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Optimization of micropropagation protocol for three cotton varieties regenerated from apical shoot

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The need for alternative strategies to obtain transgenic cotton via apical shoot was necessitated due to the recalcitrance of cotton regeneration from somatic embryogenesis, this has greatly slowed down the development of transgenic cottons. To this effect, an optimized regeneration system from apical shoot was developed for three varieties of cotton. Ninety-five percent seed surface sterility was observed in seed germination using a combination of hydrogen perioxide and Clorox as sterilizing medium. Highest shoot elongation rate was achieved on MS supplemented with 2.5 mg/L BAP + 0.1% (w/v) AC, rapid shoot growth occurred with kinetin supplemented media. Rooting efficiency of the three improved cultivars of cotton (*Gossypium hirsutum*), Samcot 9,11 and 13 were optimized using the optimum medium for rooting of difficult-to-root *in vitro* regenerated shoots of cotton which consist of MS basal salts and modified MS vitamins, supplemented with 3% sucrose, 0.2 mg/L IBA, without activated charcoal. In the end, an improved regeneration protocol with rooting efficiency up to 47% and regeneration rate up to 87% by combining rooting induction, indole acetic acid (IAA) shock and graft technique was developed.

Key words: *Allium sativum,* cotton tissue culture, transgenic plant, optimized regeneration of cotton.

INTRODUCTION

The focus of research in plant cell culture for many crop species was to be able to put species into tissue culture maintain or grow the plant cells, tissues or organs under sterile controlled laboratory conditions and ultimately regenerate a normal fertile plant. In comparison with other crops, successes in cotton tissue culture lag behind those of other crops. *In vitro* cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies (Shoemaker et al., 1986; Chen et al., 1987; Trolinder and Goodin, 1987; Kolganova et al., 1992; Zhang, 1994a; Zhang et al., 1996, 1999).

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Regenerated plants have been obtained from explants such as hypocotyls, cotyledon, root (Zhang, 1994a) and from various cotton species (Zhang, 1994b). Gould et al. (1991) reported a successful regeneration method of two cotton varieties; *G*. *barbadense* cultivars and *G*. *hirsutum* cultivars that was independent of genotype; however, rooting efficiency was low. Nasir et al. (1997), Morre et al. (1998) and Zapata et al. (1999) also reported the regeneration of cotton plants from shoot meristems. This method has also been successfully used in cotton transformation when combined with particle bombardment (McCabe and Martinell, 1993). Trolinder and Goodin (1987) reported regeneration of cotton plants from callus by somatic embryogenesis, and the efficiency of regeneration via somatic embryogenesis has been reported to improve significantly in recent years (Trolinder et al., 1989; Rajasekaran et al., 1996; Zhang et al., 2001), some difficulties still remain. Major limitations had been that only few cultivars can be induced to produce somatic embryos and regenerative plants. Most responsive lines are Coker varieties, which are no longer under cultivation (Feng et al., 1998). Aside from the genotype limitation, many of the plants regenerated from callus as somatic embryos are abnormal (Cousins et al., 1991; Trolinder and Goodin, 1987; Rajasekaran et al., 1996). Due to these shortcomings, cotton biotechnology has been a major task in cotton breeding and production. As an improved approach, Renfroe and Smith (1986) reported regeneration of cotton from isolated shoot meristem from seedlings of *G*. *hirsutum* L. cv. to obtain regenerated plants. Gould et al. (1991) extended this approach by using two *G. barbadense* cultivars and 19 *G*. *hirsutum* cultivars in his research, which showed that regeneration from shoot tips was genotype-independent. Saeed et al. (1997), Morre et al. (1998) and Zapata et al. (1999) also reported the regeneration of cotton plants from shoot meristems. However, rooting efficiencies were low in these reports (from 38 to 58%). In this report, an optimized regeneration protocol with improved rooting efficiency in shoot apex based cotton regeneration system is presented. Three factors that could affect the rooting efficiency of shoot apices were investigated in this research: 1) Effect of seed sterilization method, 2) Effect of shoot apex age, and 3) Effect of concentration of IAA shock. In the end, an improved regeneration protocol with rooting efficiency up to 87% was developed. The protocol uses cotton shoot apices as explants and combines basic rooting, IAA shock and grafting steps to increase rooting efficiency up to 47% and regeneration to 87%.

