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BRIEF COMMUNICATION

Multiple shoot induction and plant regeneration from embryo axes of six cultivars of *Gossypium hirsutum*

A.K. BANERJEE*, D.C. AGRAWAL**[1], S.M. NALAWADE**, S. HAZRA** and K.V. KRISHNAMURTHY**

*Horticulture Hall, Iowa State University, Ames, Iowa-50011 USA**
*Plant Tissue Culture Division, National Chemical Laboratory, Pune-411 008, India***

Abstract

The report describes *in vitro* plant regeneration from embryo axis explants of six cultivars of cotton. Induction of a maximum number of multiple shoots in all six cultivars could be achieved on Murashige and Skoog's (MS) salts and Gamborg's (B5) vitamins supplemented with 0.4 μM benzyladenine (BA) and 0.1 μM napthaleneacetic acid (NAA). Elongated shoots could be rooted on half strength medium supplemented with 0.5 μM NAA. Rooted shoots survived (92 %) after hardening in the greenhouse and grew to maturity (100 %) after transfer to field.

Additional key words: benzyladenine, cotton, micropropagation, napthaleneacetic acid.

In vitro plant regeneration in cotton *via* somatic embryogenesis has been reported (Shoemaker *et al.* 1986, Trolinder and Goodin 1987, Gawel and Robacker 1990, Firoozabady and De Boer 1993, Kumar and Pental 1998). However, this approach generated undesirable somaclonal variations (Stelly *et al.* 1989). Also the procedure was genotype dependent and applicable only to a few cotton cultivars. To overcome these limitations, plant regeneration from cotyledonary node (Agrawal *et al.* 1997, Gupta *et al.* 1997, Hemphill *et al.* 1998, Hazra *et al.* 2000) and embryo axis or shoot apex (McCabe and Martinell 1993, Morre *et al.* 1998, Agrawal *et al.* 1998, Zapata *et al.* 1999, Hazra *et al.* 2002) have been reported. However, in most reports on embryo axis derived plant regeneration, only a single shoot per explant was formed. Even though multiple shoot induction in a cotton cultivar Argentine with 13.3 μM BA was reported by Morre *et al.* (1998), the reported methodology could not be adopted to Indian cotton cultivars. Therefore, different BA and combinations of BA and NAA were tested to induce multiple shoots.

The present investigation deals with multiple shoot induction, proliferation and plant regeneration from embryo axes of six important Indian cultivars of cotton.

Delinted seeds of six Indian cultivars of *Gossypium hirsutum* L. (NHH-44, DCH-32, DHY-286, H-8, LRK-516, LRA-5166) were sterilized and germinated aseptically as described earlier (Agrawal *et al.* 1997). Embryo axes were excised from aseptically germinated seeds in the dark for 48 h. The explants (2 mm) were cultured in Petri dishes containing Murashige and Skoog's (MS) salts (Murashige and Skoog 1962) and Gamborg's (B5) vitamins (Gamborg *et al.* 1968) with or without BA (0.2 μM - 13.3 μM) or combinations of BA (0.2 μM - 13.3 μM) and NAA (0.1 μM - 0.2 μM). Sucrose (2 %) and agar (0.65 %) were added to all the media, and the pH of the media was adjusted to 5.8 before autoclaving. Cultures were incubated at 30 °C and irradiance 27 μmol m^{-2} s^{-1}. After incubation for 3 weeks, the elongated shoot apices and roots from explants were excised and discarded. Then the explants were transferred

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Abbreviations: BA - 6-benzyladenine; NAA - α-napthaleneacetic acid; MS medium - Murashige and Skoog's medium.
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[1] Author for correspondence; fax: (+91) 20 589 3438, e-mail: dagrawal@dalton.ncl.res.in

to fresh media 3 times more and incubated for 3, 6 and 10 weeks, respectively. For last 2 transfers, Erlenmeyer flasks containing 30 cm^3 and 100 cm^3 medium, respectively, were used. In the last transfer, medium consisted of MS salts and B5 vitamins was supplemented with 0.4 μM BA and 0.1 μM NAA. At the end, the number of shoots formed per explant was recorded. Thirty explants per treatment were cultured, and the experiment was repeated twice.

