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In Vitro Plant Regeneration through Multiple Shoot Induction in Cotton (*Gossypium* spp.)

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ABSTRACT

In the present study, two cotton cultivars viz. LD 694 (*Gossypium arboreum* L.) and LH 2076 (*Gossypium hirsutum* L.) were used to investigate the effects of MS (Murashige and Skoog) and DKW (Driver and Kuniyuki) basal media compositions supplemented with different concentrations of BA (6-benzylaminopurine) and Kin (Kinetin) on the sprouting response (development of shoot bud primordia), morphogenetic response, and induction and regeneration of multiple shoots from shoot tip explants excised from 5-7 day-old seedlings cultured *in vitro*. The mean sprouting response in both cultivars LD 694 (81.66%) and LH 2076 (79.44 %) was higher in MS medium than in DKW medium. The best treatment for the formation of adventitious shoots (73.80%) in LD 694 was MS + 2.00 mg/l BA, while MS + 1.00 mg/l BA + 1.00 mg/l Kin was optimum for the formation of adventitious shoots (74.78%) in LH 2076. LD 694 and LH 2076 produced a maximum number of shoots/explant (3.96 and 3.85, respectively) when cultured on MS supplemented with 2.00 mg/l BA. Highest root development (62.38%) was obtained when shoots were cultured on ½ MS medium supplemented with 0.05 mg/l NAA in both cultivars. Plantlets raised *in vitro* were placed in water for two weeks and then transferred to small polythene bags filled with a sand : soil mixture in the greenhouse. These plantlets grew into healthy plants and reached maturity. The results of this study will facilitate the application of mass multiplication of elite breeding material including CMS lines or transgenic cotton cultures.

Introduction

Cotton (*Gossypium* spp.) is an excellent natural source of textile fiber and is a high value commercial crop. Over 180 million people are associated with the fiber industry that produces 20 to 30 billion US dollars worth of raw cotton (Ozyigit and Gozukirmizi 2009). Both diploid (*Gossypium arboreum*) and tetraploid (*G. hirsutum*) cultivars are cultivated in different regions of India and are considered as important crop plants. Due to its high economic value, considerable attention has been paid to improve cotton plants by conventional plant breeding, which is time-consuming: commercialization of new cotton varieties often takes 6-10 years (Sheidai *et al*. 2008). Besides, conventional genetic improvement of cotton is limited due to many factors like the absence of necessary variation, especially resistance against pests and diseases. Plant tissue culture provides an alternative means of improvement to obtain somaclones, induced variants, somatic hybridization and double haploids to develop inbred lines or to introduce genes of interest against insects and different diseases through genetic engineering (Zhang and Zhao 1997; Sanghera *et al*. 2010). However, successful application of *in vitro* methodologies is mainly dependent on a reliable and reproducible regeneration system like somatic embryogenesis, which used to be quite difficult in cotton (Shoemaker *et al*. 1986; Trolinder and Goodin 1987; Zhang *et al*. 2001). An alternative to somatic embryogenesis is plant regeneration from explants having a pre-existing meristem. The culture of cotton apical meristems was first reported by Chappel and Mauney (1967). Since then, considerable work has been carried out to develop protocols for an efficient regeneration system in cotton. *In vitro* culture of apical shoot tips has been reported to give single or, sporadically, a few shoots; protocols were found to be genotype-dependent (Trolinder and Chen 1989), although the development of protocols for the regeneration of specific cultivars has intensified over the last 10 years (Ali *et al*. 2004; Jin *et al*. 2006; Sanghera *et al*. 2010). Multiple shoots have been induced *in vitro* from pre-existing meristems, cotyledonary nodes, primary and tertiary leaf nodes, and other explant sources (Saeed *et al*. 1997; Agrawal *et al*. 1997; Gupta *et al*. 1997; Hemphill *et al*. 1998; Morre *et al*. 1998; Zapata *et al*. 1999; Ali *et al*. 2004).

