

Various Hormonal Supplementations Activate Sugarcane Regeneration *In-Vitro*

Ghulam Zahra Jahangir & Idrees Ahmad Nasir

National Centre of Excellence in Molecular Biology, Punjab University, Lahore, Pakistan

E-mail: dr.idrees@gmail.com

Riaz Ahmad Sial & Muhammad Aslam Javid

Agriculture Biotechnology Institute, Ayub Agricultural Institute, Faisalabad, Pakistan

E-mail: quasem92@yahoo.com

Tayyab Husnain

National Centre of Excellence in Molecular Biology, Punjab University, Lahore, Pakistan

Abstract

Influence of different hormonal concentrations in plant growth medium on the onset of callus and somatic embryoid induction chased by plantlet regeneration and multiplication of regenerated shoots was the main goal of these studies. Results concluded that MS medium supplemented with auxin alone (3 to 4mg/l 2, 4-D) induces callus formation (3mg/l 2, 4-D alone produced embryogenic calli) and auxin-cytokinin combination like 2, 4-D and IAA (both in 1mg/l and 2mg/l concentration) with BAP (1mg/l) found very effective for somatic embryoid induction. Plantlet regeneration from embryogenic calli, as somatic embryogenesis, found good in auxin-cytokinin combination of 2, 4-D and IAA with BAP but in different concentrations i.e. 2, 4-D and IAA 2mg/l with 2 and 3mg/l BAP.

Keywords: *Saccharum officinarum*, Tissue culture, Callogenesis, Somatic embryogenesis

Abbreviations: 2, 4-D (2, 4-Dichlorophenoxy Acetic Acid), IAA (Indole Acetic Acid), BAP (Banzyle Amino Purine), MS (Murashige and Skoog) Basal Medium.

1. Introduction

Sugarcane (*Saccharum officinarum*) is a perennial member of Gramineae family. Besides sugar and 'Gur', ethanol is being produced from sugarcane for domestic and export purpose as well.

There is a dire need to increase sugarcane productivity and quality enhancement as Pakistan occupies fifth position in cane producing countries and fifteenth one among sugar producing countries (http://www.thebioenergysite.com/articles/33_5/pakistan-sugar-annual-report-2009). Despite the great demand, the desired production target per acre yield and quality improvement of sugarcane have not achieved yet. Moreover, lack of quality research and crop disease control measures manifold the problem. As sugarcane is a vegetatively propagated crop, it is vulnerable to pathogens and gets diseased during seed preparation and transportation practices. The present project was aimed to develop disease free germplasm of well adopted sugarcane cultivars being grown in Pakistan.

Plant tissue culture techniques like micropropagation and somatic embryogenesis can be used for successful sugarcane propagation by controlling a lot of problems which are faced during conventional breeding practices. The technique ensures disease free multiplication of elite varieties (khan *et al.*, 2006) and minimizes time span required for mass production.

The present project was aimed to develop disease free germplasm of well adopted sixteen sugarcane cultivars being grown in Pakistan (Table-1). The protocol for regeneration of plantlets through indirect somatic embryogenesis was optimized for genetic improvement of sugarcane germplasm against Sugarcane Mosaic Virus.

2. Materials and Methods

Indirect Somatic embryogenesis was selected for its overwhelming advantages over direct regeneration. The former produces plenty of plants at a time which is its most considerable gain. The source plants were taken from fields of National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore. Thin slices were cut from inner immature leaf whorls and used as explants. Outer leaf whorls removed in sterile zone up to 0.7 cm diameter of stalk and approximate length of 8-10 cm from base at node to up at internode and once swabbed with each of 0.0 1% mercuric chloride, absolute ethanol and autoclaved distilled water. Remaining whorls were also removed until stalk remained only 0.3-0.4cm thick and 4-5cm long then it was cut into slices of 0.2-0.3cm long and cultured on growth medium. Sterilization of growth medium was achieved by autoclaving at 121 °C and 105 KPa for 20 minutes. Incubation conditions were optimized at 16 hrs photoperiod of approximately 1000 lux alternating with 8hrs of dark at 23± 2°C.

