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Multiple Shoot Regeneration Response of Recalcitrant Cotton (*Gossypism hirsutum* L.) Cultivar CIM-443

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Abstract: Induction of multiple shoots in *Gossypium hirsutum* L. variety CIM-443 has been achieved by using meristem and cotyledonary node as explants. Meristems of size 3-4 mm were excised from embryos isolated from seeds while cotyledonary nodes were cut from seven days old seedling and the mean shoot number per explant response for total of three experiments was maximum i.e., (6.72±0.79) and (4.92±0.67), respectively for both explants in MS medium supplemented with benzyl-amino purine (BAP) 1.0 mg L^{-1} + naphthalene acetic acid (NAA) 0.05 mg L^{-1}. Shoot elongation was observed in MS medium amended with NAA 0.1 mg L^{-1}. The percentage of shoots forming roots was maximum (79.16) in case of ½ MS supplemented with NAA 0.05 mg L^{-1}. Rooted plantlets hardened in soil and normal boll formation observed.

Key words: *Gossypium hirsutum* Cv. CIM-443, micro-propagation, multiple shoot regeneration

INTRODUCTION

Cotton is the world's most important fiber crop and most valuable oil seed crop. The crop is damaged by many insects and pests thus making it an important candidate for genetic manipulation. *In vitro* regeneration is necessary prerequisite for genetic manipulations but in cotton limited to only a few cultivars possibly due to fact that tissue culture responses were genotype dependent[1]. Cotton plants have proved to be difficult to manipulate in tissue culture[2].

Many species of *Gossypium hirsutim* (L) grow into complete plants in simple MS basal medium by using different explants especially embryos and meristematic nodes[3,4]. But for shoot multiplication purpose hormonal manipulations are considered very critical[5,6]. Meristernatic shoot tip and bud cultures derived plants are phenotypically homogenous there by indicating genetic stability[5,7]. In this context there in need to develop a micropropagation protocol resulting in very little gross variability among regenerated plants. This paper reports a micropropagation protocol to produce genetically stable plants of high yielding and virus resistant *Gossypium hirsutum* variety CIM-443.

MATERIALS AND METHODS

Sterilization and explant preparation: Seeds of *Gossypium hirsutum* cultivar CIM-443 obtained from Central Cotton Research Institute, Multan. These seeds were delinted with concentrated sulphuric acid @ 10.0 ml for 100.0 g of seeds and subjected to five washings with distilled water containing few drops of tween-20. Under aseptic conditions these seeds were surface sterilized with 0.1% mercuric chloride +0.1% SDS (sodium dodecyl sulphate). The seeds were rinsed 5 times with autoclaved distilled water and incubated on soaked filter paper in petriplate. All the processes were carried out under strict aseptic conditions and then seeds were incubated in dark for 72 h at 37°C.

Mature embryos were isolated from incubated seeds after the given time interval and 3-4 mm epicotyl (meristematic) portion was excised. These mesistems were subjected to designed media with the base in the medium under aseptic conditions for induction of multiple shoots. Cotyledonary nodes of size 2-3 mm were detached from seedlings that were grown for one week on MS basal medium[8] supplemented with B5 vitamins, glucose, 3.0%; $MgCl_2$, 0.075%; myoinsitol 0.01%; phytagel, 0.3% with out any amendment of phytohormones. After excision, cotyledonary nodal explants were cultured on designated media with hypocotyl portion in the medium for induction of multiple shoots.

Media for *in vitro* micropropagation and hardening of plants: Explants were subjected to media designated as MS_{0-10}, amended with B5 vitamins along variable concentrations of growth regulators in different combinations including: MS_0, devoid of any growth

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regulator; MS_{1-3}, containing sole BAP (1.0-2.0 mg L^{-1}); MS_{4-7}, supplemented with BAP (1.0-1.5 mg L^{-1}) and NAA (0.05-0.1 mg L^{-1}). Apical meristem and axillary node explants were cultured on these media compositions for induction of multiple shoots. In the multiplication stage, each explant expended and micro shoots were produced from a basal mass. Percentage explant response was calculated by formula:

$$\text{Explant response (\%)} = \frac{\text{Total number of explants representing multiple shoot regeneration}}{\text{Total number of explants cultured}} \times 100$$

