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Efficient *in Vitro* Plant Regeneration of Cotton Cultivar Narashima (*Gossypium hirsutum* L.)

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ABSTRACT

Development of a plant regeneration protocol of an elite Indian cotton (*Gossypium hirsutum* L.) cultivar 'Narashima' could help in genetic transformation for biotic stress tolerance and improve quality characteristics. In the present study, successful callus, shoot and root induction were achieved from cotyledonary nodes, hypocotyls, cotyledons and leaf explants cultured on MS medium supplemented with B5 vitamins, as well as combinations of auxins and cytokinins. Callus proliferation was best on Murashige and Skoog (MS) medium with 0.5 mg/l 2,4 dichlorophenoxyacetic acid, 0.4 mg/l thidiazuron (TDZ), 0.5 mg/l 6-benzyl amino purine (BAP) and 1.0 mg/l kinetin (Kn). Shoots were produced from organogenic callus, cotyledonary nodes, hypocotyls cultured on MS medium with 1.0 mg/l BAP, 1.0 mg/l TDZ, 2.0 mg/l KN and 0.4 mg/l α-naphthalene acetic acid (NAA) and 1 g/l activated charcoal. Profuse rooting was achieved on MS medium with 1.0 mg/l NAA and 0.4 mg/l indole-3-acetic acid. The regenerated plants were successfully hardened in earthen pots after adequate acclimatization (68-70%) and hardened plantlets were obtained.

Keywords: acclimatization, multiple shoots, callus induction, thidiazuron
Abbreviations: BAP, 6-benzyl amino purine; **B5**, Gamborg's medium; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **IAA**, indole-3-acetic acid; **Kn**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid; **PPFD**, photosynthetic photon flux density; **TDZ**, thidiazuron

INTRODUCTION

Cotton is a high value commercial crop and its importance needs no introduction. Genetic engineering in cotton has been very painstaking as only two cultivars, 'Coker' and 'Acala', were amenable to genetic transformation and regeneration. The development of protocols for regeneration in other cultivars started recently (Ali *et al*. 2004; Aydin *et al*. 2004; Jin *et al*. 2006; Rathore *et al*. 2006). The proliferation of pre-existing meristems from cotyledonary nodes, primary and tertiary leaf nodes, among others, into elongated multiple shoots *in vitro* has been achieved (Agrawal *et al*. 1997; Gupta *et al*. 1997; Saeed *et al*. 1997; Caramori *et al*. 2001; Ali *et al*. 2004). However, the *de novo* development of shoots in a short period of time is a more advantageous approach because of the possibility of obtaining non-chimeric transgenic plants. Hypocotyls sections were used as explants for direct shoot organogenesis earlier but the efficiency of intact plantlet regeneration reported was low (Ouma *et al*. 2004) and there was no clear mention about the frequency of regeneration and rooting.

Although the efficiency of regeneration in cotton has improved significantly in recent years, some difficulties still remain. A genotype-dependent response restricts the application of cotton biotechnology to the development of cotton with better agronomic characteristics. Therefore, before genetic transformation techniques are widely applied to cotton improvement programmes, plant regeneration must be possible for a broad range of genotypes. Although in the past, several attempts were made for *in vitro* regeneration from other cultivars, little success has been achieved. Aslam *et al*. (2010) regenerated plantlets via apical meristem culture from CIM-443.