METHODOLOGY

Seed disinfection methods

Cotton seeds were de-linted in concentrated sulphuric acid (H_2SO_4) then washed in tap water. The de-linted seed were then wrapped in cheese cloth and soak in tap water for 1 h. Cotton seeds were disinfected via four methods:

Method 1: Cotton seeds were treated with 70% ethanol for 2 min prior to a 20 min exposure to 10% Clorox ® (5.25% sodium hypochlorite (NaOcCl)) solution with two drops of Tween 20 per 100 ml, and rinsed three times with sterile double-distilled water. The seeds were then placed on seed germination medium.
Method 2: Cotton seeds were treated with a 50% Clorox® (5.25% NaOcCl) solution with two drops of Tween 20 per 100 ml on a rotary shaker at 50 rpm for 20 min and rinsed at least three times with sterile double-distilled water. The seeds were then placed on seed germination medium.
Method 3: Cotton seeds were treated with 20% hydrogen peroxide for 2 h and rinsed three times with double-distilled water. The seeds were then placed overnight on a rotor shaker at 100 rpm. After removing the seed coat, the seeds were then placed on seed germination medium.
Method 4: Cotton seeds were treated as described in Afolabi-Balogun et al. (2011). After removing the seed coat, the seeds were placed on seed germination medium.

Seed germination

Three seeds were placed in each germination media (Afolabi-Balogun et al., 2011) and incubated in the dark at 28°C overnight and then in the light for 5 days. Upon removal from incubation, the number of elongated shoots was counted. Contamination was determined by visual inspection for fungal and/or bacterial growth.

Shoot apex isolation

The seedling apexes were isolated as described by Afolabi-Balogun et al. (2011). The epicotyl (shoot) was placed on MS+Kin medium. The plants were kept in growth room at 27± 2°C to 16 h light and 8 h dark at 70% humidity. The plantlets were grown for 10 days.

Shoot elongation and rooting development

Thirty shoots from each variety without root development were subjected to IAA shock at concentrations 0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml for one minute. The treated shoots were rinsed and transferred to fresh MS medium for another three weeks. The number of rooted plants was recorded and the rooted plants were transferred to Magenta boxes containing MS medium and incubated in a culture chamber for four weeks before being transferred to the greenhouse.

Plantlets graft

Grating of un-rooted elongated shoots from MS medium after IAA shock onto the seedling stocks of the same variety was done by cutting 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 were treated with 0.2 mg/L IAA + 0.1 mg/L GA. for 5 min. Then the treated scion was inserted into the slit and the cambiums were lined up. The grafted plant was then covered by a 1000 ml flask and kept in a humid chamber for a week. After which the flask was removed and the plants kept in the humid chamber for another week before being transferred to the greenhouse.

Data collection and analysis

Data were obtained at 25-30 days and at 42 days on the number

viable shoots and viable shoots with roots. Other observations made were on shoot health (on a rating scale 1 to 5 scale, with 1 being poorest chlorophyll development, and 5, best chlorophyll development), leaf abscission (on a 1 to 5 rating scale with a rating of 5 being complete leaf retention, and 1 complete leaf abscission), number of explants with callus, relative calli size, root length, and branching. Data was analyzed as a completely randomized design with three replications using ProcGLM of SAS program (SAS, 1987). Means of statistically significant (p=0.05) treatments were separated using LSD.

RESULTS

Seed disinfection methods

The extent of sterility was measured be physical examination of the culture bottle for contaminant such as mould. Maximum surface sterilization was observed with the seed disinfected with method 4 (number of contaminated seed is zero) (Figure 1). Methods 1 and 2 did not give perfect sterilization. Use of only 50% Clorox® gives the least sterilization. Combining Clorox® and hydrogen peroxide gave a better result, but this was still not as efficient as hydrogen peroxide.

Seed germination

All cotton seeds varieties germinated on MS though seedlings elongation on germination medium was very slow. For enhancement of growth, the tiny seedlings were transferred to different media supplemented with plant growth regulators (BAP, NAA, IBA) and activated charcoal (AC). The highest rate of elongation was achieved on MS supplemented with 2.5 mg/L BAP + 0.1% (w/v) AC.

Shoot apex isolation

Vigorous shoot growth was observed when kinetin is supplemented to the media.

