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Full Length Research Paper

Multiple shoot regeneration of cotton (*Gossypium hirsutum* L.) via shoot apex culture system

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Induction of multiple shoots of cotton (*Gossypium hirsutum* L.) plant in two commercial varieties (Sahel and Varamin) using shoot apex was done. Explants were isolated from 3 - 4 days old seedlings, then they were cultured on a shoot induction media, modified MS nutrient agar with combinations: 1- Kinetin (0.0, 0.5, 1.0, 1.5 mg l^{-1}) and 6-benzyaminopurine (BAP) (0.0, 0.1, 0.5, 1.0, 1.5 mg l^{-1}), 2- α-naphthaleneacetic acid (NAA) (0.0, 0.1, 1.0 mg l^{-1}) and BAP (0.0, 0.1, 0.5, 1.0, 1.5 mg l^{-1}). After 1.5 - 2 months, multiple shoot induction medium with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BAP resulted in the highest number of regenerated shoots per explant (3 - 4 shoots per explant) for the two cultivars. Elongation of multiple shoots was obtained on modified MS nutrient agar without any phytohormone. *In vitro* shoots were rooted on half-strength agar-solidified MS basal medium supplied with 0.1 mg l^{-1} lidole-3-butyric acid (IBA) and MS vitamins.

Key words: Cotton, Shoot apex, multiple shoot.

INTRODUCTION

Cotton (*Gossypium* spp.) is an excellent natural source of textile fiber and is cultivated in many countries. It is a crop of significant value throughout the world because it is not only a source of natural fiber, but also an oilseed crop. Because of its high economic importance, considerable attention has been paid to improving cotton plants by conventional plant breeding methods (Agrawal et al., 1997). Although this method (interspecific hybridization) has been difficult (Pndir, 1972), advances have been made by using biotechnological techniques such as ovule culture (McStewart and Hsu, 1977; Thengane et al., 1986; Kim and Triplett., 2001; Lee et al 2007), protoplast culture (Peeter et al., 1994), somatic embryogenesis (Shoemker et al., 1986; Trolinder and Goodin, 1987; Finer, 1988; Rao et al., 2006), shoot apex culture and genetic transformation (Firoozabadi et al., 1987; Umbeck et al., 1987; Finer and Mc Mullen, 1990; McCabe and Martinell, 1993; Abdellatef and Khalafalla, 2007). Tissue culture is a major prerequisite for the production of transformed plants.

Cotton crop has been difficult to manipulate with high efficiency, since the tissue culture method used for regenerating transgenic plants was by indirect transformation via callus. As only Coker variety was found to respond better for gene transfer, most of the desirable genes are introduced initially into Coker and back crossed into other varieties later (Satyavathi et al., 2002). Several years of backcrossing and selection are required to identify lines suitable for commercialization (Satyavathi et al., 2002). Aside from genotype limitation, many plants regenerated from cotton callus have exhibited extensive phenotypic abnormalities and cytogenetic changes (Stelly et al., 1985; Li et al., 1989). Callus induced genetic damage is observed commonly among plants regenerated (Stelly et al., 1985; Li et al., 1989). Therefore, development of tis-sue culture protocols to induce efficient proliferation in a genotype independent manner is desirable for genetic transformation of cotton.

Compared with somatic cell culture, shoot apex culture is an easier method to obtain regenerative plants (Zhang et al., 2000). Theoretically, the advantage of the shoot apex explant over other regeneration systems is that plants may be obtained from any genotype (Zapata et al., 1999a; Zapata et al., 1999b). Shoot meristem and apex

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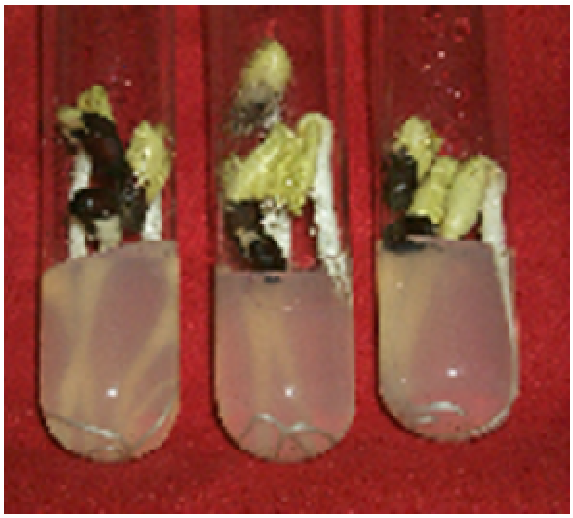

Figure 1. 4 - 5 days old cotton seedlings germinated on MS basal medium.

cultures became popular in the ornamental nursery industry after the discovery that rapidly growing shoots of many viruses infected clones could be free of virus and used to produce virus-free germplasm (Gould et al., 1998; Morel and Martin, 1952). Over time it was observed that the incidence of genetic mutations and somaclonal variations were low in plants regenerated from shoots. One of the reasons for this low mutation frequency may be the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures (Hirochika, 1993).

