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Optimization of Growth Regulators for Induction of Callus from Cotton (*Gossypium hirsutum*)

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Abstract: The objective of this study was to optimize the concentration of different plant growth regulators or hormones for callus induction of cotton (*Gossypium hirsutum* L.). Different types and concentrations of growth regulators were tested in order to obtain the best callus formation. Four growth hormones such as 1-naphthaleneacetic acid NAP), 6-Benzylaminopurine (BAP), Kinetin and 2,4-dichlorophenoxyacetic acid (2,4–D) were used in this study. It was found that growth regulator type and concentration had a significant effect on the callus induction, the increment of callus index and callus physical appearance. The higher frequency of callus growth (95-100%) were observed on both epicotyls and cotyledon explants cultured on basal medium supplemented with 0.1mg/l NAA + 0.5mg/l Bap and various concentrations such as 0.2+0.1, 0.5+0.1, 1.0+0.2mg/l of NAA + BAP also shows good callus response but at higher concentration of the same hormones shuts the callus growth. The concentration of BAP and 2,4-D also shows good callus response in higher concentration whereas low concentrations of this hormone combination show nil effect. The morphology of callus differs upon the hormonal concentration from green to white and green to brown with various textures. This protocol paves the way for the development of *in vitro* regeneration for cotton and consequently will promote the application of plant tissue culture.

Keywords: *Gossypium hirsutum* L., Epicotyl, cotyledon, MS (Murashige and Skoog) media, 1-naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BAP), Kinetin (KIN) and 2,4-dichlorophenoxyacetic acid (2,4–D), Callus.

1. Introduction

Cotton (*Gossypium hirsutum*) is great economically important crop and more important for our country as it acts cerebral column in our agricultural and industrial development, employment generation and foreign exchange earnings through export of its raw materials as well as furnished products. India became the second largest cotton producer in the world after China by pulling back US to the third position, according to a study done by the International Service for the Acquisition of Agri-Biotech Applications (ISAAA). In 2009, the cotton production in India is 23.5 million bales (National Cotton Council of America - Rankings). The biotic and abiotic stresses such as disease, draught, salinity and its vulnerability to frequent insect and pest attacks affect cotton yield adversely. Biotechnological interventions for combating the problem of insect pests through the production of transgenic cotton have gained tremendous significance in the recent past (Wilson *et al*., 1992; Flint *et al.*, 1995).

Besides producing spinnable fibers, cotton (*Gossypium*) plants produce seeds with a potential multi-product base such as hulls, oil, linters and meal. Per ton of seed crushed, cottonseed yields 540 lb of hulls (27%), 320 lb of crude oil (16%), 160 lb of linters (8%) and 900 lb of meal (45%). These cottonseed products enter markets that are highly competitive. This economic environment indicates the need for a highly efficient regeneration protocol for breeding of cotton (Hemphill *et al.*, 1998).

Somatic embryogenesis and plant regeneration have been reported from hypocotyl (Davidonis & Hamilton, 1983; Trolinder & Goodin, 1987; Finer, 1988), but cotton plants are severely limited in their regeneration *in vitro* from callus, protoplast or leaf

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tissues. Development of tissue culture protocols and optimization of media to induce efficient proliferation in a genotype-independent manner is desirable for genetic transformation of cotton (Bushra Rashid *et al.*, 2004). The aim of the present study is to optimize the media for cotton, which were cultured on MS medium with various concentrations of 2,4-D, NAA, BAP and KIN for induction of callus.

2. Materials and Methods

For this study, the seeds (*G. hirsutum* L.) were collected from Seed Centre, Tiruchirapalli, India.

2.1 Plant material and explant preparation

Cotton Seeds (*Gossypium hirsutum* L,) were collected and washed thoroughly under running tap water for 10 min and washed with an autoclaved filtered double distilled water, add 3 drops of tween 20 (liquid detergent) and add 3 drops of sodium hypochlorite (bleach) swirl it for 8 ½ min then decant or discard the water and washed it thoroughly with distilled water and add 0.1% $HgCl_2$ and swirl it for 2 ½ min, then immediately add 70% alcohol. Then wash it 3 to 4 times with distilled water and 2 times washed with autoclaved filtered double distilled water and germinated aseptically. Various explants epicotyl (excised from 7 days old seedlings), cotyledons were used for morphogenic response. Disinfection of seeds through delinting with concentrated H_2SO_4 and then followed by $HgCl_2$ has already been proved to be essential in cotton tissue culture (Abdellatef and Khalafalla, 2007; Rauf *et al.*, 2004).

