Plant Cell, Tissue and Organ Culture 51: 201–207, 1997.

A simple procedure of *Gossypium meristem* shoot tip culture

Nasir A. Saeed, Yusuf Zafar* & Kauser A. Malik
*National Institute for Biotechnology and Genetic Engineering (NIBGE), P. O. Box 577, Jhang Road, Faisalabad, Pakistan (*Corresponding author)*

Received 31 May 1996, accepted in revised form 23 September 1997

Key words: *Gossypium hirsutum* L., leaf curl virus, meristem tip culture

Abstract

In order to develop transgenic plants via the biolistic gun method regenerable embryogenic tissues are required. Meristem shoot tips of 19 cultivars of cotton were cultured on several media formulations and assessed for shoot and root development. The best shoot development was observed on media containing 0.46 mM kinetin while rooting was observed on media containing 2.68 mM NAA and 0.46 mM kinetin. No intervarietal variability was observed. A complete protocol was developed from meristem tip culture to field transfer. This methodology is simple and replaces the existing protocols for meristem tip culture of cotton.

Abbreviations: 2,4-D – 2, 4 - dichlorophenoxyacetic acid; IAA – indoleacetic-3-acid; 2iP – N^6 -[2-isopentenyl]adenine; MS – Murashige and Skoog (1962); NAA – naphthaleneacetic acid

Introduction

Tissue culture responses are highly genotype dependent and, in cotton only a few cultivars are regenerable (Trolinder and Xhixian, 1989; Cousins et al., 1992). The latest technology for delivery of genes into plant tissues is the biolistic gun which requires regenerable tissues. Target tissues may be callus, suspension cells, leaves, meristem tips or any other regenerable explant. Cotton plants have proved to be difficult to manipulate in tissue culture (McCabe and Martinell, 1993). Shoot tip culture is a possible alternative to cope with the problem of recovering plants from callus tissue (Gould et al., 1991; McCabe and Martinell, 1993).

Intact soybean meristems were bombarded *in situ* and induced to undergo organogenesis, resulting in plants whose germline has been transformed (Mc Cabe et al., 1988). Shoot regeneration from shoot apex is direct, relatively simple and is not prone to somaclonal variation and chromosomal abnormalities. Chlan et al. (1995) transferred transformed shoots to MS medium supplemented with IAA (100 mg l^{-1}) to support the formation of a vigorous root system. No multiple shooting has been observed in cotton (Gould et al., 1992) as with soybean (Mc Cabe et al. 1988) and sorghum (Bhaskaran et al., 1992). Gould et al. (1992) cultured cotton shoot explants of different lengths (0.3 to 1.0 mm) on media containing IAA and kinetin in various combinations. The auto-inhibitory response was reduced by culturing on media containing charcoal. It also helped to induce rooting by alternate subculturing on MS (Murashige and Skoog, 1962) media. All attempts to induce root formation *in vitro* were unsuccessful. Hormonal treatments tested to induce rooting in culture resulted in tissue mortality.

The protocol developed in our laboratory for shoot and root development from meristematic shoot tips is quite simple and needs less time and labor to regenerate large numbers of plants. This regeneration system will help in the screening of large numbers of biolistic gun-transformed meristematic shoot tips for desirable genes.

Materials and methods

Seed material

Seeds of 19 varieties of cotton *Gossypium hirsutum* L.; GOHAR-87, CIM-70, CIM-109, CIM-240, B-557; SL-41, BH-36, RH-1, COKER-304, MNH-93

(Source: Central Cotton Research Institute, Multan); A-1-85 A-18/87 and AEM-52 (Source: Atomic Energy Agricultural Research Center Tandojam; N-26, NIAB-78, S-12, FH-87, FH-634, FH-682 and *G. arboreum* L.; RAVI (Source: Cotton Research Institute, Faisalabad) were used in this study. Germination efficiency was checked before the start of the experiments which was more than 90% from all the cultivars tested.

