Research Article

Callus formation and somatic embryogenesis in sugarcane (*Saccharum spp* L.) using various concentrations of 2, 4-D and RAPD analysis of regenerated plants

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Abstract

The present study was carried out to build up an efficient protocol for various concentrations of 2, 4-D for “Callus formation and somatic embryogenesis in sugarcane (*Saccharum spp* L.) using various concentrations of 2, 4-D and RAPD analysis of regenerated plants in the laboratory of Biotechnology Nuclear Institute of Agriculture, Tandojam during the year 2015 to 2016. Three sugarcane varieties BL4, NIA-2010, NIA-2011 with different concentrations of 2, 4-D (0.5, 1.0, 2.0, 3.0 and 4.0 mg L⁻¹) were used respectively, while 3.0 mg L⁻¹ Kin+IAA+IBA was used for callus proliferation and shoot formations. Four different concentration of IBA were used for rooting purposes 0.5, 1.0, 2.0 and 3.0 mgL⁻¹+20 g Sugar. Embryogenic callus was obtained by culturing young apical meristem. Three sugarcane clones BL4, NIA-2010 and NIA-2011 were developed in the field area for eight month and sand was used for tissue culture somatic embryonic callus study. Apical meristematic region was used for callus formation and somatic embryogenesis induction on 0.5, 1.0, 2.0, 3.0 and 4.0 mg L⁻¹. 2, 4-D actively growing callus was subcultured on kin. IAA, IBA, 3.0 mg L⁻¹. Maximum callus proliferation and number of plantlets shoot length and regeneration growth were observed in plants that 1.0 and 2.0 mg L⁻¹ 2, 4-D callus was taken from. Maximum chlorophyll mutation frequency was observed in NIA-2010 and BL4 grown on 1.0 mg L⁻¹ 2, 4-D. Maximum number of roots were observed in BL4 when 1.0 mg L⁻¹ IBA+ 20% sugar was applied; variability was also obtained through callus culture and confirmed through random amplified polymorphic DNA (RAPD) techniques.

Keywords: Embryonic callus induction; Callus proliferation; Chlorophyll mutant regeneration and root formation

Introduction

Sugarcane (*Saccharum spp* L.) hybrid is a

profitable crop cultivated in 74 countries around the globe [1]. It is a perennial crop

and belongs to Poaceae family, which contributes approximately 75% of worldwide sugar production [2]. Other than sucrose production, it is likewise utilized as part of Ayurvedic drugs and for the treatment of skin and urinary tract diseases, bronchitis, heart conditions, loss of milk production, cough, anemia, constipation and also general debility [3]. Another essential utilization of sugarcane is the production of press mud, which is utilized as a source of organic matter and supplements for crop production [4].

Sugarcane is the world largest crop by production, the annual production of sugarcane in the world is 1.83 billion tons having an area of 26.1 million hectares. The average yield of sugarcane in the world is 70.24 tons/ha. Pakistan produces 58.4 million tons by harvesting 1.04 million hectare and the yield is 55.8 tons/ha [5]. In Pakistan, sugarcane is the biggest industrial cash crop second to cotton and is basic as a result of its huge share in agriculture sector. Various demands for sugarcane in agricultural industry has vitalized the need for continuous varietal improvement and extensive research on the crop [6]. An extensive breeding work, management and cultural practices are required to narrow down this big gap. Although, a good crop is harvested in Pakistan, it does not flower and produce seed (fuzz) under normal growing conditions in the country.

Non-sporadic flowering and natural viable and fertile seed production has usually been a problem in Pakistan. Modern sugarcane varieties have multifarious genetic characteristics and low fertility that renders their genetic improvement is very difficult through traditional breeding. Thus the substitute method for propagation is *in vitro* culture techniques and induced transformation being utilized to make the

new genetic variability for the determination of desired clones of sugarcane [7]. *In-vitro* culture technique is enriched with high cane yield, resistance to biotic and a biotic stresses. Callus culture of sugarcane has been efficiently settled utilizing shoot apices, young leaves and young inflorescences as explants.