The regeneration protocols have generally been used to obtain genetically modified plants (Rajasekraran *et al*. 1996; Trolinder *et al*. 2006; Kategri *et al*. 2007; Nandeshwar *et al*. 2009). Although the efficiency of cotton regeneration has improved significantly, difficulties still remain such as the low efficiency of somatic embryogenesis (Zhang and Zhao 1997). Regeneration in cotton is still restricted to a limited number of cultivars (Sanghera *et al*. 2009a). Furthermore, a long culture period and often complicated protocols have thus far limited the wider application of biotechnology in cotton. To be able to apply different biotechnological techniques to cotton, a broad range of genotypes must be responsive to regeneration, which is a fundamental pre-requisite for mass propagation of a germplasm

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of interest. The aim of the present investigation was to study the effect of two basal media MS (Murashige and Skoog, 1962) and DKW (Driver and Kuniyuki, 1984) and various concentrations of Kin and BA on multiple shoot induction and regeneration in two newly developed cotton cultivars, a diploid (LD 694) with high yield and a parent of the hybrid 'Moti' released for cultivation in North India and a tetraploid (LH 2076) possessing high yield, tolerance to *Cotton leaf curl virus* and with desirable fibre quality traits, and to refine a proliferation protocol for these cultivars. This is the first ever *in vitro* protocol for these cotton cultivars.

Materials and Methods

Seed germination and cultivation of sterile seedlings

Seeds of LD 694 and LH 2076 were obtained from the Cotton Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana and were delinted by using concentrated commercial H_2SO_4. The seeds were continuously stirred in H_2SO_4 by a wooden rod for 5~10 min until a shiny surface appeared on seeds. Some water was then added after decanting the acid and stirred for a few seconds. The seeds were thoroughly washed five times with tap water to remove the acid completely, left in a beaker of water for a few minutes; seeds floating on the water surface were discarded. Plump, mature seeds were chosen and washed in a solution containing a few drops of Tween 20 (surfactant) in water, shaken vigorously then washed thoroughly three times in autoclaved distilled water (ADW). Seeds were surface sterilized in a $HgCl_2$ (0.1%) + Bavistin (Indofil chemicals) (1.0%) solution for 6 min followed by 4-5 washes with ADW (Sanghera *et al*. 2009b). The seeds were soaked in ADW for 6 h and then sown in 200-ml jam jars containing25-30 ml of MS medium supplemented with 8 g/l agar (Hi-media) for germination at 28 ± 2°C in the dark. All sterilization work was performed in a laminar airflow cabinet.

Induction of multiple shoots

Shoot tips were excised under sterile conditions from 7- and 14-day-old *in vitro* raised seedlings of LD 694 and LH 2076 genotypes by removing both cotyledons. Isolated shoot apices were planted with the base inserted into basal MS or DKW media containing 6-benzylaminopurine (BA, 0.0–2.5 mg/l) or kinetin (Kin; 0.0–2.5 mg/l) or combinations of BA and Kin (0.0–2.5 mg/l) and DKW medium (Table 1). All media were supplemented with 3% sucrose and 0.8% agar and the pH was adjusted to 5.8 before autoclaving and dispensing 10-15 ml into borosilicate test tubes (15 × 125 mm). Cultures were incubated for 35 days at 25 ± 2°C under cool white fluorescent light at 30 µE m^{-2} s^{-1} under a 16-h photoperiod. After 3-5 days, only those explants which formed a minimum of one shoot were considered to be responsive (expressed as the percentage sprouting response of explants), while the formation of > 2 shoots by an explant was considered as the induction of multiple shoots (percentage multiple shoot induction).

Elongation and rooting of shoots

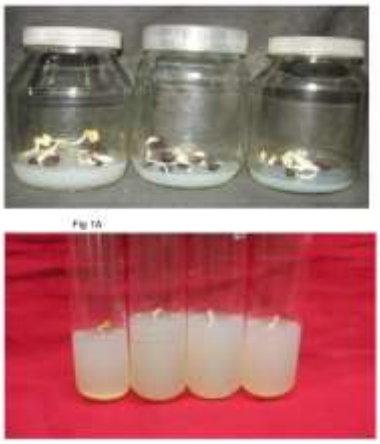
Multiple shoot masses forming shoots (MS2 and MS7 media) were trimmed at the base and single shoots were transferred to hormone-free MS basal medium containing 0.8% agar and 3% sucrose in test tubes and left to elongate for 4 weeks. All cultures were incubated in the same temperature and light conditions as shoot induction experiments. Elongated shoots of uniform size (8-10 mm) were selected, trimmed slightly at the base and transferred into four rooting media (Table 1) comprising half-strength MS basal medium (containing 0.8% agar and 3% sucrose) with or without 0.05-0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.2% (w/v) activated charcoal (Gargas *et al.* 2007) and incubated for 6 weeks. The number of elongated shoots was recorded for both genotypes after 4 weeks while the number of shoots inducing roots (per cent root induction) was recorded after 6 weeks in culture.

