and combinations of cytokinins (BAP and Kin ranging from 0.5 to 3.0 mg/L) with and without auxins was used for proliferation of shoots. Subculturing was done at every four weeks interval. The cultures were incubated in culture room at 25 ±2 °C temperature and 40 μmol m⁻²s⁻¹ SFPD light intensity for 12 h/d.

2. 5. *In vitro* rhizogenesis and hardening of plantlets

For *in vitro* root induction, the elongated shoots were separated from multiplied shoots and inoculated on different strengths of MS medium supplemented with auxins (IBA, indole-3 acetic acid and Naphthalene acetic acid) with varying concentrations (1.0-4.0 mg/L). Initially the cultures were maintained under diffused light (20 μmol m⁻²s⁻¹ SFPD) for a week and then shifted to photon rich light conditions for three weeks. The well established rooted plantlets were carefully separated from the culture vessels, washed with water to remove the medium and transferred to eco-friendly paper cups containing sterile soilrite[®] (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India) and moistened with 1/4th MS salts aqueous solution. The set up was maintained in the greenhouse for 4 weeks. Thereafter the well developed plantlets were shifted to earthen pots containing soilrite[®], organic manure and garden soil (1:1:1) and maintained under the

greenhouse environment for two weeks. These were regularly irrigated with 1/4th MS salts. The plantlets were placed initially under low temperature (26-28 ±2 °C) and high humidity (70-80 %) conditions, and gradually shifted to high temperature (30-32 ±2 °C) and low humidity (55-65 %) area of the greenhouse. The acclimatized plantlets were finally transferred to the field.

2. 6. Statistical analysis

All the experiments were set up with ten replicates per treatment. Each experiment was repeated thrice. The percentage of response, number and length of shoots, number and length of roots were recorded after four weeks interval. Data were analyzed by analysis of variance. Differences between mean values were compared using DMRT at 0.05% level of probability.

3. RESULTS AND DISCUSSION

3. 1. Induction of shoots from the explants

Nodal explants were responded better than the apical bud explants in this study with MS medium and BAP. Extra care has been taken to sterilize the explants because the explants with soft tissues were used to establish the cultures. Surface sterilization of explants using 0.1 % HgCl₂ for 3 and ½ min was optimum with limited contamination (2-5%). The prolonged duration of sterilization caused blackening of the explants and these explants were unable to respond in culture induction experiments. Sadio (2000) regenerated *H. subdariffa* shoots through auxillary branching.

About 96% explants were responded in bud breaking within ten days after inoculation. Maximum response in bud break was recorded on MS medium supplemented with 1.0 mg/L BAP than the rest of the concentrations tested. This combination proved more appropriate for initiation of cultures in terms of the number and length of shoots. Full strength MS medium coupled with the presence of additives enhanced the initial culture response from the explants. BAP 1.0 mg/L yielded maximum 4.2 ±0.24 shoots (2.40 ±0.18 cm in length) from each node (Figs. 1A to C, Table 1). The increase in the concentration of BAP gradually increased the callus formation from the lower part of the explants with less number of shoots. The callus was found to be non-regenerative and hindered further shoot elongation. Comparing to the results obtained from BAP, Kin was found to be less effective than all the concentrations of BAP in induction of number of shoots. But, Kin (2.0 mg/L) enhanced length of the shoots length (2.08 ±0.30 cm).

The stability and superiority of BAP over Kin in terms of culture response and number of shoots have been reported in number of plant species, such as *Carum copticum* (Salehi et al., 2014), *Morinda coreia* (Shekhawat et al., 2015) and *Peperomia pellucida* (Shekhawat and Manokari, 2015). But, Soulange et al., (2009) reported higher percentage of shoot induction in *H. subdariffa* when MS medium was augmented with Kin than BAP. Lower concentrations of BAP resulted in moderate callus formation, but higher concentrations of BAP as well as Kin resulted in the formation of high intensity of callus.

Callus formation was found to be the limiting factor in the regeneration capacity of the explants at initial stage itself in this study. Maximum 78% bud breaking response with the regeneration of 3.0 ±0.32 shoots (1.98 ±0.21 cm length) was achieved using 1.5 mg/L Kin.