Media composition

Seedlings were grown on MS basal salts (Murashige and Skoog, 1962) containing B_5-Vitamins, 3% sucrose and 0.8% agar. In all other experiment, MS-salts, B_5-Vitamins, 3% glucose, 2.5% phytagel (Sigma P-8169) and 0.075% $MgCl^{-2}$ were used. Meristem shoot tips of three cotton varieties, S-12, NIAB-78 and MNH-93 were first cultured on 21 media combinations for shoot, root or callus development (Table 1). Effects of various growth regulator combinations were noted.

Subsequently, all the other cultivars (mentioned above) were cultured on the following 6 pre-selected defined media and growth regulator combinations. 1/2MS, MS, MS + 0.46 mM kinetin,. M + 0.93 mM kinetin, MS + 0.45 mM 2,4-D + 2.32 mM kinetin and MS + 0.45 mM 2,4-D + 2.46 mM 2iP.

For rooting, regenerated shoots were transferred to media containing the following combinations. 1/2MS, MS, MS + IBA (0.98 mM, 2.46 mM, 4.92 mM, 9.84 mM and 24.60 mM) and MS + 2.68 mM NAA + 0.46 mM kinetin. Rooted plants were transferred to sterile sand and then to the soil.

Preparation of plant material

Seeds were delinted using concentrated H_2SO_4 @ 15 ml per 100 gms of seeds. Lint was removed completely. Seeds were washed with water, sun dried and used as and when required. For sterilization, the seeds were first dipped in 70% ethanol for 1–2 minutes and then in 15% commercial bleach (5.25% active ingredient) with one drop of Tween-80 for 25–30 minutes. Seeds were washed with sterilized distilled water for three times and placed in Petri plates with a few drops of water. These seeds were cultured on media (described earlier) in culture tubes (22 × 2.5 cm) with cotton plug closures and incubated at 28±2 °C in the dark for 5–7 days. Culture tubes were then transferred to a 16-h photoperiod (60–80 μmol m^2 s^{-1}) at the same temperature. These seedlings were grown for 2–10 days to get the meristem tips.

Meristem tip culture

Aseptically raised cotton seedlings were removed from the culture tubes and placed in petri plate. Cotyledon and hypocotyl were cut at the point of attachment. Petri plate along with the plant material was then put under the dissecting microscope to further reduce the size of the tip. With the help of the surgical needles, these tips were then cultured on media in petri plates and incubated in the growth room with a 16-h photoperiod (60–80 μmol m^{-2} s^{-1}), at 28±2 °C for 2–3 weeks. Shoots which attained 1.0 cm height were taken out and cultured on fresh media in small culture tubes (size 10 cm × 2 cm with cotton plug closures). After another 2–3 weeks, these tips were transferred to rooting media (described earlier) for root induction.

Hardening of plants

Rooted shoots were taken out, washed with warm water (50 °C), dipped in 0.2% Dithane M-45 for 1 minute and planted in 250 ml glass jars having sterilized sand. Hoagland solution was applied to moisten the sand and covered with glass sigma jars. These jars were placed under high light intensity at a 16-h photoperiod for one week (100–120 μmol m^{-2} s^{-1}). Meanwhile, the covered jars were removed gradually. More Hoagland solution was applied if required. After 15 days, the upper jars were completely removed.

Rooted plants were put in earthen pots containing soil, transferred to the glasshouse and grown to maturity. The temperature of the glasshouse was adjusted to 30±2 °C with 65% relative humidity. Data was collected for all the parameters under considerations.

Results

Plant material and age of explant

Cotton seeds from the field, are highly contaminated as the seeds contain large number of small hairs which hold spores of fungus and bacteria. Delinting with H_2SO_4 is highly effective to remove the hairs and reduce the risk of contamination in the cultures. Germination rate was more than 90% from all the cultivars tested. The procedure will help to reduce the precision and availability of shoot tip plant material for large scale experimentation. In preliminary experiments, seed borne contamination caused serious problems. For any tissue culture study, the explant material must

Table 1. Effect of different media formulations on shoot, root and callus induction from meristem shoot tips of three cotton (*G. hirsutum* L.) varieties