The rooted shoots were then transferred to test tubes (one rooted plantlet/tube) containing a little cotton soaked with a small volume (5-10 ml) of water and incubated in a culture chamber (27°C) for 2 weeks (the hardening period); water was substituted daily. The hardened plantlets were then transferred to small polythene bags filled with a sand soil mixture (1:3) in the greenhouse and finally transferred to earthen pots in a glasshouse and allowed to grow to maturity.

The number of shoot tips used for each treatment and genotype was n = 36-48. All experiments were repeated three times. Data recorded was analyzed according to completely randomized design analysis (Snedecor and Cochran 1967) using statistical software CPCS-1 package developed by Cheema and Singh (1990). The data of percent were converted to arc sine value for the analysis of variance (ANOVA). The significance of variation among the treatments were observed by applying the *F*-test and critical differences (CD) at the 5% level of significance were calculated and used to compare the means of treatments.

Results and Discussion

To obtain the best method for isolating shoot tips, delinted seeds were disinfected with 1.0% Bavistin + 0.1% $HgCl_2$ (Sanghera *et al*. 2009b), which resulted in surface disinfection with minimum seed contamination (0-2%) and germination as good as (69-80%) on MS medium when treatment duration was 6 min in both cotton genotypes. Germination was observed after 72 h. The germination of cotton seeds on agar-solidified MS media has also been observed earlier (Shoemaker *et al*. 1986; Zhang 1994; Rashid *et al*. 2004; Sanghera *et al*. 2009b). Within 5 days, well developed axenic seedlings (root system with an expanded cotyledon) formed (Fig. 1A). At this stage only hypocotyls 2-3 cm in length emerged. Shoot apices (0.5-0.6 cm) were excised from 5-7-days old seedlings (Fig. 1B) and cultured on different media compositions having MS and DKW basal media supplemented with or without plant growth regulators (Table 1). Cultured shoot apices started to grow within 3-4 days on different media. Further results obtained on the effect of basal medium on explant sprouting response, effect of different plant growth regulators on morphogenetic response and effect of BA and Kin on induction of multiple shoots in two cotton varieties (LD 694 and LH 2076) are described in the following sections.



Effect of basal medium on explant sprouting response

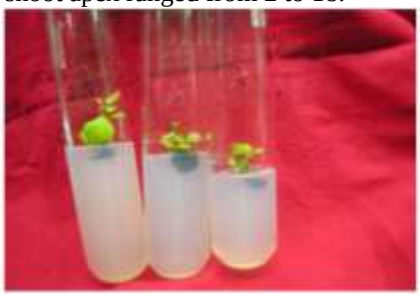
Shoot tips of LD 694 and LH 2076 cotton varieties excised aseptically from *in vitro* grown seedlings after 5-7 days of seed germination were used to induce multiple shoots on two basal media (MS and DKW) supplemented with different concentrations of BA and Kin (Table 1). Shoot tips excised from

5-7 day old seedlings were used for multiple shoot induction based on the studies by Gupta *et al.* (1997), who reported that the number of multiple shoots increased as the age of *G. hirsutum* cv. Khandwa-2 seedling increased and was maximum when explants were excised from seedlings obtained 6 days after seed germination. The variable age of seedlings for excision of explants and multiple shoot induction has also been reported (Banerjee 2001; Ouma *et al.* 2004). An experiment was carried out to assess the effect of two basal media formulations (MS and DKW) on explant sprouting response of shoot tips with varying medium formulations and for both cultivars (Table 2). Medium containing MS salts and vitamins supported a higher sprouting response in both cultivars compared to DKW medium. The use of MS salts and modifications of the basal medium meant for *in vitro* plant regeneration in cotton from pre-existing meristems has been reported by several groups (Gould *et al.* 1991; Gupta *et al.* 1997; Hemphill *et al.* 1998; Zapata *et al.* 1999; Rashid *et al.* 2004; Sanghera *et al*. 2010). In MS medium, the mean percentage of sprouting response was higher than DKW medium for both varieties (Table 2).

However, multiple shoot induction (%) was higher in DKW medium for both varieties than MS medium. LD 694 and LH 2076 recorded 33.05 and 45.50% multiple shoot induction, respectively on MS medium while the corresponding figures in DKW medium were 46.97 and 48.64%. Sharma *et al.* (2007) achieved maximum sprouting (58%) from shoot apices of cotton by culturing on MS salts + modified B5 vitamins without plant growth regulators.