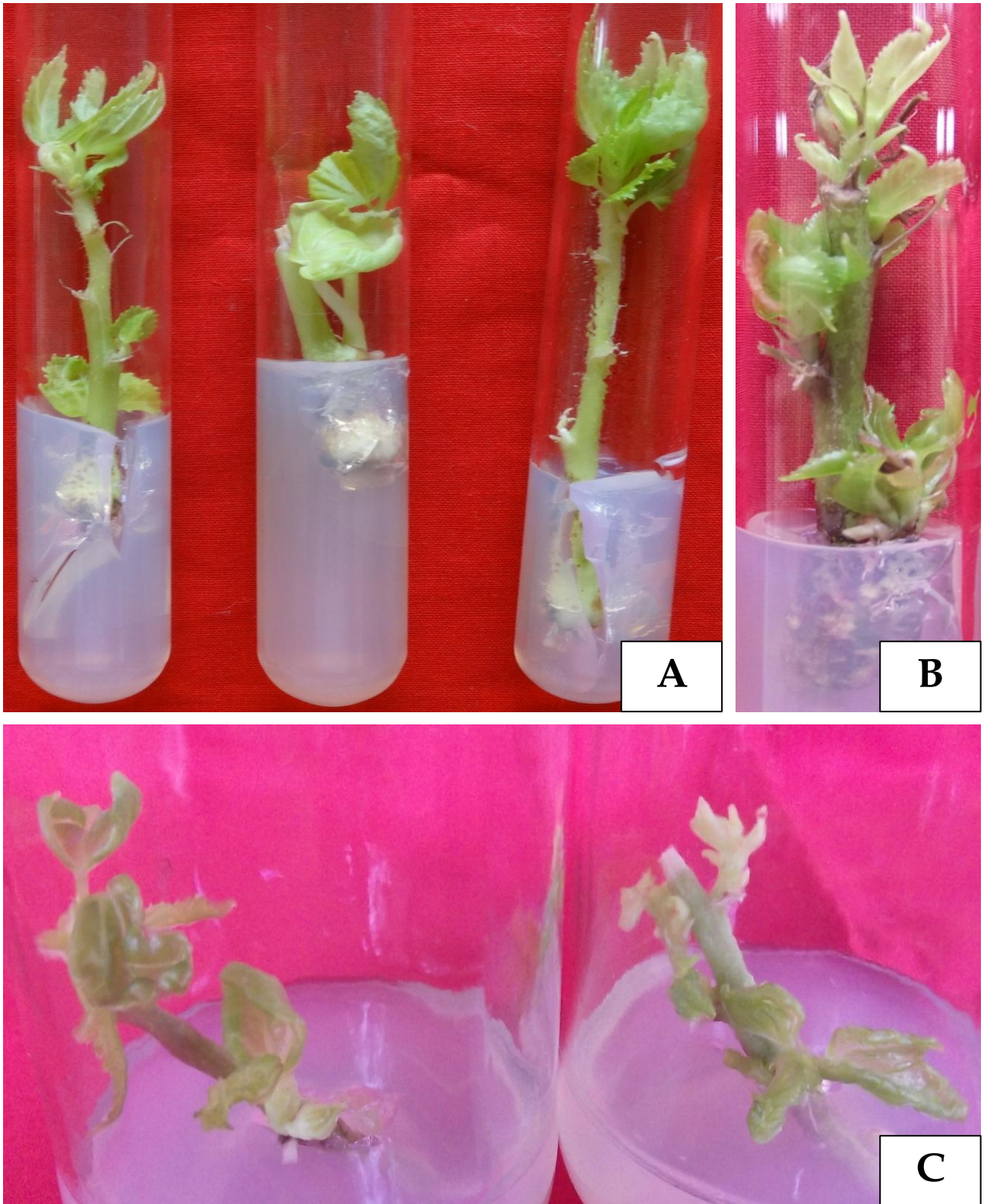


Fig. 1. Different stages in the establishment of cultures using nodal explants.

Callus formation was found to be the limiting factor in the regeneration capacity of the explants at initial stage itself in this study. Maximum 78% bud breaking response with the regeneration of 3.0 ± 0.32 shoots (1.98 ± 0.21 cm length) was achieved using 1.5 mg/L Kin. There was no callus formation observed at 0.5 mg/L both BAP and Kin. Soulange et al. (2009) regenerated maximum 3.2 shoots with 81% response from *H. subdariffa* on MS medium devoid of growth regulators.

They recorded 0.6 shoots with 39.6% shoot regeneration capacity on BAP 1.0 mg/L. These findings were contrast to the present results where hormone free MS medium did not induce any shoot from the nodal explants, and BAP 1.0 mg/L induced maximum number of shoots without callus formation. Soulange et al. (2009) also reported formation of callus at initial stages of culture establishment. Gassama-Dia et al. (2004) reported genetic transformation in the embryos of *H. subdariffa* using *Agrobacterium tumifaciens* transformation.

Table 1. Effect of different concentrations of cytokinins (BAP and Kin) on responses of bud breaking from nodal explants of *H. subdariffa*.