Growth medium used for induction of callus, somatic embryoids and for plant regeneration was same except hormonal supplementation. It contained 4.43g/l MS medium based on Murashige and Skoog's (1962) basal medium (MS SIGMA, M 5519) with 3% sucrose, 0.3% phytigel as gelling agent and pH maintained at 5.5 to 5.8 (with 1.0 normal HCl and NaOH). For callogenesis four auxin concentrations (Table-2) were tested. For somatic embryogenesis and plant regeneration, calli were subjected to eight auxin-cytokinin combinations (Table-2).

Callus cultures were kept under complete darkness for first three weeks and on induction of somatic embryoids were exposed to 16/8 hrs light/dark conditions. Callus cultures were maintained in disposable sterile Petri plates and on regeneration of plantlets the cultures were shifted and maintained in test tubes. Cultures were subcultured and transferred to fresh medium after every 15 days. Time taken to induce callus, somatic embryoids and plantlet regeneration in specific hormonal supplementation was recorded (in days) on daily basis inspection.

3. Results and discussion

According to the skeleton of experiment the results observed are presented in succession.

Callogenesis:

The four concentrations of 2, 4-D used for callus induction were (1.0, 2.0, 3.0, and 4.0mg/l). A specific supplementation was considered more or less suitable on the basis of time (in days) the explant took to onset the process of callus formation with appearance of minute nodule like cellular mass at cut edges (Figure 4 a & b) of explant; from the day of inoculation. On all supplementations different varieties produced calli of different forms like tough, compact, nodular, friable and soft foam like, and of varying shades like greenish yellow, fresh yellow, pale yellow and even in some golden yellow and whitish in shade. Although no generalization was concluded yet tough, compact and yellow calli with whitish shade were good embryogenic. All varieties showed approximately similar and good results with 3.0mg/l and 4.0mg/l 2,4-D supplementations but best in former among all varieties. (Figure 1 depicts the lowest values of time (mean number of days) taken to induce callus formation in SC-3 and SC-4). Medium with 2,4-D 3.0mg supplementation was recorded most efficient as it induced the explant to be changed into callus (a mass of undifferentiated cells; Figure 4 c & d) completely but others were observed slightly less effective as the conversion was comparatively slow and incomplete. It was observed in all cultivars that within a period of six to nine weeks the piece of explant cultured had developed into callus tissue completely, even embryo induction (whitish shade) was also observed in some varieties on same supplementation but this was not the case with any other concentration (like 2,4-D 2.0 or 4.0mg/l).

Many scientists have used 2,4-D for callus formation and found effective like Athar, A. *et al* (2009) obtained 100% callus induction in 3.0 mg/L of 2,4-D. Badawy, O. M *et al* (2008) and Gandonou *et al* (2005) also obtained embryogenic callus from leaf bases at 3mg/l 2,4-D. Ramanand *et al* (2005) observed maximum percentage of callus induction from leaf sheath explants within 10-14 days at 2,4-D 4.0mg/l. Alam *et al* (2003) also produced callus from leaf sheath at 4.0mg/l concentration of 2,4-D.

Some other researchers have reported variety of callus colors and texture types (forms) in many varieties of sugarcane on different media like Anbalgan *et al*; (2000) reported two types of callus (one loose, friable and embryogenic other compact, white, nodular and embryogenic). Fitch and Moor (1990) observed white, nonregenerative and green, regenerative callus. Escalona *et al*; (1995) found compact and nodular callus forms.