Effect of BA and Kin on induction of multiple shoots

Different concentrations of BA, Kin and BA+Kin in MS and DKW media induced multiple shoots in both LD 694 and LH 2076 (Fig. 1C). For LD 694, maximum multiple shoot induction (73.80%) was recorded in MS + 2.0 mg/l BA medium followed by 72.45% (MS + 1.0 mg/l BA + 1.0 mg/l Kin), and 70.68% (DKW + 1.0 mg/l BA). The medium MS + 1.0 mg/l BA + 1.0 mg/l Kin showed the highest multiple shoot induction (74.78%) closely followed by DKW + 1.0 mg/l BA (74.59%) for LH 2076. The higher concentration of BA and Kin (2.5 mg/l) alone and in combination showed a low percentage of multiple shoot induction (Table 3). There were significant differences among media with respect to multiple shoot induction (CD = 12.20). Sharma *et al*. (2007) reported that when regenerated plants were transferred to different multiple shoot induction media, 65% of shoot apices induced multiple shoots on DKW medium supplemented with 1 mg/l BA and the number of shoots per shoot apex ranged from 2 to 18.



The number of multiple shoots formed per explant among various concentrations of BA, Kin and BA + Kin in MS and DKW media varied from 1.06 to 3.96 depending on the cultivar and medium composition (Table 3).

The maximum number of shoots (3.96) was recorded for LD 694 on MS medium containing 2.0 mg/l BA followed closely by 3.38 when 1.0 mg/l BA was used. Similarly, the number of multiple shoots formed per explant in LH 2076 was highest (3.85) on MS medium containing 2.0 mg/l BA followed by 3.41 with 1.0 mg/l BA. Further proliferation of these shoots was observed on transfer of shoot masses to fresh medium and incubated for 6 weeks.

The number of explants exhibiting multiple shoots was higher than when cultured on MS medium supplemented with 2.0 mg/l BA. As the concentration of BA and Kin increased, shoots and leaves swelled and shape was distorted. When BA and Kin at 2.5 mg/l were used, the growth of shoots slowed and most of the shoots became desiccated, eventually leading to death of the plant. Chinchane *et al*. (2005) observed that MS medium containing 2 mg/l BA and 1 mg/l Kin was best for induction of multiple shoots in *G. arboreum* (cv. PA 255 and PA 402). Likewise, Nandeshwar *et al*. (2002) obtained 9-10 shoots per explant on MS medium supplemented with 2 mg/l BA and 1 mg/l Kin in cv. RG8.

Effect of plant growth regulators on morphogenetic response

Explants excised from 5-7-day old seedlings when cultured on MS basal medium supplemented with BA (1.0 – 2.5 mg /l), Kin (1.0 – 2.5 mg/l), a combination of BA and Kin (1.0 – 2.5 mg/l) and DKW with BA (1.0 mg/l) and a combination of BA and Kin (1.0 – 1.0 mg/l) resulted in shoot formation with differing degrees of response depending on the plant growth regulator tested (Table 4). BA was used to induce multiple shoots based on studies on shoot proliferation conducted by Gupta *et al.* (1997); they found BA to be most effective in inducing multiple shoots. Morre *et al.* (1998) also reported that BA is responsible for producing multiple buds and subsequent shoot development. BA alone at 1.0 mg/l, in combination with 1.0 mg/l Kin and DKW with 1.0 mg/l BA resulted in 2-3 shoots/explant. Although the media containing lower concentrations of BA and Kin (1.0 mg/l each) developed 2-3 shoots/explant, the percentage response decreased as their concentration in the media increase (Table 4). These results are in agreement with the findings of Ganesan and Jayabalan (2006) who studied the influence of different forms of cytokinins, auxins and polyamines for mass multiplication and regeneration of cotton and reported that concentrations of BA and Kin above 1.0 mg/l showed poor response for multiple shoot induction. Zapata *et al.* (1999) observed similar findings with cotton shoot apices in which plant regeneration was suppressed with higher concentrations of BA. Kin alone at 1.0-2.5 mg/l developed 1-2 shoots/explant after 5 weeks of incubation. All Kin concentrations induced callus at the base of explants. Kin at 1.0 and 2.5 mg/l resulted in profuse callusing and developed stunted shoots (Fig. 1D). At higher levels of Kin (2.5 mg/l), explants did not respond and turned brown (Fig. 1E). In a similar study, Gould *et al.* (1991) observed blackening of tissues at 2.0 mg/l Kin. After 20-25 days of culture, new axillary shoots had formed and the primary shoot started to degenerate in this study. The result of the present study is in congruence with that of Sharma *et al*. (2007), who found that pre-formed shoots degenerated when multiple shoots initiated development from the base.