Sugar yield of Pakistan is small as compared to other countries of the world and genetic improvement through customary hybridization in Pakistan is hampered due to the complex flowering nature of sugarcane [8]. The genetic variability created through traditional breeding techniques is slow and depends on recombination [9]. In conventional breeding method, development of selected sugarcane cultivars with high sugar yield and disease resistance is often defeated by tight linkage between cane quality, cane yield and disease resistance etc. The developments in plant tissue culture have opened up new possibilities in creating genetic variability. Somatic embryogenesis is a common regeneration pathway which is being stated by many scientists [7, 10, 11]. Plant tissue culture is the best technique through callusing of sugarcane for variation, genetic advancement, quality and phytosanitary planting material at a speedier rate in a smaller duration of time. Callus prompting was seen few weeks after explants under proper condition and the differentiation of either shoots from callus of sugarcane assortments easily acquired [12]. Leaf sheath were utilized as explants to induce callus on modified MS supplemented with various concentrations of 2, 4-D as growth regulators [13].

The major area of utilization of tissue culture in sugarcane improvement are for the production of somaclones through callus culture for disease resistance [14] and variety in plant morphology due to

dissimilarity in chromosome number [15]. These studies uncover that the effective use of tissue culture technology for the improvement of sugarcane is subject to the capacity to start, keep up and control and regenerate plants from callus [16]. The genetic variability through conventional breeding techniques is slow and depends on recombination [17]. The achievements in plant tissue culture have opened up new possibilities in creating genetic variability. Auxin is reflected to be the most essential hormone in managing somatic embryogenesis [10]. Different biotechnological techniques are used to modify the existing plant characteristics or to generate new ones. For this purpose, plant tissue culture techniques, predominately somatic embryogenesis are being practiced. Somatic embryogenesis is well renowned and most famous method to produce numerous plantlets from callus in limited time span. Somatic embryoids induced on embryogenic callus regenerate into several numbers of plantlets. The plants regenerated from somatic embryoids are indistinguishable in all genetic and agronomic characteristics in light of single cell [6]. Molecular markers are the most efficient tool to evaluate genetic reliability of the regenerated plantlets. This could be a down to earth way to deal with screen somaclonal variations created in tissue cultured plants, for example, in sugarcane [12]. PCR based molecular markers, such as RAPD technique most often is being utilized for assessment of somaclonal variation and estimation of genetic fidelity. DNA investigation in sugarcane embryogenesis regenerated plants [18].

Optimized conditions for callus induction in sugarcane have been studied investigated in the present study. This optimized protocol will help in establishing efficient method for the genetic transformation in callus of the important sugarcane varieties for resistance to various stresses. Present study was conducted to induce the callus and somatic embryogenesis in different genotypes of sugarcane (BL4, NIA-2010 and NIA-2011) with the use of different concentrations of 2, 4-D (0.5, 1.0, 2.0, 3.0 and 4.0 mg L⁻¹). Embryonic calli then regenerated and analyzed through DNA RAPD marker for genomic variations among the somaclones and its mother plant.

Materials and methods

The research was carried out at Nuclear Institute of Agriculture, Tandojam for callus formation and somatic embryogenesis in sugarcane (*Saccharum* sppL.) using various concentrations of 2, 4-D and RAPD investigation of regenerated plants of BL4, NIA-2010 and NIA-2011. Laboratory used was well equipped and facilitated by culture and washing room, laminar airflow cabinet, culture vessels, incubation room, autoclave, pH meter, electric oven, glass house, centrifuge machine, spectrophotometer, PCR and Gel documentation and miscellaneous needs.

Preparation of micronutrient stock solution

The quantities each of salts (Table 1) were measured and transferred into the conical flask and sterilized (ddH₂O), distilled water 700ml was then delivered into conical flask. Solution was stirred to dissolve properly and transferred to volumetric flask (1L) and made up to volume. The flask was labeled and stored in the refrigerator.

