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In vitro Morphogenic Response of Cotton (*Gossypium hirsutum* L.) From Apical Meristem Culture

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Abstract: A study was conducted for regeneration of cotton (*Gossypium hirsutum*) through meristem culture in order to produce transgenic plants through microinjection technology. Five locally adapted commercial cotton varieties (CIM-443, CIM-446, CIM-473, NIAB-Karishma and FH-900) and a candidate variety NIAB-98 was used in this study. Seeds were grown on MS medium supplemented with B_5 vitamins and apical meristem was obtained from *in vitro* grown 5-7 days old cotton seedlings. Apical meristem was cultured on different concentration of Kinetin and Benzyl Adenine (1-2-3-4-6 mg L^{-1}) and various concentrations of auxins and cytokinins to investigate the effect of these hormones for shoot, root and callus induction. The best medium was 2 mg L^{-1} BA alone for shoot meanwhile, 2 mg L^{-1} Kin. + 1.5 mg L^{-1} IAA for root induction. Varietal response of various growth regulators for regeneration was also noted which revealed that all varieties showed regeneration on above media but the maximum regeneration was observed incase of CIM-443 whereas FH-900 showed the minimum response. It was noted that low concentration of phyto hormones was beneficial for plant regeneration and high concentration of these led to abnormal shoot formation and induced callus formation. Most of the regenerated plants could not produce root, therefore, these were grafted to seedlings.

Key words: *In vitro* · Plant regeneration · Meristem culture · Cotton · *G. hirsutum* · Grafting

Abbreviations: K - Kinetin; BA - benzyl adenine; IAA - Indole acetic acid; NAA - α naphthalene acetic acid; MS medium [13]; B_5 medium [14].

INTRODUCTION

Cotton (*Gossypium* spp.) is a unique and intriguing non-food cash crop through out the world for its fiber. It is the largest cash crop of Pakistan cultivated over 3125,000 hectares area [1]. Besides earning huge amount of foreign exchange through its export and providing fiber to the textile industry, it provides food (oil) and feed (seed cake) for human and animals respectively. During the last decade the leaf curl virus (CLCuV) disease emerged as the most important disease in Pakistan for cotton. This viral disease was appeared near Multan during 1967 [2]. This disease reached economic importance in 1987-1988 and became epidemic in 1992. Cotton leaf curl virus belongs to Gemini group of viruses transmitted by white fly (*Bamisia tabaci*) as a vector [3]. Control of insect vector and crop rotation are used as measures but resistant varieties must be developed to overcome this epidemic.

Cotton breeders have continuously sought to improve through cultivar genotypes that are resistant to disease but genetic improvement of cotton through conventional breeding is limited by several factors like lack of useful variation and longer time period that are required. Although plant biotechnology is an attractive mean for improving cotton plant but its use requires an effective regeneration system from somatic tissue of cotton plant. Cotton like many genera is recalcitrant to regeneration from protoplast, leaf or callus tissue which restrict improvement through genetic engineering because few cultivars are regenerable and somatic embryogenesis is restricted to few genotypes e.g. Cocker and Siokra [4-8]. Therefore the development of tissue culture protocol to induce efficient proliferation in a genotype-independent manner is required for producing genetic transformations in cotton. Apical meristem culture has become an important tool for rapid propagation of plant species.

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Meristem tissue is best for regeneration because these tissues are programmed for shoot organogenesis. Therefore compared with somatic cell culture, shoot and meristem culture is an easier method to obtain true to type plants.

Meristem culture makes a wide range of germplasm accessible to improve by DNA transformation [9, 10]. Currently cotton is being transformed by two methods: particle bombardment [11] and co cultivation with *Agrobacterium tumifacience* [12]. DNA microinjection is a recent technique to deliver DNA into animals and plant cells through a very fine syringe. The transformation efficiency of microinjection is as against 1 transgenic plant per 1000 bombardments [11]. All these transformation procedures require somatic embryogenesis, which is restricted to few cultivars and method involves transformation of regenerable cells or callus and regeneration, collection of transgenic seed and back crossing of the desired traits into the adopted cultivars.

