OPEN ACCESS

International Journal of
Plant Physiology and Biochemistry

March 2018
ISSN 2141-2162
DOI: 10.5897/IJPPB
www.academicjournals.org

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International Journal of Plant Physiology and Biochemistry

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academicJournals

Vol. 10(2), pp. 10-18, March 2018
DOI: 10.5897/IJPPB2016.0255
Article Number: 3D5364F56565
ISSN 2141-2162

http://www.academicjournals.org/IJPPB

International Journal of Plant Physiology and Biochemistry

Full Length Research Paper

Estimation of auxins and cytokinins requirements in sugarcane soma clones for effective *in vitro* regeneration procedure

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Received 25 September, 2016; Accepted 6 July, 2017

Three sugarcane varieties were used in this experiment to optimize specific plant hormones. The present work was conducted in 2014 at Nuclear Institute of Agriculture (NIA), Tando jam. The experiment was designated with three sugarcane varieties (NIA-2012, NIA-105 and GULABI-95) obtained from Nuclear Institute of Agriculture (NIA) Tando jam. Regeneration of plantlets was compared under different concentration of auxins and cytokinin (IAA, IBA and kinetin (2.0, 3.0 mg1^{-1}), highly significant ($p<0.05$) variations were observed for all parameters of regeneration and root formation. Interactive effect of variety x treatment x concentration was non-significant for number of regenerated plantlets. Auxins and cytokinins at 3.0 + 3.0 mg1^{-1} concentration were most optimized and effective for regenerated plantlets and number of shoots. These concentrations should be used in the future for *in vitro* culture of sugarcane.

Key words: *In vitro*, regeneration, cytokinins, sugarcane, growth regulators.

INTRODUCTION

Sugarcane is the most important agro-industrial crop, belonging to the Poaceae family with chromosome number = 80 (Khan et al., 2005). This crop provides many byproducts such as gur, sugar, biofuel and energy (Garacia et al., 2007). The yield of sugarcane is low in Pakistan as compared to other countries of the world (Chengalrayan and Gallo-Meagher, 2001). The main reason for this low yield is genetic improvement which takes place through conventional hybridization in Pakistan.

Plant *in vitro* regeneration technique offers successful sugarcane propagation (Franklin et al., 2006; Roy et al., 2007). This procedure is most helpful in controlling bundle of problem which is faced during conventional breeding practices. These techniques ensure disease free multiplication of elite varieties (Khan et al., 2004) and minimizes time span required for mass production.

There are many researchers from different countries

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Table 1. ANOVA for regeneration of sugarcane plantlets modulated by different concentration auxin and cytokinin.

Source	DF	Mean square			
		Number of plantelets	Number of shoots/plantelet	Length of shoots/plantelets	Number of mutant plantelets
Varieties	2	43.400**	273.756**	165.267**	1.38148**
Treatment	2	152.270**	888.804**	174.804**	0.20741 ns
Concentrations	4	82.756**	111.80 **	62.289 **	0.20741 ns
V x T	4	7.409**	20.098**	21.887 **	0.61481 ns
V x C	8	10.456 **	16.222**	57.189**	0.33704 ns
T x C	8	5.876**	26.559**	14.187**	0.54074**
V x T x C	16	1.923 ns	18.662**	7.879**	0.67037**
Error	88				
Total	134	CV 23.56	CV 18.13	CV 26.46	CV 313.98

In each column, means followed by common letter are not significantly different at 5% probability level. V x T= varieties x treatment, V x C= varieties x concentration. T x C= Treatment x concentration, V x T x C= varieties x treatment x concentration. CV= critical value, ns= non-significant.

who have used tissue culture for genetic improvement of sugarcane (Dibax et al., 2011;, Takahshi and Takamizo, 2013). It was also observed that callus derived from different auxins have different regeneration potential (Solangi et al., 2016). Plant regeneration from regenrable callus obtained from meristem have been exploited by number of researchers (Blanco et al., 1997 and Vickers et al., 2005) using (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins (Sughra et al., 2014). Regeneration from callus culture creates genetic as well as epigenetic variations induced by enforced hormonal stimuli (Nawaz et al., 2013; Karim et al., 2015). To fulfill sugar need of the increasing population, this study was planned to induce soma clonal variation which is a necessary component of any conventional crop breeding program (Mathur, 2013). The typical crop improvement cycle takes 8 to 16 years to complete and includes germplasm manipulations, variety selection and stabilization. Regeneration of plantlets through callus culture provides genetic variability known as somaclonal variation. Genotype increases, testing variety protection of crop and production stages. Plant tissue culture is an enabling technology from which many novel tools have been developed to assist plant breeders with improved *in vitro* regeneration ability of these three sugarcane varieties influenced by auxins, cytokinins and sucrose.