Medium	cultivars[a]								
	S-12			N-78			M-93		
	S	R	C	S	R	C	S	R	C
1.2 MS+No growth regulators	2.8	0.0	0.0	3.0	0.0	0.0	2.9	0.0	0.0
MS+No growth regulators	2.7	0.0	0.0	2.7	0.0	0.0	2.8	0.0	0.0
MS+0.46 mM kinetin	7.0	0.0	0.0	7.1	0.0	0.0	8.6	0.0	0.0
MS+0.93 mM kinetin	7.8	0.0	0.0	8.5	0.0	0.0	7.3	0.0	0.0
MS+2.32 mM kinetin	7.5	0.0	0.0	6.4	0.0	0.0	5.4	0.0	0.0
MS+4.65 mM kinetin	2.1	0.0	0.0	2.4	0.0	0.0	3.0	0.0	0.0
MS+9.30 mM kinetin	1.0	0.0	0.0	1.9	0.0	0.0	1.9	0.0	0.0
MS+0.45 mM 2,4-D+2.3 mM kinetin	1.0	0.0	2.2	1.2	0.0	3.0	2.8	2.1	0.0
MS+0.45 mM 2,4-D+4.65 mM kinetin	0.8	0.0	2.1	1.5	0.0	3.0	1.5	2.1	0.0
MS+0.28 mM IAA+4.65 mM kinetin	1.5	0.0	0.0	2.0	0.0	0.0	1.1	0.0	0.0
MS+0.57 mM IAA+4.65 mM kinetin	1.1	0.0	0.0	1.9	0.0	0.0	1.7	0.0	0.0
MS+2.85 mM IAA+4.65 mM kinetin	3.0	0.0	0.0	2.5	0.0	0.0	2.4	0.0	0.0
MS+5.70 mM IAA+4.65 mM kinetin	2.5	0.0	0.0	2.5	0.0	0.0	2.8	0.0	0.0
MS+0.28 mM IAA+9.30 mM kinetin	0.8	0.0	0.5	1.8	0.0	0.0	1.8	0.0	0.0
MS+0.57 mM IAA+9.30 mM kinetin	1.2	0.0	0.0	1.7	0.0	0.0	1.4	0.0	0.0
MS+2.85 mM IAA+9.30 mM kinetin	2.5	0.0	0.0	2.8	0.0	0.0	2.7	0.0	0.0
MS+5.70 mM IAA+9.30 mM kinetin	1.8	0.0	0.0	1.1	0.0	0.0	1.9	0.0	0.0
MS+5.70 mM IAA+23.23 mM kinetin	1.7	0.0	0.0	1.5	0.0	0.0	2.0	0.0	0.0
MS+5.70 mM IAA+92.93 mM kinetin	1.8	0.0	0.0	1.0	0.0	0.0	2.0	0.0	0.0
MS+0.28 mM IAA+46.46 mM kinetin	1.0	0.0	0.0	1.4	0.0	0.0	1.2	0.0	0.0
MS+11.41 mM IAA+2.32 kinetin	1.5	1.8	1.4	1.0	1.7	1.0	1.7	1.5	0.6
MS+11.41 mM IAA+4.65 mM kinetin	1.5	0.0	1.0	1.8	0.0	0.0	1.5	0.0	0.0
MS+2.68 mM NAA+0.46 mM kinetin**	2.0	3.0	0.8	1.5	3.0	0.0	2.0	2.9	0.0
MS+5.37 mM NAA+2.32 mM kinetin	0.9	1.4	0.0	1.0	1.2	0.0	1.6	0.9	0.0
MS+10.74 NAA+0.46 mM kinetin	1.1	1.9	1.0	1.1	1.8	1.1	1.0	1.6	1.1
MS+10.74 mM NAA+2.32 mM kinetin	1.1	1.9	1.0	1.2	1.9	1.1	1.4	1.7	0.9
MS +10.74 mM NAA+4.65 mM kinetin	2.0	0.0	0.0	1.9	0.0	0.0	1.5	0.0	0.0
MS+5.37 mM NAA+2.46 mM 2iP	0.9	0.0	0.0	2.0	0.0	0.0	1.9	0.0	0.0