We have been able to develop a simple meristem culture protocol alongwith grafting of regenerated plants into already existing plants to induce efficient proliferation in genotype independent manner in the local cotton genotypes, which will help us to screen large number of DNA transformations in cotton obtained through microinjection technology.

MATERIALS AND METHODS

Plant Material: True to type, selfed seeds of the following local cotton varieties/lines were used in the study.

Preparation of Plant Material: Seeds were delinted with H_2SO_4@ 10 mL per 100gm and washed with excessive water to remove acid. These seeds were dried under shade and stored for further studies.

Media Composition: The basic culture media was MS mineral salts [13] plus B_5 vitamins [14], 30 g L^{-1} Sucrose and Difco Batco agar at 10 g L^{-1} and various concentrations and combinations of phyto hormones (Table 2) were added to the medium in order to determine the optimum conditions of plant regeneration. The medium was adjusted to pH of 5.8 with 1N HCl or

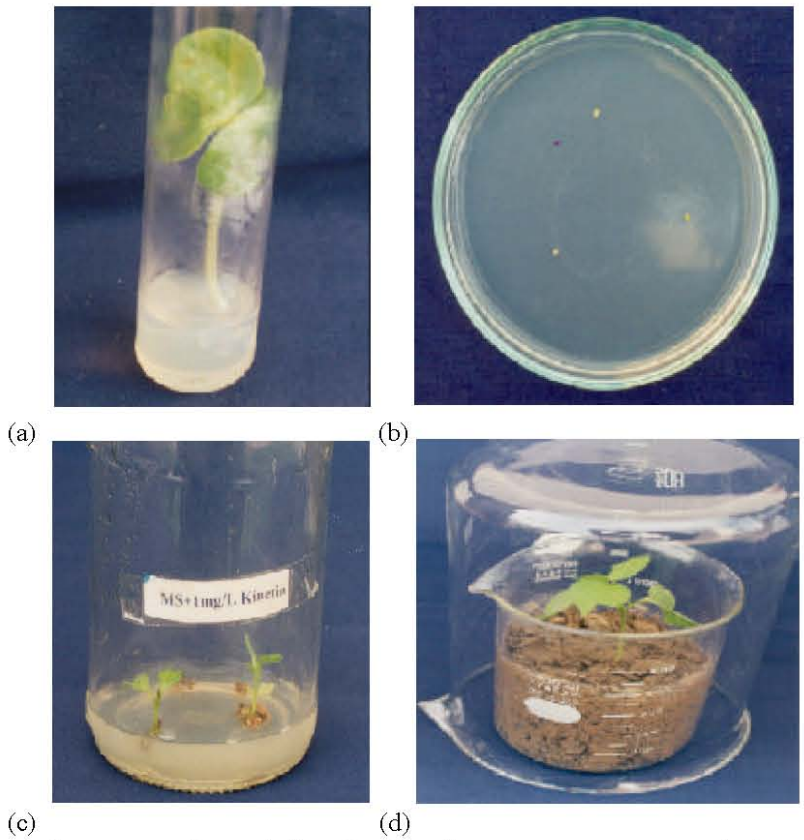

Fig. 1: Different stages of *in vitro* culture a) Germination of cotton genotype to obtain meristem shoot tip. b) Culturing of meristem on modified MS medium for regeneration. C) Regeneration of cotton meristem on modified MS media. d) Hardening of cotton plant obtained through meristem culture

Table 1: Different cotton genotypes used in meristem culture studies

Sr No.	Origin	Cotton genotypes
1	NIAB, Faisalabad	NIAB-98
2	NIAB, Faisalabad	NIAB-Karishma
3	CRI, Multan	CIM-443
4	CRI, Multan	CIM-473
5	CRI, Multan	CIM-446
6	CRI, AARI, Faisalabad	FH-900