2.2 Preparation of Media with different concentration of growth regulators

Leaflets were cultured in basic MS medium (Murashige & Skoog, 1962) supplemented with Potassium nitrate (1425.00mg/l), Ammonium nitrate (1237.50mg/l), Calcium chloride.$2H_2O$ (330mg/l), Magnesium sulphate (135.52mg/l), Potassium phosphate monobasic (127.50mg/l), Manganese sulphate.H_2O (16.90mg/l), Boric acid (6.20mg/l) Potassium Iodide (0.83mg/l), Molybdic acid (sodium salt), $2H_2O$ (0.25mg/l), Zinc sulphate.$7H_2O$ (8.60mg/l), Copper sulphate.$5H_2O$ (0.025mg/l), Cobalt chloride.$6H_2O$ (0.025mg/l), Ferrous sulphate $7H_2O$ (27.80mg/l), Na_2-EDTA (37.30mg/l), myo-inositol (100mg/l), Thiamine hydrochloride (0.10mg/l), Pyridoxine hydrochloride (0.50mg/l), Nicotinic acid (0.50mg/l), Glycine (2mg/l), agar as a gelling agent (9g/l). In addition, 4 combinations of growth regulators were added such as NAA + BAP, KIN + NAA, BAP + 2,4–D and KIN + 2,4-D presented in Table 1 was added in culture medium in order to form callus from leaves. In addition, medium of a control group without growth regulators were used.

2.3 Sterilization Protocol

Culture media were sterilized at 121°C for 15 minutes at 15 p.s.i., in an autoclave. Equipment were sterilized for 1 hour at 170°C in an autoclave.

2.4 Inoculation

The sterile leaves were taken with the help of sterile forceps and place it in a sterile paper and remove the corners of the leaves with the help of sterile blade. Approximately 1cm of leaf sample was cut and inoculated in the media. Each Phytajar was inoculated with 4 leaves samples.

2.5 Incubation

The inoculated phyta Jar was incubated at 24°C for 16:8 lights and dark cycle. The morphology of leaves was observed daily and recorded. Photographs were taken after 10 days. The morphology of callus was represented (Table 1).

3. Results and discussion

The use of modern techniques of cell, tissue and organ culture is central to many crop improvement programs in both industrialized and developing countries. Indeed the limiting step to the successful development of transgenic plants of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explants material (Murphy, 2003). Hence developing an efficient *in vitro* regeneration protocol for medium staple cotton cultivar is very important.

Surface sterilized seeds cultured directly on MS basal agar medium without any growth regulators in the phyta jars showed 40-50% germination in 4 days; 95-100% germination was observed after 7 days of seedling (Fig. 1A). This seedling plant explants like epicotyl (excised from 7 days old seedlings), cotyledons were used for morphogenic response. Higher germination rate is an important factor for establishing cotton tissue culture and be particularly useful when there is a need to submit a uniform set of seedlings to a treatment (Sakhanokho *et al.*, 2001).

Fig. 1(A). 7 days old seedling plant.

Table 1. Callus formation in MS Media with Different concentrations of NAA, BAP, KIN and 2, 4-D.

S. No.	NAA (mg/lit)	BAP (mg/lit)	Kinetin (mg/lit)	24-D (mg/lit)	Morphology of Callus	% of callus formation Epicotyl	% of callus formation Cotyledon
1	0.1	0.5	0.0	0.0	Compact, Yellowish Green	100	90
2	0.2	1.0	0.0	0.0	Brittle, yellow	72	78
3	0.5	0.1	0.0	0.0	Brittle, white	68	63
4	1.0	0.2	0.0	0.0	Brittle, white	64	63
5	2.0	2.0	0.0	0.0	Absence	Nil	Nil
6	0.5	0.0	0.1	0.0	Brittle, light green	68	68
7	1.0	0.0	0.2	0.0	Brittle, white with root	68	34
8	0.1	0.0	0.5	0.0	Absence	34	68
9	0.2	0.0	1.0	0.0	Brittle, white	34	34
10	2.0	0.0	2.0	0.0	Brittle, white	68	68
11	0.0	0.1	0.0	0.5	Absence	Nil	Nil
12	0.0	0.2	0.0	1.0	Compact, white	63	34
13	0.0	0.5	0.0	0.1	Compact, white	60	34
14	0.0	1.0	0.0	0.2	Compact, white	68	68
15	0.0	2.0	0.0	2.0	Brittle, Green	68	34
16	0.0	0.0	0.1	0.5	Brittle, white	34	68
17	0.0	0.0	0.2	1.0	Brittle, white with green	68	72
18	0.0	0.0	0.5	0.1	Absence	Nil	Nil
19	0.0	0.0	1.0	0.2	Brittle, light green	68	34
20	0.0	0.0	2.0	2.0	Brittle, Brown	68	68

For callus induction leaf of about 1 sq.cm were excised and cultured on MS medium supplemented with different concentrations of 2,4–D, Kin, BAP and NAA. The epicotyl explant induced callusing in the presence of auxin, on the other hand, failed to produced callus in media free auxins, this declared that the presence of auxins was capable to inducing callus. However, the callusing percentage, degree of callusing and callus appearance is auxin types and concentration dependant Callus initiation was observed after 12 – 15 days of inoculation. The result showed that among different combinations 0.1mg/l NAA with BAP (0.5mg/l) was found to be best for callus induction (Fig. 1B) than any other combinations, this combination show 100% of callus formation from leaves. These calli were yellowish green and compact (Fig. 1C). Essentially effects of auxins on cotton callus induction already have been reported (Trolinder and Goodin, 1987; Firoozabady *et al.*, 1987; Sakhanokho *et al.*, 1998. Among the auxins types tested, NAA induced the highest callusing rate and best callus appearance, compared to other types (Table 1).