In this study we selected a cotton (*Gossypium hirsutum* L.) cultivar 'Narasimha' developed by our University with very good agronomic characteristics and has been used in many of the cotton hybrids produced in India by both public and private sectors. 'Narasimha' is a versatile *hirsutum* cultivar (Ravindranath *et al.* 2004) in Southern India and has shown not only wide adaptability in several regions of south India, but also exhibits high tolerance to drought. It has high yield potential, which has made it useful as a parent in the hybrid cotton and transgenic hybrid cotton programme in southern and central India. 'Narasimha' is the existence of somewhat higher residual variability for boll weight (3.5-5.0 g), highly sympodial nature and higher staple length (29-30 mm) and Very skillful breeding procedures like intense selection towards high GOT, high drought tolerance, high jassid (*Amrasca devastans*) and bacterial blight (*Xanthomonas axonopodis* pv. Malvacearum) resistance, high boll number coupled with high potential and medium staple were practiced at all stages and in the evaluation 'Narasimha' (NA 1325) stood out, so much so that it was released in the state of Andhra Pradesh, which has a total area of 0.8 to million ha under cotton. After the release of 'Narasimha', whitefly problem was eradicated. 'Narasimha' has recorded 20 Q (Quintais)/ha seed cotton yield under rainfed conditions and 30-36 Q/ha under irrigated conditions with best management practices. Even under adverse drought conditions, as witnessed in 2002-03, 'Narasimha' provided higher and stable performance. 'Narasimha' recorded 36 to 37% ginning turn out, 27-28 mm staple length (29 mm 2.5% span length) with spinning potential of 40s HSC. It has a high boll load bearing potential in normal as well as stress situations (Ravindranath *et al*. 2004). We report here the formation of callus, shoot regeneration and efficient rooting by using cotyledonary nodal meristems and hypocotyls as explants of 'Narasimha'.

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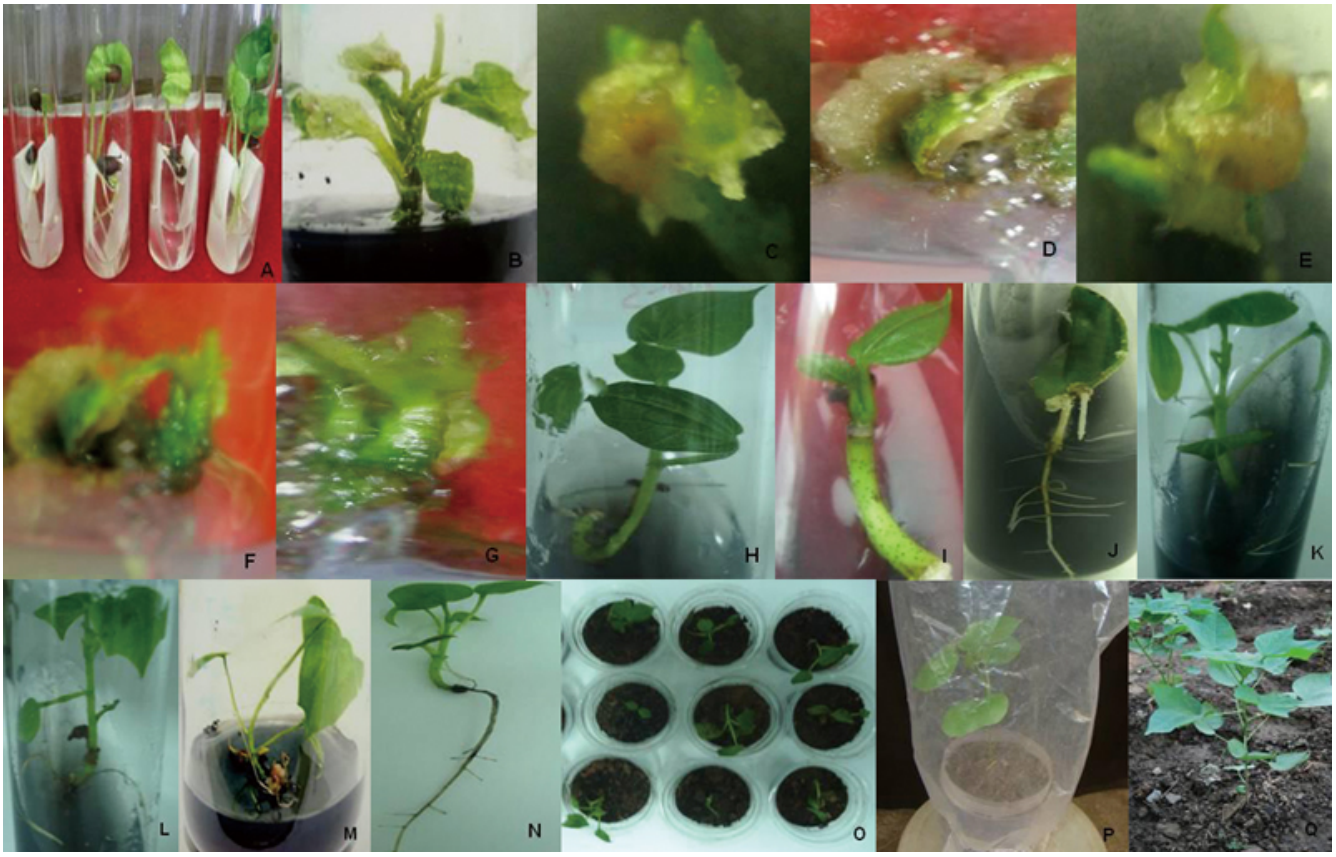