Earlier studies on cotton and kenaf shoot apex regeneration established a method to obtain a single shoot from a shoot meristem explant (Zapata et al., 1999a). In previous studies, transformation frequencies of a cotton shoot apex explant producing a single shoot were low (Zapata et al., 1999b). A shoot apex explant developing multiple shoots directly without an intervaling callus stage would provide multiple targets for transformation yet maintain the genotype fidelity that can be lost with shoots arising from callus (Srivatanakul et al., 2000). Recently, Satyavathi et al. (2002) demonstrated that shoot apices derived from 3 - 5 days old cotton seedlings (in three varieties) yielded multiple shoots when cultured on MS medium plus BAP and NAA (0.1 mg L^{-1} each). In the present study, a rapid micropropagation protocol in Sahel and Varamin cotton cultivars, which are highly yielding *G. hirsutum* varieties, is examined.

MATERIALS AND METHODS

Plant materials

Seeds of two commercial varieties (Sahel and Varamin) were obtained from cotton research institute of Varamin, Iran. Seeds were sterilized by soaking in 15% (w/v) solution of $HgCl_2$ for 2 min and washed subsequently at least tree times with sterile double-distilled water, following by 10 s keeping in 90% Ethanol. Three seeds per test tubes were germinated on 10 ml of MS (Murashige and Skoog, 1962) basal medium consisting of 30 g l^{-1} sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. Seeds were maintained at room temperature (25 ± 2 ºC) at 16 h photoperiod (60 - 80 μ mol m^{-2} s^{-1}) for 3 - 4 days (Figure 1).

Shoot apex isolation

Shoot apices were isolated from 3 - 5 days old seedlings with the aid of a dissecting microscope as described by Zapata et al. (1999a). One cotyledon was removed by pushing down on it until it snaps off to expose shoot apex, then another cotyledon was removed, shoot apex was excised from hypocotyls by cutting at the base of it. The unexpanded and primordial leaves were left in place to supply hormones and other growth factors (Smith and Murashige, 1982; Shabde and Murashige, 1977) (Figure 2).

Plant media for multiple shoot induction

Shoot apices of the two cultivars were placed on MS basal medium supplemented with 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl, 3% sucrose (Zapata et al., 1999b) and different combinations of plant growth regulators as follows.: 1**-** Kinetin (0, 0.50, 1.00, 1.50 mg l^{-1}) and 6-benzyaminopurine (BAP) (0, 0.1, 0.50, 1.00, 1.50 mg l^{-1}), 2**-** α-naphthaleneacetic acid (NAA) (0.0, 0.1, 1.0 mg l^{-1}) and BAP (0, 0.1, 0.5, 1.0, 1/50 mg l^{-1}). The pH of the medium was adjusted to 5.8 before autoclaving, and all media were solidified with 2 g l^{-1} phytagel. Seeds and all *in vitro* plant materials were incubated at 25 ± 2°C under a l6 h photoperiod. Light was provided by cool white fluorescent lamps with an intensity of 60 μE m^{-2} s^{-1} (Zapata et al., 1999b). After 6 weeks, the responses of plant regeneration were recorded (shoot apices) that were able to initiate a shoot or clumps. The percentage of regenerated plants was determined for each cultivar. The elongated shoots (3 – 4 cm) were transferred to culture tubes containing the same initial medium but half strength MS medium supplemented with 0.1 mg l^{-1} indole-3-butyric acid (IBA) for rooting (Mohammadi-Bazargani et al., 2004; Mohammadi-Bazargani et al., 2009).