For histology, embryo axis explants (2 mm) were fixed in formalin:acetic acid:ethanol (5:5:90) for 48 h. Paraffin embedding of the tissue was carried out. Longitudinal sections of 10 μm thickness were cut, de-waxed and stained with haematoxylin-eosin, mounted with DPX-4 189- [2-chloro-N-(4-methoxy-6-methyl-1,3-5-triazin-2yl)-aminocarbonyl] benzene sulphonamide and were observed under the microscope.

For *in vitro* rooting, elongated shoots (3 - 4 cm in length) were isolated and transferred onto medium containing half strength MS salts and vitamins supplemented with 0.5 μM NAA. Each treatment consisted of 20 shoots. Rooted shoots (60) were hardened and transferred to the greenhouse as described in our earlier report (Agrawal *et al.* 1997). Plants (20) were transferred to the field after 3 weeks of hardening in the greenhouse.

All the media combinations employed (Table 1), including the medium without plant growth regulators supported the development of at least one shoot from the embryo axis explants. The percentage of responding explants (explants forming at least one shoot) after 12 weeks of culture was maximum on the basal medium (MS salts + B5 vitamins) without any phytohormones. With other media combinations with growth regulators, the response varied depending on the concentrations of BA and NAA (Table 1). Addition of BA concentrations higher than 0.2 μM caused decrease in percentage of response in all the cultivars tested. With increasing concentrations of BA, decrease was more pronounced. Combination of 0.4 μM BA and 0.1 μM NAA resulted in higher percentages of response than on media with other BA and NAA concentrations (Table 1).

There was no induction of multiple shoots on the basal medium (MS salts + B5 vitamins) devoid of plant growth regulators. Different concentrations of BA, BA + NAA induced multiple shoot formation in embryo axes varying

Table 1. Effect of BA and NAA [μM] on percentage of responding explants in six cultivars of cotton (data scored after 12 weeks of incubation, experiment repeated twice with thirty replicates).

BA	NAA	NHH-44	DCH- 32	DHY- 286	H-8	LRK-516	LRA- 5166
0.0	0.0	98.3	85.0	85.0	78.3	96.7	80.0
0.2	-	91.7	78.3	71.7	65.0	80.0	70.0
0.4	-	76.7	75.0	63.3	56.7	76.7	60.0
2.2	-	50.0	46.7	53.3	40.0	43.0	36.7
4.4	-	40.0	36.7	36.7	31.7	30.0	35.0
13.3	-	30.0	31.7	20.0	25.0	15.0	18.3
0.2	0.5	76.7	71.7	66.7	63.3	80.0	63.3
0.4	0.1	81.7	81.7	80.0	71.7	78.3	73.3
2.2	0.2	43.3	45.0	43.3	35.0	55.0	33.3
4.4	0.2	35.0	33.3	28.3	26.7	20.0	23.3

Table 2. Effect of BA and NAA [μM] on average number of shoots per responding explant (means ± SD) in embryo axes of six cultivars of cotton. (Data scored after 12 weeks of incubation, experiment repeated twice with thirty replicates).

BA	NAA	NHH-44	DCH- 32	DHY- 286	H-8	LRK-516	LRA- 5166
0.0	0.0	1.90 ± 0.69	1.98 ± 0.62	1.57 ± 0.58	1.41 ± 0.49	1.21 ± 0.58	1.40 ± 0.58
0.2	-	3.38 ± 0.85	3.24 ± 0.85	2.61 ± 0.95	2.02 ± 0.73	2.45 ± 0.74	2.31 ± 0.86
0.4	-	3.96 ± 0.69	4.63 ± 1.09	3.15 ± 0.77	2.85 ± 0.90	2.91 ± 0.85	3.64 ± 1.24
2.2	-	1.54 ± 0.51	1.96 ± 0.64	1.65 ± 0.55	1.42 ± 0.59	1.46 ± 0.51	1.55 ± 0.52
4.4	-	1.16 ± 0.37	1.48 ± 0.51	1.18 ± 0.41	1.16 ± 0.49	1.11 ± 0.33	1.29 ± 0.43
13.3	-	1.00 ± 0.00	1.22 ± 0.45	1.17 ± 0.43	1.06 ± 0.19	1.00 ± 0.00	1.18 ± 0.47
0.2	0.5	4.34 ± 0.89	4.65 ± 0.95	3.12 ± 0.93	2.86 ± 0.90	2.98 ± 0.77	3.28 ± 1.10
0.4	0.1	5.95 ± 1.16	6.04 ± 1.38	4.55 ± 1.25	3.88 ± 1.11	4.44 ± 0.95	4.77 ± 1.51
2.2	0.2	1.80 ± 0.69	2.37 ± 1.05	1.55 ± 0.58	1.47 ± 0.70	1.38 ± 0.49	1.76 ± 0.75
4.4	0.2	1.26 ± 0.42	1.62 ± 0.58	1.29 ± 0.49	1.25 ± 0.48	1.17 ± 0.41	1.21 ± 0.46