Fig. 1 (**A**) Seed germination on MS liquid medium. (**B**) Shoot elongation of hypocotyls. (**C**) Callus induction from cotyledonary nodal meristem. (**D**) Callus induction from cotyledons. (**E**) Callus induction from leaf. (**F**) Multiple shoot induction from cotyledonary nodal meristem-derived callus. (**G**) Multiple shoot induction from cotyledon derived calli. (**H, I**) Shoot elongation of hypocotyls. (**J**) Root initiation from leaf explants. (**K-M**) Shoot elongation and root induction. (**N**) Shoot and root elongated plantlet. (**O, P**) Acclimatization of well-developed plantlets. (**Q**) Plantlets in the field.

MATERIALS AND METHODS

Source of seeds

Mature seeds were obtained from the Regional Agricultural Research Station, ANGRAU, Nandyal, Andhra Pradesh, India.

Seed germination and explants preparation

Mature seeds were delinted with conc. sulphuric acid (H_2SO_4) for 3 min and placed on a rotary shaker (50 rpm) for 30 min (Rao *et al*. 2006). The seeds were washed three times with sterile double distilled water (SDSW), 5 min each wash. The seeds were surface sterilized using 70% ethanol (v/v) for 2 min and 2-3 times washed with SDSW. The seeds were sterilized by agitation in 4% sodium hypochloride (v/v) (HiMedia Pvt. Ltd. Mumbai, India) for 15 min followed by 3-4 washes with SDSW (Divya *et al*. 2008).

Surface-sterilized seeds were inoculated in 6″ × 1″ borosilicate tissue culture test tubes containing moist filter paper with half-strength Murashige and Skoog (MS) medium. The pH of the media was adjusted to 5.7 with 1 M NaOH before autoclaving. After radical emergence, the tubes were transferred to a growth chamber at 24 ± 2°C with a 16-h photoperiod (PPFD = 83.6 $\mu Em^{-2} S^{-1}$) using cool white fluorescent tubes (6500° K, 36 W, B9, Bajaj). Explant material, namely cotyledons, hypocotyls, cotyledonary nodal meristems, shoot tips and leaf segments were obtained from 10-14 days *in vitro* seedlings.