RESULTS AND DISCUSSION

The number of shoots regenerated from isolated shoot apices of two cotton cultivars in 27 different media is shown in Tables 1 to 4. Medium without growth regulators did not support the induction of multiple shoots. The percentages of explants responding on this medium were 90% (18/20) and 95% (19/20) for Sahel and Varamin, respectively, and one shoot per responding explant was regenerated (Figure 3). The medium including BAP alone did not induce multiple shoot formation at any concentration and only abnormal seedlings were regenerated from that in 0.1 and 0.5 mg l^{-1} concentration (Figure 4a, Tables 1 and 3). High concentration of BAP (1 - 1.5 mg l^{-1}) resulted in callus formation from the shoot apex. Similar to the report of Zapata et al. (1999a), multiple shoots failed to be stimulated from the kenaf and cotton shoot apex using

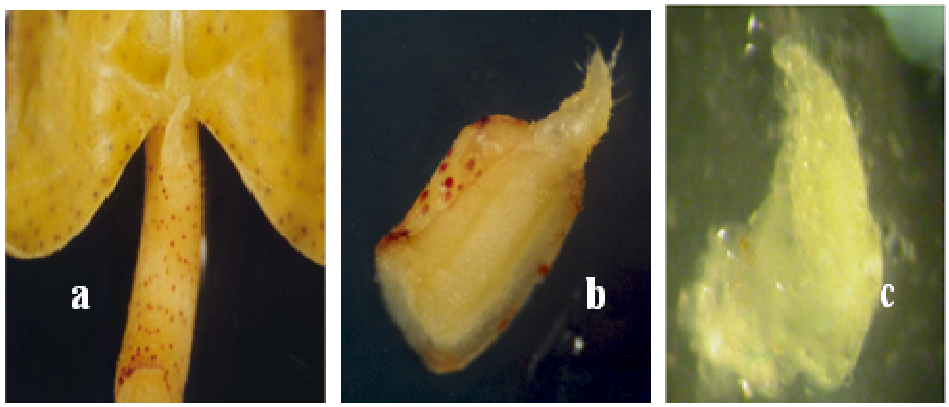

Figure 2. Shoot apex isolation scheme for cotton (*Gossypium hirsutum* L.). **a**- One cotyledon was removed by pushing down until it snaps off to expose shoot apex. **b-** Another cotyledon was removed. **c-** Cut shoot apex and remove from seedling.

Table 1. Comparison of the effect of benzyaminopurine (BAP) on percent shoot response and frequency of shoots per explant (given in parentheses) in shoot tip culture of cotton (Varamin) when used alone or in combination with naphthalene acetic acid (NAA).

BAP (mg l^{-1})	NAA (mg l^{-1})		
	0	0.1	1
0	95% (1)	86% (1)	80% (1)
0.1	33% (1)	65% (3)	52% (2)
0.5	10% (1)	42% (1)	64% (1)
1	0	40% (1)	43% (1)
1.5	0	0	0

1 = Single shoot; 2 = two shoots; and 3 = three or (four) shoots per explant.

Table 2. Comparison of the effect of benzyaminopurine (BAP) on percent shoots response and frequency of shoots per explant (given in parentheses) in shoot tip culture of cotton (Varamin) when used alone or in combination with cytokinin (kinetin).

BAP (mg l^{-1})	Kin (mg l^{-1})		
	0.5	1	1.5
0.1	63% (1)	55% (1)	44% (1)
0.5	40% (2)	23% (2)	11% (1)
1	5% (1)	0	0
1.5	0	0	0

1 = Single shoot; and 2 = two shoots per explant.

Table 3. Comparison of the effect of benzyaminopurine (BAP) on percent shoots response and frequency of shoots per explant (given in parentheses) in shoot tip culture of cotton (Sahel) when used alone or in combination with nphthaleneacetic acid (NAA).

BAP (mg l^{-1})	NAA (mg l^{-1})		
	0	0.1	1
0	90% (1)	73% (1)	70% (1)
0.1	29% (1)	61% (3)	49% (2)
0.5	0	40% (2)	50% (1)
1	0	36% (1)	39% (1)
1.5	0	0	0

1 = Single shoot; 2 = two shoots; and 3 = three or (four) shoots per explant.

Table 4. Comparison of the effect of benzyaminopurine (BAP) on percent shoots response and frequency of shoots per explant (given in parentheses) in shoot tip culture of cotton (Sahel) when used alone or in combination with cytokinin (kinetin).

BAP (mg l^{-1})	Kin (mg l^{-1})		
	0.5	1	1.5
0.1	59% (1)	47% (1)	41% (1)
0.5	40% (2)	21% (2)	6% (1)
1	17% (1)	3% (1)	0
1.5	0	0	0

1 = Single shoot; and 2 = two shoots per explant.

