Pure Appl. Biol., 12(2):948-957, June, 2023
http://dx.doi.org/10.19045/bspab.2023.120096

Research Article

Investigation of genetic variability in the direct regeneration population of sugarcane

Aamir Ali Shaikh[1,2], Ghulam Sughra Mangrio[1], Muharram Ali[1], Shakeel Ahmed Soomro[3*], Nighat Seema Soomro[1], Zhenping Yang[2], Akhtiar Ahmed Kalhoro[4], Nida Jabeen[5], Usman Ali[6], Aamir Hamid Khan[6], Aafaque Ahmed Keerio[1] and Natasha Iqbal[7]

1. Faculty of Crop Production, Sindh Agriculture University, Tandojam, 70060, Pakistan
2. College of Agriculture, Shanxi Agricultural University, Taigu, Shanxi 030800, China
3. Department of Farm Structures, Sindh Agriculture University, Tandojam, 70060, Pakistan
4. Department of Botany, Shah Abdul University, Khairpur Mirs, Pakistan
5. College of Information and Computer, Taiyuan University of Technology, 030024, Taiyuan, Shanxi, China
6. College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan, China
7. Department of Botany, Government College University Faisalabad, 37000, Faisalabad, Pakistan

**Corresponding author's email: ssoomro@sau.edu.pk*

Citation

Aamir Ali Shaikh, Ghulam Sughra Mangrio, Muharram Ali, Shakeel Ahmed Soomro, Nighat Seema Soomro, Zhenping Yang, Akhtiar Ahmed Kalhoro, Nida Jabeen, Usman Ali, Aamir Hamid Khan, Aafaque Ahmed Keerio and Natasha Iqbal. Investigation of genetic variability in the direct regeneration population of sugarcane. Pure and Applied Biology. Vol. 12, Issue 2, pp948-957. http://dx.doi.org/10.19045/bspab.2023.120096

Received: 19/12/2022 Revised: 17/02/2023 Accepted: 02/03/2023 Online First: 17/03/2023

Abstract

This study aimed to examine the genetic variation in sugarcane plantlets which were derived from direct regeneration. The objective of this study was to optimize the effect of different phytohormones on direct regeneration in sugarcane, and to extract and optimize DNA isolation from sugarcane plantlets for molecular diagnostics. Direct regeneration potential on different concentrations of plant growth regulators IBA, NAA, Kinetin and BAP were studied for four sugarcane clonal lines i.e. BL4, NIA-2010, NIA-2004 and Thatta-10, using five different media's. 4 mg L^{-1} IAA + 0.5 mg L^{-1} 2, 4-D + 1.0 mg L^{-1} Kin medium resulted with best regeneration. Maximum plantlet regeneration rate was observed for Clone NIA-2010, while minimum was for Thatta-10. The best primary and secondary roots were observed when treated with 1 mg L^{-1} IBA + 4% sugar, followed by 2 mg L^{-1} IBA + 4% sugar. A decrease in primary and secondary number rootlets of NIA-2004, Thatta-10, BL4 and NIA-2010 soma clone was observed with increasing IBA concentration (4 mg L^{-1} IBA + 4% sugar). Development of chlorophyll mutants affirmed that direct regeneration of genetic fidelity cannot be maintained, but is a good source of exploring existing aneuploidy. It was concluded that MS medium containing 4 mg L^{-1} IAA + 0.5 mg L^{-1} 2,4-D + 1.0 mg L^{-1} Kin proved to be the rapid method for obtaining plantlets through direct regeneration. The RAPD study confirmed the genomic difference in plantlets population which were obtained through direct regeneration. The results of this research will help in the identification of superior parental lines for hybridization, which can be used to produce better sugarcane varieties.

Keywords: BAP; direct regeneration; IBA; RAPD; Sugarcane

Introduction

Sugarcane is an important commercial crop on which a number of people around the World depends on [1]. It is being cultivated across the World at an area of 28.3 M ha, with a production of 1.69 M tonnes [2]. Sugarcane in Pakistan is considered to be the second largest cash crop [3], with an total cultivated area of 1040 thousand hectares in 2019 [4]. In recent years its

importance has increased as raw material to sugar related industries [5, 6]. Press mud production is another crucial use of sugarcane, which for crop production is being used as source of organic matter and nutrients [7]. Less sugar recovery from the sugarcanes being cultivated in Pakistan are contributing in main cause of high cost of production [8].

