Pak. J. Bot., 36(4): 817-823, 2004.

IN VITRO SHOOT TIP CULTURE OF COTTON (*GOSSYPIUM HIRSUTUM*)

BUSHRA RASHID*, TAYYAB HUSNAIN AND SHEIKH RIAZUDDIN

Centre for Applied Molecular Biology,
87-West Canal Bank Road, Thokar Niaz Baig Lahore-53700, Pakistan.

Abstract

Shoot apices from *in-vitro* germinated seedlings of 22 cotton varieties were cultured on basal MS salts and vitamins (Murashige & Skoog, 1962). No phytohomones were used. Growth response of different varieties varied. Root and shoot formation, was observed in all varieties. After plantlet formation, *in vitro* plantlets were shifted to pots containing a specific composition of soil, sand and peat moss. Pots were covered with polythene bags to maintain the humidity level and uncovered for a limited period for aeration for three weeks. These plants grew into healthy green plants and reached to maturity.

Introduction

Cotton is a major cash crop in Pakistan. It has given a big boost to agricultural industry and is a major source of foreign exchange earnings. Domestic edible oil production is also supplemented from cotton. It is considered that cotton is recalcitrant to *in vitro* proliferation (Gupta *et al*., 1997). Somatic embryogenesis and plant regeneration has been reported from hypocotyl (Davidonis & Hamilton, 1983; Trolinder & Goodin, 1987; Finer, 1988), but the response is highly genotype dependant (Trolinder & Xhixian, 1989; Cousins, *et al*., 1992; Firoozabady & DeBoer, 1993). Cotton plants are severely limited in their regeneration *in vitro* from callus, protoplast or leaf tissues. This widespread problem presently restricts improvement of the few potential commercial genotypes through genetic engineering (Gould *et al*., 1991, McCabe & Martinell, 1993). Development of tissue culture protocols to induce efficient proliferation in a genotype independent manner is desirable for genetic transformation of cotton. Shoot tip culture is an alternative to obtain plants and to solve the problems of recovering plants from callus tissues (Gould, *et al.,* 1991; McCabe & Martinell, 1993; Saeed *et al.,* 1997). Hence, to improve the potential of commercial cotton cultivars for genetic transformation, an alternative procedure was developed for regeneration of cotton plantlet *in vitro* through shoot apices. This procedure is quite simple and less expensive and needs less labour to regenerate cotton plants. In all the methods described previously for regeneration in cotton through shoot apices, different types and levels of phytohormones were used (Gould *et al*., 1991; Gupta *et al*., 1997; Saeed *et al*., 1997) but in our procedure no phytohormone was used.

Materials and Method

Seeds of 22 cotton varieties *Gossypium hirsutum* L.; FH-631, FH-672, FH-673, FH-679, FH-658, FH-87, MNH-93, Coker 312, K-68-9, SLH-41, CIM-109, Cotton-156, CIM-443, CIM-446, B-557, S-12, CIM-70, NIAB-92, NIAB-86, V3, NIAB-26, Krishma were obtained from different sources.

*E-mail: bushra_2002@lycos.co.uk
Tel: 042-5423942-45, Fax: 042-5421316

Culture procedure

Seeds of all varieties of *Gossypium hirsutum* were treated with concentrated H_2SO_4 for 1-2 min. Seeds were thoroughly mixed with acid to remove the lint completely, then washed with tap water at least 4-5 times to remove the acid. For sterilization purpose approximately 500 seeds were dipped in distilled water with one drop of Tween-20 per 100ml of water, shaken well for 3 min., on a shaker at 40-50 rpm and rinsed for 3-4 times with autoclaved distilled water. The seeds were then treated with 0.1% Mercuric chloride and 0.1% SDS for 3-5 min. These seeds were washed with autoclaved distilled water for 4-5 times. After sterilization procedure, the seeds were placed on sterilized filter papers in Petri plates and moistened with 2-3 ml of autoclaved distilled water. The seeds were covered with moist filter paper and then with lid of Petri plate. Plates were sealed with parafilm and kept at 30ºC in dark for 72 hrs. Germination percentage was checked for each variety. All the cultivars showed more than 80% germination. After 3 days the germinated seeds were cultured on MS basal salts and vitamins in glass test tubes (Fig. 1). Cultures were kept in a growth room at 27±2ºC to 16h photoperiod. Seedlings were allowed to grow for 10 days.