Shoot elongation and rooting development

The rooting efficiency of the three varieties was significantly different in different concentrations of IAA (p=0.027) (Figure 2). The effect of different IAA shock concentrations varied from 6.7 to 47%. The highest efficiency (47%) was observed for a 1.5 mg/ml IAA and the lowest efficiency (12%) was observed for 0.1 mg/ml IAA. So the concentration of 1.5 mg/ml IAA was chosen for regeneration. The rate of rooting of elongated shoots cultured on various media is presented in Table 1. Optimum rooting was observed using ERM 4 which was about (47%) while the lowest rooting was observed with ERM 2 giving only (6.7%). Hence, a concentration of 1.5 mg/ml IAA was chosen in the regeneration system. The difference of rooting efficiency was not significantly different in all varieties (p=0.08). This result indicated that rooting efficiency is genotype independent.

Plantlet grafting

Rooting efficiency of plantlet as well as survival rate was improved to eighty five percent when plantlets were kept humid, pre-treating the scion and stock with 0.1 nmg/L IAA + 0.2 mg/L GA (Figure 3). Grafting is a very useful technique and is commonly used in horticultural crops.

DISCUSSION

Recently, several researchers have regenerated plants from shoot tip meristems (Zapata et al., 1999). Gould et al. (1991) reported that the yield of shoots *in vitro* from isolated apices depends on the incidence of contamination and rooting efficiency. In recent years, protocols involving proliferation of cotton shoots (Agrawal et al., 1997; Hemphill et al., 1998) have been published. The rooting efficiency ranged from 38 to 58% in their reports. Here we report an optimized regeneration protocol involving shoot tips regenerated directly without a callus phase, this method has the advantage of being genotype-independent; almost all cultivars can be regenerated from shoot tips. The use of shoot tips as explants in an *Agrobacterium*-mediated transformation system is a good way to overcome the obstacles in traditional *Agrobacterium*-mediated transformation. From the germination results, all seeds sterilized by hydrogen peroxide germinated in 5 days (Figure 1); seeds sterilized by both Clorox® methods had a lower germination rate (95 and 37%, respectively). The reason for those results may be that the residual of Clorox, specifically, chlorine, suppressed the germination of cotton seeds, while the residual of hydrogen peroxide is water and CO_2, did not affect the germination of cotton seeds.

The age of explants has a significant effect on shoot tip elongation (Table 2). The elongation rates of the three varieties were not significantly different from each other (p=0.1573). The elongation rate was also affected by the size of isolated tips. It was observed that if the starting size of the apex was less than 1 mm, the tips would not grow at all.

The efficiency of the rooting media was evaluated based on the increase in length and number of roots developed per seedling. The highest rate of elongation was achieved on MS supplemented with 2.5 mg/L BAP + 0.1% (w/v) AC however, MS + 3% (w/v) sucrose + 1.5 mg/IAA proved more effective for the development of better root system and the rooting of the plantlet was by grafting procedure.

The type and concentration of plant growth regulators

Figure 1. Effect of sterilization method on seed germination.

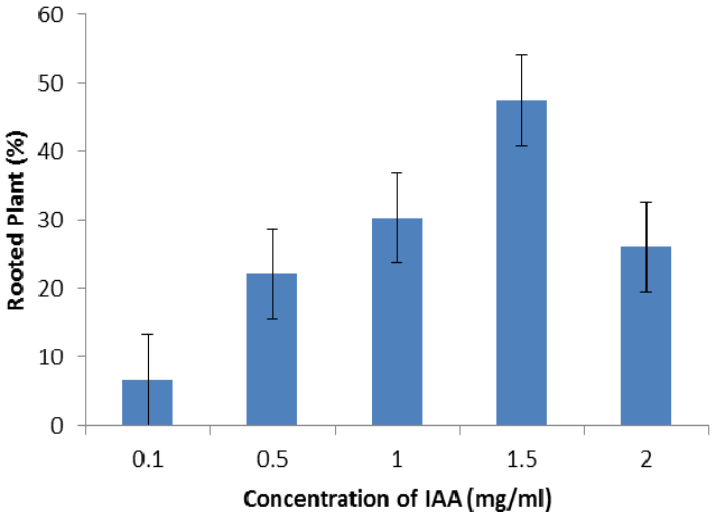

Figure 2. Effect of IAA shock on stimulating the rooting of previously unrooted Cotton shoot apices. Vertical bar represents the standard error of the 5 treatments of IAA.