The word multiple shoot regeneration was used when the explant regenerated more than two shoots. Micro shoots of 2-3 cm size were cut from the explant and cultured on MS basal medium amended with gibberellic acid (GA) 0.1 mg L^{-1} for elongation. Micro shoots when attained suitable length 5-6 cm in culture vessel then transferred to half strength MS basal medium amended with NAA (0.05-0.10 mg L^{-1}) for rooting. After three weeks rooted shoots were removed from the medium and washed thoroughly the root portion to remove the potential source of contamination before transferring to pot soil containing soil, sand and peat moss 1:1:1 ratio. Plants were covered with polythene bags and placed under green house conditions with 16 h photoperiod and 30±2°C temperature. Plants were uncovered on weekly basis till these were become compatible to natural environment.

RESULTS

Meristem and cotyledonary node explants were subjected to various tissue culture media designed as MS_{0-10} (Table 1). MS_0 medium is devoid of any plant phytohormone and served as control for other media containing different concentrations of cytokinins (BAP, kinetin) and auxin (NAA). MS basal medium did not support the induction of multiple shoots however response of both explants was found to be 100% (Table 1). In the MS basal medium as the concentration of sole BAP gradually increased from 1.0- 2.0 mg L^{-1} in media (MS_{1-3}) then rate of multiple shoot response was also increased and found to be maximum i.e., (2.75 shoots per explant) for 2.0 mg L^{-1} concentration of BAP in case of meristerm while (3.08 shoots per explant) for 1.5 mg L^{-1} concentration of BAP when cotyledonary node was used as explant (Table 1). Multiple shoot response was further increased for both explants when kinetin was amended along BAP in media (MS_{5-7}) (Table 1) in balance concentration from 1.0-2.0 mg L^{-1}. Maximum shoot multiplication response (3.87 shoots per meristem explant) (Fig. 1A) was induced by 1.5 mg L^{-1} concentration of both cytokinin however this was further increased to 4.09 shoots per cotyledonary explant (Fig. 1B) when both cytokinins were added in 1.0 mg L^{-1} concentration. At low concentration of both cytokinins BAP (1.0 mg L^{-1}) and kinetin (0.5 mg L^{-1}) in case of MS_4 medium, multiple shoot response for meristem and cotyledonary node explant was found to be 3.32 and 3.88%, respectively (Fig. 1A). Explant response in MS_4 medium was 100% for both explants however this explant response was declined to 60 and 76% for meristem and cotyledonary node explants when both cytokinins in MS_7 medium were used in high concentration i.e 2.0 mg L^{-1}.

Table 1: Effect of phytohormones on shoot induction of meristem and cotyledonary node explants of cotton cultivar (CIM 443)

Medium code	Phytohormones (mg L^{-1})	Meristem explant response (%)	Cotyledonary node explant response (%)
MS_0	0.00 +0.00	100	100
MS_1	BAP 1.0	100	100
MS_2	BAP 1.5	100	100
MS_3	BAP 2.0	80	92
MS_4	BAP 1.0+Kin 0.50	100	100
MS_5	BAP 1.0+Kin 1.0	92	88
MS_6	BAP 1.5+Kin 1.5	64	96
MS_7	BAP 2.0+Kin 2.0	60	76
MS_8	BAP 1.0+NAA0.05	100	100
MS_9	BAP 1.0+NAA 0.1	72	76
MS_{10}	BAP 1.5+NAA 0.1	80	72

Table 2: The effect of NAA on rooting of *in vitro* derived shoots of the cotton cultivar (CIM 443) after 25 days of culturing

Medium	Number of shoots rooted	Rooting (%)
½ MS	28.33±0.62	70.83
1/2MS+NAA(0.05 mg L^{-1})	31.66±0.57	79.16
1/2MS+NAA(0.10 mg L^{-1})	18.33±0.50	45.83

Among all the tested media maximum shoot multiplication response (6.72 and 4.92 per meristem and cotyledonary explant, respectively) was observed in MS_8 medium where combination of BAP (1.0 mg L^{-1}) and NAA (0.05 mg L^{-1}) was used (Table 1). In this medium both explants response was 100%. No further increase in multiple shoot response per explant and percentage explant response could be observed with an increase in concentrations of BAP and NAA in media (MS_9 and MS_{10}) (Table 1). The multiple shoots obtained on various shoot multiplication media were transferred to shoot elongation medium containing MS basal medium supplemented with gibberellic acid (0.1 mg L^{-1}) (Fig. 1B).