The other combinations showed different growth of callus formation. The higher NAA+BAP (0.2 & 1.0mg/l) combination also produces yellowish green but friable callus which is compared to BAP + 2,4-D (0.2 & 1.0mg/l and 0.5 & 0.1mg/l) combination, showed white compact callus (Fig. 1D, E & F). The texture of the calli ranged from very hard and compact to friable was observed in different combinations.

Development of an efficient tissue culture and plant regeneration protocol for elite cotton varieties is the first step towards the application of transgenic technology to improve cotton breed and is, thus, the foundation of the cotton biotechnology research program (Zhang *et al*., 2001). In the present study, we report an efficient protocol by using epicotyls and cotyledon region of seedling, which can be induced to differentiate into adventitious callus that can be used for efficient production of transgenic Cotton.

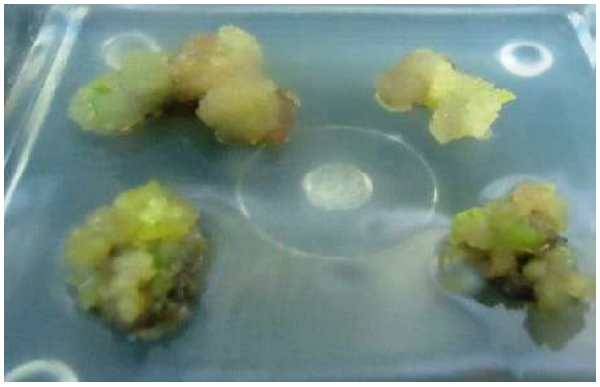

Fig. 1(B). Calli formed from NAA + BAP (0.1 + 0.5mg/l) combination after 10 days.

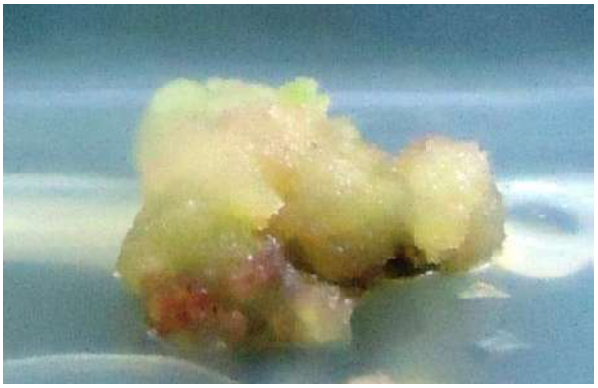

Fig. 1(C). Magnified calli showed yellowish green compact characteristics.

Fig. 1(D). Calli of BAP + 2,4-D (0.2+1.0mg/l) combinations.

Fig. 1(E). Enlarged image of compact White to Green calli of BAP+2,4-D.

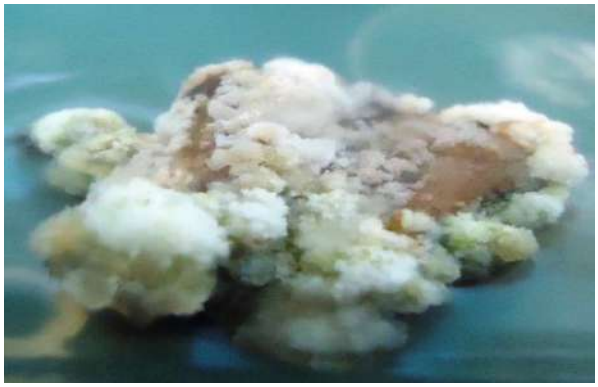

Fig. 1(F). Enlarged image of compact White to Brown calli of BAP+2,4-D.

4. Conclusion

The results of the present study would suggest that NAA + BAP (0.1 + 0.5mg/l) combinations were best for callus formation whereas, the higher concentration of NAA and BAP leads variations in callus formation. Hence the above mentioned concentration is good for callus induction from cotton. The other combinations produce discrete white colonies also recommended. This protocol will pave the way for the development of *in vitro* regeneration system for this elite cultivar and consequently will promote the application of plant tissue culture technology in the area of selection resistance, production of artificial seeds, and genetic transformation.

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