MATERIALS AND METHODS

Three varieties selected for this experiment are early maturing NIA-2012, mid maturing NIA-105 and late maturing Gulabi-95. Selected callus was shifted to the fresh media for regeneration of the plantlets. Different tissues were selected as explants sources like roots, leaves, and stems which produce more variations than explants with pre-existing meristems such as shoot tips and axillary buds.

The mass of regenerable calli produced with the help of five callus induction media were transferred to 5 types of regeneration media. MS modified with various concentration of cytokinins and auxins include indole-3- acetic acid IAA, indole-3- butyric acetic acid IBA 2,4-D, picloram and NAA. Shoot regeneration started with the appearance of green dots on callus within two weeks after regeneration medium and generally produced normal micro shooting. Regeneration results were not obtained in control or hormone free MS medium. After two subcultures (4 weeks of each), calli were transferred to bottle containing the medium of regeneration (MS modified 2,4-D 2, 4-dichloro-phenoxyacetic acid, Picloram, NAA naphthalene acetic acid, indole-3-acetic acid IAA, indole-3-butyric acid IBA and kinetin 2.00, 2.50 and 3.0 mg l^{-1}) of each growth regulators. Cultures were incubated in growth room with 2000-3000 lux under 16th photo period at 25±2°C. The effects of callus age on regeneration were predictable by transferring the calli to regeneration media after 15, 20, 26, and 37 days of culture.

The plantlets obtained were aseptically transferred to the same regeneration medium for 5 other weeks. Shoot emerging takes place from green calli. Multiplication of shoots was continuously till healthy plantlets obtained for this purpose trimmed off shoots and transferred into fresh media. Data analysis was done by ANOVA on collected number of plantlets, micro shoot, and length of shoot using computer software Statistics version 8.1(Table 1). Experiment design was complete randomized design (CRD with three treatments and five different concentrations through two factorial designs, and the regenerated shoots from callus were counted for calculation of the shoot organogenesis. The regenerated shoots were scored for chlorophyll mutations. When the regenerated plantlets reached 7 to 8 cm height, these were subjected to rooting by culturing on rooting media.

RESULTS AND DISCUSSION

An efficient protocol supporting grooming of callusing and regeneration potential is essential for successful genetic transformation of the commercial clones with somaclonal variation (Asad et al., 2009; Solangi et al., 2016). Sugarcane plantlets were regenerated successfully from all the tested clones.

Number of regenerated plantlets/callus

The 3 sugarcane varieties were cultured on MS modified

Table 2. Effect of different concentrations of auxin and cytokinin on regeneration of plantlets in three different genotype of sugarcane.

Growth regulators	Conc (mg l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2, 4-D + ABK	0.0 + 2	2.66^{o-q}	1.33^{q}	$1.66p^{-q}$	2.00^{f}
	0.5 + 2	6.66^{e-i}	5.00^{i-m}	5.00^{i-m}	4.22^{d-e}
	1.0 + 2	$9.33^{b–c}$	7.00^{e-h}	$4.33k^{-o}$	6.88^{c}
	2.0 + 3	11.00^{b}	5.66^{g-l}	5.33^{h-l}	8.22^{b}
	3.0 + 3	13.33^{a}	9.66^{b-c}	9.00^{c-d}	10.66^{a}
Picloram + ABK	0.0 + 2	$1.66p^{-q}$	$2.00p^{-q}$	2.66^{o-q}	2.00^{f}
	0.5 + 2	$3.00n^{-q}$	2.66^{o-q}	6.00^{g-k}	3.44^{e-d}
	1.0 + 2	6.33^{f-j}	4.00^{l-o}	5.00^{l-m}	5.11^{d}
	2.0 + 3	8.00^{c-f}	5.00^{i-m}	4.66^{j-n}	4.66^{d}
	3.0 + 3	9.00^{c-d}	8.33^{c-e}	$7.33^{d-}g$	8.00^{b-c}
NAA + ABK	0.0 + 2	1.66^{p-q}	1.33^{q}	1.66^{p-q}	1.55^{f}
	0.5 + 2	3.00^{n-q}	2.66^{o-q}	2.00^{p-q}	4.22^{d-e}
	1.0 + 2	5.00^{i-m}	4.33^{k-o}	$3.00n^{-q}$	4.11^{d-e}
	2.0 + 3	5.66^{g-l}	3.33^{m-p}	5.00^{i-m}	5.00^{d}
	3.0 + 3	7.00^{e-h}	6.00^{g-k}	4.66^{j-n}	7.00^{c}
Mean		6.46^{a}	5.04^{b}	3.75^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. LSD = least significant difference, SE= standard error. Varieties SE 0.2528; LSD 5% 0.0524; Concentrations: SE 0.5653; LSD 5%: 1.1134; V x C SE 0.9791; LSD 5%: 1.9457.