Area of about two third of sugarcane is kept under raton crops which causes reduction in output, particularly in northern areas [9]. Low yield and sucrose recovery from cane are caused by different reasons, where non-availability of disease free elite stock for seeding is considered as an important among others [10, 11]. Sugar cane is a genetically complex crop that has a number of variable chromosomes. Being highly cross-pollination in nature, for flowering it necessitates hot and specific climate [12]. The genetic improvement of sugarcane is based on the production of hybrids by controlled pollination techniques [13]. Genetic improvement in Pakistan through traditional hybridization is difficult due to the complex nature of sugarcane flowering. The in vitro culture technique provides a unique opportunity to exploit genetic circumstance and rapid isolation clones with preferred trait [14]. Plants that has been attained via in vitro culture can testify phenotypic variability that is because of the real genetic changes [15]. Lakshmanan *et al*. [16] reported that plants that were obtained directly from explants had an effective strategy for significantly reducing the somaclonal variation.

Molecular marker technique i.e. random amplified polymorphic DNA (RAPD) markers are observed to be highly useful for detecting genetic changes in deoxy ribonucleic acid (DNA) levels amidst others [17]. RAPD analysis technique is considered to be quick, simple, and easy to perform which requires a small amount of DNA for analysis [18]. Repeated application of the technique is the justification of benefits in genetic variability studies [19]. The purpose of current study was to examine the genetic variation in sugarcane plantlets which were derived from direct regeneration, with objectives to optimize the effect of different phytohormones on direct regeneration in sugarcane, and to extract and optimize DNA isolation from sugarcane plantlets for molecular diagnostics.

Materials and Methods

The study aiming to investigate the genetic variability in direct regeneration population of sugarcane plants was carried out at Nuclear Institute of Agriculture (NIA), Tandojam. Four sugarcane varieties i.e. BL4, NIA-2010 NIA-2004 and Thatta-10 were obtained from various sugarcane research institutes of Sindh province of Pakistan.

Preparation of micronutrient and vitamin based stock solution

The flask containing salt (Table 1) was sterilized by adding 700 ml of distilled water, and was stirred continuously to dissolve properly. The solution prepared was labeled and stored in the refrigerator at 4 ºC. The amount of the ingredients for vitamin stock (Table 1) was added with distilled water, and was then mixed thoroughly. The solution prepared was transferred to volumetric flask, labelled and stored in the refrigerator at 4 ºC for further experimentation.

Preparation of MS basal medium

The (Table 1) shows the amount of macronutrients used. Micro nutrients added were sugar 20 g L^{-1}, agar 11 to 13 g L^{-1} and vitamins. pH of media was adjusted to a range 5.5 to 5.7. The beaker was kept in the microwave for 12 to 15 min, until it became transparent. The beaker was autoclaved for 2 hours at a temperature of 121 ºC and 15 psi, and was then cooled at the laboratory temperature.

Selection of explants

Four cane varieties i.e. BL4, NIA-2010, NIA-2004 and Thatta-10 were used in the present study. The Fresh plant material was collected from NIA Tando Jam Experimental Farm. Explants were obtained from the apical meristematic

region, which were sterilized and cultured on modified MS medium.

Root induction from shoots

Healthy long shoots of 6-7 cm were transferred and supplemented with various concentrations of IBA i.e., 1, 2 and 3 with 4 mg L^{-1}. Root induction duration, induced root's nature and frequency were recorded during root induction from shoots experiment. The cultures for all experiments were placed at 20 ºC under 16 photoperiods.