BAP.

The percentages of responding explants on medium containing only 0.1 - 1.0 mg l^{-1} NAA were 80 - 86% and 70 -73% for Varamin and Sahel, respectively, which compared to growth regulator-free medium was not obviously lower in the number of regenerated plants . Therefore, NAA might have no inhibiting effect on the explants for single shoot regeneration (Tables 1 and 3).

The medium supplemented with kinetin alone at concentration of 0.5, 1.0, 1.5 mg l^{-1} support the induction of single shoot regeneration (44, 55, 62%, respectively, for

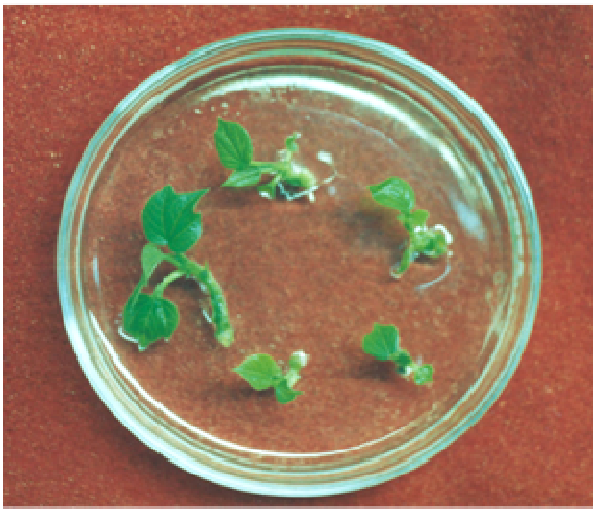

Figure 3. Cotton shoots obtained from medium with no plant growth regulator (after 3 - 4 weeks).

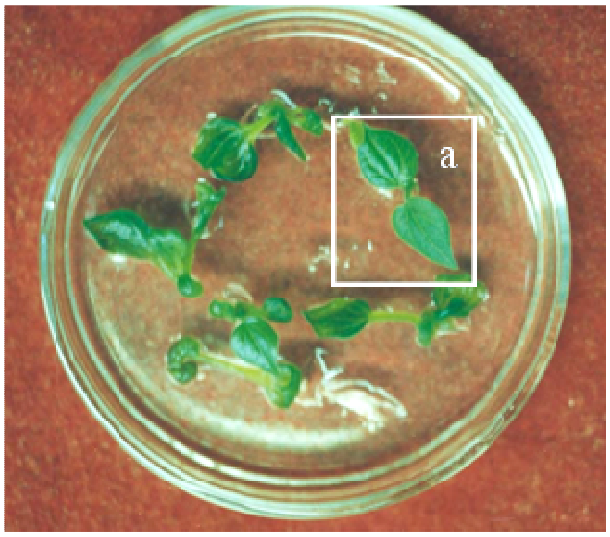

Figure 4. Explants at lower concentration BAP (0.1 – 0.5 mg l^{-1}) produced abnormal cotton seedlings. **a)** showed explants responding (percentages low).

Varamin explants and 41, 47, 59%, respectively, for Sahel explants), which were lower than that of growth regulator-free medium. These results were in agreement with Agrawal et al. (1997). Although increasing the concentration of kinetin diminished normal plantlets, the negative effects were not as bad as that of BAP. MS medium supplemented with BAP and kinetin (0.5 mg l^{-1} for each and 0.5 mg l^{-1} BAP + 1 mg l^{-1} Kin) induced low percentages of two shoots per explant in both cultivars (Figure 6). Higher concentration of the two cytokinins (BAP+Kin), induced abnormal seedling production and exhibited browning of explants as characterized by hormonal stress (Figure 5). Because the combined

Figure 5. Cotton explant at higher (BAP + Kin) concentration exhibited browning as characterized by hormonal stress.

synergistic effect of the two cytokinins intensifies the cell division, it seems the higher concentration of the hormones inflicts a hormonal stress in explants. Therefore, low concentration of cytokinin/cytokinin was found to be more effective than high concentration for shoot induction (Tables 2 and 4) which was in agreement with Satyavathi et al. (2002).