After 10 days the aseptically grown cotton seedlings had formed well grown shoot tips that were isolated for shoot tip culture. The seedling was removed from the test tube, placed on a sterilized tile and cotyledons removed from the point of attachment. The expanding leaves were also cut down with the help of blade. After that shoot tip of approximately 2.0, 4.0 and 6.0mm were excised and cultured on MS basal medium in glass culture vessels with plastic closures (Fig. 2). Shoot apex of 6.0mm long gave maximum shoot and root formation (Fig. 3a). The density of the shoot apices was 4 shoot apices per culture vessel, and a total of three replicates was cultured. Shoot apices were cultured only on MS salts and vitamins (Murashige & Skoog, 1962). For rooting, the shoots were subcultured on the same MS medium, although the shoot and root formation response varied for different varieties

The culture conditions for shoot and also for root formation were maintained at 27°± 2ºC with a photoperiod of 16h under the light regime, 100-120$\mu mm^{-2}Sec^{-1}$. Normal healthy, green plants were shifted to a specific composition of soil.

Transfer of rooted plantlets to soil

A soil mixture was prepared to shift the plants from culture vessels to soil. The mixture composed of clay + sand + peat moss in a ratio of 1:1:1, mixed well, moistened with distilled water, filled in an autoclavable bag and autoclaved. After autoclaving the soil composite was cooled and filled in plastic pots having a drain hole at the bottom.

The rooted shoots were taken out of the culture vessel, washed with tap water to remove the medium from roots and planted into the pot filled with soil mixture. Twenty to 25 ml of Hoagland's solution was added and pots were covered with a polythene bag. Plants were kept in a growth room at 30°± 2°C at 16 hrs., photoperiod (250-300 umol m^{-2} S^{-1}) (Fig. 3b). After 3-4 weeks the covers were removed completely. Hoagland solution was added if required. The plants were ready for shifting to glasshouse in earthen pots (Fig. 3c).

