Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

ANSInet
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http://ansinet.com

Asian Journal of Plant Sciences 4 (3): 249-254, 2005
ISSN 1682-3974

Optimization/Standardization of Tissue Culture Technology Suitable for Inducing Genetic Transformations through Microinjection in the Locally Adopted Cotton Genotypes

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Abstract: In the present study, a series of experiment were carried out to investigate the influence of explant type and media combination on the induction of embryogenic callus, plant regeneration and suitable explant for DNA microinjection. The size of explant played major role in the plant regeneration. Mortality rate was high when smaller size explant was used. 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 phenotypic characters in these studies. With the increase in kinetin concentration up to 2 mg L^{-1}, shoot formation was enhanced and callus initiation and proliferation was observed which was maximum at 6 mg L^{-1} concentration. Best media for callus induction, shoot and root regeneration was MS medium supplemented with 2 mg L^{-1} K and 1.5 mg L^{-1} IAA. All varieties showed higher degree of root formation except FH-900 and CIM-446 that showed least rooting. The MS medium without growth regulators produced moderate shoot and root formation while medium containing different levels of phytohormones effected significantly in shoot and root formation. Charcoal had positive effect on callus induction and browning was reduced. It is concluded that cotyledon and root from the local genotypes are not suitable explants for callus induction and proliferation. Ovules and hypocotyls are the best for callus induction. The efforts are underway to develop plant regeneration procedure for gene transfer leading to rapid introduction of leaf curl virus resistance into the higher yielding cotton genotypes and vice versa.

Key words: Optimization, tissue culture technology, genetic transformation, locally adopted, cotton genotypes, explant

INTRODUCTION

Cotton (*Gossypium* spp.) is a unique and intriguing non-food cash crop throughout the world for its fibre. It is the largest cash crop of Pakistan cultivated over an area of 3125, 000 ha[1]. Besides earning huge amount of foreign exchange through its export, it provides fiber to the textile industry. It also provides food (oil) and feed (seed cake) for human and dairy animals. During the past decade, the Cotton Leaf Curl Virus (CLCuV) disease emerged as the most important disease of cotton in Pakistan. This disease was first noticed near Multan during 1967[2] reached to economic importance in 1987-1988 and became epidemic in 1993-1994. Control of insect vector and crop rotation are the control measures to minimize losses but resistant varieties must be developed to overcome this epidemic. Cotton breeders have continuously sought to improve the situation through developing cultivars/genotypes that are resistant to disease. However, the genetic improvement of cotton through conventional breeding method is limited by several factors like, lack of desirable genetic variability, undesirable gene linkage and longer time period required to develop a desired genotype. Although plant biotechnology is an attractive tool for improving crop plant but its use requires an effective regeneration system from somatic tissue of plants. Cotton like many genera is recalcitrant to regeneration from cell, organ, protoplast, leaf or callus tissue, which restrict its improvement through genetic engineering. Because few cultivars are regenerable and somatic embryogenesis is restricted to few genotypes e.g. Cocker and Siokra[3-5] and most of the programmes utilizing transgenes rely heavily or exclusively on back crossing to incorporate foreign genes into a desired genotype, a strategy that does nothing to expand genetic diversity.

Therefore, the development of tissue culture protocol to induce efficient callus induction and regeneration 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. Other explants like roots, root segments, epicotyls and cotyledons are also used for somatic

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embryogenesis and plant regeneration[6] but callus induction and somatic embryo formation is required which is restricted only to few cultivars of cotton[7,8]. Meristem tissue is the best for regeneration because these tissues are programmed for shoot organogenesis and compared with somatic cell culture, shoot and meristem culture is an easier method to obtain true to type plants.

Currently cotton is being transformed most widely by two methods: particle bombardment[9] and co-cultivation with *agrobacterium tumifacience*[10,11]. DNA microinjection is used to deliver donor DNA into animals and plant cells through an ultra fine syringe. The transformation efficiency of microinjection is higher than other transformation techniques[12]. These transformation procedures require somatic embryogenesis, involving transformation of regenerable cells or callus and regeneration, collection of transgenic seed and back crossing of the desired traits into the adopted cultivars. In the present study, a series of experiment were carried out to investigate the influence of explant type and media combination on the induction of embryogenic callus, plant regeneration and suitable explant for DNA microinjection. Efficient procedures to induce plant regeneration from meristem of indigenous genotypes have been reported but regeneration of plants from callus has not yet been reported from the local cotton genotypes.