Table1. Preparation of MS basal medium, first discovered by Murashige, T; Skoog, F (1962). [19]

Ingredient	Chemical composition	Weight in mg/L
Micronutrients		
Manganese sulphate	MnSO ₄ .4H ₂ O	22.2
Zinc sulphate	ZnSO ₄ .7H ₂ O	8.2
Boric acid	H ₃ BO ₃	6.2
Potassium Iodide	KI	0.83
Sodium molybdate	Na ₂ MoO ₄ . 2H ₂ OHH	0.025
Copper sulphate	CuSO ₄ . 5H ₂ O	0.025
Cobalt chloride	CoCl ₂ . 6H ₂ O	0.025
Iron		
Iron sulphate	FeSO ₄ . 5H ₂ O	27.8
Sodium EDTA (Sodium ethylene diamine tetra acetic acid)	Na ₂ ETDA.2H ₂ O	37.26
Vitamins		
Thiamine HCl	C ₁₂ H ₈ N ₄ O ₅ Cl ₂	0.5
Myo-inositol		0.1 g/l
Macronutrients		
Potassium nitrate	KNO ₃	1.09g/l
Ammonium nitrate	NH ₄ NO ₃	1.65g/l
Calcium chloride	CaCl ₂ .2H ₂ O	0.44g/l
Potassium phosphate	KH ₂ .PO ₄	0.17g/l
Magnesium sulphate	MgSO ₄ . 7H ₂ O	0.37g/l
Sugar	C ₁₂ H ₂₂ O ₁₁	25.00g/l
Casein		2.00g/l
AGAR		8.00g/l

Preparation of vitamin based stock solution

The quantities of ingredients for the vitamin stock presented (Table 1) were measured into conical flask having some quantity of sterilized distilled water and mixed thoroughly using magnetic stirrer. The solution was transferred into volumetric flask 1l and made up to volume and the flask labeled and stored in refrigerator till use. The following are the growth regulators stock solution for somatic embryogenesis callus formation shoot regeneration and rooting forming culture media: a) 2, 4-Dichloro phenoxy acetic acid; b) Kinetin; c) Indol-3- acetic acid and d) IBA Indole-3-butiric acid.

Preparation of laminar air flow cabinet

The laminar air flow cabinet was switched on for 30 min.

Before starting the process of culturing, it was uncontaminated with absolute alcohol like 70% ethanol. After laminar air flow cabinet was further sterilized with all required materials and kept inside the cabinet.

Selection of explants, surface sterilization and culture

Ten (10) explants containing apical meristems were taken from every genotype. Superfluous part of cane top was uprooted and the remaining initially washed with refined water and afterward disinfected with

70% Alcohol (ethanol) for 1 min and 20% sodium hypochloride solution for 20 min. After sanitization explants material were washed with double refined water 2 to 3 times to uproot any hints of disinfectant under aseptic conditions in laminar air flow cabinet. These sterilized meristem or shoot tip was cut or isolated from stem and a V-shaped cut with sterilized knife applied. In the meristem tip culture, the cut was a small piece applied 0.3 to 0.5 mm below the tip of the dome and the excised tissue (explants) was removed along portion of procambial tissue and immediately planted on media.

Molecular marker RAPD analysis of somatic embryo-derived plants among mother plants

RAPD is any DNA Segment that is

amplified using short oligodeoxy nucleotide primers of arbitrary nucleotide sequence and polymerase chain reaction procedure.

Buffer solution genomic DNA extraction

Total genomic DNA was separated from samples of young leaf tissue of all the three varieties BL4, NIA-2010, NIA-2011; three specimens of that recovered from somatic embryo callus were taken from field plantlets' and determined through nodal shoot multiplication and somatic embryogenesis from every accession. Leaf tissues were ground into fine powder in liquid nitrogen using a mortar and pestle. Buffer chemicals for 500X reactions used are: 50 ml Tris-HCl, 140 ml MATAB, 20 ml EDTA, 10 g NaCl, 5 g PEG 6000 and 2.5 g Na₂SO₃ (Table 2).