Somatic embryogenesis and plantlet regeneration:

Some compact calli were whitish in shade and embryogenic while their white appearance was because of presence of somatic embryoids (Figure 4g), in some even before transfer to embryogenic medium. Five week old calli were selected and subjected to embryogenic media (containing auxins and cytokinins in combinations, Table-2) to investigate the role of hormonal combinations. Most selection was made from SC3 as the calli were

compact and embryogenic which supported somatic embryoids and persuaded by plantlet regeneration from them. Among different combinations used 2,4-D+BAP and IAA+BAP both in 1+1, 2+1 showed excellent results (SS1 & SS5, SS2 & SS6 respectively in Figure 2) as the white granule like somatic embryoids (Figure 4g) assured appearance within one to two weeks after transfer. Minute plantlets were observed to be regenerated from nodular structure (Figure 4h) within three weeks of transfer to hormonal combination as described in Table-2. Among all concentrations 2,4-D+BAP and IAA+BAP both in 2+2 and 2+3 showed very good results as the whole of the callus tissue supported plenty of plants (Figure 4i,j) in all varieties with no exception but in some varieties growth was late and slow (SS3 & SS7, SS4 & SS8 respectively in Figure 3). A general behavior noted was that high auxin concentration in combination supported callus proliferation and embryo induction but high level of cytokinin in combination supports plant regeneration.

Review of related research by other workers also supports the presented results. Ali *et al.*; (2007) reported indirect somatic embryogenesis on auxin-cytokinin combination and found best results in 2,4-D +BAP (1+0.25mg/l) in CP 77,400 and BL-4 (1+0.5mg/l). Manickavasagam and Ganapathi (1998) also reported best effect of auxin-cytokinin interaction (2,4-D+BAP) on somatic embryogenesis and plant regeneration in sugarcane variety Co 671. They obtained best results with 2,4-D+BAP in concentration of 2+1mg/l. Ho and Vasil (1983) observed that many embryoids (somatic embryos) were formed when the callus was allowed to remain on the high 2,4-D medium for a prolonged period. They reported somatic embryogenesis from embryogenic callus formed from segments of young leaves cultured on MS supplemented with auxin-cytokinin combination (0.53 mg/l 2,4-D, 5% coconut milk). Nadar *et al.*; (1978) raised embryogenic callus on MS with 3.0 mg/liter 2,4-D and suggested that the process of dedifferentiation and embryogenic-cell initiation required relatively high auxin concentrations. They further found that auxin concentrations may be required for advanced embryogenesis stages. According to literature on HEC website (<http://pr.hec.gov.pk/Chapters/1370-5>), lower 2,4-D (auxin) concentration i.e. 1.0 or 0.5mg/l does not support somatic embryogenesis rather at 1.5-3.0mg/l induces up to 80% somatic embryogenesis.

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Table 1. Sugarcane cultivars used in the experiment

Variety Code	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16
Variety Name	NSG-555	NSG-6	NSG-311	NSG-59	NSG-646	HSF-240	HSF-242	HSF-245	CSSG-66	CSSG-676	SPSG-26	BF-162	M-93	CP-77/400	SPF-234	SPF-213

Table 2. Media compositions used in the experiment

Medium code	Medium composition	
	Callogenesis	Somatic embryogenesis and Plant regeneration
SC1	MS+2,4-D 1.0 mg/l	
SC2	MS+2,4-D 2.0 mg/l	
SC3	MS+2,4-D 3.0 mg/l	
SC4	MS+2,4-D 4.0 mg/l	
SS1		MS+2,4-D+BAP 1+1 mg/l
SS2		MS+2,4-D+BAP 2+1 mg/l
SS3		MS+2,4-D+BAP 2+2 mg/l
SS4		MS+2,4-D+BAP 2+3 mg/l
SS5		IAA+BAP 1+1 mg/l
SS6		IAA+BAP 2+1 mg/l
SS7		IAA+BAP 2+2 mg/l
SS8		IAA+BAP 2+3 mg/l

