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High Frequency Regeneration and Multiple Shoot Induction in Indian Cotton (*Gossypium hirsutum* L.) Cultivar

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ABSTRACT

In present investigation an attempt has been made on regeneration and multiple shoot induction from meristamatic tissues like seedling coteledonary nodal explants in MCU-11. Improvement of regeneration method to induce the efficient transformation system is very important in many Indian local cotton cultivars are desirable. However, in the previous reports rooting efficiencies were genotype independent and the data was inadequate. The objective of the investigation is to improve shoot initiation, multiple shoot induction, *in vitro* grafting for the un rooted micro shoots for maximum recovery of regenerated shoots with 90% for greatly improve the crop yield and gene transfer of desirable genes for biotic and abiotic aspects of cotton.

Key words: Cotton, Genotype, Regeneration, Shoot induction, *In vitro* grafting

Cotton (*Gossypium hirsutum* L.) is an important agricultural and commercial crop for fiber, feed, and edible oil in the world. Plant tissue culture plays an important role in modern cotton breeding and genetic engineering; it has been a significant impact on production. To take advantage of this promising technology, a reliable and genotype-independent regeneration system is essential for *in vitro* studies and genetic manipulation for crop improvement. *In vitro* culture of meristamatic tissues and shoot tips has been reported to give single or sporadically, a few shoots (Bajaj and Gill 1986, Gould *et al*. 1991). *In vitro* shoot regeneration from meristamatic tissue (shoot tip, axillary buds and coteledonary nodal explants) were direct, relatively simple and is not prone to somaclonal variations and chromosomal aberrations (Chlan *et al*. 1995, Nasir *et al*. 1997, Morre *et al*. 1998, Zapata *et al*. 1999).

India is the third largest cotton producing country and also holds third place in the world in terms of cotton cultivated land. In vitro regeneration and genetic engineering studies of Indian local cotton cultivars lag far behind with only few regenerable varieties are reported and direct regeneration attempted in so many species in cotton but not expect very limited reports (Sathyavathi *et al*. 2002, Katageri *et al*. 2007). Earlier many scientists reported and used meristamatic tissues and axilary buds as explants (Price and Smith 1979). However, the previous reports mainly on regeneration protocols of cotton pertain to either wild or Coker varieties of *Gossypium hirsutum* which are not in routine agricultural cultivation practices. Cotton is a resilient species and very difficult to manipulate *in vitro* response and very limited number of cultivars are in regenerable manner (Trolinder and Goodin 1987, Trolinder and Xhixian 1989, Cousin *et al*. 1991). Improvement of regeneration method to induce the efficient transformation system is very important in many Indian local cotton cultivars are desirable. An attempt has been made on regeneration and multiple shoot induction from meristamatic tissues like seedling coteledonary nodal explants in MCU-11.

MATERIALS AND METHODS

Seed germination and plant material

Seeds of sotton (*Gossypium hirsutum* L.) cultivars were kindly provided by Agricultural Research Centre, Warangal Region, N. G. Ranga Agricultural University, Hyderabad, India. Seed were decoated and sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 10 minutes then washed four times subsequently with double distilled water. The sterile seed were inoculated in 100 ml conical flasks containing 50ml ½ strength MS (Murashing and Skoog 1962) medium consisting of 15gr/l sucrose and 0.8% (w/v) agar, the *p*H of the medium was adjusted to 5.8 before autoclaving and the seeds were incubated at 28°C in dark for 7 days.

Shoot regeneration from seedling coteledonary nodal explants

In the preliminary experiments nearly fifteen Indian cotton cultivars were evaluated in tissue culture for response with the explants seedling coteledonary nodal explants (7-12 days old) were tried. Among those cultivars, three best responsive cultivars in tissue culture i e (RAH9750, TCH1569 and MCU11) were selected. Seedling coteledonary nodal explants were excised from aseptically grown seedlings and cut into 5-7 mm size explants placed on the medium containing MSB1 (MS basal salts, B_5 vitamins supplemented with 3% (w/v) glucose with different hormones supplemented with BA 0.5-2.0 mg/l and NAA 0.1 mg/l and Kn 0.5-2.0 mg/l with NAA 0.1 mg/l for initiation,

proliferation and shoot elongation. The viable shoots were sub cultured onto different media i e MSB2 (MS basal salts supplemented with the low concentration of BA (1.5 mg/l) combination with NAA (0.1 mg/l) for shoot elongation. The explants initiation and elongation of shoots data were recorded after 30 days of the inoculation.