Table 2. Buffer chemicals

Chemicals	For 500X reactions
Tris-HCl	50 ml
MATAB	140 ml
EDTA	20 ml
NaCl	10 g
PEG 6000	5 g
Na ₂ SO ₃	2.5 g

Random amplified polymorphic DNA (RAPD) analysis of plants regenerated from embryogenic calli

Deoxyribonucleic acid (DNA) was extracted using the protocol of [12, 20]. Sample for DNA extraction were the control, which is propagated through sets and seven randomly selected somaclones of BL4, NIA-210 and NIA-2011. Genomic DNA was quantified spectrometrically. Polymerase chain reaction (PCR) amplification was done using RAPD primers (Operon Technologies, Alameda, USA). Four (4) operon primers were selected for the study. The selected primers A2, A3, A8, A10 PCR was performed in 15 ml reaction. 12 ng of the template DNA was

amplified with one unit of taq DNA polymerase (0.1 µl) in a 15 µl reaction mixture containing 10 × PCR buffer (1.5 µl), 0.2 mM of dNTPs (0.33 µM) and 1 µM of random primer. Amplification was performed in a BioRad PTC 200 Thermal Cycler System and initiated by a denaturation of 3 min at 95°C followed by 40 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 2 min. The amplification was completed with 1 cycle of 7 min at 72°C.

Results and discussion

The results on weight of explants/bottle, weight of Callus/bottle, weight of proliferation /bottle, type of callus, number of shoots per bottle, shoot length per bottle,

number of chlorophyll mutants per bottle and number of roots of sugarcane callus induction under various concentrations of 2,

4-D recorded are presented in Tables 1, 3 to 8 and their analysis of variance and data shown in Table 9.

Table 3. Analysis of variance weight of somatic callus in the various concentration of 2, 4-D with different varieties

Concentrations of 2,4-D mg l ⁻¹	BL4	NIA-2010	NIA-2011		Mean
0.5	0.79 ^{b-d}	0.84 ^{bc}	0.57 ^{c-e}		0.73 ^{bc}
1.0	0.95 ^b	0.45 ^{de}	1.38 ^a		0.92 ^{ab}
2.0	0.86 ^{bc}	0.40 ^e	1.65 ^a		0.97 ^a
3.0	0.38 ^e	0.55 ^{c-e}	0.84 ^{bc}		0.59 ^{cd}
4.0	0.82 ^{b-d}	0.36 ^e	0.24 ^e		0.47 ^d
Mean	0.76 ^a	0.52 ^c	0.93 ^a		
Source	Degrees of freedom	Sum of squares	F value	P	Remark
Rep	5	0.5457	-	-	-
Varieties	2	2.1703	12.44	0.0000	
Treat	4	2.7215	7.80	0.0000	**
Varieties*Treat	8	5.6749	8.13	0.0000	**
Error	56	4.8845	-	-	-
Total	74	15.9969	-	-	-

Table 4. Effect of different growth regulator concentrations of 2, 4-D on callus induction of sugarcane somaclones

Growth regulator concentration	Type of callus
BL4	
2,4-D (0.5mg L ⁻¹)	Nodular Compact (A)
2,4-D (1.0mg L ⁻¹)	Nodular Compact (A)
2,4-D (2.0mg L ⁻¹)	Nodular Compact (A)
2,4-D(3.0mg L ⁻¹)	Nodular Compact (A)
2,4-D(4.0mg L ⁻¹)	Nodular Compact (A)
NIA-2010	
2,4-D (0.5 mg L ⁻¹)	Nodular Compact /smooth Compact (A and B)
2,4-D (1.0 mg L ⁻¹)	Nodular Compact /smooth Compact (A and B)
2,4-D (2.0 mg L ⁻¹)	Nodular Compact (A)
2,4-D(3.0 mg L ⁻¹)	Nodular Compact (A)
2,4-D(4.0 mg L ⁻¹)	Nodular Compact (A)
NIA-2011	
2,4-D (0.5 mg L ⁻¹)	Nodular/smooth Compact (A and B)
2,4-D (1.0 mg L ⁻¹)	Nodular/smooth Compact (A and B)
2,4-D (2.0 mg L ⁻¹)	Nodular/smooth Compact (A and B)
2,4-D(3.0 mg L ⁻¹)	Nodular/smooth Compact (A and B)
2,4-D(4.0 mg L ⁻¹)	Nodular/smooth Compact (A and B)

Table 5. Analysis of variance weight of somatic callus proliferation in the various concentration of 2, 4-D with different varieties