Induction and proliferation of organogenic callus

Different explants, namely hypocotyls (3-5 mm in length), cotyledon pieces (8-10 mm^2 surface area), cotyledonary nodal meristems (2-4 mm in length) and shoot tips (3-4 mm long), were inoculated onto MS medium in tissue culture test tubes containing 3% sucrose, 0.8% agar, B5 vitamins, 1 g/l activated charcoal (AC) (Divya *et al.* 2008) and various concentrations of cytokinins, namely 6-benzyl amino purine (BAP; 0.1-2.0 mg/l), kinetin (Kn; 0.5-3.0 mg/l), thidiazuron (TDZ; 0.2-2.0 mg/l) and auxins, namely 2,4 dichlorophenoxyacetic acid (2,4-D; 0.1-2.0 mg/l), α-naphthalene acetic acid (NAA; 0.1-2.0 mg/l), indole-3-acetic acid (IAA; 0.1-2.0 mg/l). Heat-labile TDZ was filter sterilized and added to the autoclaved media. The effect of these different combinations on callus induction was evaluated after 28-30 days and the appropriate combinations for optimal callus growth were determined. Callus growth was expressed as fresh and dry weight (g). Callus (0.5-0.6 g of fresh weight) was transferred to MS medium for shoot and root induction.

Shoot induction and elongation

Explants (hypocotyls and cotyledonary nodal meristems) and organogenic callus from all the explants were cultured on basal MS medium with B5 vitamins (Gamborg *et al.*1968), 1 g/l AC and different combinations of cytokinins [BAP (0.5-3.0 mg/l), Kn (1.0-5.0 mg/l), TDZ (0.5-3.0 mg/l)] and low concentrations (Rao *et al*. 2006; Divya *et al.* 2008) of an auxin, α-naphthalene acetic acid (0.2-1.0 mg/l). All the tubes were incubated at 24 ± 2°C under a 16-h photoperiod (PPFD = 83.6 $\mu Em^{-2} s^{-1}$). Shoot growth and development (4-6 mm) was observed after 24 days.

Root induction and acclimatization

Elongated and well-developed shoots (2-3 cm) were excised and transferred to MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, 1 g/l AC and different concentrations of auxins, NAA (0.5-2.0 mg/l), indole-3-acetic acid (0.2-1.0 mg/l) for root induction. After root development (10-14 days), plantlets with well-developed roots were rinsed with water to remove adhering agar medium and grown in a mixture of Soilrite®, farmyard manure (Ag. Technologies (Manures & Fertilizers) and sand (1: 1: 1), in plastic pots (180 mm Torino Square 3.2 L. CP180ST). The pots were covered with a plastic cover with four perforations each for aeration. All the cultured plantlets grown in a controlled environment were gradually acclimatized for their survival in the field.

Plantlets were left for 18 days in the plastic pots at 24 ± 2°C with 60% relative humidity. After 4 weeks of acclimatization, all the plants were shifted to a greenhouse (natural daylight, 15-18°C and 55-60% relative humidity).

Statistical analysis

Cultures were observed on a visual basis and regeneration percentage was calculated as the percentage of responding explants with a minimum of one shoot and two roots. All treatments were carried out three times with 10 replicates each using a completely randomized design. Tests of significance were carried out by ANOVA and the data (means ± SD (standard deviation)) were analyzed by Duncan's multiple range test using SAS/STAT version 9.1.

RESULTS AND DISCUSSION

Induction and proliferation of organogenic callus

Explants (i.e., cotyledons, hypocotyls, cotyledonary nodal meristems and shoot tips) were obtained from 10-14 days-old seedlings (**Fig. 1A**) and inoculated onto MS medium, B5 vitamins, 30 g/l sucrose, 1 g/l AC and different PGRs for callus induction and regeneration. High frequency callus proliferation was observed 20-25 days after inoculation onto MS medium with 1 mg/l KN, 0.4 mg/l TDZ, 0.5 mg/l BA and 0.5 mg/l 2,4-D (**Table 1**). The frequency of callus induction ranged from 25.0 to 72.8% with the explants used and further increase in the 2,4-D concentration above 0.5 mg/l reduced the callusing frequency and induced rhizogenesis. After 6 weeks, highly friable greenish-white callus were obtained from cotyledons, hypocotyls and cotyledonary nodes; these were selected for shoot initiation (**Fig. 1B-E**). Similar results were observed by other groups (Trolinder *et al*. 1987; Ikram-ul-Haq *et al*. 2005; Sun *et al.* 2006). Regeneration via somatic embryogenesis was reported earlier (Finer *et al*. 1988; Shoemaker *et al*. 1986). For further development of organogenic callus into shoot buds, callus was transferred to regeneration media. Cotton callus cultures are prone to browning, and in order to prevent browning, we followed a step-wise transfer of callus from induction to proliferation medium in which they turned organogenic. Callus 3-4 weeks-old showed good regeneration. The inclusion of certain urea derivatives with a cytokinin like TDZ to the culture media leads to profuse callus formation (Sucheta *et al.* 2002; Jones *et al*. 2007).