Using combinations of BAP and NAA in the medium (0.1 mg l^{-1} for each) resulted in 3 shoot regeneration at the frequencies of 65 and 63% for Varamin and Sahel, respectively (Figure 7). It should be noted that four shoots were regenerated in a few explants, which was desirable compared to that reported by Satyavathi et al. (2002). At concentration of 0.1 mg l^{-1} BAP + 1.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA about half of explants produced two shoots per explants (Figure 8). So multiple shoot induction medium with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BAP resulted in the highest number of regenerated shoots per explant (3 - 4 shoots per explant) for two tested cultivars (Sahel and Varamin) and was found to be optimum for inducing shoots. Shoots were rooted on half-strength agar-solidified MS basal medium supplied with 0.1 mg l-1 Iidole-3-butyric acid (IBA) and MS vitamins (Figure 9).

It is generally believed that hormonal balance is a key factor in regulating morphogenesis in explants (Satyavathi et al., 2002; Ebrahimi et al., 2006). The balance and ratios of hormones present is what helps to influence plant reactions. The hormonal balance possibly regulates enzymatic reactions in the plant by amplifying them, leading to the results the grower wants to see. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and

Figure 6. Two cotton shoots per explants formed on the induction medium with hormonal combination (0.5 mg l^{-1} BAP + 1 mg l^{-1} Kin)

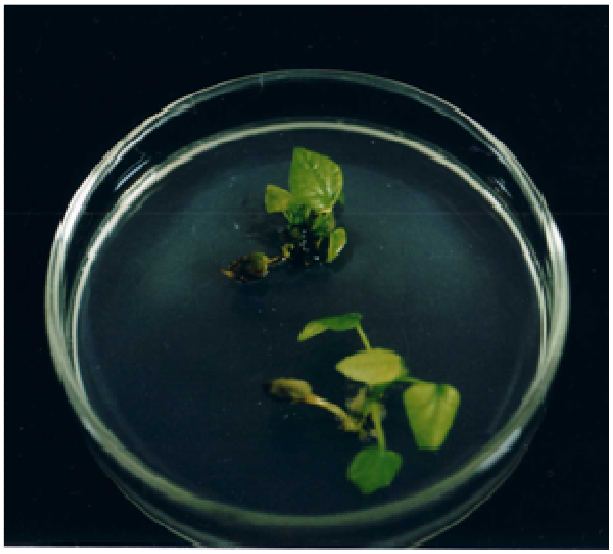

Figure 8. Two–three shoots per cotton explant formed on the induction medium with hormonal combination (0.1 mg l^{-1}BAP + 1 mg l^{-1}NAA) and (0.5 mg l^{-1}BAP + 0.1 mg l^{-1} NAA).

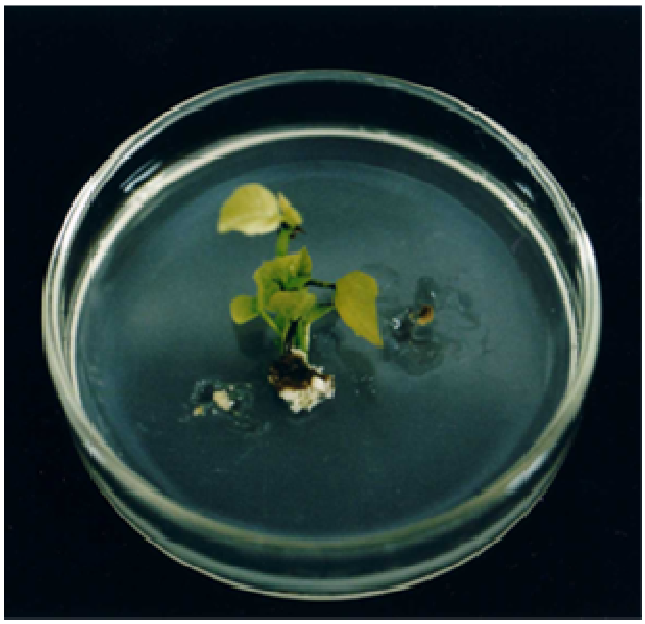

Figure 7. A multiple cotton shoot clumps (three shoots per explant) formed on the induction medium with combinations of BAP and NAA, at concentration 0.1 mg l^{-1} of the two hormones.

cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue. Since hormone balance is presumably important to the overall effect on growth and morphological changes. Our result indicated that the formation of multiple shoot will be promoted by a balance of cytokinin and auxin.