MATERIALS AND METHODS

True to type, self seeds of local cotton varieties/lines selected under Pak-Kazakh project were used in this study. For ovule culture NIAB-98, CIM-443, L-31, DNH-49 and K-599 were used while for meristem, cotyledons, hypocotyls and root culture of NIAB-98, NIAB-K, CIM-443, CIM-473, CIM-446 and FH-900 were used.

Preparation of plant material: Seeds were delinted with commercial H_2SO_4@ 10 mL 100^{-1} g and washed with excessive water to remove acid. These seeds were dried under shade and stored for further studies.

Media composition: The basic culture medium was MS mineral salts[13] plus B_5 vitamins[14], 30 mg L^{-1} Sucrose and Difco Batco agar 10 mg L^{-1} and various concentrations and combinations of phytohormones were added to the medium in order to determine the suitable medium and optimum conditions for callus induction and plant regeneration. The medium was adjusted to pH 5.8 with 1 N HCl or 1 N NaOH prior to autoclaving for 15 min at 121°C. The cultures were incubated at 28± 2°C with 16 h photoperiod.

Meristem tips of all genotypes were cultured on the following media combinations: MS, MS+1 mg L^{-1} K, MS+2 mg L^{-1} K, MS+mg L^{-1} K, MS+4 mg L^{-1}K, MS+6 mg L^{-1} K, MS+ 4 mg L^{-1} K +2 mg L^{-1} NAA, MS +2 mg L^{-1} K +1.5 mg L^{-1} IAA.

Ovules were cultured on MS+1 mg L^{-1} BAP+1 mg L^{-1} GA; MS + 1.5 mg L^{-1} BAP + 1 mg L^{-1} GA; MS + 2 mg L^{-1} BAP+1 mg L^{-1} GA; MS + 3 mg L^{-1} BAP + mg L^{-1} GA; MS+4 mg L^{-1} BAP+1 mg L^{-1} GA; MS+5 mg L^{-1} BAP+1 mg L^{-1} GA) while roots, hypocotyls and cotyledons explants were cultured on MS+0.1 mg L^{-1} 2, 4, D+ 0.5 mg L^{-1} Z; MS+0.5 mg L^{-1} 2, 4, D+ 1 mg L^{-1} Z; MS+1.25 mg L^{-1} 2, 4, D+ 2 mg L^{-1} Z+ 1 mg L^{-1} activated charcoal; MS+0.5 mg L^{-1} Z+ 0.5 mg L^{-1} IAA; MS+0.1 mg L^{-1} 2,4,D+ 1 mg L^{-1} K+1 mg L^{-1} activated charcoal; MS+0.1 mg L^{-1} 2, 4, D+ 0.25 mg L^{-1} BAP; MS+1 mg L^{-1} 2, 4, D+3 mg L^{-1} Z.

Preparation of explant: Delinted cottonseeds were surface sterilized by agitation (5 min) in 0.1% $HgCl_2$ and 0.5% SDS (as wetting agent). Seeds were washed with excessive sterile distilled water to remove all the traces of $HgCl_2$ and soaked in sterile water at 25°C to soften seed coat. After soaking, seed coat was removed and kernels were cultured on MS medium supplemented with B_5 vitamins. These cultured embryos were grown on growth room at 16 h day/night length and 28±2°C to obtain explants. Cotyledons, hypocotyls, root and meristem explants were separated. Transverse sections of hypocotyls ranged from 3-5 mm thickness were taken from lower half of the seedling. Cotyledons were separated and cut in 4 mm^2 pieces. Meristem tips were also dissected from aseptically raised seedlings.

Culturing of explants: All explants were cultured on MSB media containing a range of auxins and cytokinins at various concentrations in petriplates. Two to three days old self sterilized bolls were collected and surface sterilized by immersing for 5 min with ethanol and 5 min with $HgCl_2$ 0.1% and 0.5% SDS followed by three rinses with distilled water and dissected with scalpel. These dissected ovules were floated in sterile dionized water until all ovules had been isolated[15] and placed vertically onto the medium with cut surface in contact with the medium. Ten explants were placed in each petriplates and 10 petriplates were used per treatment. Petriplates were sealed with parafilm and incubated at 16 h day length at 28±2°C. Explant preparation and culturing was manipulated in the laminar airflow (Model, Biocyt Flu France Zac Du Vaulorin-91320 Wissous-France).