Table 1. Components of MS medium

Chemical composition / Ingredient	Weight in mg L^{-1}
Micro Nutrients	
$MnSO_4$ $4H_2O$	22.2
$ZnSO_4$ $7H_2O$	8.2
H_3BO_3	6.2
Kl	0.83
Na_2 MoO_4 $2H_2O$	0.025
$CuSO_4$ $5H_2O$	0.025
$CoCl_2$ $6H_2O$	0.025
Iron	
$FeSO_4.5H_2O$	27.8
Na_2 ETDA $2H_2O$	37.26
Vitamins	
$C1_2H8N_4OSCl_2$	0.5
Myo-inosital	0.1 g L^{-1}
Macro Nutrients	
KNO_3	1.09 g L^{-1}
NH_4 NO_3	1.65 g L^{-1}
$CaCl_2$ $2H_20$	0.44 g L^{-1}
KH_2 PO_4	0.17 g L^{-1}
$MgSO_4$ $7H_20$	0.37 g L^{-1}
$C_{12}H_{22}O_{11}$	25.00 g L^{-1}
Casein	2.00 g L^{-1}
AGAR	8.00 g L^{-1}

Direct regeneration media

1. 4 mg L^{-1} IAA + 1 mg L^{-1} Kinetin + 0.5 mg L^{-1} 2,4,D + Sugar 4% *(T_0)*
2. 4 mg L^{-1} IAA + 1 mg L^{-1} Kinetin + 0.2 mg L^{-1} 2,4,D + Sugar 4% *(T_1)*
3. 1 mg L^{-1} NAA + 2 mg L^{-1} BAP + Sugar 4% *(T_2)*
4. 1 mg L^{-1} NAA + 3 mg L^{-1} BAP + Sugar 4% *(T_3)*
5. 1 mg L^{-1} NAA + 4 mg L^{-1} BAP + Sugar 4% *(T_4)*

Isolation of genomic DNA and PCR amplification

Modified Gentra Kit method was used to isolate total genomic DNA from fresh leaves. The samples of DNA were quantified using a spectrophotometer. DNA was diluted in a TE buffer to a concentration of 10ng μ^{-1}. Eppendorf Master cycler was used for amplification reactions with initial denaturation for 5 minutes at 94 ºC temperature, for 1 min at 94 ºC; 1minute annealing at 52 ºC and 2 minutes extension at 72 ºC. The Final extension was performed at 72 ºC for 10 min. The primer pairs used in study are given in (Table 2).

Buffer solution genomic DNA extraction

Young leaf tissue samples were selected for the isolation of total genomic DNA for all the four varieties i.e. BL4, NIA-2010, NIA-2001 and Thatta-10. Three samples of that

were regenerated from somatic embryo callus, and three were taken from field plantlets.

Procedure of buffer solution

Beaker and conical flask were washed and dried in oven for 10 min. Tris-HCl 15.76 g was added to the beaker and dissolved in 100 ml ddH_2O, which was then kept on the magnetic stirrer for 15 min. NaCl 58.5 g was dissolved in 170 ml ddH_2O. EDTA 9.306 g was dissolved in 50 ml ddH_2O, pH was maintained as the EDTA pH (8) should be.

Buffer solution

It is a solution that resists changes in pH resulting by accumulation of acid or alkali. The solution was made by the combination of several acids, and verifying the strength with the conjugated base (Table 3).

Results

Weight of explants per bottle (g)

Statistical analysis of variances was found to be significantly different. The maximum weight (g) of explants was observed in clone BL4 followed by clone NIA-2010 (Fig. 1). Whereas the minimum weight (g) of explant was observed in NIA-2004. Comparison of means are presented in (Table 4).

Shoot regeneration

Statistical analysis of the variance for plantlets was observed to be significantly different (Table 5). It was observed that plantlets as affected yielded greater number of plantlets (77.00) in NIA-2010 soma clone on medium T_0, followed by NIA-2004. Whereas, minimum plantlets were noted on medium T_4 in all four clones viz. BL4, NIA-2010, NIA-2004 and Thatta-10.

Shoot length (cm)

The maximum elongated shoots (13.56cm & 13.52 cm) were found in NIA-2004 and NIA-2010 soma clones when grown on medium T_0. Whereas, minimum plantlets were noted on medium T_4. Comparison of means are presented in (Table 6).

No. of chlorophyll mutant

Maximum numbers of chlorophyll mutants (12.66) was observed for NIA-2010 soma clone, which was grown on medium T_0 (Table 7). The minimum chlorophyll mutant was recorded in NIA-2004 soma clone on medium T_3 (Fig. 2).