Shoot induction and elongation

Well developed organogenic callus, hypocotyls and cotyledonary nodal meristems were selected for regeneration and inoculated onto MS basal medium with B5 vitamins (Gamborg *et al*. 1968), 3% sucrose, 1 g/l AC and different concentrations of PGRs. Higher frequency shoot induction was observed on medium containing BAP (1.0 mg/l), KN (2.0 mg/l) and NAA (0.4 mg/l) (**Table 2**). Significantly higher frequency of shoot regeneration was observed on medium containing BAP (1.0 mg/l), KN (2.0 mg/l), TDZ (1.0 mg/l) and NAA (0.4 mg/l) compared to medium without TDZ. Lower concentrations of TDZ yielded more shoot primordia than at higher concentrations (Huetteman and Preece 1993). Divya *et al.* (2008) reported that maximum number of shoots per explant was obtained using 2.0 mg/l TDZ in combination with 1.0 mg/l BAP and 2.0 mg/l KN (**Fig. 1F, 1G**). After sub-culturing on regeneration medium shoot buds were initiated between 22 and 25 days and maintained on the same medium for 3-4 weeks to allow sufficient growth (**Fig. 1H, 1I**). The maximum percent regeneration frequency was 72.6, 76.8 and 78.2 for organogenic callus from cotyledonary, nodal meristems and hypocotyls explants, respectively. Differences were observed in shoot regeneration frequency among different concentrations and combinations of auxins and cytokinins. Combinations of BAP, TDZ, KN and NAA were more successful than any other combination (**Table 2**). Ali *et al*. (2004) reported similar results i.e. 1.0 mg/l BAP and 0.05 mg/l AC has been used for *in vitro* tissue culture to adsorb inhibitory compounds such as phenolics, excessive PGRs and to release growth-promoting substances (van Winkle *et al*. 2003).

Table 1 Comparative morphogenetic responses of cotyledons, hypocotyls, cotyledonary nodal meristems aseptically grown cotton (*Gossypium hirsutum* L.) seedlings cultured in MS medium supplemented with various PGRs.