RESULTS AND DISCUSSION

Cottonseeds as such are highly contaminated with spores of fungus and bacteria. Therefore, treating them with H_2SO_4 is a highly effective method to remove the hairs and reduce the risk of contamination in the

Table 1: Effect of media combination on meristem culture of different cotton varieties

Plant growth regulator supplement	Morphogenic response																	
	NIAB-98			NIAB-Karishma			CIM-443			CIM-473			CIM-446			FH-900		
mg L^{-1}	C	R	S	C	R	S	C	R	S	C	R	S	C	R	S	C	R	S
MS	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
MS+1 mg L^{-1} K	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
MS+2 mg L^{-1} K	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
MS+3 mg L^{-1} K	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
MS+4 mg L^{-1} K	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
MS+6 mg L^{-1} K	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
MS+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
MS+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

0.0 to 0.5 cm = Very little growth, 1.0 to 1.5 cm = Large growth, 0.5 to 0.1 cm = Medium growth, 1.5 to 2.0 cm = Very large growth

cultures because in any tissue culture study the explant material must be fully sterilized. The effect of different phytohormones on plant regeneration was observed. In all these experiments, the concentration of various plant growth regulators was kept at lower level because high concentration of plant hormones can act at molecular level and produce adverse effects on plants. The size of explant plays major role in the plant regeneration. Mortality rate was high when smaller size of explant was used. The objective is to inject DNA into embryonic cells present at the tip of the stem. Therefore, it is not necessary to reduce the size of explant because it supports the meristem growing on the medium. The data revealed that at different concentrations of kinetin, all the genotypes showed diverse response. With the increase in kinetin concentration up to 2 mg L^{-1} shoot formation was enhanced and callus initiation and proliferation was observed which was maximum at 6 mg L^{-1} concentration (Table 1). Similar results have been reported by Nasir *et al.*[16] who found best shoot regeneration from *Gossypium* meristem at MS medium supplemented with 0.46 mM kinetin. Kinetin enhances cell division of meristematic region and favours leaf formation. A very small mass of callus was observed at the base of meristem on all kinetin containing media, which turned black, medium supplemented with 2 mg L^{-1} kintein showed root regeneration along with shoot formation.

Media combination of auxins and cytokinin showed very diverse response. Best media for callus induction shoot and root regeneration was MS medium supplemented with 2 mg L^{-1} K and 1.5 mg L^{-1} IAA (Table 1). All varieties showed higher degree of root formation except FH-900 and CIM-446 that showed least rooting. Chan *et al.*[17] transferred transformed shoots to MS medium supplemented with IAA (100 mg L^{-1}) to support the formation of a vigorous root system. However no multiple shooting has been observed in cotton[18] as incase of soybean[19] and sorghum[20]. Gold *et al.*[18] cultured cotton shoot explants of different sizes (0.3 to 1.0 mm) on media containing IAA and kinetin in various combinations and obtained regenerable plant. From the above studies it is concluded that MS medium without growth regulators produced moderate shoot and root formation while medium containing different levels of phytohormones effected significantly for shoot and root formation. Some plants produced root and transferred to autoclaved soil. But most of the regenerated shoots could not regenerate roots and hence were grafted successfully onto a well-established cotton plant. All the grafted plants prolonged well, set bolls and bore seed.

Table 2: Effect of media combination on callus induction of cotton ovule of different cotton varieties

Medium used	No. of explants	No. of calluses	Callus induction	Colour of callus	Mortality rate (%)
L-311	50	33	66	Light yellow	34
2	50	46	92	Light yellow	8
3	50	35	70	Cream	30
4	50	22	44	Whitish brown	56
5	50	12	24	Brown	68
6	50	11	22	Brown	78
NIAB-98					
1	50	36	72	Light yellow	28
2	50	47	94	White	6
3	50	40	80	Light yellow	20
4	50	21	42	Greenish brown	58
5	50	18	36	Brown	64
6	50	15	30	Brown	70
CIM-443					
1	50	31	62	Light yellow	38
2	50	37	74	Light yellow	26
3	50	32	64	Light yellow	36
4	50	21	42	White	58
5	50	14	28	Greenish brown	72
6	50	12	24	Brown	76
DNH-49					
1	50	33	66	Light yellow	34
2	50	44	88	Yellow	12
3	50	36	72	Light yellow	28
4	50	20	40	Greenish brown	60
5	50	12	24	Brown	76
6	50	11	22	Brown	78
K-599					
1	50	33	66	White	34
2	50	46	92	Light yellow	8
3	50	34	68	White	32
4	50	16	32	Brownish white	68
5	50	19	38	White	62
6	50	10	20	White	80