S = Shoot, R = Root and C = Callus. 0.0 = No growth of shoot, root or callus, 0.1–2.0 cm = Small growth/blackening of tissues/stunted growth, 2.1–3.0 cm = Medium growth of shoot/root/callus and 3.1–10.0 cm = Large growth of shoot, root and callus. *&** were selected for shoot and root development subsequently.

be fully sterilized. Cotton seeds were found 95% contamination free when dipped in 70% ethanol for 1–2 minutes and then in 15% commercial bleach with one drop of Tween –80 for 25–30 minutes. Cotton seeds germinated in one week and hypocotyl enlarged up to 5–10 cm in height. Cotyledons expanded and covered an area of 3 cm, which required large culture tubes (22 cm × 2.5 cm) with cotton plug enclosures, and all other types of test tubes and culture vessels were not fit for cotton seedling growth. Cotton seeds which grew for 7 days in the dark and then transferred to a 16-h photoperiod (60–80 μmol m^{-2} s^{-1}) at 30 °C gave maximum germination and plant yield. Above the cotyledons, meristem tip growth started after one week of seed culture and dome dividing into leaf primordia and rudimentary leaves (Gould et al., 1991). Meristem tips older than 4–5 days attained many leaf primordia, rudimentary leaves and mature leaves.

Media composition for shoot and root development

Unlike many other crop plants, which can be grown easily in tissue culture, cotton has proved to be very difficult. Addition of sucrose (30 g l^{-1}) and agar (8 g l^{-1}) in media, tissues/cells readily became brown/black, watery and ultimately loose the capacity of division. The problem was circumvented by the replacement of sucrose agar media with glucose (30

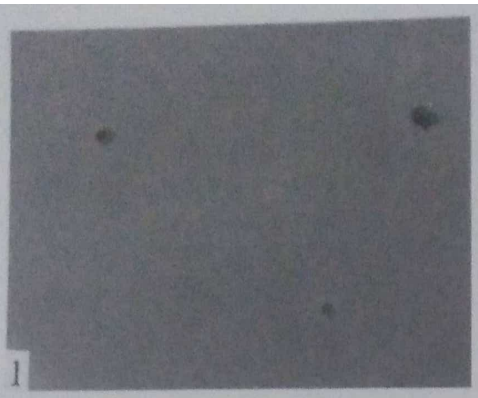

Figure 1. Isolated meristem shoot tips of cotton (*G. hirsutum* L) var. NIAB-78

Figure 2. Shoot tips of cotton (*G. hirsutum* L) var. NIAB-78 at different stages of shoot growth

g l^{-1}) and phytagel (2.5 g l^{-1}) and $MgCl_2$ (0.75 g l^{-1}) and raising of temperature from 25 to 30 °C. Basal portions of tips did not turn brown and grew normally. Browning problem was more severe when tissues were cultured for callus induction and suspension cultures.

In the first experiment, three cultivars viz. S-12, NIAB-78 and MNH-93 were tested on 8 different media and growth regulator combinations. As the media formulations were very diverse, response were also quite different. Size of meristem/shoot tip were also contributed significantly to the rate of plant formation. Mortality rate was highest (50%) when meristem of less than 0.5 mm size were cultured (Figure 1).

For the first two weeks the tips grew normal on all 28 media. In later stages, some proved detrimental and caused death of tip tissues. On 1/2 MS, MS, MS + 0.46 mM kinetin, MS + 0.93 mM kinetin an MS + 2.32 mM kinetin, shoots grew but callus and root formation was not observed (Figure 2). On media containing 2,4-D alongwith kinetin and 2iP, callus developed at the base of the meristem tip, tip growth was normal in the first two weeks and then retarded, leaves shed and tip degenerated. Media containing higher levels of kinetin also did not show good response. Tips cultured on