However, when these cultures were transferred to basal medium without a cytokinin, explants assumed normal growth and elongation of shoots was achieved in

both varieties (Fig. 1F). Morre *et al.* (l998) also observed that BA reprograms apex development and multiple bud formation; however, it also inhibited shoot development and elongation.

***In vitro* rooting**

Multiple shoots started to develop 3 weeks after culture and 3-4 shoots were produced per explant. Individual shoots were detached from the proliferating basal shoot mass and were transferred to half-strength MS medium fortified with NAA and activated charcoal to induce rooting (Table 1) for 3 weeks. The experiment was repeated twice. Roots initiated after 15 days of shoot regeneration in both genotypes. Fig. 1G shows root induction and well developed plantlets in LH 2076 and LD 694, respectively. Statistical analysis of data showed that the genotypic differences with respect to percentage root induction were not significant in all the media tested.

However, significant differences existed among different rooting media tested (Table 5). Half-strength MS + 0.05 mg/l NAA resulted in the highest percentage root induction (62.38%) while half-MS exhibited the least root induction (42.03%). Banerjee (2001) observed a high percentage of rooting (85%) in cv. LRK-516 followed by NHH-44 (82.50%) and H-8 in half-MS + vitamins medium supplemented with 0.05 mg/l NAA. However, Sanghera *et al*. (2010) reported that root induction in Indian cotton cultivars ranged from 55-75%. After 3 weeks' culture, plantlets were then transferred to test tubes containing a little cotton soaked with a small amount of water (Fig. 1H) and incubated in a culture chamber (27°C) for 2-3 weeks and the water from these tubes was changed daily during hardening.

The hardened plantlets were then transferred to small polythene bags filled with a sand: soil mixture in the greenhouse (Fig. 1I-J) and finally transferred to earthen pots in a glasshouse and grown to maturity. The plants appeared normal (Fig. 1K-L). In this study, multiple shoot induction and regeneration from this apical shoot tip procedure was used for the regeneration of cotton plants in LD 694 (*arboreum*) and LH 2076 (*hirsutum*) varieties. Since cotton is recalcitrant and has proved difficult to manipulate in tissue culture (McCabe and Martinell 1993), this procedure can be used for multiplication of elite cotton breeding material (transgenic or male sterile lines) as the formation of multiple shoots has now been demonstrated.

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Table 1 List of media used for multiple shoot and root initiation

Media code	Composition
Shoot Regeneration	
MS0	MS basal
MS1	MS + 1.0mg/l BA
MS2	MS + 2.0mg/l BA
MS3	MS + 2.5mg/l BA
MS4	MS + 1.0mg/I Kin
MS5	MS + 2.0mg/I Kin
MS6	MS + 2.5mg/I Kin
MS7	MS + 1.0mg/l BA + 1.0mg/l Kin
MS8	MS + 2.5mg/l BA + 2.5mg/I Kin
MS9	DKW + 1.0mg/l BA
MS10	DKW + 1.0mg/l BA + 1.0mg/l Kin
Root Induction	
R1	Half MS
R2	Half MS + 0.1mg/l NAA
R3	Half MS + 0.05mg/l NAA
R4	Half MS + 0.2 % activated charcoal
Abbreviations: BA, 6-benayleaminopurine; DKW, Driver and Kuniyuki; Kin, kinetin; MS, Murashige and Skoog; NAA, α-naphthalene acetic acid	

Table 2 Effect of basal media on sprouting and morphogenetic response in cotton

Basal medium	Sprouting response (%)		Multiple shoot induction (%)		Morphogenetic response	
	LD 694	LH 2076	LD 694	LH 2076	LD 694	LH 2076
MS salts and vitamins	81.66 (64.64)	79.44 (63.03)	33.05 (35.09))	45.50 (42.41)	1-2 shoots, Elongated	2-3 shoots, Elongated
DKW	78.50 (62.38)	75.50 (60.34)	46.97 (43.26)	48.64 (44.20)	1-2 shoots, stunted	1-2 shoots, stunted

Table 3 Effect of phytoharmone on multiple shoot induction and morphogenic response in LD 694 and LH 2076 varieties of cotton