Table 2: Morphogenic response of meristem tips from cotton varieties cultured on Ms- media containing different concentrations from plant growth regulators

	NIAB-98			NIAB-Karishma			CIM-443			CIM-473			CIM-446			FH-900		
Plant growth regulator	C	R	S	C	R	S	C	R	S	C	R	S	C	R	S	C	R	S
Zero	0.0	0.5	1.0	0.0	0.4	1.2	0.3	1.0	0.5	0.2	0.5	0.3	0.0	0.2	0.5	0.0	0.3	0.4
1 mg L^{-1} Kin	0.2	0.0	0.8	0.1	0.0	0.5	0.3	0.0	0.5	0.1	0.0	0.6	0.4	0.0	0.4	0.1	0.0	0.2
2 mg L^{-1} Kin	0.0	0.2	1.2	0.3	0.4	1.5	0.2	0.8	1.8	0.5	0.9	1.3	0.4	0.3	0.9	0.1	0.3	0.9
3 mg L^{-1} Kin	0.3	0.0	0.6	0.2	0.0	0.8	0.5	0.2	0.9	0.4	0.1	0.6	0.3	0.0	0.8	0.4	0.0	0.9
4 mg L^{-1} Kin	0.2	0.0	0.6	0.3	0.0	0.9	0.5	0.0	0.8	0.4	0.0	1.0	0.4	0.0	0.5	0.3	0.0	0.7
6 mg L^{-1} Kin	0.4	0.0	0.5	0.2	0.0	0.8	0.1	0.0	0.7	0.5	0.0	0.5	0.2	0.0	0.5	0.1	0.0	0.3
1 mg L^{-1} BA	0.5	0.0	1.5	0.5	0.0	1.5	0.3	0.0	1.6	0.2	0.0	1.5	0.4	0.0	1.0	0.2	0.0	1.3
2 mg L^{-1} BA	0.5	1.0	2.5	0.8	0.0	2.0	0.5	0.0	1.8	0.5	0.0	1.8	0.2	0.0	2.1	0.4	0.0	2.2
4 mg L^{-1} BA	1.0	0.5	1.0	1.2	0.2	1.39	1.2	0.2	1.2	0.8	0.0	0.	0.7	0.0	1.0	0.8	0.0	0.8
6 mg L^{-1} BA	1.8	0.0	0.5	1.5	0.0	0.2	1.6	0.0	0.6	1.6	0.0	0.3	1.6	0.0	0.5	1.8	0.0	0.2
2 mg L^{-1} BA+1 mg L^{-1} NAA	0.7	0.2	1.1	0.5	0.0	0.6	0.4	0.0	0.2	0.6	0.0	0.5	0.4	0.0	0.3	0.2	0.0	0.3
1 mg L^{-1} BA+0.5 mg L^{-1} NAA	0.8	0.0	1.0	0.6	0.0	0.8	0.5	0.0	0.7	0.5	0.0	0.6	0.4	0.0	0.5	0.2	0.0	0.6
1 mg L^{-1} BA+1 mg L^{-1} NAA	0.5	0.0	1.1	0.9	0.0	1.2	1.0	0.0	1.6	0.8	0.0	1.4	0.5	0.0	0.8	0.4	0.0	0.6
0.5 mg L^{-1} K+1 mg L^{-1} NAA	0.5	0.0	1.0	0.7	0.0	1.2	0.7	0.0	1.6	0.5	0.0	1.5	0.6	0.0	1.1	0.3	0.0	1.1
4 mg L^{-1} K+2 mg L^{-1}NAA	1.0	0.0	1.5	0.7	0.0	0.9	0.5	0.0	0.8	1.1	0.0	1.0	0.7	0.0	1.2	0.5	0.0	0.9
2 mg L^{-1}K+1.5 mg L^{-1} IAA	1.4	1.1	1.8	0.8	1.0	1.7	0.9	1.5	1.9	1.0	0.8	1.5	0.9	0.5	1.1	0.8	0.4	1.4