Concentration of 2,4-D with different varieties						
Concentrations of 2,4-D mgL ⁻¹	BL4	NIA-2010	NIA-2011	Mean		
0.5	1.43 ^d	1.10 ^{de}	1.25 ^{de}	1.26 ^b		
1.0	1.07 ^d e	0.95 ^{ef}	2.45 ^a	1.49 ^{ab}		
2.0	1.21 ^d e	0.89 ^{ef}	2.06 ^{ab}	1.39 ^{ab}		
3.0	1.94 ^{bc}	1.53 ^{cd}	1.49 ^{cd}	1.65 ^a		
4.0	0.83 ^{ef}	0.52 ^{fg}	0.33 ^g	0.56 ^c		
Mean	1.29 ^b	1.00 ^c	1.52 ^a			
Analysis of variance for weight of callus proliferation/bottle						
Source	Degrees of freedom	Sum of squares	Mean square	F-value	P	Remark
Rep	5	0.1269	0.03172	-	-	-
Varieties	2	3.4224	1.71122	12.09	0.0000	**
Treat	4	10.7018	2.67546	18.91	0.0000	**
Varieties*Treat	8	8.7704	1.09630	7.75	0.0000	**
Error	56	7.9239	0.14150	-	-	-
Total	74	30.9454	-	-	-	-

Table 6. Analysis of variance number of plants /bottle that regenerated from in the various concentration of 2, 4-D with different varieties

Concentrations of 2,4-D mgL ⁻¹	Bl4	2010	2011	Mean	
0.5	22.000 ^f	20.000 ^g	16.000 ⁱ	19.333 ^d	
1.0	30.000 ^c	22.000 ^f	21.000 ^{fg}	24.333 ^c	
2.0	30.000 ^c	33.400 ^a	22.000 ^f	28.467 ^a	
3.0	25.200 ^d	31.800 ^b	21.800 ^f	26.267 ^b	
4.0	24.000 ^e	30.000 ^c	17.600 ^h	23.867 ^c	
Mean	26.24 ^b	27.44 ^a	19.68 ^c		
Analysis of variance for number of plants / bottle					
Source	Degrees of freedom	Sum of squares	Mean square	P	Remark
Rep	5	153.12	38.28	-	-
Treat	4	689.52	172.38	0	**
Varieties	2	872.43	436.213	0	**
Treat*Varieties	8	449.84	56.23	0	**
Error	56	37.68	0.673	-	-
Total	74	2202.59	-	-	-

Table 7. Analysis of variance Size of shoot length plant-1 that regenerated from in the various concentration of 2, 4-D with different varieties

Concentration s of 2,4-D mgL ⁻¹	BL4 Shoots length	NIA-2010 shoots length plant ⁻¹	NIA-2011 shoots length	Mean		
	plant ⁻¹ (c)	(c)	plant ⁻¹ (c)			
0.5	2.60 ^{fg}	2.00 ^g	1.60 ^g	2.06 ^e		
1	9.20 ^a	8.00 ^{ab}	3.80 ^{ef}	7.00 ^a		
2	6.80 ^{bc}	6.00 ^{cd}	4.00 ^{ef}	5.60 ^b		
3	7.20 ^{bc}	4.00 ^{ef}	2.80 ^{fg}	4.66 ^c		
4	5.00 ^{de}	2.60 ^{fg}	3.00 ^{fg}	3.53 ^d		
Mean	6.16 ^a	4.52 ^b	2.36 ^c			
Analysis of variance for number of shoot / plantlets						
Source	Degrees of freedom	Sum of squares	Mean square	F-value	P	Remark
Rep	5	36.613	9.1533	-	-	-
Varieties	2	121.787	60.8933	46.72	0.0000	**
Treat	4	214.747	53.6867	41.19	0.0000	**
Varieties*Treat	8	50.213	6.2767	4.82	0.0002	**
Error	56	72.987	1.3033	-	-	-
Total	74	496.347	-	-	-	-

Table 8. Analysis of variance number of chlorophyll mutant /bottle that regenerated from in the various concentrations of 2, 4-D on kinetin, IBA, IAA 3.0 mg l-1 concentrations with different varieties