media. Cultivars under exploration showed highly significant differences for various variables of *in vitro* callus of three different genotypes of sugarcane, NIA-2012, NIA-105 and Gulabi-95. Data for number of regenerated plantlets are presented in Tables 2, Figure 1a, b and c. Significant variations ($P< 0.05$) for regenerated plantlets were observed for all genotypes. Highest result was obtained in NIA- 2012 (6.46), and minimum in Gulabi- 95. According to the treatment variance, picloram with ABK gave best result for grooming of regeneration of plantlets while NAA and ABK gave no positive result for the sugarcane varieties. Mostly, NAA phytohormone preferable for root induction is not suggested for improvement of regeneration of sugarcane plantlets (Table 2, Figure 1a, b and c). The maximum proliferation of regeneration of plantlets was observed in the concentration of 3.0 mg /l 2,4-D +3.0 mg/l ABK.

In the present investigation, shoot apical regeneration of different sizes increased with improved dose of all the treatment of auxins and cytokinins. As shown in Table 2, time for shoot formation was increased by enhancing the dose of phytohormone. Maximum rate of survival was achieved when concentration of 3.0 mg/l was used. This size exhibited 100% survival with 90% regeneration potential within 12 days of inoculation. These results are similar to that of Ali et al. (2010) and Ijaz et al. (2012). The present results vary as compared to that of the previous worker (Khan et al., 2008; Seema et al., 2001). They obtained good results of regeneration of plantlets at lower concentration of auxins and higher concentration of cytokinins.

Maximum number of shoots/plant

About 5-6 weeks shoot, vigorously growing regenerated plantlets were transferred to fresh medium in bottles for further growth and proliferation. Both stages of regeneration of plantlets were tested. Highest result was obtained in NIA-2012. Better results for shoot burgeoning were obtained in MS medium at the concentration of 3.0 mg/l. Proliferation of shoot started and during secondary proliferation stage, lateral shoots developed from the base of newly initiated shoot. As a result, a dense mass of shoots was developed in NIA-2012 followed by Gulabi-95 and minimum in NIA-105. After about 20 days, these shoots were further sub-divided in small shoots containing 4-5 shoots and were transferred into fresh medium in bottles (Table 3). Best treatment was optimized (Picloram) for the variety, NIA-2012 followed by NIA- 105 and minimum by Gulabi-95.

The present result of shoot formation and proliferation were obtained with concentration of 3.0 mg/l. Recent

Figure 1a. Effect of different concentration of 2, 4-D (cytokinin) on regeneration of plantlets.

study also demonstrates the outcome of phytohormone for shoot formation and multiplication described positve correlation (Rocha, 2012). All the results are consistent with that of Torque et al. (2010) Sughra et al. (2014) and Solangi et al. (2016). The current study is quite different due to change in the variety or concentration of hormones (Roy and Kabir, 2007; Duminil and Di Michele, 2009).

Albino mutant plant production

The present study also describes the role of cytokinins, particularly kinetin in shoot formation and number of albino mutant plant. The main mode of action of plant growth hormone involves binding of active substances to a specific receptor molecule which bind either on cell surface or within the cytoplasm. Different growth regulators responsible for albino mutation, maximum albino mutant are obtained in the concentration of 3.0 mg/l (Table 4 and Figure 2). Highest albino plant was obtained in NIA-2012 and minimum in NIA-105. The results also suggest that shoot multiplication and albino formation in sugarcane depends on the genotype and media concentration. However, the best albino mutant plant was achieved when cultured on MS medium supplemented with 3.0 mg/L 2,4-D (Khan et al., 2004; Roy et al., 2010; Begum et al., 2011) and decreased concentration reduced shootlets induction and albino plantlets (Biradar et al., 2009; Ali et al., 2010).

Figure 1b. Effect of different concentration of picloram (cytokinin) on regeneration of plantlets.

Length of shoots/day

Among the three varieties of sugarcane, on average, NIA-105 requires minimum days (21) for the induction of the shoot, while the maximum days were recorded in NIA-2012 (19) and Gulabi-95 (18). However, more vigorous shoot length development was achieved when the plantlets were separated and cultured on MS medium supplemented shoot induction with 3 mg/L of Picloram. The overall highest length of shoots for NIA-105 (15 cm) was seen with medium containing Picloram + ABK.