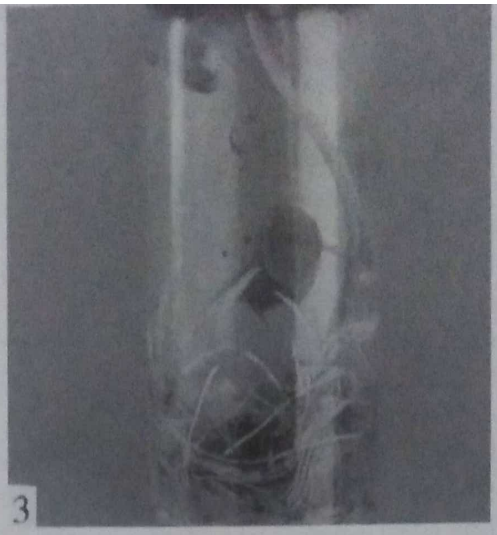

Figure 3. Rooted shoot tips of cotton *G. hirsutum* L) var. NIAB-78

media containing IAA (11.41 mM) with kinetin (2.32 mM) initiated roots from S-12, NIAB-78 and MNH-93 (Figure 3) but callus was also present at the base and tip growth was not too good as on simple kinetin (0.46 mM and 0.93 mM) media.

In media containing NAA (10.74 mM) and kinetin (0.46 mM and 2.32 mM) rooting response was higher than shoot development. Callus was also present at the base. Very little shoot growth was observed but not rooting on media containing NAA (5.37 mM) and 2iP (2.46 mM). Highest rooting was observed when (2.68 mM) with kinetin (0.46 mM) was used. All the three varieties cultured on this concentration developed roots (Table 3).

From the above studies, data of shoot, root and callus was collected. Some media were selected only for shoot tip growth and some others were included in the rooting media formulations. In the second experiment, all the other 16 cultivars were cultured on 6 defined media. MS simple medium, MS with 0.46 mM and 93 mM kinetin were found highly suitable for shoot tip growth in cotton. Kinetin enhanced the division of tip cells and favor the formation of leaves. Media containing 2,4-D and 2iP enhanced the callus formation and no root formation respectively. Shoots from 10 cultivars which were developed on kinetin media were transferred to rooting media. Root initiation was not observed on MS or 1/2MS medium. Varieties S-12, SL-41, FH-682 and BH-36 also developed roots on media containing 2.46 mM IBA in the media. While all the varieties tested developed roots only on media containing 2.68 mM NAA and 0.46 mM kinetin (Table 2). Shoot tips developed roots more readily than hypocotyl segments which indicates the response of leaf and tip material for root development.

Table 2. Rooting of shoot tips from 10 cultivars of cotton (*G. hirsutum* L) on 8 different media compositions.

Cultivar	MS-Media with growth regulators (mM)							
	1/2 MS	MS	0.98 IBA	2.46 IBA	4.92 IBA	9.84 IBA	24.6 IBA	2.68 NAA 0.46 kin
1. N-78-	-	-	-	-	-	-	-	ROOT
2. S-12	-	-	-	ROOT	-	-	-	ROOT
3. Coker-312	-	-	-	-	-	-	-	ROOT
4. B-557-	-	-	-	-	-	-	-	ROOT
5. CIM-109-	-	-	-	-	-	-	-	ROOT
6. RH-1-	-	-	-	-	-	-	-	ROOT
7. N-26-	-	-	-	-	-	-	-	ROOT
8. SL-41	-	-	-	ROOT	-	-	-	ROOT
9. FH-682	-	-	-	ROOT	-	-	-	ROOT
10. BH-36	-	-	-	ROOT	-	-	-	ROOT

[a] = No root formation, ROOT = Roots developed.

Transfer of plants to sand and soil

Meristem tips raised to complete plants were transferred to sand and soil for further growth. Conditions were optimized for the whole process and a protocol was developed (Table 3). Plant height should be at least 2–3 cm at the time of transfer to sand and soil. Sand media hardened the roots and gradual uncovering hardened the cuticle layer that was helpful to withstand in the adverse outside environment out of test tube. For growth of cotton plant, a temperature of 29–31 °C with 65% relative humidity and a photoperiod of 14/10 hours was required in the green house.