Basal media	Phytohormone Conc (mg/l)		Varieties				Nature of response
	BA	Kin	LD 694		LH 2076		
MS			Multiple shoot induction (%)	Shoots/ explant	Multiple shoot induction (%)	Shoots/ explant	
	0.0	0.0	33.05 (35.09)	1.38	45.50 (42.41)	1.25	Elongated shoots
	1.0		69.65 (56.56)	3.38	70.68 (57.21)	3.41	Elongated shoots
	2.0		73.80 (59.19)	3.96	71.93 (57.98)	3.85	Stunted shoots
	2.5		55.49 (48.13)	1.54	50.10 (45.03)	2.02	Stunted shoots
	0.0	1.0	60.40 (57.66)	1.16	58.87 (57.96)	1.42	Elongated shoots
		2.0	55.76 (48.30)	1.25	50.07 (45.04)	1.16	Stunted shoots, callus at base
		2.5	46.97 (43.26)	1.21	48.64 (44.20)	1.06	Stunted shoots, callus at base
	1.0	1.0	72.45 (58.33)	2.91	74.78 (59.84)	2.86	Elongated shoots
	2.5	2.5	48.64 (44.20)	1.17	50.10 (45.03)	1.25	Stunted shoots, callus at base
DKW	0.0	0.0	46.97 (43.26)	1.26	48.64 (44.20)	1.21	Elongated shoots
	1.0	0.0	70.68 (57.21)	2.98	74.59 (59.70)	2.45	Elongated shoots
	0.0	1.0	61.40 (57.66)	1.16	58.77 (57.96)	1.52	Elongated shoots
	1.0	1.0	62.25 (52.09)	1.90	71.16 (57.51)	1.46	Stunted shoots, callus at base
CD at (0.05) in LD 694 for per cent multiple shoot induction 12.32; number of shoots/ explant is 0.48 CD at (0.05) in LH 2076 for per cent multiple shoot induction 13.65; number of shoots/ explant is 0.55							

Table 4 Effect of phytoharmone on sprouting and morphogenetic response in LD 694 and LH 2076 varieties of cotton

Basal media	Phytohormone Conc (mg/l)		Varieties			
	BA	Kin	LD 694		LH 2076	
MS			Explant response (%)	Shoots/ explant & Nature of response	Explant response (%)	Shoots/explant & Nature of response
	0.0	0.0	81.60 (64.60)	1-2 shoots, Elongated	79.40 (63.01)	1-2 shoots, Elongated
	1.0		100.00 (90.00)	2-3 shoots, Elongated	100.00 (90.00)	2-3 shoots, Elongated
	2.0		82.00 (64.90)	1-3 shoots, stunted	80.00 (63.43)	2-3 shoots, stunted
	2.5		70.00 (56.79)	1-2 shoots, stunted	65.00 (53.73)	1-2 shoots, stunted
	0.0	1.0	90.00 (71.57)	1-2 shoots, Elongated	85.00 (67.21)	1-2 shoots, Elongated
		2.0	69.00 (56.17)	1-2 shoots, stunted callus at base	65.00 (53.73)	1-2 shoots, stunted callus at base
		2.5	59.00 (50.18)	1-2 shoots, stunted callus at base	60.00 (50.77)	1-2 shoots, stunted callus at base
	1.0	1.0	100.00 (90.00)	2-3 shoots, Elongated	90.00 (71.57)	2-3 shoots, Elongated
	2.5	2.5	65.00 (53.73)	1-2 shoots, stunted callus at base	70.00 (56.79)	1-2 shoots, stunted callus at base
DKW	0.0	0.0	78.50 (62.37)	1-2 shoots, Elongated	75.50 (60.33)	1-2 shoots, Elongated
	1.0	0.0	85.00 (67.21)	2-3 shoots, Elongated	74.59 (59.73)	2-3 shoots, Elongated
	1.0	1.0	72.00 (58.05)	1-3 shoots, stunted, callus at base	71.16 (57.52)	1-2 shoots, stunted, callus
CD at (0.05) in LD 694 for explant response 13.42 CD at (0.05) in LD 2076 for explant response 15.69						

Table 5 Effect of different media composition on root induction in cotton

Genotype/ Media	Root Induction (%)		
	LD 694	LH 2076	Mean
Half MS	42.06 (40.43)	42.00 (40.39)	42.03
Half MS + 0.1mg/l NAA	55.72 (48.28)	54.44 (47.54)	55.08
Half MS + 0.05mg/l NAA	64.49 (53.41)	60.28 (50.92)	62.38
Half MS + 0.2 % activated charcoal	46.25 (42.84)	47.15 (43.36)	46.70
Mean	52.13 (46.21)	50.96 (45.54)	
CD at (0.05) Genotypes NS; Media 4.30 ; Genotype x Media 6.75			

Values in the parentheses are arc sine transformation