0.0cm-0.5cm = very little growth, 1.0cm-1.5cm = large growth, 0.5cm-0.1cm = medium growth, 1.5cm-2.0 = very large growth

C: Callus, R: root, S: shoot

1N NaOH prior to autoclaving for 15 min at 121°C. All the varieties were cultured on the following media combinations: MS, MS+1 mg L^{-1} K, MS+ 2 mg L^{-1} K, MS+ 3 mg L^{-1} K, MS+ 4 mg L^{-1} K, MS+6 mg L^{-1} K, MS+1 mg L^{-1} BA, MS+ 2 mg L^{-1} BA, MS+ 4 mg L^{-1} BA, MS+ 6 mg L^{-1} BA, MS+2 mg L^{-1} BA+1 mg L^{-1} NAA, MS+1 mg L^{-1} BA+ 0.5 mg L^{-1} NAA, MS+1 mg L^{-1} BA+ 1 mg L^{-1} NAA, MS+ 0.5 mg L^{-1} K +1 mg L^{-1}NAA, MS+4 mg L^{-1} K +2 mg L^{-1} NAA, MS+2 mg L^{-1} K +1.5 mg L^{-1} IAA.

Preparation of Explants: Delinted cotton seed were surface sterilized by agitation (5min) in 0.5% $HgCl_2$ and 0.1% SDS (as wetting agent). Seeds were washed with excessive sterile distilled water to remove all the traces of $HgCl_2$ and soaked in dark for 10 hrs to moisten with sterile water at 25°C. After soaking, seed coat was removed and embryos were cultured on MS medium supplemented with B_5 vitamins, which were grown on growth room at 16-hr day length and 28±2°C to get apical meristem. Seed sterilization was manipulated in laminar airflow (model, Biocyt Flufrance Zac Du Vaulorin-91320 Wissous-France),

Culturing of Meristem Tip: Meristem tips were obtained from 5-7 days old aseptically raised cotton seedling. Cotyledons and hypocotyls were removed and meristem tips were separated, cultured on MS media containing a range of auxins and cytokinins at various concentrations in petriplates. Meristem tips were cultured on these petriplates, various growth regulators on shoot, root and callus. When shoots grew to a height of 1-2 cm, these were transferred to test tubes containing same medium. The shoots having regenerated roots, when attained height of 2-3 cm were transferred to autoclaved soil and covered with inverted glass beaker that was removed after 10-15 days. The plants, which could not develop root but high shoot, were grafted on already growing seedling.

Grafting of Plant: A preexisting plant was selected for the grafting of new regenerated plants. An internodal section of any pre-existing plant having same age and diameter was selected for grafting and regenerated plant was inserted in same orientation. The naked portion of attachment was wrapped with Parafilm and covered with

glass jar. The plant was watered whenever required. The jar was gradually raised and after 10-15 days was removed completely and plant was transferred to glasshouse.

RESULTS AND DISCUSSION

Cottonseeds germinated within one week, attaining the height of 8-10 cm. seeds, which grew for 7 days in the dark and then transferred to a 16 hr photoperiod (60-80-μmol m^{-2} s^{-1}) at 30° C, gave maximum germination and plant yield. Meristem tip growth started after one week of seed culture and dome dividing into leaf perimodia and rudimentary leaves [9] Meristem tips older than 4-5 days attained many leaf primodia, rudimentary leaves and mature leaves. In the above experiment, 5 days old explants was used as a source of meristem.