Concentrations of 2,4-D mgL ⁻¹	BL4	NIA-2010	NIA-2011	Mean		
0.5	1.60 ^{c-e}	1.20 ^{de}	1.00 ^e	1.26 ^c		
1.0	6.00 ^a	6.40 ^a	2.80 ^{bc}	5.06 ^a		
2.0	3.00 ^{bc}	5.40 ^a	2.20 ^{b-e}	3.53 ^b		
3.0	3.60 ^b	2.60 ^{b-d}	3.20 ^b	3.13 ^b		
4.0	2.60 ^{b-d}	3.00 ^{bc}	2.60 ^{b-d}	2.73 ^b		
Mean	3.36 ^b	3.72 ^a	2.36 ^c			
Analysis of variance for chlorophyll mutant/bottle						
Source	Degrees of freedom	Sum of squares	Mean square	F value	P	Remark
Rep	5	4.187	1.0467	-	-	-
Varieties	2	24.827	12.4133	8.75	0.0005	**
Treat	4	113.120	28.2800	19.94	0.0000	**
Varieties*Treat	8	45.840	5.7300	4.04	0.0008	**
Error	56	79.413	1.4181	-	-	-
Total	74	267.387		-	-	-

Table 9. Genetic polymorphism among the different varieties of sugarcane sequence of the primers

Primer code	Sequence (5' -3')
A2	TGACCGAGCTG
A3	AGTCAGCCAC
A8	GTGACGTAGG
A10	GTGATCGCAG

Weight of explants /bottle (g)

Statistical analysis of variances was found significantly different at 5% probability level. The maximum weight of explants in

clone BL4, however, no significant difference was observed in weight of explants variety and treatment (Table 10).

Table 10. Weight of explants per bottle (g)

Concentrations of 2,4-D mg ⁻¹	BI4	NIA-2010	NIA-2011	Mean
0.5	0.84 ^{ab}	0.35 ^{ef}	0.44 ^{d-f}	0.54 ^{ab}
1.0	0.92 ^a	0.36 ^{ef}	0.73 ^{a-c}	0.67 ^a
2.0	0.91 ^a	0.37 ^{ef}	0.67 ^{a-d}	0.65 ^a
3.0	0.32 ^f	0.47 ^{c-f}	0.49 ^{c-f}	0.43 ^b
4.0	0.73 ^{a-c}	0.56 ^{b-f}	0.62 ^{b-e}	0.64 ^a
Mean	0.74 ^a	0.42 ^c	0.59 ^b	

Analysis of variance for weight of explants/bottle						
Source	Degrees of freedom	Sum of squares	Mean square	F-value	P	Remark
Rep	5	0.36977	0.09244	-	-	-
Varieties	2	1.28376	0.64188	12.67	0.0000	**
Treat	4	0.60018	0.15005	2.96	0.0273	*
V × T	8	1.06325	0.13291ns	2.62	0.0163	ns
Error	56	2.83643	0.05065	-	-	-
Total	74	6.15339	-	-	-	-

Weight of callus / bottle (g)

The concentrations of 2,4-D 1.0 and 2.0 mg L⁻¹ respectively demonstrated best for greatest callus induction (1.38 and 1.65g) in clone NIA-2011, trailed by BL4(0.95g) through the application of 2,4-D (1.0 mg L⁻¹). Increase and decrease in the concentration of 2, 4-D adversely influenced the ratio of callus development and growth. At the higher concentration medium (4.0 mgL⁻¹ 2, 4-D), the ratio of callus formation decreased in clones NIA-2010 and NIA-

2011 (Table 1 and Figure 1). Comparative results were accounted for by Shahid *et al.* [21]. In addition, [22] had additionally reported that the callus induction capacity in sugarcane is genotype dependent.

Types of callus (A) regenerable and (B) non-regenerable

Study of callus type is also important for the identification of various responses of callus tissues. Two types of callus were discovered (and) signified in the study, as re-regenerable callus Type-A and non-regenerable callus

Type-B. The regenerable callus were distinguished and perceived as dry nodular compact and light yellowish in color as wild, however, B type callus was watery, translucent and non-nodular in shape (Figures 1 and 2). Whereas, Type A (dry nodular and compact callus) was found in clones of BL4, though, NIA-2010 and NIA - 2011 had smooth conservative callus denoted as Type A and B (Table 1). The outcomes are upheld by Seema *et al.* [8].