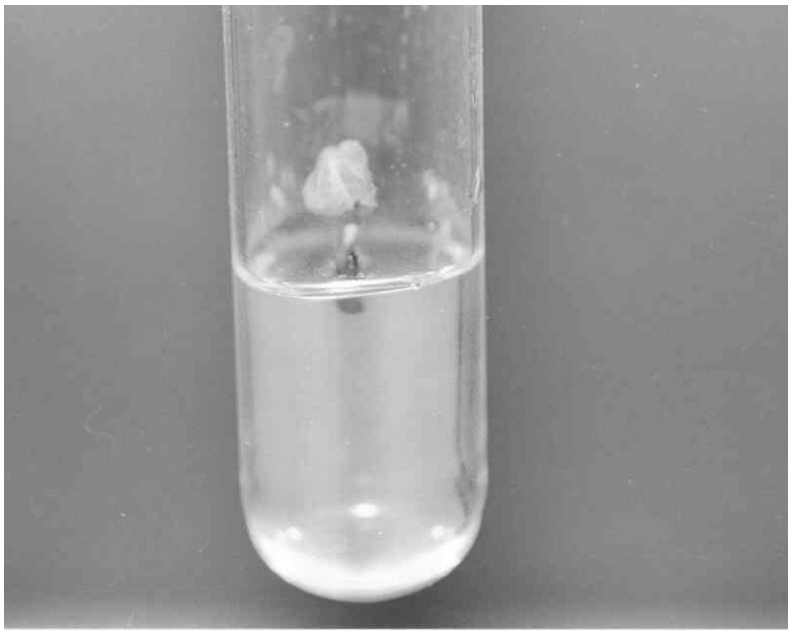

Fig. 1. Seed germination in cotton
Var. CIM-443
7-days old seedling

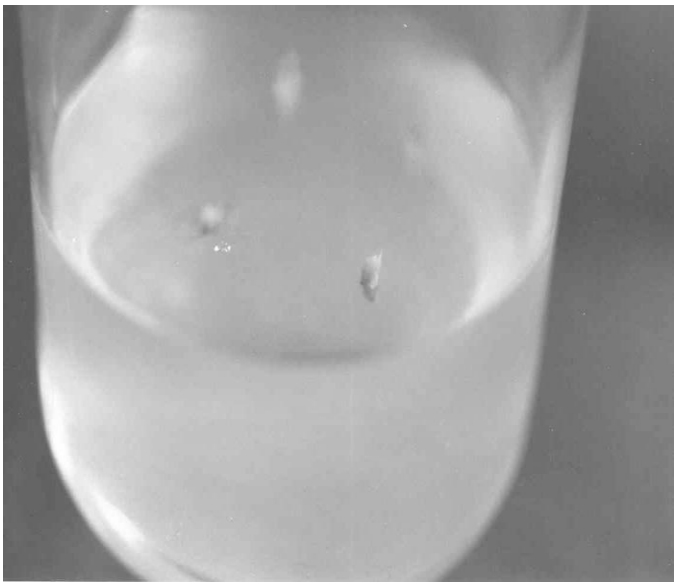

Fig. 2. Shoot apices cultured on MS medium
Var.CIM-443
10-DAYS OLD

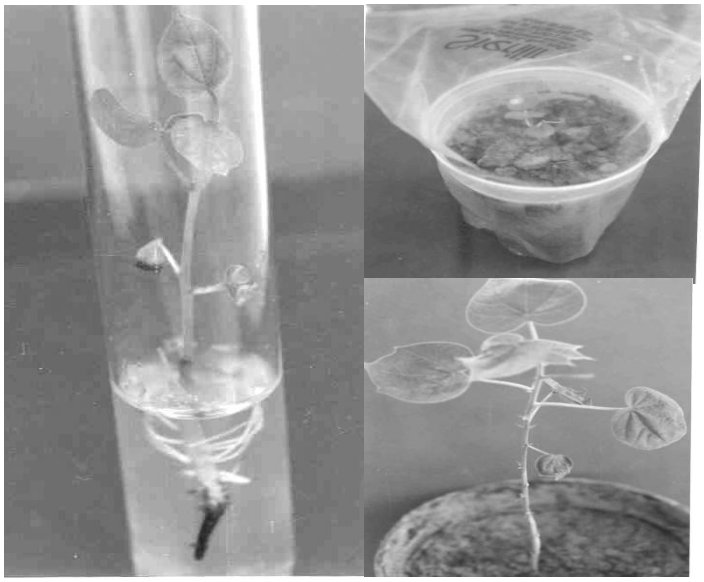

Fig. 3. Stages of plant regeneration from shoot apex/ var. CIM-443

a. 4 weeks old regenerated plant growing on MS medium.
b. 6 weeks old plant shifted to soil & covered with polythene.
c. 10 weeks old regenerated plant established in soil.

Media composition

Medium used for seedling growth and shoot apex culture was MS basal salt mixture (Murashige & skoog, 1962) which composed of all Macro and Micro salts essential for plant growth. Moreover MS vitamins were also added. Sucrose was added as a carbon source @ 30 g $l^{-1.}$ Phytagel was used as a gelling agent. The pH of the medium was adjusted to 5.7 before autoclaving. No phytohormone was added. After autoclaving approximately 20 ml medium was dispensed in each test tube (20 x 2.5 cm) and approximately 50 ml medium was dispensed in glass culture vessels (9 x 4.5 cm) for shoot apex culture and allowed to solidify at room temperature.