The micro shoots of 4-5 cm length were then transferred to different rooting media containing different concentrations of auxins and half strength MS basal medium (½ MS) (Table 2; Fig.1C). ½ MS supplemented

Fig. 1: *In vitro* induction of multiple shoots and plant regeneration in cotton
A: Induction of multiple shoots B: Elongated multiple shoots C: Rooted shoots (3-4 cm)
D: Hardened plants in soil E: Plant showing normal bool formation

with NAA (0.05 mg L^{-1}) showed maximum rooting (79.16%) however further increase in concentration of NAA (1.0 mg L^{-1}) decreased the rooting (45.83%) as well as number of rooting shoots from 31.66 to 18.33 (Table 2). MS alone resulted in 70.83% rooting and number of shoots rooted on this medium were found to be 28.33 (Table 2).

Rooted shoots were transferred to pots containing soil, sand and peat moss (1:1:1) under green house condition. Survival rate of plants in soil was 80% after two months. The survived plants were successfully established in soil (Fig. 1D) and normal boll formation was observed (Fig. 1E) in all the established plants.

DISCUSSION

The results of present study describe a simple micropropagation procedure of recalcitrant cotton (*Gossypium hirsutum* L) cultivar CIM-443 which despite of high yielding is also virus resistant. Cotyledonary nodes and meristem explants were subjected to various tissue culture media amended with different concentrations of cytokinins and auxins. Hormonal amendments are necessary because developmental capacity of meristem is due to subjacent leaf and stem tissues while for cotyledonary node growth reduction in attributed to detached apical meristem and cotyledons. It is therefore unlikely that the meristem and cotyledonary node explants have sufficient cytokinin to support growth and development with out hormonal amendment in culture media. The significant role of phytohormones was also documented by other workers in regeneration studies of various species of cotton including Gossypium herbaceum[9], *G. hirsutum*[10]. Cytokinins played a predominant role in multiple shoot regeneration. The effect is even more pronounced when two types of cytokinins (BAP and kinetin) were used in combination. Thus synergistic effect of two cytokinins leads to enhanced shoot regeneration as reported by Jayasree *et al.*[11] in potato. However, correct concentration of combination of growth regulators in necessary because unfavourable concentration may inhibit the growth of cellular mass as reported by Moore[12].

Multiple shoot regeneration response for both meristem and cotyledonary node explants was increased tremendously when BAP (1.0 mg L^{-1}) was used along NAA (0.05 mg L^{-1}). However, further increase in concentration of cytokinin and auxin resulted in no any further increase in shoot multiplication response from 6.72 shoots per meristem explant and 4.92 shoots per cotyledonary node explant. This is possibly due to the reason that normal development of somatic tissue required a fine temporal and spatial regulation of cell division, enlargement and differentiation that could be achieved by correct concentration of cytokinin to auxin as also reported by Ammirato[13]. High multiple shoot regeneration response is also due to the reason that supplementation of cytokinins during the histodifferentiation phase can be compensated for the detrimental effects of auxins on the meristematic tissue development. This observation was in agreement with Merkle[14] who proposed the same fact in Magnoliaceae.

In conclusion present study developed a simple and efficient condition for micropropagation that may results in production of genetically stable plants by using meristems and cotyledonary node as explants. As establishment of definite regeneration condition is necessary perquisite for genetic improvement of plants therefore this procedure may be helpful for genetic improvement of *Gossypium hirsutum* cultivar CIM-443 with very low gross abnormalities

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