Grafting of micro shoots

Among in vitro grown micro shoots, some of them did not develop roots on the MS medium supplemented with different auxins concentration and also MS ½ strength media. Unrooted micro shoots were grafted onto the seedling stocks of the same variety grown in vitro. These seedlings stocks were the healthy normal plantlets with two primary leaves grown from *in vitro* grown seedlings of the age between 8-10 days. The first step was to cut the bottom of the scion into a wedge with a scalpel blade then the upper part of the seedling stocks was cut under the first true leaf; and a slit (about 1.0 cm) on the stem was cut vertically. The decapitated end of the root stocks and matching cut ends of the scions. Then the scion was inserted into the slit and the cambiums were lined up. Final step was to bind the grafted parts together with Parafilm then inculated in the conical flask with medium supplemented with ½ strength MS basal salts with B_5 vitamin and the cultures maintained in an automatically controlled incubation room at 26 ± 2° C under a 16-h (day) / 8-h (night) photoperiod with light provided by cool white fluorescent lamps at an irradiation of 135 µ mol m^{-2} s^{-1} for 30 days. Next step was to remove the fully rooted plant from conical flask then thoroughly washed in running tap water for 5 minutes for remove the media residues then transferred into pot with sterilized soil and sand (1:1) and kept in incubation chamber maintained with temperature of 26 ± 2°C and humidity of 70-80% for another 15 days before being transferred to the greenhouse. It was important to keep proper humidity in the chambers. Then transferred the plantlets to green house for further growth and establishment.

RESULTS AND DISCUSSION

Plant material

In the preliminary experiments we conducted general tissue culture experiments with fifteen Indian local cultivars for screening for the best cultivars for early response in *in vitro* (data not shown) based on the above experiment we are selected three cultivars namely (RAH-9750, TCH-1569 and MCU-11) for further experimental studied.

Shoot induction and multiple shoot induction

Among the different three cultivars tried in tissue culture with the seedling coteledonary nodal as explants of MCU-11 found the best cultivar for shoot initiation and multiple shoot induction. In all the treatments with all cultivars bud proliferation initiation occurred within 5 to 7 day. All media with different hormones effective in inducing sprouting of the existing bud and producing of shoots of each explant. It seems to all the treatments assayed were able to cause shoot formation nearly 79% efficiency. Seedling coteledonary nodal explants were more responsive to BA compared to the other explants. During the first seven days of culture, there was no differences find in response between treatments and the control. Shoot regeneration occurred via adventitious proliferation from the axillary portion. Meristem based regeneration methods have been used successfully in transformation in cotton (Mc-Cabe and Martinell 1993). Rapid shoot growth and multiple shoot induction performed after 4 weeks of culture MS media supplemented with BA (1.5 mg/l) with combination auxin like NAA (0.1 mg/l) concentrations in MCU-11.

Table 1 Effect of different cytokinins on shoot induction and multiple shoots of seedling coteledonary nodal explants of the cultivar MCU-11 after 30 days of culture

Cultivar	Phytohormones mg/l			Explants response	Shoot/explants	Length of shoot (cm)
	BA	Kn	NAA	%	(Mean ± SD)	(Mean ± SD)
MCU11	0.5	0.0	0.1	85	1.02 ± 0.06	2.46 ± 1.06
	1.0	0.0	0.1	90	1.15 ± 0.31	2.44 ± 1.06
	1.5	0.0	0.1	98	6.86 ± 1.02	3.22 ± 1.28
	2.0	0.0	0.1	92	2.33 ± 0.68	2.84 ± 1.29
	0.0	0.5	0.1	60	0.75 ± 0.18	1.40 ± 0.38
	0.0	1.0	0.1	62	0.84 ± 0.13	1.71 ± 0.30
	0.0	1.5	0.1	75	1.06 ± 2.42	2.05 ± 0.48
	0.1	2.0	0.1	86	1.37 ± 0.42	2.40 ± 0.52