Results

Seed germination, seedling age and size

Sterilized cotton seeds of all varieties when incubated in dark on moist filter papers for at least 72 hrs., gave good germination about 80% or more (Table 1). At this stage only hypocotyl of 2-3 cm in length had emerged. The cotyledons were still in testa of the seed. At this stage of development, testa was removed and cotyledons expanded after 2-3 days of culturing on medium (Fig. 1). The 10 days old seedlings had the fully expanded cotyledons with meristem tip, leaf primordia and rudimentary leaves. At this stage the seedlings were ready for excision of the shoot tip. Beside the age of seedling, size of shoot tip was also very important. We used 6.0mm size shoot apex in our experiments (Fig. 2).

Table 1.Regeneration of cotton plants from apical meristem.

Varieties	% of plants developed
S-12	13
FH-631	22
FH-672	33
FH-673	42
FH-679	14
CIM-70	34
CIM-685	20
CIM-87	20
MNH-93	75
COKER-312	40
NIAB-92	40
NIAB-86	85
V3	25
NIAB-26	25
Krishma	65
K-68-9	34
SLH-41	30
Cotton-156	20
CIM-443	100
CIM 446	29
B-557	59
CIM-109	12

Data is based on 10 plants of each variety.

Shoot and root development

Six mm sized shoot apex when cultured on MS hormone free medium, the shoot elongation and leaf formation started within 5-10 days. Sub-culturing was carried out at least 4 weeks after the first culture because an earlier sub-culturing disturbs the growth phenomenon. Browning and blackening of tissues was also observed during frequent sub-culturing. Root formation started after 4-5 week of inoculation. All the varieties cultured, formed the plants, however the response was different for different varieties e.g., the variety CIM 443 formed 100%, MNH-93 75% and NIAB-86 85% plantlets with shoots and roots but the varieties CIM-109 showed 12%, FH-679, 14% and S-12, 13% plants with shoots and roots (Table 1). When the shoots attained a height of 4-5 inches with 4-5 leaves and had an extensive root system, the plantlets were successfully shifted to a specific composition of soil i.e., clay + sand + peat moss in a ratio of 1:1:1. All the plants grew normally. All this procedure was completed in approximately 8-10 weeks.

Discussion

In this study the shoot tip culture procedure was used for the regeneration of cotton plants, because the cotton is recalcitrant and has proved difficult to manipulate in tissue culture (McCabe & Martinell, 1993). In a simple procedure *in vitro* grown seedlings were used. For healthy seedling formation, good quality seeds and proper seed germination is

required. Gould *et al*., 1991 observed that the contamination rate in seedlings is different for seed lot and genotype. Age and size of explant is the most important factor in this study. Shoot tip from less than 5 days old seedlings are difficult to isolate, due to small size and tenderness. Likewise it was also difficult to excise apices of more than 10 days old seedlings due to maturity and hardness of tissues, because if the tissues become more mature, hard or woody, the phenolic compounds will be difficult to control (Saha, *et al*., 1997). In the present work, 6.0 mm sized shoot apex from 10 days old seedling showed best response for shoot and root formation on MS basal medium. In other reports, the smaller shoot apex size was taken but in those cases seedling age was also less than 10 days (Bajaj & Gill, 1986; Gould *et al*., 1991; Gupta, *et al*., 1997; Saeed, *et al.*, 1997). The response of different varieties was 12-100% for plant formation (Fig. 3a). The different response for different varieties may be due to seed lot and genotype.

Several naturally occurring hormones work in the cotton plant to adjust the plant growth, so there is no need to add any phytohormone in the medium. Photosynthesis supplies the carbon and respiration supplies the energy for plant growth. The medium itself has all the essential elements for plant growth because in some cases the addition of auxin and cytokinin form the callus tissues at the base of shoot tip prove detrimental and may cause the death of tip (Saeed *et al*., 1997) as root is not formed due to callus formation at base of excised shoot tip. When the plant growth regulators are applied to the cotton plant, they work in much the same way as the endogenous regulants. In many ways, they supplement or destroy the natural hormone (Albers & Schnakenberg, 1994).

