BIOLOGIA PLANTARUM 45 (2): 317-319, 2002

BRIEF COMMUNICATION

A rapid and simple method for *in vitro* plant regeneration from split embryo axes of six cultivars of cotton

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Abstract

Plant regeneration was achieved from the seed derived decotyledonated split embryo axes of six Indian cultivars of cotton (*Gossypium hirsutum* L.). Explants were cultured on Murashige and Skoog's basal medium supplemented with 2 % sucrose and 0.65 % agar. Incorporation of 0.25 % charcoal in the medium and incubation of the cultures at 30 ± 2 °C had synergistic effect on the frequency of shoot and root formation. The method employed is genotype independent, simple and rapid.

Additional key words: *Gossypium hirsutum*, micropropagation, tissue culture, activated charcoal.

Most of the cotton cultivars are susceptible to pests and diseases, causing considerable loss in the crop production. Since introgression of resistance traits *via* interspecific hybridization is difficult (Pundir 1972), genetic transformation of cotton has been attempted (Umbeck *et al.* 1987, Firozabady *et al.* 1987, Perlak *et al.* 1990, McCabe and Martinell 1993). *In vitro* regeneration of plants *via de novo* organogenesis or embryogenesis though an essential pre-requisite for genetic improvement of crops involving *Agrobacterium* mediated transformation method has limitations because of low regeneration frequency, chimeras resulting from a group of cells and somaclonal variations. To overcome these limitations, regeneration procedures in cotton have been developed from explants with pre-existing meristems (Gould *et al.* 1991, Zapata *et al.* 1999). Both these reports describe the use of dissection microscope for the explant preparation.

In this communication, we report a simple, rapid plant regeneration protocol using split embryo-axis as explants without the aid of microscope. Influence of charcoal in the basal medium and two incubation temperatures were also examined.

Delinted cotton seeds (*Gossypium hirsutum* L.) of six Indian cultivars (CNH-36, DCH-32, DHY-286, LRA-5166, LRK-516 and NHH-44) were surface sterilized as described in our earlier reports (Hazra *et al.* 2000). The sterilized seeds were then soaked in sterile water for 4 h, thereafter water was decanted and seeds were transferred to 85 mm plastic Petri dishes containing moist, sterile filter paper. The seeds were incubated in dark at 25 ± 2 °C for 24 h. Embryo axes explants were isolated with a mild squeeze of germinated seeds and were split longitudinally along the mid-axis into two equal halves without the use of microscope. These split axes were cultured in pairs in culture tubes containing 20 cm^3 of MS (Murashige and Skoog 1962) basal medium, supplemented with or without 0.25 % activated charcoal. Cultures were divided into two groups and incubated at 25 ± 2 or 30 ± 2 °C under 16-h photoperiod (irradiance of 40 μmol $m^{-2}s^{-1}$). The experiment was repeated thrice. A response in terms of explants forming shoots or shoots and roots and shoot length were recorded after 4 weeks of incubation. Explants that developed shoots and roots were transferred

Received 5 March 2001, *accepted* 8 August 2001.
Acknowledgments: We acknowledge the financial support by the Department of Biotechnology (DBT), Government of India and the award of senior research fellowship to A.K. Banerjee and S.M. Nalawade by the Council of Scientific and Industrial Research, New Delhi.
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to a sand:soil (1:1) mixture in earthen pots. Plantlets were hardened at 25 ± 2 °C in 16-h photoperiod at irradiance of 30 μmol $m^{-2}s^{-1}$ for 6 weeks in greenhouse. Twenty plants of each cultivar were transferred to earthen pots. The statistical analysis, three-way analysis of variance of data was carried out as described by Steel and Torrie (1987).

Two types of responses were noted: *1*) explants formed both shoots and roots which indicates that both the root and shoot meristems were present in these explants either partially or fully and were regenerative; *2*) explants formed only either shoot or root indicates that the incision was oblique. Eventually only those explants which formed shoots (with or without roots) were used for establishment of shoot cultures or plants.