(Agarwal *et al*. 1997) was reported similar results in Indian cultivar Ajali. With comparison to BA, the Kn show very slow growth of shoot and some cases stunted growth observed in all the cultivars and also no difference observed in micro shoot height with different concentration of Kn. Low level concentrations of Kn with NAA resulted formation of callus at the cut end of the explants, naturally Kn and NAA were promote cell division and aid in growth, it leads to shoot cause leaf turned to yellow color and fall down finally its leads to die. This problem was observed in all the cultivars. But in MS media supplemented higher concentration of Kn with NAA effect on coteledonary nodal explants was similar in all the cultivars with shoot initiation take place within 3-5 day and did not induce multiple shoot formation at any concentration with any aged explants, it leads to develop single shoot only (Plate 1B). Vigorously growing viable micro shoots transfer for multiple shoot formation observed when elongated shoots were maintained

on MS media supplemented with hormonal concentration of BA (1.5 mg/l) with NAA (0.1 mg/l) and in first and second subculture produced multiple shoots of six to eight per explants (Plate 1C). Multiple shoot induction and mean length of the shoot height observed in (Table 1).

Grafting of micro shoots

The unrooted microshoots were grafted onto in vitro grown seedling (Jinhua and Jean 1999) then inculated in the conical flask with medium supplemented with ½ strength MS basal salts with B_5 vitamin and the cultures maintained in an automatically controlled incubation room at 26 ± 2°C under a 16-h (day) / 8-h (night) photoperiod with light provided by cool white fluorescent lamps at an irradiation of 135 µ mol m^{-2} s^{-1} for 30 days. Well rooted plantlets washed in running top water to remove the medium residues and transferred into pots (1:1 sand: soil) and incubated in incubation chamber for establishing the plant for 15 days with maintained of 70 to 80% humidity and at 27°C temperature, then transfer to green house for further growth and plant establishment. With this technology were able to recovery nearly 90% plants from in vitro regenerated plants (Plate 1E).