All the above-mentioned genotypes of cotton were tested against 16 media comprising various combinations of plant hormones. From the above studies the data of root, shoot and callus were recorded. From the data it is revealed that at different concentrations of Kinetin and benzyl Adenine all genotypes showed diverse response. With the increase in Kinetin concentration upto 2 mg L^{-1} shoot formation was enhanced while further increasing concentration, shoot regeneration was suppressed. At concentration above 2 mg L^{-1} Kinetin callus initiation and proliferation was observed which was maximum at 6 mg L^{-1} concentration. Similar results were reported by Nasir *et al.*, [10] who found best shoot regeneration for Gossypium meristem at MS medium supplemented with 0.46 mM Kinetin. Kinetin enhances cell division of meristematic region and favours the shoot formation. A very small mass of callus was observed at the base of meristem on all Kinetin containing media but medium supplemented with 2 mg L^{-1} Kintein + 1.5 mg L^{-1} IAA showed root regeneration alongwith shoot formation.

The above data revealed that meristem tip cultured on MS medium without any growth regulator showed moderate shoot formation along with root formation in all varieties. No callus was observed in any genotype except very little in case of CIM-443 and CIM-473. Meristem cultured on BA showed that at concentration of 1 and 2 mg L^{-1} shoot formation was favoured but as concentration was increased abnormal shoot was formed and at concentration of 6 mg L^{-1} black watery callus was formed. Therefore, higher concentration of BA produced detrimental effect and inhibited shoot regeneration BA activated root formation in all varieties with wide range of concentration. Rooting was observed at 2 mg L^{-1} BA in NIAB-98 and 4 mg L^{-1} in NIAB-98, NIAB-Karishma and CIM-443. The results are not in agreement with Hemphill *et al.*, [15] who used BA for shoot regeneration and elongation and reported that maximum shoot formation was at concentration of 0.3 μ M BA but failed to regenerate root. Media combination of various concentrations of auxins and cytokinin showed very diverse response. Best media for shoot and root regeneration was MS medium supplemented with 2 mg L^{-1} K and 1.5 mg L^{-1} IAA for all cotton lines. Rooting was not observed on any media combination of cytokinins and auxins except the above-mentioned media. All varieties showed higher degree of root formation on the above media but FH-900 and CIM-446, which showed least, rooting response. Chlan *et al.*, [16] transferred transformed shoots to MS medium supplemented with IAA (100 mg L^{-1}) to support the formation of a vigorous root system. A reduced auto inhibitory response was noted by culturing on media containing charcoal (unpublished data). Gloud *et al.*, [9] were unsuccessful in root induction from cotton shoots isolated apices but there was high frequency of root formation when cultured on media containing charcoal and then transferring it to potting soil. From the above studies it is concluded that MS medium without growth regulators promoted moderate shoot and root formation. Lower level of these hormones like 2 mg L^{-1} Kinetin and Benzyl Adenine showed shoot and root regeneration Some of these media showed higher degree of root formation and plants formed were directly transferred to soil for hardening. Shoot was regenerated on different media but rate of shoot formation was different on all media. In the most of the experiments meristem tips produced callus and turned brown at the base. The results revealed that over all it was difficult to regenerate root than shoot from apical meristem.

Some plants produced root and transferred to autoclaved soil. But most of the regenerated shoots could not regenerate roots and grafted successfully into a well-established plant. All the grafted plants prolonged well, set bolls and bore seed.

The plants developed through meristem culture did not undergo any type of malformation because the meristems are highly organized tissues and showed no variation for the genotypic/phenotypic characters in these studies. Normal roots and shoots were developed which bore flowers and fruits and set seeds on them. Overall there was no difficulty in raising plants from meristem tips of cotton and grafting on already established plants. However the varietal differences were quite obvious to regenerate into plants through meristem culture. DNA microinjection was previously used for transformation in

protoplast [17], microspore [18] and cell [19] but in meristem no attempt was made in the past. The efforts are underway to develop this plant regeneration procedure with gene transfer for the rapid introduction of leaf curl virus resistant traits into the higher yielding cotton genotypes and vice versa.

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