Incorporation of charcoal in MS basal medium and incubation of explants at 25 ± 2 °C induced formation of roots and shoots simultaneously at higher percentages than on MS basal medium alone in all genotypes except DCH-32 (Table 1). However, the overall frequency of shoot formation in explants in medium with charcoal was lower than without charcoal due to the decrease in the number of explants forming only shoots. The total number of responding explants was slightly higher at 30 ± 2 °C compared to 25 ± 2 °C in MS medium with or without charcoal (Table 1) in all the cultivars (except DCH-32 and NHH 44 in MS medium alone).

Table 1. Effect of 0.25 % activated charcoal (Char) and incubation temperature on frequency of shoot/root formation in split embryo axis of six cotton cultivars on MS medium. Data scored after four weeks of incubation.

Cultivars	Media	Incubation at 25 °C			Incubation at 30 °C		
		number of explants	explants forming shoots [%]	explants forming shoots and roots [%]	number of explants	explants forming shoots [%]	explants forming shoots and roots [%]
CNH-36	MS	119	17	37	66	6	45
	MS+Char	42	0	57	95	4	66
DCH-32	MS	60	16	53	60	0	54
	MS+Char	62	0	37	76	0	68
DHY-286	MS	120	14	32	67	9	40
	MS+Char	40	0	44	112	3	53
LRA-5166	MS	59	27	27	60	4	50
	MS+Char	58	0	31	108	0	53
LRK-516	MS	130	22	35	64	9	53
	MS+Char	124	3	37	138	0	54
NHH-44	MS	119	17	56	60	5	48
	MS+Char	40	0	62	115	6	56

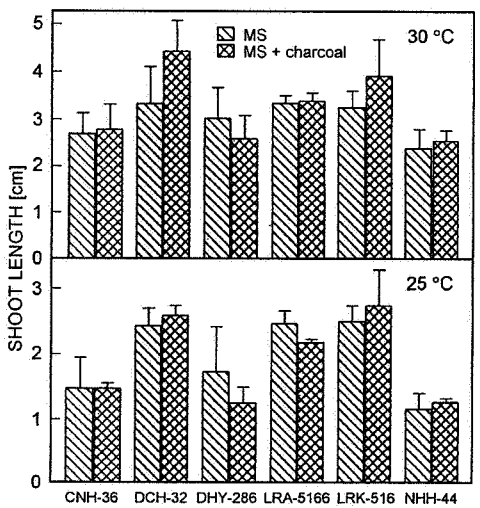

Fig. 1. Effect of charcoal and incubation temperature on elongation of shoots.

Shoot length was measured with a scale to assess the vigor of the plantlets. It was observed that in general, incubation of explants at 30 ± 2 °C and incorporation of charcoal in the medium had positive influence on shoot length (Fig. 1). The beneficial effects of charcoal in plant tissue cultures have been reported earlier (Fridbourg *et al.* 1978, Wann *et al.* 1997, Madhusudhanan and Rahiman 2000). To the best of our knowledge, the synergistic effect of charcoal with increase in incubation temperature has not been reported earlier.

Plants raised from the split embryo axes survived in soil in low frequency of 30 - 35 %. On transfer of 120 plants (20 of each genotype) to earthen pots with sand+soil mixture (ratio 1:1), the plants were thin and crooked. This was probably due to the partial stem development in the split axis. After three months in greenhouse, these plants became erect and normal. Thirty-five plants raised by this method survived in the field and formed bolls on maturation.

The protocol involving the split embryo axis, described in this report is simple, fast and reliable as the natural morphogenetic ability of the mature zygotic embryo axis is exploited. Activated charcoal in the medium and incubation of cultures at high temperature have synergetic effect on improvement of regeneration potential of the split zygotic embryo axes.

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