BAP (mg/L)	Kin (mg/L)	Response (%)	Number of shoots (Mean ± SD)	Shoot length (cm) (Mean ± SD)	Frequency of callus (%)
0.00	0.00	0	0.0 ± 0.00^a	0.00 ± 0.00^a	No response
0.50	-	82	3.3 ± 0.71^f	2.52 ± 0.30^j	No callus
1.00	-	96	4.2 ± 0.24^g	2.40 ± 0.18^i	No callus
1.50	-	77	3.4 ± 0.60^f	2.60 ± 0.42^j	Moderate callus
2.00	-	54	2.9 ± 1.00^e	2.16 ± 0.38^h	Moderte callus
2.50	-	50	2.1 ± 0.29^c	2.00 ± 0.33^f	High callus
3.00	-	37	1.0 ± 1.19^b	1.06 ± 0.12^b	High callus
-	0.50	66	2.1 ± 0.14^c	1.32 ± 0.43^d	No callus
-	1.00	71	2.9 ± 0.76^e	1.57 ± 0.64^e	Moderate callus
-	1.50	78	3.0 ± 0.32^e	1.98 ± 0.21^f	Moderate callus
-	2.00	59	2.6 ± 0.45^d	2.08 ± 0.30^g	High callus
-	2.50	51	2.0 ± 0.26^c	1.12 ± 0.36^b	High callus
-	3.00	44	2.1 ± 0.40^c	1.17 ± 0.17^b	High callus

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 2. Multiplication of shoots *in vitro*

The *in vitro* regenerated shoots were repeatedly subcultured to fresh nutrient medium enriched with additives and growth hormones. Multiple shoots amplification was achieved by subculture of *in vitro* regenerated shoots with mother explants on fresh medium after 4 weeks. Full strength MS medium supplemented with cytokinins alone enhanced the number of shoots in the present study.

Among the various growth regulators tested, the combination of 0.5 mg/L each of BAP and Kin was found most appropriate for shoots amplification. Maximum 8.2 ± 1.37 shoots with 5.9 ± 1.05 cm length was achieved from each shoot clump within 4 weeks of incubation at 25 ± 2 °C temperature (Figs. 2A and B).

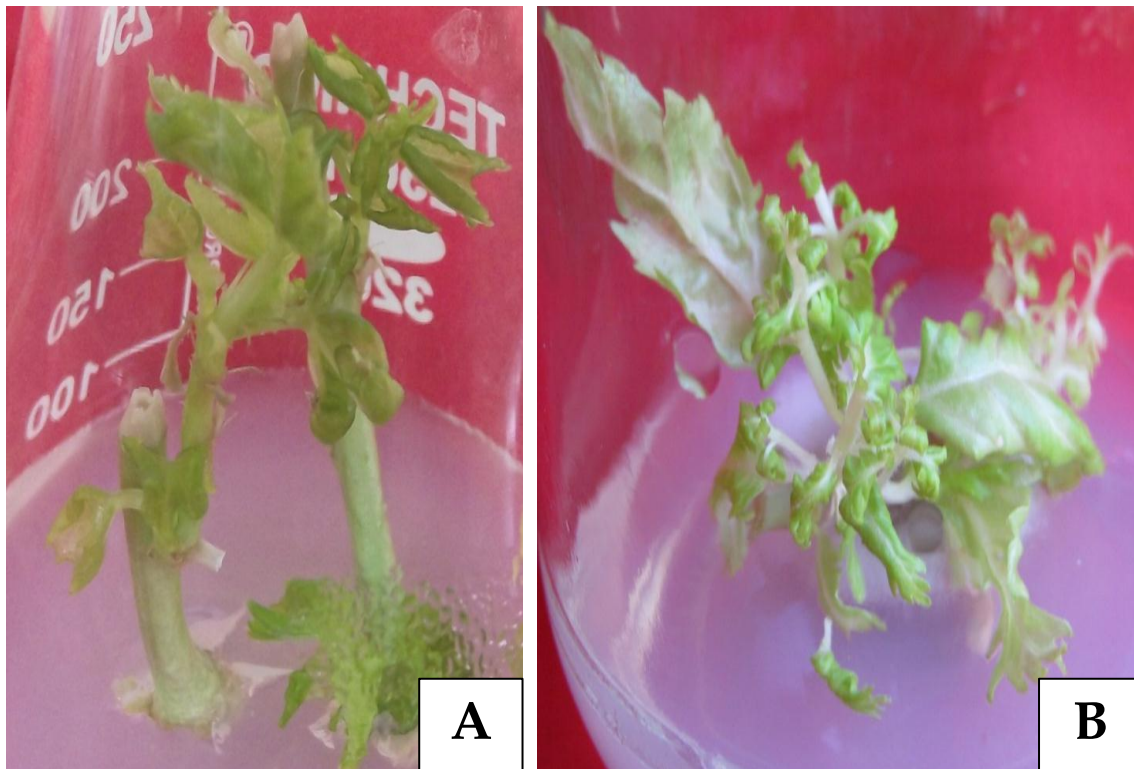


Fig. 2. Multiplication of shoots on MS medium augmented with BAP and Kin.