Table 2. Sequence of the primers (RAPD)

Primer	Sequence	Primer	Sequence
A-01	CAGGCCCTTC	B-06	TGCTCTGCCC
A-02	TGCCGAGCTG	B-12	AGGGAACGAG
A-04	AATCGGGCTG	C-02	GTGAGGCGTC
A-13	CAGCACCCAC	C-05	GATGACCGCC
A-18	AGGTGACCGT	C-07	GTCCCGACGA
A-20	GTTGCGATCC	C-08	TGGACCGGTG
B-10	CTGCTGGGAC	C-09	CTCACCGTCC

Table 3. Buffer chemicals

Chemicals	For 500 X reactions
Tris-HCL	50 ml
MATAB	140 ml
EDTA	20 ml
NaCL	10 g
PEG 6000	5 g
Na_2SO_3	2.5g

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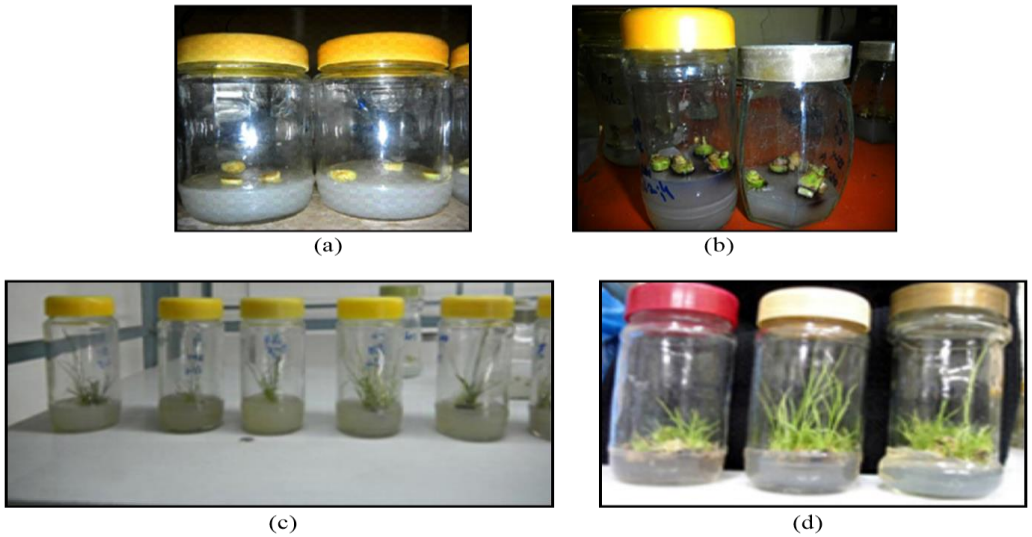


Figure 1. Effect of different phytohormones on direct regeneration in Sugarcane {a= Weight of explant (g), b= Weight of regenerated plant (g), c= No. of shoots per bottle and d= Shoot length per bottle (cm)}

Table 4. The weight (g) of explant under the influence of growth regulators

MS + Different concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
T_0	0.51^{k}	0.71^{d}	0.56^{ij}	0.66^{ef}	0.61^{C}
T_1	0.51^{k}	0.74^{c}	0.57^{hi}	0.65^{efg}	0.61^{BC}
T_2	0.51^{k}	0.80^{a}	0.58^{h}	0.64^{g}	0.63^{A}
T_3	0.51^{k}	0.76^{b}	0.55^{j}	0.67^{e}	0.62^{AB}
T_4	0.51^{k}	0.73^{c}	0.58^{h}	0.64^{fg}	0.61^{BC}
Mean	0.51^{D}	0.75^{A}	0.56^{C}	0.65^{B}	

Table 5. Number of shoot regeneration on different concentrations of MS + BAP Media