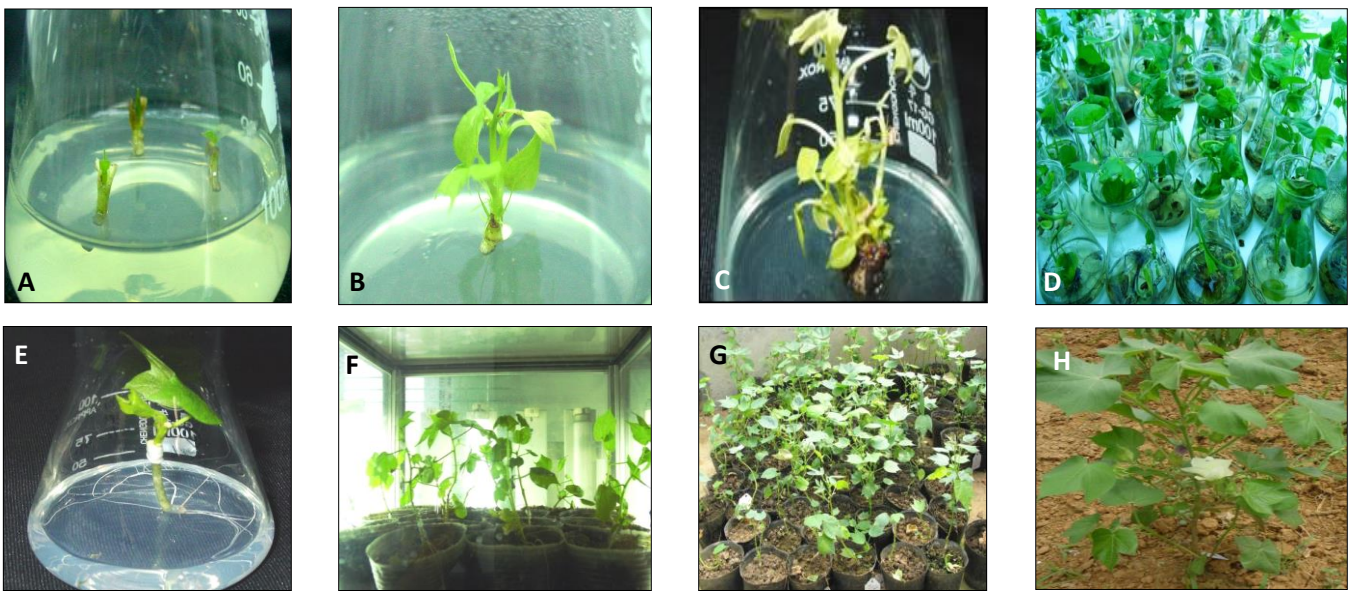

Plate 1 Induction of multiple shoots, plant regeneration and rooting from seedling coteledonary nodal explants of cotton (*Gossypium hirsutum* L.) (A). Coteledonary nodal explants initiation (B). coteledonary nodal explants proliferation (C). Multiple shoots induction (D). *In vitro* rooting of micro shoots (E). *In vitro* grafted plant rooting (F) Plant Acclimatization and establishment in growth chamber (G). Further growth in greenhouse (H). Morphologically normal plants in the field with flowers and bolls

The advantage of genetic engineering techniques for crop improvement for transfer the desirable genes, there is need to develop a reliable, reproducible and efficient regeneration system for cotton. In recent years, there has been a focus in the development of regeneration systems through shoot meristamatic tissues (Hemphill *et al*. 1998, Nasir *et al*. 1999, Sathyavathi *et al*. 2002). Regeneration from the pre existing meristamatic tissues as explants like seedling coteledonary nodal explants, shoot tips, nodal explants, shoot axillary buds explants and shoot meristems were direct and simple, true to type, no somaclonal variations, and no chromosomal aberrations. Theoretically, each excised meristamatic tissue should develop into a rooted plant; it is already programmed tissue for further growth, However, the yield of shoots in vitro from isolated meristamatic tissues depends on the incidence of contamination and rooting efficiency (Gould *et al*. 1991). The induction of multiple shoots in explants varied with hormonal concentration and was also influenced by the age of explants. MS basal medium without BAP or Kn did not support the induction of multiple shoots. In the shoot proliferation and multiple shoot induction BA is the very important cytokinin with single or combination with auxins in many reports (Sathyavathi *et al*. 2002).

We observed in our research low level of BA concentration should promote the explants high rate proliferation and induce the multiple shoot induction where as higher concentrations of BA low level proliferation and single shoot production observed. With the age of 7 days old seedling coteledonary nodal explants yield the maximum multiple shoot induction (6-8 shoots/explants) on MS (BA 1.5mg/l + NAA0.1mg/l) (Plate 1C). Regarding Kn promotes the mostly single shoot proliferation with low and higher level concentrations. Sometimes we observed higher concentration of Kn promotes the proliferate the callus induction at the cut ends of the explants and its shows the stunted growth, the leaf become yellow color and fall down and its leads to die, this may be due to endogenous hormonal regulations. For overcome by this problem finally we adopted in vitro technology to micro shoots. Grafting is a very useful technique and is commonly used in horticultural crops. Un rooted micro shoots, were grafted on to normal in

vitro grown seedlings as previous described method (Jinhua and Jean 1999) in this procedure we absorbed the good rooting efficiency, maximum plants recovery with 90% survival rate (Plate 1E).

Efforts have been made to couple this regeneration procedure with Agrobacterium mediated transformation for rapid introduce of agronomical value added traits like disease resistance, herbicidal resistance and drought resistance directly into high fiber yielding cotton germplasm. These results are now being exploited to other Indian cultivars so that regeneration protocols for economically valuable cotton verities can be made available for cotton genetic transformation. Through this technique we are in progress introducing agrominacally important genes through agrobacterium mediated transformation for qualitatively and quantitatively desirable traits.

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