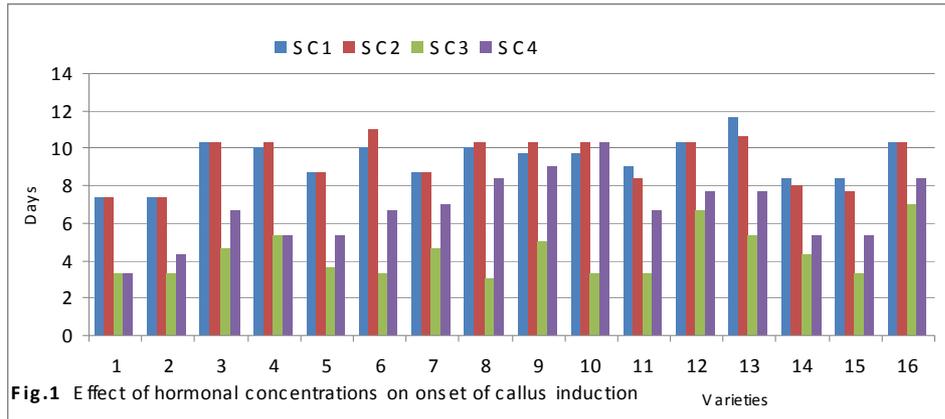


Figure 1. Effect of hormonal concentrations on onset of callus induction

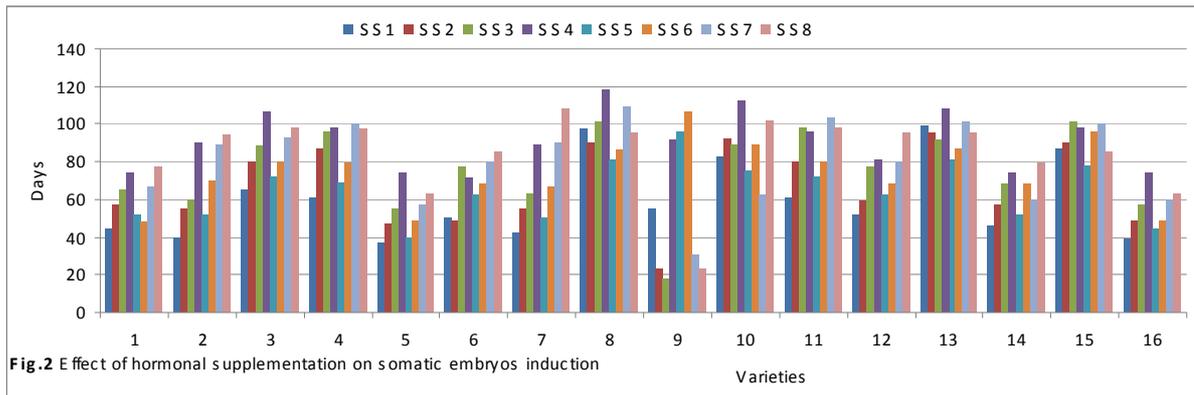