For transformation a rapid, reliable regeneration system is required so this procedure can be used for gene delivery either by *Agrobacterium* transformation or particle bombardment. Regeneration through shoot apex has been used successfully in *Agrobacterium* mediated transformation of petunia (Ulian *et al*., 1998); sunflower (Schrammeijer *et al.,* 1990); corn (Gould *et al.,* 1991) and cotton (Gould *et al*., 1998; Wilkins *et al., 2000*).

References

Albers, D.W. and C.T. Schnakenberg. 1994. Agricultural Publications G4258.

Bajaj, Y.P.S. and M. Gill. 1986. Preservation of cotton (*Gossypium* spp.) through shoot tip and meristem culture. *Indian J. Exp. Biol*., 24: 581-583.

Cousins, Y.L., B.R. Lyon and T. Lleusellyn. 1992. Transformation of an Australian cotton cultivar: Prospects for cotton improvement through genetic engineering. *Aust. J. Plant Physiol*., 18: 481-494.

Davidonis, G.H. and R.H. Hamilton. 1983. Plant regenration from Callus tissue of *Gossypium hirsutum* L., *Plant Sci. Lett*., 32: 89-93.

Finer, J.J. 1988. Plant regeneration from somatic embryogenic suspension cultures of cotton (*Gossypium hirsutum*). *Plant Cell Rep*., 7: 399-402.

Firoozabady, E. and D.L. DeBoer. 1993. Plant regeneration via somatic embryogenesis in many cutlivars of cotton (*Gossypium hirsutum* L.) *In vitro cell Dev. Biol*., 29: 166-173.

Gould, J., S. Banister, O. Hasegawa, M. Fahima and R.H. Smith. 1991. Regeneration of *Gossypium hirsutum* and *G. barbadense* from shoot apex tissues for transformation. *Plant Cell Rep*., 10: 12-16.

Gould, J.H. and M.M. Cedeno. 1998. Adaptation of cotton shoot apex culture to *Agrobacterium* mediated transforamtion. *Plant Mol. Biol. Rep*., 16: 1-10.

Gupta, S.K., A.K Srivastava, P.K. Single and R. Tuli. 1997. *In vitro* proliferation of shoots and regeneration of cotton. *Plant cell tissue and organ culture*., 51: 149-152.

McCabe, D.E. and B.J. Martinell. 1993. Transformation of elite cotton cultivars via particle bombardment of meristems. *Bio/technol*., 11: 596-598.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco cultures. *Phyiol. Plant*., 15:473-497.

Saeed, N.A., Y. Zafar, and K.A. Malik. 1997. A simple procedure of *Gossypium* meristem shoot tip cutlure. *Plant Cell, Tissue and Organ Culture*, 51: 201-207.

Saha, S.E. Franklin, C. Douglas, A. Dollar and J.B. Creech. 1997. Effect of lyophilization of cotton tissue on quality of extractable DNA, RNA and protein. *J. Cotton Sci*., 1: 10-14.

Schrammeijer, B., P.C. Sijmons, E. Vanden and A. Hoekema. 1990. Meristem transformation of sunflower via *Agrobacterium*. *Plant Cell Rep*., pp. 55-60.

Trolinder, N.L. and J.R. Goodin. 1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). *Plant Cell Rep*., 6: 231-234.

Trolinder, N.L. and C. Xhixian. 1989. Genotype specificity of the somatic embryogenesis response in cotton. *Plant Cell Rep*., 8: 133-136.

Ulian, E.C., R.H. Smith, J. Gould and T. McKnight. 1998. Transformation of plants via the shoot apex., *In vitro Cell Dev. Biol*., 24: 951-954.

Wilkins, T.A., K. Rajasekran and D.M. Anderson. 2000. Cotton Biotechnology. *Crit. Rov. Plant Sci*., 19: 511-550.

(Received for publication 23 July 2004)