Results of this study indicated that suitable combination of auxins and cytokinins are important for multiple shoot regeneration of cotton from shoot apex. Similar observations were made by earlier authors for shoot meristem culture in cotton (Zapata et al., 1999a; Hemiphill et al., 1998; Gould et al., 1991; Satyavathi et al., 2002), and in Cumin (Ebrahimi et al., 2006), but compared to the values obtained by Satyavathi et al. (2002), these were slightly lower percentages. The methods which have been practiced here revealed some advantages in comparison with others: 1- Multiple shoot regeneration from each explant, shoot apex explant used contain essentially apical meristem and two axillary meristems giving rise to three or more shoots if cultured on appropriate medium. 2- The advantages of shoot tip culture over other regeneration systems are many fold.

Shoot regeneration from shoot apex is direct, relatively simple and needs less time to regenerate large number of plants (Nasir et al., 1997). Plants regenerated from shoot apices are true to phenotype with low incidence of somaclonal variation and chromosomal abnormalities (Bajaj, 1998). One of the major draw backs of the earlier reports of *Agrobacterium* mediated transformation of cotton is that only limited cultivars can be regenerated into fertile plants through somatic embryogenesis (McCabe et al., 1998). However, in this study, genotype seems to have not much effect on shoot induction and shoot prolixferation. This is evident from the similar response of the two varieties on different hormonal concentrations studied. Gould et al. (1991) also reported genotype independent regeneration using similar type of explant but reported no multiple shoots. Shoot apex explant has few genotype limitations and considered as more appropriate because meristematic cells are programmed for direct

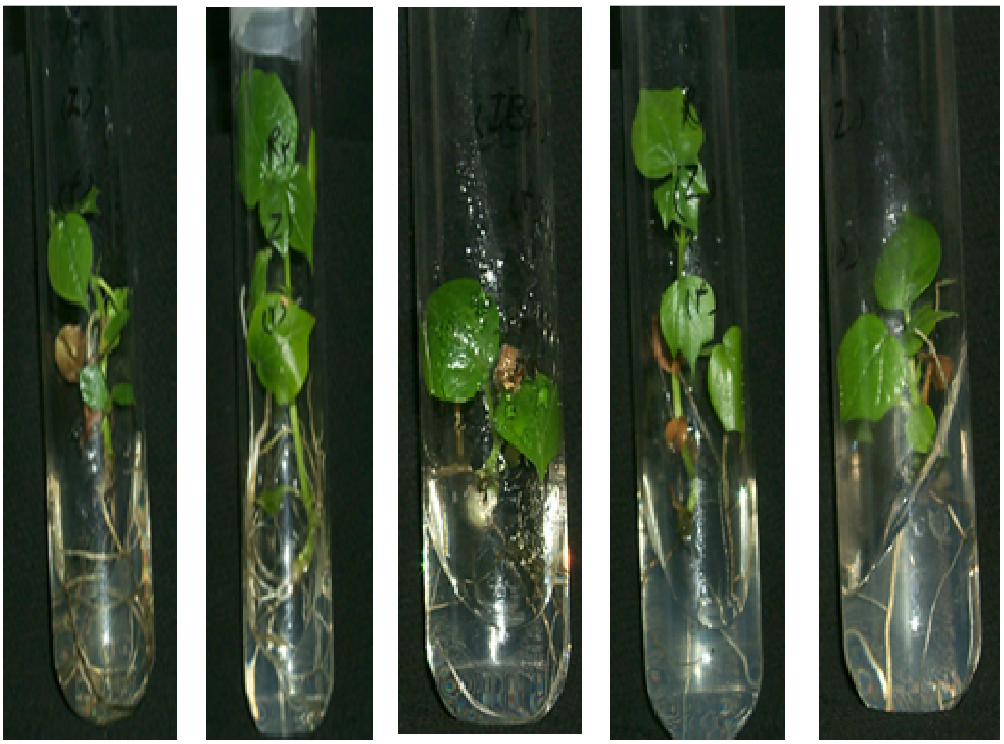

Figure 9. Elongated cotton shoots were induced to form roots on half strength MS medium supplemented with 0.1 mg l^{-1} IBA.

shoot organogenesis without an intervening callus stage. (Zapata et al., 1999b) On the other hand, development of transgenics via somatic embryogenesis requires 6 – 12 months to obtain mature transgenic plants and an addition of 6 - 10 years are necessary to backcross the added value traits into the desired agronomic lines (Bajaj, 1998). In view of the limitations, the need for alternate technology as reported in this paper seems to be an answer to develop transgenics in a short time in any variety.

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