The increased concentration of BAP and Kin resulted in decreased number of shoots with shoot length. The additives played crucial role in prevention of tip burning of the shoots and formation of healthy and lengthy shoots. The positive role of additive in the enhancement of shoots have been reported in *Terminalia catappa* (Phulwaria et al., 2011), *Salvadora oleoides* (Shekhawat et al., 2012) and *Leptadenia reticulata* (Patel et al., 2014).

Continuous subculture enhanced the growth rate by breaking apical dominancy. The cultures were subcultured regularly after 4 weeks of interval. Delayed subculture resulted in the loss of vigor and health of the shoots. The number of shoots was decreased with length on higher concentrations of BAP and Kin (Table 2).

Table 2. Effect of different concentrations and combinations of cytokinins on shoot multiplication of *H. subdariffa*.

BAP (mg/L)	Kin (mg/L)	Shoot numbers (Mean \pm SD)	Shoot length (cm) Mean \pm SD
0.1	0.1	5.0 \pm 1.30 ^a	5.6 \pm 1.53 ^c
0.5	0.5	8.2 \pm 1.37 ^c	5.9 \pm 1.05 ^c
1.0	0.5	7.5 \pm 0.84 ^b	4.5 \pm 0.47 ^b
1.5	0.5	6.9 \pm 1.59 ^b	4.1 \pm 0.72 ^a
2.0	0.5	5.3 \pm 0.22 ^a	3.6 \pm 0.45 ^a