Figure 3. 1-Rooting plant, 2-Effect of sterilization method 1 & 2 showing microbial growth,3-Germinating seed,4-Plantlet before grafting,5-Elongating apical shoot, 6-Grafted plantlet,7-Elongating apical shoot, 8- Cross section of work area, 9- Regenerated Plant in soil, 10-Plantlets in greenhouse.

Table 1. Elongation and rooting media composition used in optimization.

Medium	Media composition
ERM 1	½ MS + 1.5 % (w/v) sucrose
ERM 2	MS + 3 % (w/v) sucrose + 0.5 mg/L IAA
ERM 3	MS + 3 % (w/v) sucrose + 1.0 mg/L IAA + 0.1% (w/v) AC
ERM 4	MS + 3 % (w/v) sucrose + 1.5 mg/ IAA
ERM 5	0.1mg/l GA3 + 1.0 mg/L IAA

All media were solidified with 0.4% phytagel (Sigma).

Table 2. Mean number of explants elongated on elongation medium from 3 cotton varieties at 4 different ages.

Cotton variety	Age of Explant				
	5 days	7 days	9 days	11 days	Mean
Blec-Samcot 9	11.00±2.00++	25.33±2.08	28.67±0.57	30±0.0	23.75[a]
Blec-Samcot 11	13.33±3.06	26.70±0.57	28.00±1.029	33±0.57	24.33[a]
Blec-Samcot 13	14.67±3.21	26.67±2.08	28.67±0.57	30±0.0	25.00[a]
Mean	12.75[c+]	25.75[b]	28.41[a]	29.75[a]	

+ Different letter label significant at p=0.05 level using LSD method, ++ Mean±Std.

Table 3. Regeneration response of apical shoot explant and split cotyledon node from cotton to the concentration of IAA.

Explant	Media composition	% Rooting (days)	
		14	27
	½ MS + 1.5% (w/v) sucrose	0[a]	0[b]
	MS + 3% (w/v) sucrose + 0.5 mg/l IAA	8[a]	54[a]
SA	MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.1% (w/v) AC	4[a]	63[a]
	MS + 3% (w/v) sucrose + 1.5 mg/ IAA	18[a]	38[a]
	0.1 mg/l GA_3 + 1.0 mg/l IAA	17[a]	56[a]
Explant Mean		9.4[a]	42.2[b]
	½ MS + 1.5% (w/v) sucrose	8[a]	0[ab]
	MS + 3% (w/v) sucrose + 0.5 mg/l IAA	0[a]	4[a]
SCN	MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.1% (w/v) AC	6[a]	23[a]
	MS + 3% (w/v) sucrose + 1.5 mg/ IAA	32[a]	67[a]
	0.1 mg/l GA_3 + 1.0 mg/l IAA	32[a]	29[a]
Explant Mean		25.6[a]	24.6[a]

Means followed by the same letter are not significantly different at p=0.05. Glu- Glucose, Suc- Sucrose, Ac. Char- Activated Charcoal. SA- Shoot apices SCN- Splited Cotyledon Node.

strongly influenced the organogenic potential of the apical shoot explant. The responding frequency of shoot seemed to depend more on concentration of indole-3-acetic acid (IAA). The regeneration response of apical shoot explant and split cotyledon node from cotton to the concentration of IAA is shown in Table 3. It is evident that without IAA regeneration of apical shoot is low and maximum shoot regeneration response has been observed with 1.5 mg/L IAA concentration along with MS. The further increment in IAA concentration to 2.0 mg/L along with MS showed decreased shoot regeneration response. We observed maximum number of shoots when GA was combined with IAA in all variety.

Conflict of Interest

The authors have not declared any conflict of interest.

REFERENCES

Afolabi-Balogun NB, Inuwa HM, Sani I, Ishiyaku MF, Bakare-Odunola MT, Nok AJ, van Emmenes L (2011). Effect of age of explant on transgenic cotton (Gossypium) plant due to expression of mannose-binding Lectin Gene from Allium sativum. Asian J. Agric. Sci. 3:393-396.