MS media + PGR concentration (mg/L)	Cotyledons	Hypocotyls	Cotyledonary nodal meristems	Leaf
MS basal	NG	NG	NG	NG
0.1 NAA	NG	NG	NG	NG
0.5 NAA	FR	FR	FR	NG
1.5 NAA	FR	FR	FR	FR
2.0 NAA	FR	FR	FR	FR
0.1 IAA + 0.1 NAA	NG	NG	NG	NG
0.5 IAA + 0.5 IAA	C + FR	C + FR	FR	FR
1.0 IAA + 1.0 IAA	C + FR	C + FR	R	C + FR
1.5 IAA + 1.5 IAA	FR	R	C + FR	R
2.0 IAA + 2.0 IAA	C + FR	C +F R	C + FR	C + R
0.1 BAP + 0.1 IAA + 0.1 NAA	C + R	C + R	C + R	C+ FR
0.5 BAP + 0.5 IAA + 0.5 NAA	C + MR	C	C + Sh	FR
1.0 BAP + 1.0 IAA + 1.0 NAA	C + Sh + R	C + Sh +R	C + Sh	R
1.5 BAP + 1.5 IAA + 1.5 NAA	C + Sh	C + Sh	C + M.Sh	C + M.Sh
2.0 BAP + 2.0 IAA + 2.0 NAA	C + Sh	C + M.Sh	C + M.Sh	C + M.Sh
0.1 BAP + 0.2 TDZ + 0.1 NAA	C	C	C + Sh	C + Sh
0.5 BAP + 0.4 TDZ + 0.5 NAA	C	C	C	C + Sh
1.0 BAP + 0.6 TDZ + 1.0 NAA	C +Sh	C	C + Sh	C
1.5 BAP + 0.8 TDZ + 1.5 NAA	C + Sh +R	C + M Sh	C + M.Sh	C + MR
2.0 BAP + 1.0 TDZ + 2.0 NAA	C + Sh + R	C + Sh +R	C + M.Sh +R	C + R
0.1 BAP + 0.2 TDZ + 0.1 2,4-D + 0.5 KN	C^{+} + Sh + R	C^{+} + Sh	C^{+} + Sh + R	C +Sh
0.5 BAP + 0.4 TDZ + 0.5 2,4-D + 1.0 KN	C^{++}	C^{++} + FR	C^{++}	C^{++}
1.0 BAP + 0.6 TDZ + 1.0 2,4-D + 1.5 KN	C + M.Sh+R	C + M.Sh + R	C + M.Sh + R	M.Sh + R
1.5 BAP + 0.8 TDZ + 1.5 2,4-D + 2.0 KN	C + M.Sh	C + M.Sh	C + Sh + FR	C + Sh + FR
2.0 BAP + 1.0 TDZ + 2.0 2,4-D + 3.0 KN	C + Sh + R	C + Sh + R	C + M.Sh + R	C + Sh + R

BAP = 6-benzyl amino purine; C = callus; C^{+} = Good callus; C^{++} = excellent callus; 2,4-D, 2,4-dichlorophenoxyacetic acid; FR = few roots; IAA = indole-3-acetic acid; KN = kinetin; MR = more roots; M.Sh = multiple shoots; NAA = α-naphthalene acetic acid; NG = no growth; PGR = plant growth regulator; R = roots; Sh = shoots; TDZ = thidiazuron.

Table 2 Effect of combinations of BAP, KN, TDZ and NAA on shoot development of cotyledonary meristem, hypocotyls and callus in cotton (*Gossypium hirsutum*).

MS media + PGR concentration (mg/L)	Cotyledonary nodal meristems	Hypocotyls	Callus
0.5 BAP + 1.0 KN + 0.2 NAA	1.1 ± 0.57 d	1.3 ± 0.48 e	0.8 ± 0.63 e
1.0 BAP + 2.0 KN + 0.4 NAA	3.2 ± 0.78 ab	3.5 ± 0.52 b	2.8 ± 0.78 b
1.5 BAP + 3.0 KN + 0.6 NAA	2.2 ± 0.63 c	2.4 ± 0.51 cd	1.5 ± 0.52 de
2.0 BAP + 4.0 KN + 0.8 NAA	1.5 ± 0.52 cd	2.1 ± 0.57 cde	1.4 ± 0.51 de
3.0 BAP + 5.0 KN + 1.0 NAA	2.1 ± 0.73 c	1.8 ± 0.63 de	1.1 ± 0.31 de
0.5 BAP + 1.0 KN + 0.5 TDZ + 0.2 NAA	2.1 ± 0.73 c	2.3 ± 0.67 cd	1.7 ± 0.48 cd
1.0 BAP + 2.0 KN + 1.0 TDZ + 0.4 NAA	4.1 ± 0.73 a	4.4 ± 0.51 a	3.6 ± 0.51 a
1.5 BAP + 3.0 KN + 1.5 TDZ + 0.6 NAA	3.6 ± 0.51 a	3.5 ± 0.52 b	2.3 ± 0.48 bc
2.0 BAP + 4.0 KN + 2.0 TDZ + 0.8 NAA	2.4 ± 0.51 bc	2.7 ± 0.67 bc	1.7 ± 0.48 cd
3.0 BAP + 5.0 KN + 3.0 TDZ + 1.0 NAA	2.2 ± 0.63 c	2.3 ± 0.48 cd	1.7 ± 0.48 cd