Figure 2. Effect of hormonal supplementation on somatic embryos induction

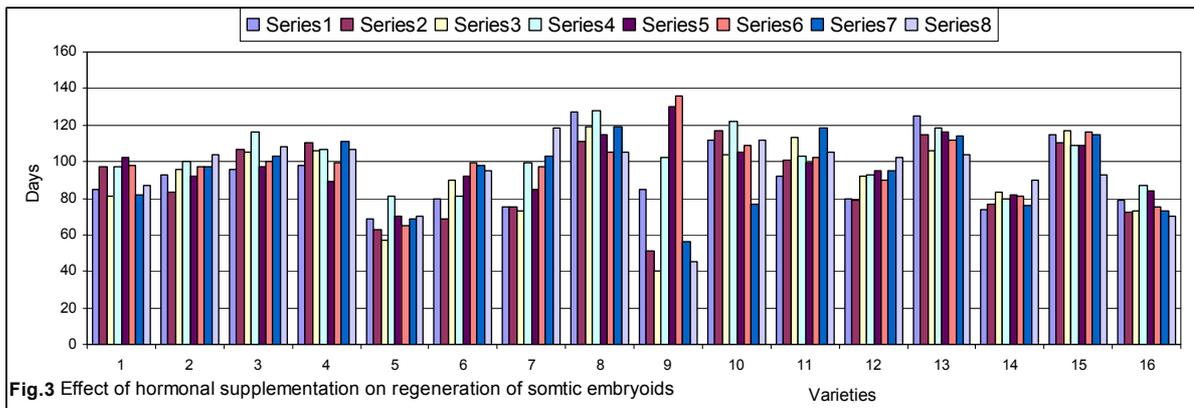
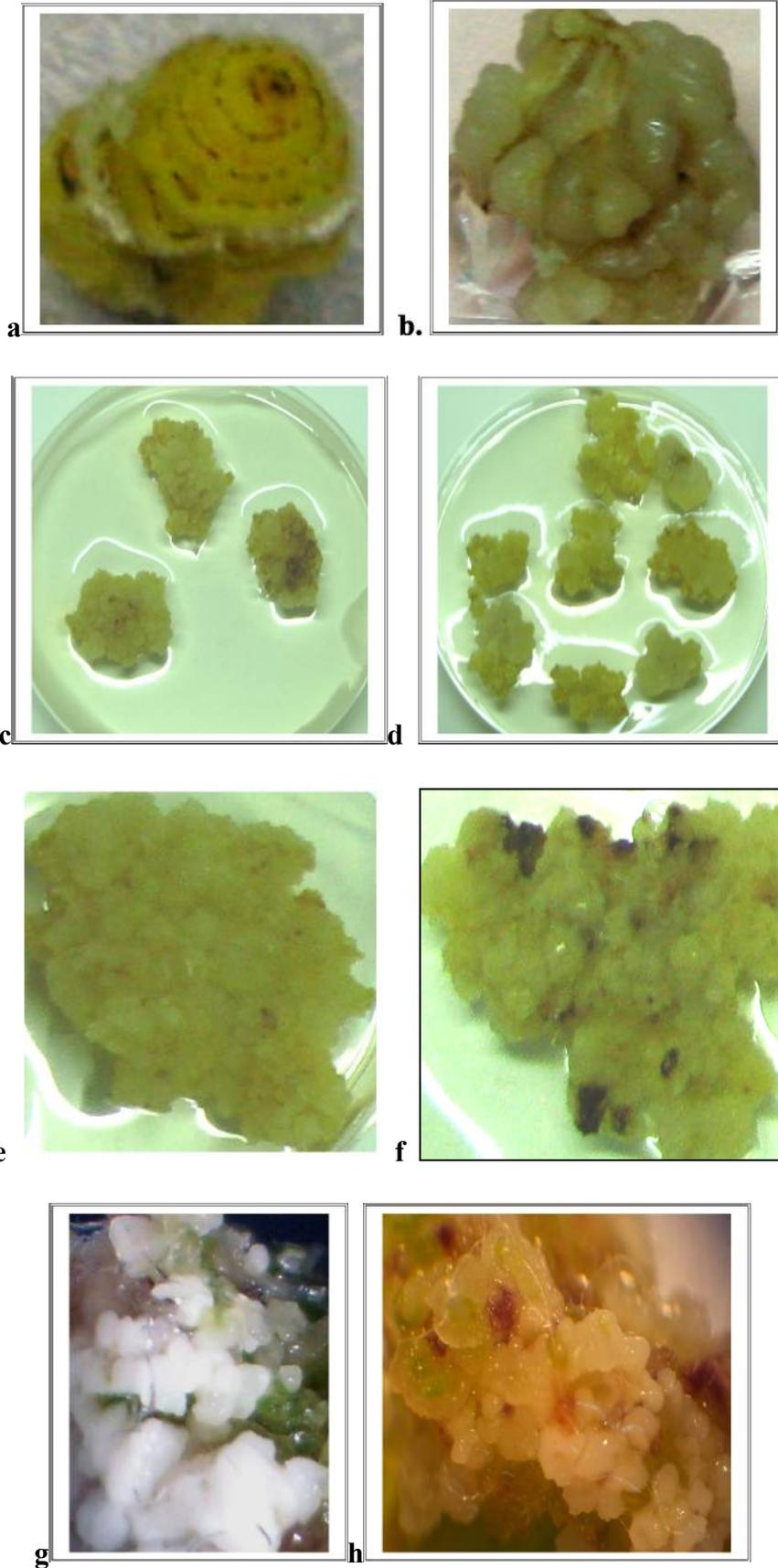