Who reported three clones of sugarcane with the utilization of 2,4-D (2.0 mg L^{-1}) dry nodular callus in BL4. According to the study by Gunderson *et al.* [23] the embryogenic sort A callus and non-embryogenic sort B callus was seen on the premise of callus external. It was observed that sort B callus of sugarcane did not show the same characteristics, rather, its development generously decreased.

Figure 1. RAPD-PCR with primer A-3, M=DNA marker, 1= NIA 2010, 2=2010SC, 3=NIA-2011P, 4=NIA-2011SC, 5=BL4P, 6=BL4SC, B=Control

Figure 2. RAPD-PCR with primer A-02, M=DNA marker, 1=2010P 2=2010SC, 3=2011P 4=2011SC, 5=BL4P, 6=BL4SC, B=Control

Weight of callus proliferation / bottle (g)

The proliferation reaction callus varied regarding sub Culturing. It decreased significantly with an increase in growth

regulator concentration. Maximum proliferation of callus (2.45g) was observed in NIA-2011 somaclone with medium containing 1.0 mg L^{-1} 2, 4-D. However, the

minimum value (0.33 g) was observed in same somaclone using concentration of 4.0 mg L⁻¹ 2, 4-D (Table 5 and Figure 3). These outcomes are trailed by Dibax and Bomfim [24] whose perceptions portrayed the

embryogenic masses proliferation on MS culture medium with 2, 4-D, the diminishment of 2, 4-D concentration to (2.0 mg L⁻¹) Effectively double the number of embryogenic masses in every subculture.

Figure 3. Maximum proliferation of callus (2.45g) was seen in NIA-2011 somaclone with medium containing 1.0 mg L⁻¹ 2, 4-D. However, the least (0.33 g) was observed found in same somaclone using application of 4.0 mg L⁻¹ 2, 4-D

Number of plants/bottle

Regeneration potential was specific and a genotype dependent phenomenon. The plantlets as affected by different growth regulators containing higher number of plantlets (33.400) in NIA-10 somaclone on callus derive from 2, 4-D (2.0 mg L⁻¹), followed by, 31.800 plantlets through the

application of 2, 4-D (3.0 mg L⁻¹) in the same clone. Minimum numbers of plantlets were observed on callus derived from 2, 4-D in all three clones namely; BL4, NIA-2010 and NIA-2011 (Table 6 and Figure 4). These information affirmed the outcomes reported in *Primula ssp.* [25] *Oryza sativa* [26].

Figure 4. Plantlets were observed on callus derived from 2, 4-D in all three clones namely; BL4, NIA-2010 and NIA-2011

Size of shoots length plant⁻¹ (c)

A significant decrease in size of shoot length was observed with an increase in growth regulator concentration. Stretched shoots (9.20 cm) were observed in BL4 somaclone with 1.0 mg L⁻¹ 2, 4-D concentration used. Maximum shoot elongation was also noted in NIA-2010 somaclone with the application of 2, 4-D (1.0, 2.0 and 3.0 mg L⁻¹). The clones were non-significant. However, minimum shoot length (1.06 cm) was noted in NIA-11 somaclone on callus derived from 0.5 mg L⁻¹ 2, 4-D (Table 3 and Figure 4). Comparable results were likewise by Raza *et al.* [16] Gunderson *et al.* [23] in sugarcane.

Number of Chlorophyll mutant / bottle

Regeneration of albino and viridis plantlets displayed the presence of chlorophyll changes *in-vitro* plantlets. The chlorophyll mutant under the influence of growth regulator concentrations showed maximum numbers of chlorophyll mutants (6.40) when NIA-10 somaclone was grown on callus derived from 1.0 mg L⁻¹ 2,4-D. Minimum chlorophyll mutant was recorded in NIA-2011 somaclone through application of 0.5 mg L⁻¹ 2, 4-D (1.00) (Table 8 and Figure 4). A chlorophyll lacking phenotype can be responsible for passive transformations [3, 27] additionally reported high genetic similarity among sugarcane varieties using RAPD marker.