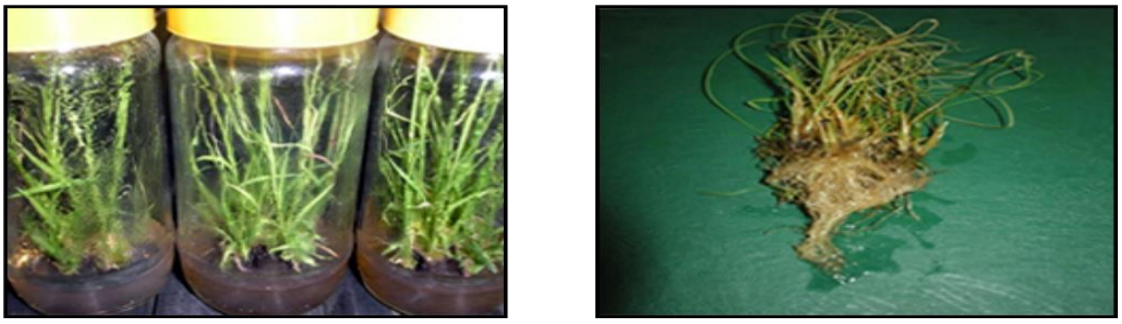
MS + Different concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
T_0	45.00^{f}	37.66^{g}	31.33^{hi}	55.66^{d}	42.41^{B}
T_1	63.33^{b}	59.33^{c}	52.33^{e}	77.00^{a}	63.00^{A}
T_2	25.00^{kl}	39.33^{g}	29.33^{ij}	32.00^{h}	31.41^{C}
T_3	20.00^{m}	27.00^{jk}	23.33^{l}	38.00^{g}	27.08^{D}
T_4	17.00^{n}	18.00^{mn}	11.00^{o}	22.66^{l}	17.16^{E}
Mean	34.06^{C}	36.26^{B}	29.46^{D}	45.06^{A}	

Table 6. Length of shoot regeneration on different concentrations of MS + BAP Media

MS + Different concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
T_0	12.92^{bc}	11.50^{e}	10.40^{gh}	13.33^{ab}	12.03^{A}
T_1	13.56^{a}	10.77^{fg}	10.25^{gh}	13.52^{a}	12.03^{A}
T_2	12.10^{d}	10.77^{fg}	9.45^{ij}	12.41^{cd}	11.18^{B}
T_3	11.30^{ef}	9.96^{hi}	9.21^{j}	12.11^{d}	10.65^{C}
T_4	11.45^{e}	9.55^{ij}	8.06^{k}	11.50^{e}	10.14^{D}
Mean	12.27^{B}	10.51^{C}	9.47^{D}	12.57^{A}	

Table 7. No. of chlorophyll mutant of shoot regeneration on different concentrations of MS + BAP Media

MS + Different concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
T_0	5.33^{de}	9.66^{b}	3.66^{f}	7.66^{c}	6.58^{A}
T_1	3.66^{f}	5.00^{e}	2.66^{fg}	12.66^{a}	6.00^{B}
T_2	3.66^{f}	3.66^{f}	2.33^{g}	3.66^{f}	3.33^{C}
T_3	2.66^{fg}	2.33^{g}	2.33^{g}	2.66^{fg}	2.50^{D}
T_4	2.33^{g}	6.33^{d}	7.66^{c}	5.66^{de}	5.50^{B}
Mean	3.53^{C}	5.40^{B}	3.73^{C}	6.46^{A}	



(a) (b)

Figure 2. Effect of different phytohormones on rooting in Sugarcane (a= No. of chlorophyll mutants per bottle and b= No of primary roots per bottle)

Primary roots

Statistical analysis of variances for primary roots as affected by various concentrations of indole butyric acid and their interactive effects (somaclones x indole butyric acid) had been enormously distinct at 5% probability level (Table 8). Higher number of primary roots were recorded (9.66) in NIA-2004 soma clone applying medium 1 mg L^{-1} IBA + 4 % sugar, followed by 2 mg L^{-1} IBA + 4 % sugar (Fig. 2). An increase in IBA concentration (4 mg L^{-1} IBA + 4 % sugar) significantly resulted a decrease in number of primary rootlets of BL4, NIA-2010, NIA-2004 and Thatta-10 soma clones.

Secondary roots

Secondary roots with 21.00 were observed to be greater in NIA-2004 soma clone when treated with 1 mg L^{-1} IBA+ 4% sugar, followed by 18.33 secondary roots within the identical type applying 2 mg L^{-1} IBA + 4% sugar (Table 9). The lower number of secondary roots (10.00) was observed in NIA-2010 soma clone grown on medium containing 3 mg L^{-1} IBA + 4% sugar.