from 1 to 6.04 per explant depending upon the cultivar and the medium composition (Table 2). Even though the maximum number of shoots in all six cultivars was observed in the medium containing 0.4 μM BA and 0.1 μM NAA, the number of multiple shoots per explant varied among the six cultivars (Table 2). The highest number of multiple shoot formation was observed in cultivar DCH-32, followed by NHH-44, LRA-5166, DHY-286, LRK-516, and H-8. Further proliferation of these shoots was observed on their transfer to a fresh medium containing 0.4 μM BA and 0.1 μM NAA and on prolonged incubation for 10 weeks. This resulted in more than a two-fold increase in multiple shoots. The highest number of proliferated shoots was recorded in cultivar DCH-32 (15.04) followed by NHH-44 (13.08), DHY-286 (11.12), H-8 (9.72), LRA-5166 (8.04) and LRK-516 (7.84).

It was observed that non-decapitated explants did not form multiple shoots (data not published) and it is the reason for excising and discarding the elongated shoot apices after an initial incubation of explants for 3 weeks. Our earlier experience with cotyledonary nodes (Agrawal *et al.* 1997) also prompted us to decapitate the explants. This approach is in contrast to the findings of an earlier report of Morre *et al.* (1998) on Argentine cotton cultivar, where multiple shoot induction in similar explants could be achieved without decapitation and with the use of 13.3 μM BA alone. The maximum number of multiple shoots formed, however, was restricted to an average of 3.4, and also, the report is limited only to one genotype. In the present study, we found that for Indian cultivars of cotton, decapitation of explants and their culturing on a medium with 0.4 μM BA + 0.1 μM NAA was essential to maximize the induction of multiple shoots. Inclusion of 13.3 μM BA alone in the medium did not induce multiple shoots. The effect of shoot apex decapitation in cotyledonary nodes and plant growth regulators on induction of multiple shoots has been discussed earlier (Agrawal *et al.* 1997).

The elongated shoots rooted on their transfer to the medium containing half strength MS salts and vitamins supplemented with 0.5 μM NAA. The maximum percentage of rooting (90 %) was observed in cultivar DCH-32 and DHY-286 followed by LRK-516 (85 %), NHH-44 (82.50 %), LRA-5166 (80 %) and H-8 (80 %). The rooted shoots survived (92 %) after hardening in the greenhouse and grew to maturity (100 %) after transfer to the field.

Due to ease of regeneration, in dependence from genotype, the embryo axis has become the explant of choice for *in vitro* plant regeneration and for delivering the target genes *via Agrobacterium* (McKently *et al.* 1995) and particle bombardment methods (McCabe and Martinell 1993, Sautter *et al.* 1995). The embryo axis has been used to induce multiple shoots and for development of transgenic plants in peanut (McKently *et al.* 1995).

Histology of the cotton embryo axis in the present study revealed that the apical meristem covered with primordial leaves was overgrown by cotyledonary leaf bases. Therefore, for particle bombardment mediated transformation of cotton embryo axis, it is essential that tissues surrounding the apical meristem be removed so that the dome is directly exposed to the shower of DNA coated particles.

The present investigations have enormous importance since Indian cotton cultivars are recalcitrant under cultural conditions as cotton cultivars elsewhere. The use of the embryo axis as an explant with the present procedure should overcome this limitation and boost the ongoing efforts in genetic transformation of Indian cotton cultivars by microprojectile as well as *Agrobacterium* mediated techniques. The present method circumvents the genotype dependent plant regeneration and the possibility of somaclonal variation usually encountered in plants obtained *via* somatic embryogenesis.

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