Table 3: Effect of media combination on callus induction of cotton cotyledon of different cotton varieties

Medium used	No. of explants	No. of calluses	Callus induction	Colour of callus	Mortalitye rate (%)
NIAB-98					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	5	10	Brownish green	90
4	50	0	0	Brown	100
5	50	5	10	Brownish green	90
6	50	0	0	Brown	100
7	50	5	10	Brown	90
CIM-443					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	3	6	Light brown	94
4	50	0	0	Brown	100
5	50	10	20	Green	80
6	50	5	10	Green	90
7	50	8	16	Brown	84
CIM-473					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	5	10	Greenish brown	90
4	50	0	0	Brown	100
5	50	15	30	Greenish brown	70
6	50	0	0	Brown	0
7	50	15	30	Green	70
L-31					
1	50	0	0	Brown	0
2	50	3	6	Brown	94
3	50	10	20	Green	80
4	50	3	6	Brown	94
5	50	10	20	Light brown	90
6	50	0	0	Brown	0
7	50	3	6	Brown	94
CIM-1100					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	0	0	Brown	100
4	50	0	0	Brown	100
5	50	0	0	Brown	100
6	50	0	0	Brown	100
7	50	0	0	Brown	100

In case of ovule culture all the genotypes showed best callus induction with medium containing MS+1.5 mg L^{-1} BAP+ mg L^{-1}GA (Table 2). NIAB-98 showed overall highest callus induction in this medium i.e. 94% while CIM-443 showed least i.e. 74%. Overall L-31 showed best callus induction and proliferation. The colour of callus ranged from light yellow, creamy white to brown. Ovules of all genotypes cultured on BA showed that concentration of 1-2 mg L^{-1} favoured callus induction but as the concentration was increased the rate of callus formation was decreased. At concentration of 5 mg L^{-1} black watery callus was formed. Therefore, higher BA concentration produced detrimental effect on callus initiation. Medium supplemented with BA has been used for callus induction and regeneration of roots in cotton[21].

Medium used:

1. MS+1 mg L^{-1} BAP+1 mg L^{-1} GA
2. MS+1.5 mg L^{-1} BAP+1 mg L^{-1} GA
3. MS+2 mg L^{-1} BAP+1 mg L^{-1} GA
4. MS+3 mg L^{-1} BAP+ 1 mg L^{-1} GA
5. MS+4 mg L^{-1} l BAP+1 mg L^{-1} GA
6. MS+5 mg L^{-1} l BAP+1 mg L^{-1} GA

Cotyledons cultured on media combination of auxins and cytokinins showed very low callus induction rate and most of the explants turned brown and dead and did not proliferate (Table 3). CIM-473 showed highest degree of callus induction but CIM-1100 showed no callus induction on any of media combination with this explant but all varieties did not proliferate further and most of them showed 100% mortality rate.

Table 4: Effect of media combinations on callus induction of cotton roots of different cotton varieties

Medium used	No. of explants	No. of calluses	Callus induction	Colour of callus	Mortality rate (%)
NIAB-98					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	5	10	Greenish brown	90
4	50	0	0	Brown	100
5	50	3	6	Whitish brown	94
6	50	0	0	Brown	100
7	50	0	0	Brown	100
CIM-443					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	5	10	Green	90
4	50	0	0	Brown	100
5	50	3	6	Whitish brown	94
6	50	0	0	Brown	100
7	50	0	0	Brown	100
CIM-473					
1	50	0	0	Brown	100
2	50	3	6	Brown	94
3	50	8	16	Green	84
4	50	3	6	Brownish white	94
5	50	0	0	Brown	100
6	50	0	0	Brown	100
7	50	0	0	Brown	100
L-31					
1	50	0	0	Brown	100
2	50	3	6	Brown	94
3	50	10	20	Whitish brown	80
4	50	3	6	Whitish brown	94
5	50	10	20	Brown	80
6	50	3	6	Brown	94
7	50	0	0	Brown	100
CIM-1100					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	0	0	Brown	100
4	50	0	0	Brown	100
5	50	0	0	Brown	100
6	50	0	0	Brown	100
7	50	0	0	Brown	100