Characters of regenerated plants

Cotton meristem tissues did not undergo any form of malformation. Meristems are highly organized tissues and showed no variation in genotypic and phenotypic characters in the present study. Normal shoots and roots were developed which bore flowers and bolls and set seeds. Only a small fraction of meristems developed into abnormal shoots. Overall there was no difficulty in raising plants from the meristem tips of cotton. Varietal differences were also negligible.

Discussion

The starting material for these experiments was the seeds of different cotton varieties. Availability of good quality and true to type seed is the prerequisite. Poorly germinated seeds hinder the raising of seedlings in large scale and supply of meristems in the required quantity for biolistic gun transformation experiments. Seed borne contamination also effects significantly but in these studies it was controlled up to maximum level. Explant is the major source of contamination and in certain cases, tissue sterilization could not be properly done. Gould et al., (1991) found that the rate of uncontaminated seedlings varied between seed lot and genotype.

Age of explant found to be major factor for successful micropropagation of cotton. Explant seedlings less than 2-days and more than 5-days old did not develop good type of meristems. It is difficult to excise and detect the meristem dome from the tips of less than two days age. As dome formation has just started, even it is difficult to detect and surgery at this microscopic level is even more difficult. Extra care is required to remove the leaf primordia and tip must not be injured in any case. Root hairs also hinder the exposure of the tip. Tissue older than 4–5 days, developed a small stem beyond the base of cotyledons. At this stage, the tissue become smaller in size and more watery with lots of hairs thus making the excision of the tip a tedious exercise. Only the explant tissues between the age of 3–5 days is best for the excision of meristem tips. Chlan et al. (1995) excised embryonic axis from overnight soaked seeds and exposed the meristem sections for bombardment. These embryonic axis are the alternative explants for bombardments and found to be more suitable for transgenic experiments (unpublished data). Even in such studies this methodology is applicable. These tips are required for the direct

Table 3. Hardening procedure for cotton plants regenerated from apical meristem.

0–day	When shoot (2–3cm) and roots develop *in-vitro*, take out the plant, remove agar with warm water. Dip the roots for a few minutes in fungicide solution (0.2% Dithane M-45). Place the plants in jars containing sterilized fine sand, apply Hoaglands solution and cover the pots with another small jar/polythene.
4–5 days.	Uncover the jars gradually
7–10 days.	New roots develop.
12–14 days.	Transfer plants to earthen pots and place in the glass house with a temperature 29–31 °C and 16/8 hour photoperiod.
90–95 days.	Plants reach the flowering stage and bear seeds.

DNA transfer by biolistic gun, exposure of the dome to the tungsten particles is required. It is necessary to clear the dome and rudimentary leaves and primordia must be removed. Size of the tissue is not as important as the exposure of dome and Tungsten + DNA must be reached up to the embryogenic cell which is beneath the tunica (double layer of cells) layer. Replacement of sucrose and agar media with glucose and phytagel helped in the control of browning and tissue mortality. These results are in consistent with the findings of Shoemaker et al. (1986) and Trolinder and Goodin (1987) who used the glucose and phytagel in the medium for various types of callus induction and plant regeneration experiments.

Tips are very delicate, watery aggregates of cells and during side tissues removal, the meristematic cell portions may be disturbed that cause death/malformation of tissues. The objective is to hit the regenerable meristem tips which remain exposed even when the base tissues are present, therefore, no need to reduce its length to a very small size. These basal tissues also help to grow the shoot tip properly and have no limitation.

Acknowledgements

The authors wish to thank Cotton Research Institute, AARI, Faisalabad; Atomic Energy Agri. Research Center, Tandojam and Central Cotton Research Institute, Multan, Pakistan for providing seeds of cotton varieties. This research work has been partially supported by research grants from Common Fund for Commodities (CFC), Netherlands/International Cotton Advisory Commodities (ICAC), Washington, USA and from Federal Ministry of Food, Agriculture and Livestock, Pakistan/Asian Development Bank.

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