Figure 3. Effect of hormonal supplementation on regeneration of somatic embryos



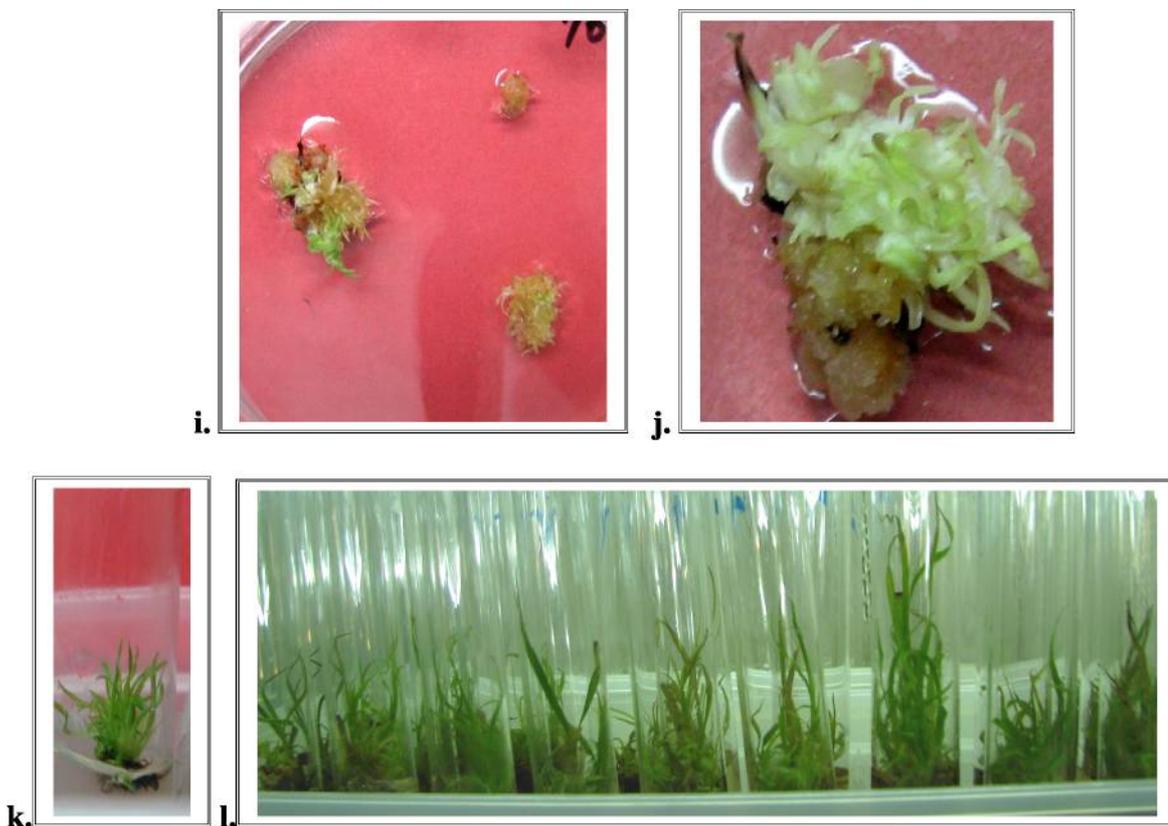


Figure 4. (a to l)

a. 5 days old culture on MS+2,4-D 3mg/l showing nodular structure at cut edges (HSF-245). b. 21 days old callus in MS+2,4-D 4mg/l (HSF-240). c. 4 weeks old callus on MS+2,4-D 3mg/l (NSG-555) d. 4 weeks old callus on MS+2,4-D 3mg/l (SPF-213) e. 5 week old callus of BF-162 f. 6 week old CP-77/400 g. white somatic embryoids on yellow callus of SPF-234 in 2,4-D+BAP 2+1mg/l h. minute plantlets ready to emerge from nodular structure of NSG-31 1 in 2,4-D+BAP 1+1mg/l i. plant regeneration from callus of CSSG-676 in 2,4-D+BAP 2+2 mg/l j. plant regeneration in SPF-234 in IAA+BAP 2+3 mg/l. k. 3 months old plant of M-93 l. 4 months old plants of different varieties in table-1.