Table 5: Effect of media combination on callus induction of cotton hypocotyls of different cotton varieties

Medium used	No. of explants	No. of calluses	Callus inducion	Colour of of callus	Mortality rate (%)
NIAB-98					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	25	50	Brownish green	50
4	50	0	0	Brown	100
5	50	30	60	Green	40
6	50	0	0	Brown	100
7	50	0	0	Brown	100
CIM-443					
1	50	0	0	Brown	100
2	50	10	20	Brownish green	80
3	50	25	50	Brown	50
4	50	15	30	Green	70
5	50	30	60	Brown	40
6	50	10	20	Light green	80
7	50	5	10	Brown	90
CIM-4731					
1	50	0	0	Brown	100
2	50	5	10	Brownish green	80
3	50	23	46	Green	50
4	50	0	0	Brown	70
5	50	25	50	Green	40
6	50	0	0	Brown	80
7	50	0	0	Brown	90
L-31					
1	50	0	0	brown	100
2	50	5	10	brown	90
3	50	30	60	green	40
4	50	15	30	green	70
5	50	33	66	Light green	34
6	50	15	30	green	70
7	50	8	16	green	84
CIM-1100					
1	50	0	0	Brown	100
2	50	0	0	Brown	100
3	50	15	30	Brownish green	70
4	50	0	0	Brown	100
5	50	20	40	Green	60
6	50	0	0	Brown	100
7	50	0	0	Brown	100

Root explants of all genotypes on all media combination showed least response (Table 4). Of all the genotypes, L-31 showed highest callus induction rate in medium supplemented with 1.25 mg L^{-1} 2, 4, D+2 mg L^{-1} Z and I mg L^{-1} activated charcoal There was no callus induction on medium containing 0.1 mg L^{-1} 2, 4, D+ 0.5 mg L^{-1} Z and MS medium containing 1 mg L^{-1} 2, 4, D and 3 mg L^{-1} Z.

Hypocotyls explants showed highest frequency of callus induction and proliferation on all media combinations (Table 5). MS containing 1.25 mg L^{-1} 2, 4, D+2 mg L^{-1} Z +I mg L^{-1} activated charcoal and MS+0.1 mg L^{-1} 2, 4, D+ 1 mg L^{-1} K+ 1 mg L^{-1} activated charcoal. L-31 showed highest frequency of callus induction on these media and CIM-1100 showed least response and highest mortality ratio on all media combinations.

Medium used:

1. MS+0.1 mg L^{-1} l 2, 4,D+0.5 mg L^{-1} Z
2. MS+0.5 mg L^{-1} 2, 4,D+1 mg L^{-1} Z
3. MS+2 mg L^{-1} Z+1.25 mg L^{-1} 2, 4,D+ 1 mg L^{-1} activated charcoal
4. MS+0.5 mg L^{-1} IAA+0.5 mg L^{-1} l Z
5. MS+0.1 mg L^{-1} 2, 4, D+1 mg L^{-1} K+ 1 mg L^{-1} activated charcoal
6. MS+0.1 mg L^{-1} 2, 4, D+0.25 mg L^{-1} l BAP
7. MS+1 mg L^{-1} 2, 4, D+3 mg L^{-1} Z

A reduced auto inhibitory response was noted by medium containing charcoal. Charcoal had positive effect on callus induction and browning was reduced. Charcoal in low concentration is used to regenerate root from shoot apices[18]. All calluses obtained from all explants were sub cultured after 8 weeks of culture and produced very small amount of embryogenic callus, which did not show regeneration on subculture and turned black. Embryogenic callus, which proliferated further, did not show regeneration. High concentration in general produced deleterious effect on explant as reported by Trolinder and Goodin[4]. Most of the callus regenerated from these explants did not generated somatic embryos. Callus induction was significantly effected by genotype, culture conditions and explants type. There was significant callus induction among genotypes.

From the above study it is concluded that cotyledon and root are not suitable explants for callus induction and proliferation for the local genotypes. Ovule and hypocotyls are best for callus induction. 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 phenotypic characters in these studies. DNA microinjection has been previously used for transformation of protoplast[22], microspore[23] and cell[24]. 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.

ACKNOWLEDGMENTS

The research studies are being carried out under Pak-kazakh project, "Development of leaf curl virus tolerant varieties of cotton" awarded to the first author. All authors are highly grateful to the Ministry of Science and Technology, Islamabad, Pakistan for providing financial support.

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