BAP = 6-benzyl amino purine; IAA = indole-3-acetic acid; KN = kinetin; NAA = α-naphthalene acetic acid; PGR = plant growth regulator; TDZ = thidiazuron. Different letter indicate significantly different treatments (between rows) ($P < 0.05$, critical difference = 0.2144). No significant differences were observed between explant types (between columns) for all treatments ($P > 0.05$). Values = means ± S.D.

Table 3 Effect of combinations of NAA and IAA on root development of shoots from cotyledonary meristem, hypocotyls and callus in cotton (*Gossypium hirsutum*).

MS media + PGR concentration (mg/L)	Cotyledonary nodal meristems	Hypocotyls	Callus
0.5 NAA + 0.2 IAA	2.1 ± 0.73 c	2.4 ± 0.51 c	2.2 ± 0.63 c
1.0 NAA + 0.4 IAA	5.1 ± 0.87 a	5.2 ± 0.78 a	4.6 ± 0.51 a
1.5 NAA + 0.6 IAA	3.4 ± 0.69 b	3.7 ± 0.48 b	3.2 ± 0.78 b
2.0 NAA + 0.8 IAA	2.1 ± 0.73 c	2.0 ± 0.66 c	1.7 ± 0.48 c
2.5 NAA + 1.0 IAA	1.8 ± 0.63 c	0.7 ± 0.66 d	0.8 ± 0.63 d

IAA = indole-3-acetic acid; NAA = α-naphthalene acetic acid; PGR = plant growth regulator; Different letter indicate significantly different treatments (between rows) (($P < 0.05$, critical difference = 0.2144). Values = means ± S.D.

Root development of regenerants and acclimatization

The well developed shoots from callus, hypocotyls and cotyledonary nodes were transferred to rooting medium containing different concentrations of NAA (0.5–2.5 mg/l) and IAA (0.2–1 mg/l) (**Table 3**). A significant level of rhizogenesis was observed in the three explants i.e., 74, 76.2 and 78.4 at 1.0 mg/l NAA and 0.4 mg/l IAA (**Fig. 1K-M**). Root induction occurred between 15 and 18 days after transfer to rooting medium and rooting frequency was higher in all these explant regenerants. Ozyigit *et al*. (2007) and Ibrahim *et al*. (2008) reported similar results. In cotton tissue culture, rooting is also one of the major problems and many different methods were applied to induce rooting (Ouma *et al*. 2004). However, the results of rooting success in the literature is not quite clear and there is less data on rooting.

After the development of roots the plantlets were first transferred to plastic pots having a 1: 1: 1 mixture of Soilrite, farmyard manure and sand (**Fig. 1N**). The pots were covered with a plastic cover with 4 perforations for aeration at 25 ± 2°C under a 16-h photoperiod (PPFD = 83.6 $\mu Em^{-2} S^{-1}$) (**Fig. 1O, 1P**). After 15-18 days of hardening, these plantlets were transferred to earthen pots and then to the field. The hardened plants showed a survival rate of 78.2 ± 2.0% under field conditions (**Fig. 1Q**).

The present study also indicates the genotype specificity for callus induction and plant regeneration in cotton regeneration. Earlier reports on cotton regeneration was mostly with few cotton genotypes i.e., Coker lines, T series (Davidonis *et al*. 1983) and there were scanty reports on Indian cotton cultivars.

Though success has been obtained for cotton regeneration with one Indian genotype of cotton it is important to replicate the success in other cotton cultivars of India and establish a genotype-independent protocol for regeneration and genetic transformation. This will pave the way for cotton improvement through transgenic approach for many of the important agronomic characteristics of this crop.

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