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

3. 3. *In vitro* rhizogenesis

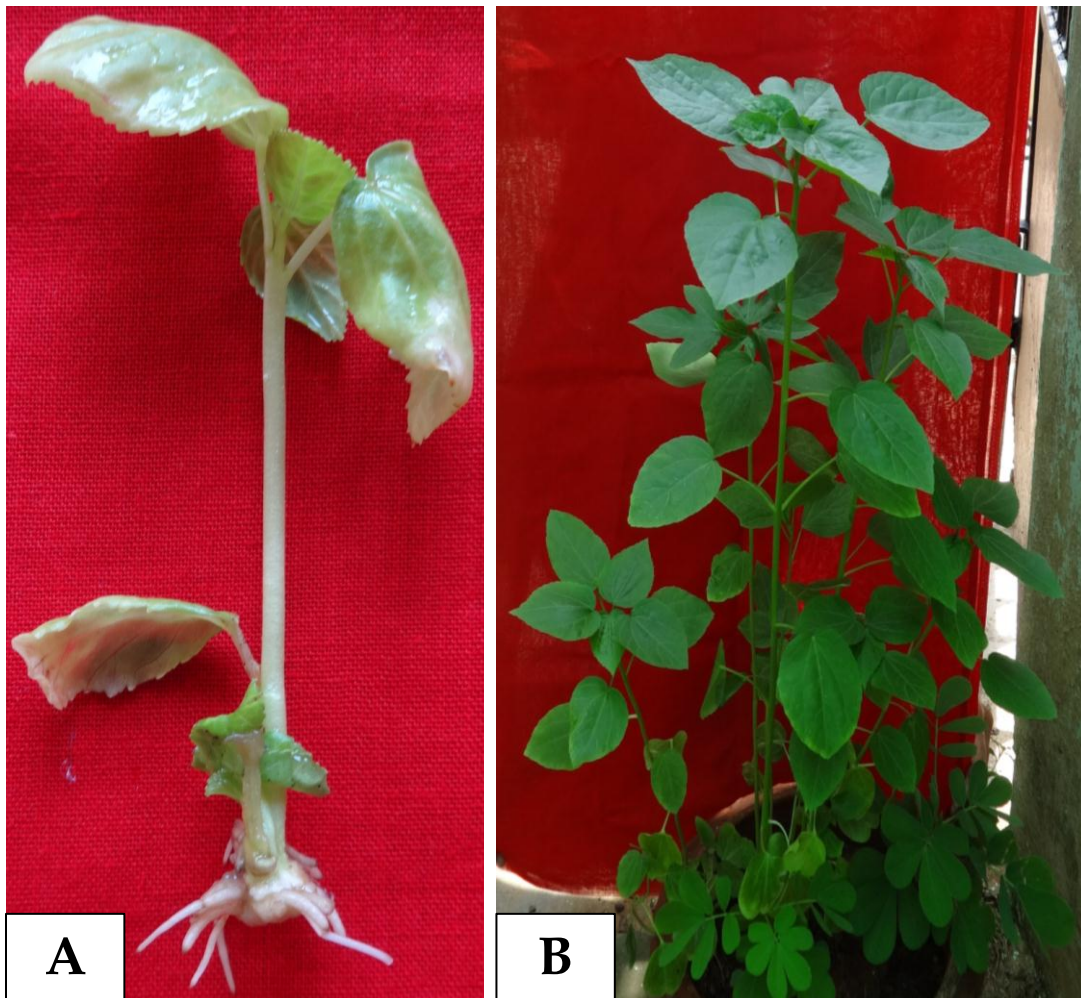


Fig. 3. *In vitro* rooted shoot and hardened plantlet in the greenhouse.

The healthy multiplied shoots measuring 2-3 cm in length were excised and cultured on half strength MS medium with 15 g/L sucrose to induce roots. Among the auxins tested, IBA induced maximum percentage of *in vitro* rooting. Half strength MS medium augmented with 2.0 mg/L IBA was found most favorable for root induction (98%). On this medium 7.5 ±0.61 roots with 4.8 ±0.23 cm length of roots were recorded within 4 weeks (Fig. 3A, Table 3). This is agreed with the results of *Alternanthera sessilis* (Gnanaraj et al., 2011) and *Morinda coreia* (Shekhawat et al., 2015).

Table 3. Effect of auxins (IBA, IAA and NAA) on *in vitro* root induction, number and length of roots from the shoots on half strength MS medium.

IBA (mg L ⁻¹)	IAA (mg L ⁻¹)	NAA (mg L ⁻¹)	Response (%)	Number of Roots (Mean ± SD)	Length of Roots (cm) (Mean ± SD)
1.0	-	-	63	5.2 ± 0.46 ^e	2.1 ± 0.20 ^b
2.0	-	-	98	7.5 ± 0.61 ^f	4.8 ± 0.23 ^h
3.0	-	-	71	4.0 ± 0.34 ^c	4.3 ± 0.31 ^h
4.0	-	-	59	3.7 ± 0.28 ^c	3.2 ± 0.49 ^g
-	1.0	-	26	2.9 ± 0.11 ^b	2.8 ± 0.25 ^e
-	2.0	-	40	4.6 ± 0.43 ^d	3.0 ± 0.17 ^f
-	3.0	-	34	3.5 ± 0.24 ^c	2.3 ± 0.24 ^c
-	4.0	-	31	2.8 ± 0.40 ^b	2.0 ± 0.26 ^b
-	-	1.0	50	1.5 ± 0.30 ^a	1.8 ± 0.13 ^b
-	-	2.0	63	3.6 ± 0.47 ^c	2.0 ± 0.10 ^b
-	-	3.0	59	2.9 ± 0.21 ^b	1.4 ± 0.19 ^a
-	-	4.0	48	2.0 ± 0.10 ^a	1.0 ± 0.42 ^a

Note: Mean separation was analyzed by ANOVA using SPSS software (version 16) and the superscripts of values in the column followed by same letters are not significantly different according to DMRT at 0.05%.

Number of roots was less on higher concentrations of IBA, IAA and NAA. This might be related to the fact that higher concentrations of IBA and NAA is inhibitory both to root induction and elongation (Biradar et al., 2009). Maximum 3.6 ±0.47 roots with average 2.0

± 0.10 cm lengths with 63% response were observed on 2.0 mg/L NAA. IAA at 2.0 mg/L induced maximum 4.6 ± 0.43 shoots (3.0 ± 0.17 cm length) with 40% response. Soulange et al. (2009) reported rooting of shoots on Kin as well as IBA 2.5 mg/L. In the present study no roots were observed on MS medium supplemented with cytokinins. The plantlets were hardened in the greenhouse for two months and finally transferred to the field conditions.

4. CONCLUSION

The present investigation describes an efficient *in vitro* propagation protocol for plantlet regeneration of *H. subdariffa* from somatic tissues. It was found that lower concentrations of cytokinins are effective in shoot regeneration and amplification. MS medium augmented with additives enhanced better growth of the shoots. Half strength MS medium and auxins induced efficient root regeneration. This protocol could overcome the drawbacks of traditional conventional plant propagation methods.

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