Agrawal DC, Banerjee AK, Kolala RR, Dhage AB, Kulkarni WV, Nalawade SM, Hazra S, Krishnamurthy KV (1997). In vitro induction of multiple shoots and plant regeneration in cotton (Gossypium hirsutum L.). Plant Cell Rep. 16: 647-653. http://dx.doi.org/10.1007/BF01275508

Chen ZX, Li SJ, Trolinder NL, Goodin JR (1987). Some characteristics of somatic embryogenesis and plant regeneration in cotton cell suspension culture. Sci. Agric. Sin. 20(5):6-11.

Cousins YL, BR Lyon, Llewelly DJ (1991). Transformation of an Australian cotton cultivar: prospects for cotton improvement through genetic engineering. Aust. J. Plant Physiol. 18:481-494. http://dx.doi.org/10.1071/PP9910481

Feng R, Zhang BH, Zhang WS, QL Wang (1998). Genotype analysis in cotton tissue culture and plant regeneration. In P. J. Larkin (ed.). Proceedings of the 4th Asia-Pacific Conference on Agricultural Biotechnology, Darwin 13-16 July 1998. Canberra, UTC Publishing, pp. 161-163.

Gould J, Banister S, Fahima M, Hasegawa O, Smith RH (1991) Regeneration of Gossypium hirsutum and G. barbadense from the shoot apex. Plant Cell Report 10: 12–16. http://dx.doi.org/10.1007/BF00233024

Hemphill JK, Maier CGA, Chapman KD, (1998). Rapid in vitro plant regeneration of cotton (Gossypium hirsutum L.), Plant Cell Report 17: 273-278. http://dx.doi.org/10.1007/s002990050391

Kolganova TV, Srivastava DK, Mett VL (1992). Callusogenesis and regeneration of cotton (Gossypium hirsutum L. cv 108-F). Sov. Plant Physiol. 39: 232-236.

McCabe DE, Martinell BJ (1993) Transformation of elite cotton cultivars via particle bombardment of meristems. Biotechnology 11: 596-598. http://dx.doi.org/10.1038/nbt0593-596

Morre JL, Permingeat HR, Maria VR, Cintia MH, Ruben HV (1998). Multiple shoots induction and plant regeneration from embryonic axes of cotton. Plant Cell Tiss. Organ Cult. 54: 131-136. http://dx.doi.org/10.1023/A:1006170529397

Nasir AS, Zafar Y, Malik KA (1997). A simple procedure of Gossypium meristem shoot tip culture. Plant Cell Tiss. Organ Cult. 51: 201-207. http://dx.doi.org/10.1023/A:1005958812583

Rajasekaran K, Grula JW, Hudspeth RL, Pofelis S, Anderson DM (1996). Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. Mol. Breed. 2:307-319. http://dx.doi.org/10.1007/BF00437909

Renfroe MH, Smith RH (1986). Cotton shoot tip culture. Beltwide Cotton Prod. Res. Conf. Proc. pp. 78-79.

Saeed NA, Zafar Y, Malik KA (1997). A simple procedure of Gossypium meristems shoot tip culture. Plant Cell Organ Cult. 51:201-207. http://dx.doi.org/10.1023/A:1005958812583

Shoemaker RC, Couche IJ, Galbraith DW (1986). Characterization of somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.). Plant Cell Rep. 3:178-181. http://dx.doi.org/10.1007/BF00269112

Trolinder NL, Xhixian C (1989). Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep. 8:133-136. http://dx.doi.org/10.1007/BF00716824

Trolinder NL, Goodin JR (1987). Somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.) Plant Cell Rep. 14:758-776.

Zapata C, Park SH, El-Zik KM, Smith RH (1999). Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex. Theor. Appl. Genet. 98: 252-256. http://dx.doi.org/10.1007/s001220051065

Zhang BH (1994a). A rapid induction method for cotton somatic embryos. Chin. Sci. Bull. 39: 1340-1342.

Zhang BH (1994b). List of cotton tissue culture (Continuous). Plant Physiol. Commun. 30: 386-391.

Zhang BH, Feng R, Li XL, Li FL (1996). Anther culture and plant regeneration of cotton (Gossypium klotzschianum Anderss). Chin. Sci. Bull. 41:145-148.

Zhang BH, Feng R, Liu F, Wang QL (2001). High frequency somatic embryogenesis and plant regeneration of an elite Chinese cotton variety. Bot. Bull. Acad. Sin. 42:9-16.