Number of roots plant⁻¹

The number of roots affected by various concentrations of IBA recorded higher number of primary and secondary roots (13.40) in NIA-2010 somaclone when 1.0 mg L⁻¹ IBA + 20% sugar was applied and trailed by BL4 somaclone (13.00) provided with 2.0 mg L⁻¹ IBA + 2% sugar. The increased IBA concentration (3.0 mg L⁻¹ IBA + 20% sugar) significantly decreased number of primary rootlets of BL4, NIA-2010, and NIA-2011 somaclone (Table 4).

Analysis of variance of number of root plant⁻¹ regenerated from various concentrations of IBA with different varieties. [7] Shoed or depicted that utilization of IBA with 6% sucrose in growth medium incited fiery root improvement. The plantlets with all around created shoots and roots were transferred to jiffy pots having sterilized perlite. After acclimatization the plantlets were initially transferred to the pots for hardening and later on in the field. Highest number of bands was obtained with Primer A-02, while the lowest numbers were obtained with primers B-10 and B-08 (Figures 1 and 2). Some specific bands were also identified, thus, reflecting the RAPDs application for the identification of sugarcane mutants. Results revealed that somaclone 2010 and NIA-2011 somaclone contains a specific band of 451bp amplified with primer A-2. In the relationship between sugarcane somaclones and parent, the highest similarity was obtained between BL4P and BL4SC (96%), while minimum similarity was found between NIA-2011 and NIA - 2010SC (69%).

Molecular markers have turned out to be efficient tools in the characterization and estimation of genetic diversity within the species and population [18] RAPD markers have turned out to be a solid marker framework for genetic fingerprinting and furthermore, for identifying genetic diversity [19, 28] reported that some RAPD bands were acquired in sugarcane families, showing that RAPD bands could effectively be utilized as genetic markers. While RAPD-PCR fingerprints have been generally utilized in diverse studies, the utility of RAPD- based bands as markers is confounded as they may not be locus-specific [29] and products of various sequences or concentrations can co-migrate

with other amplification products [3, 30]. Polymorphism in repetitive DNA sequences has habitually been seen amid plant propagation by tissue culture [31] and experiences a bigger number of changes than the coding sequences. *In vitro* stress may incite changes at particular sites, for example, tedious DNA, in this way activating transposable components. High rate of molecular polymorphism was

observed in tissue culture in the era of new variability by Shahid *et al.* [21] that developed a technique to obtain intact somatic metaphase sugarcane cells. Together, these observations recommended either that a few genotypes are more susceptible to somaclonal variation, or that the *in vitro* unsteadiness is really a result of a genotype versus culture medium interaction.

Table 11. Effect of different concentration of indolebutric acid (IBA) + sugar on root induction

Concentrations of IBA mgL ⁻¹	BL4	NIA-2010	NIA-2011	Mean
0.5	6.40 ^c	5.00 ^{cd}	3.80 ^d	5.067 ^b
1.0	12.00 ^a	13.40 ^a	8.60 ^b	11.33 ^a
2.0	13.00 ^a	12.40 ^a	9.60 ^b	11.66 ^a
3.0	5.80 ^c	6.20 ^c	6.00 ^c	6.00 ^b
Mean	9.3 ^b	9.15 ^a	7.00 ^c	

Conclusion

Callus culture of three somaclones NIA-2011, NIA-2010 and BL4 produced genetic variability in the clone. During the research study, dry nodular and compact callus were found in two clones BL4 and NIA-2010 somaclones, while the smooth compact callus recognized type A and B callus in NIA-2011. Maximum callus and plant regeneration were observed with used 1.0 and 2.0 mg L⁻¹ 2, 4-D. However, maximum proliferation of callus was also observed in same concentration of 2, 4-D, while increased concentration of IBA + sugar decreased primary and secondary roots. The NIA-2010, somaclone produced vigorous plants during the hardening process in greenhouse as well as, has outstanding performance in field during acclimatization process.

Authors' contributions

Conceived and designed the experiments: M Naz, Performed the Experiments: M Naz, Analyzed the Data: M Naz, Contributed

reagents / materials/ analysis tools: M Naz, Wrote the paper: M Naz & F Hayat.

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