Discussion

The RAPD method has often been used to analyze the soma clonal variation and assess the genetic fidelity of sugarcane [20]. Plant regeneration in Poaceae species is facilitated by recognition of appropriate explant and invitro culture considerations [21]. In vitro plant regeneration is considered to be the vital step in carrying out various biotechnology strategies for improvement of crop [22]. Embryogenic calluses are formed in occurrence of 2, 4-D, where the regeneration is either achieved by minimizing the auxin, or by removing it from the medium [23], while there are few reports directly related to regeneration by different explants [12]. The fast, and effective protocol for in-vitro SDR of sugarcane immature leaves has been reported without a callus intervention procedure [24]. Similar findings for without callus phase has also been stated by various researchers i.e. for orchard grass [25], for

garlic [26] and for minor millet [27]. The current study was carried out to detect explants and a combination of growth regulators for induction of direct regeneration in four cultivars of sugarcane varieties of Pakistan i.e. BL4, NIA-2010, NIA-2004 and Thatta-10. This research was carried out to screen tissue culture (TC)-induced genetic alterations in regenerated sugarcane via direct regrowth in the immature leaf segments of cane clone by RAPD analysis using PCR. The cytokinin used in the culture medium was observed to have a clear effect on shoot proliferation. It was further observed that in occurrence of BAP, the shoots fused, developing green leaves which were thick and dark. Svetleva [28] reported that chlorophyll synthesis is controlled by nuclear and cytoplasmic genes, where to assess the genetic fidelity the chlorophyll mutants are used. Chlorophyll mutant production confirms that this process allows us to take advantage of the natural background heterozygosity, which can be achieved by access to aneuploid cells. For root number the concentrations of IBA and sugar resulted with significant differences. Clone NIA - 2004 produced the highest number of primary and secondary roots at 1 mg L^{-1} IBA 4% sugar. Dawood et al. [29] reported that if the plantlets are well developed the roots will grow from nodal primordia. Bhau & Wakhlu [30] reported that IBA is the effective auxin for root induction, while higher auxin levels stimulate callus formation from the cut end of the extract. Similar findings have also been stated by Khan *et al*. [31] and Tolera *et al*. [32]. Effective rooting was observed from the shoots of all four cultivars with 1 mg L^{-1} IBA + 4% sucrose. The protocol developed in current study can be useful for creating new genetic variability. The RAPD technique was used to detect variations in the directly regenerated plants of sugarcane. Plants directly regenerated from tissue of BL4, NIA-2010 NIA-2004 and Thatta-10 were recorded for existing genetic variability within in the tissue in the form of aneuploidy (Fig. 3).

Table 8. Number of primary roots regeneration on different concentrations of MS + BAP Media

MS + BAP concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
MS+1 mg L^{-1} BAP +4% Sugar	9.66^{a}	7.66^{b}	8.00^{b}	6.00^{c}	7.83^{A}
MS+2 mg L^{-1} BAP +4% Sugar	8.66^{ab}	6.33^{c}	6.33^{c}	6.33^{c}	6.91^{B}
MS+3 mg L^{-1} BAP +4% Sugar	8.00^{b}	4.00^{d}	4.00^{d}	4.00^{d}	5.00^{C}
Mean	8.77^{A}	6.00^{B}	6.11^{B}	5.44^{B}	

Table 9. Number of secondary roots regeneration on different concentrations of MS + BAP Media

MS + BAP concentrations	Varieties				Mean
	NIA-2004	BL4	Thatta-10	NIA-2010	
MS+1 mg L^{-1} IBA +4%Sugar	21.00^{a}	17.66^{b}	18.00^{b}	15.66^{c}	18.08^{A}
MS+2 mg L^{-1} IBA +4%Sugar	18.33^{b}	15.66^{c}	15.66^{c}	12.33^{d}	15.50^{B}
MS+3 mg L^{-1} IBA +4%Sugar	16.00^{c}	13.33^{d}	13.33^{d}	10.00^{e}	13.16^{C}
Mean	18.44^{A}	15.55^{B}	15.66^{B}	12.66^{C}	

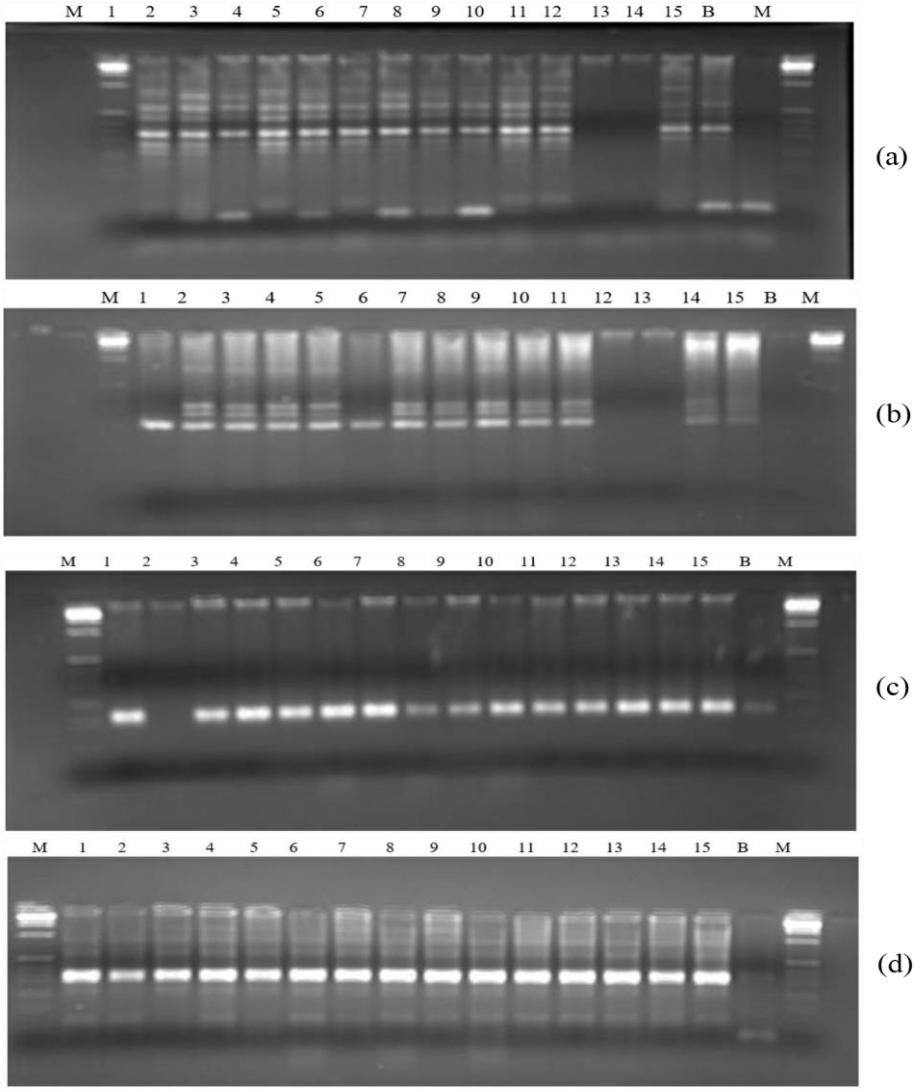


Figure 3. RAPD study of soma clone in NIA-2004 through direct regeneration (a); RAPD study of soma clone in NIA-2010 through direct regeneration (b); RAPD study of soma clone in BL4 through direct regeneration(c); RAPD study of soma clone in Thatta 10 through direct regeneration (d) {L1= (Parent), L2= (Somaclone), L3= (Somaclone), L4= (Somaclone), L5= (Somaclone), L6= (Somaclone), L7= (Somaclone), L8= (Somaclone), L9= (Somaclone), L10= (Somaclone), L11= (Somaclone), L12= (Somaclone), L13= (Somaclone), L14, = (Somaclone), L15}

Conclusion

The present study concluded that direct regeneration can be used for the improvement of sugarcane, as the genetic architecture of sugarcane can potentially be altered. The MS medium containing 4 mg L^{-1} IAA+ 0.5 mg L^{-1} 2, 4-D + 1.0 mg L^{-1} Kin resulted as rapid method to obtain plantlets through direct regeneration. The study further concluded that the RAPD study confirmed the genomic variation in the plantlets population obtained through direct regeneration.

Authors' contributions

Conceived and designed the experiments: AA Shaikh & GS Mangrio, Performed the experiments: AA Shaikh, NS Soomro & AA Keerio, Analyzed the data: U Ali & AH Khan, Contributed materials/ analysis/ tools: M Ali, Z Yang, N Jabeen & AA Kalhoro, Wrote the paper: SA Soomro & N Iqbal.

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