Among the variability parameters studied, there was significant variation at the probability level ($p<0.05$) in all the genotypes. First factor indicated the magnitude of variations exclusively due to the gene action which occurred due to the change in the concentration of hormones (Table 5), whereas, the latter indicated the total variations generated and was attributed to the conditions provided during environmental component together with the genotypic variations. The results indicated resemblance with the results of Zamir et al. (2012) and varied from that of Sahoo et al. (2011).

Conclusion

The basic phytohormones influence and enhance cell division, cell elongation and cell differentiation, and integrates the overall development of shooting properly. Efficient regeneration potential of callus is helpful for transformation of genetic variation in commercial elite sugarcane replicates. Albino mutant occur due to genetic

Figure 1c. Effect of different concentration of NAA (cytokinin) on regeneration of plantlets.

Table 3. Effect of different concentrations of auxin and cytokinin on number of shoots in regeneration of plantlets in sugarcane.

Growth regulators	Concentration (mg l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
s2, 4-D + ABK	0.0 + 2	5.00^{o-q}	4.66^{p-q}	3.66^{p-q}	4.11^{i}
	0.5 + 2	15.00^{f-i}	15.00^{f-i}	6.00^{nm}	11.89^{e-f}
	1.0 + 2	15.66^{e-h}	13.33^{g-k}	14.00^{f-j}	14.55^{c-d}
	2.0 + 3	18.66^{d-e}	13.00^{h-k}	15.00^{f-i}	19.22^{b}
	3.0 + 3	23.66^{b}	19.66^{c-d}	17.33^{d-f}	23.33^{a}
Picloram + ABK	0.0 + 2	2.33^{q}	2.33^{q}	3.00^{p-q}	3.44^{i}
	0.5 + 2	12.33^{h-l}	6.00^{nm}	10.33^{k-m}	8.89^{g-h}
	1.0 + 2	13.00^{h-k}	11.66	9.00^{i-n}	12.11^{e-f}
	2.0 + 3	22.33^{b-c}	14.66^{f-j}	13.67^{g-k}	14.44^{c-d}
	3.0 + 3	29.66^{a}	$15.00f^{-i}$	15.33^{e-f}	16.11^{c}
NAA + ABK	0.0 + 2	5.00^{q}	3.33^{p-q}	2.66^{p-q}	3.11^{i}
	0.5 + 2	8.33^{m-o}	5.66^{o-q}	4.66^{p-q}	7.00^{h}
	1.0 + 2	15.00^{f-i}	11.33^{l-m}	8.66^{m-n}	10.56^{f-g}
	2.0 + 3	16.66^{d-g}	15.66^{e-h}	12.67^{h-k}	13.78^{d-e}
	3.0 + 3	16.66^{d-g}	13.66^{h-k}	12.67^{h-k}	15.11^{c-d}
Mean		13.31a	12.04b	10.17^{c}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.4527); LSD 5% 0.8995; Concentrations SE 1.0122; LSD 5% 2.0115; V x C SE 1.7531; LSD 5% 3.4839.

Table 4. Effect of different concentrations of auxin and cytokinin on number of mutant in regeneration of plantlets in sugarcane.

Growth regulators	Concentration (mg l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2, 4-D + ABK	0.0 + 2	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	0.5 + 2	1.00^{abc}	0.66^{abc}	0.00^{c}	0.53^{ab}
	1.0 + 2	0.66^{abc}	0.00^{c}	0.00^{c}	0.22^{d}
	2.0 + 3	1.00^{abc}	0.00^{c}	0.00^{c}	0.33^{c}
	3.0 + 3	1.66^{a}	1.33^{ab}	0.00^{c}	0.99^{a}
Picloram + ABK	0.0 + 2	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	0.5 + 2	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	1.0 + 2	0.00^{c}	0.00^{c}	1.66^{a}	0.53^{ab}
	2.0 + 3	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	3.0 + 3	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
NAA + ABK	0.0 + 2	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	0.5 + 2	0.00^{c}	0.00^{c}	0.33^{bc}	0.11^{e}
	1.0 + 2	0.00^{c}	0.66^{abc}	0.00^{c}	0.22^{d}
	2.0 + 3	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{f}
	3.0 + 3	0.33^{bc}	0.00^{c}	0.00^{c}	0.11^{e}
Mean		0.13^{a}	0.22^{a}	0.26^{a}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.1373; LSD 5% 0.2728; Concentrations SE 0.5317; LSD 5% 1.0567; V x C SE 0.3070; LSD 5% 0.6106.

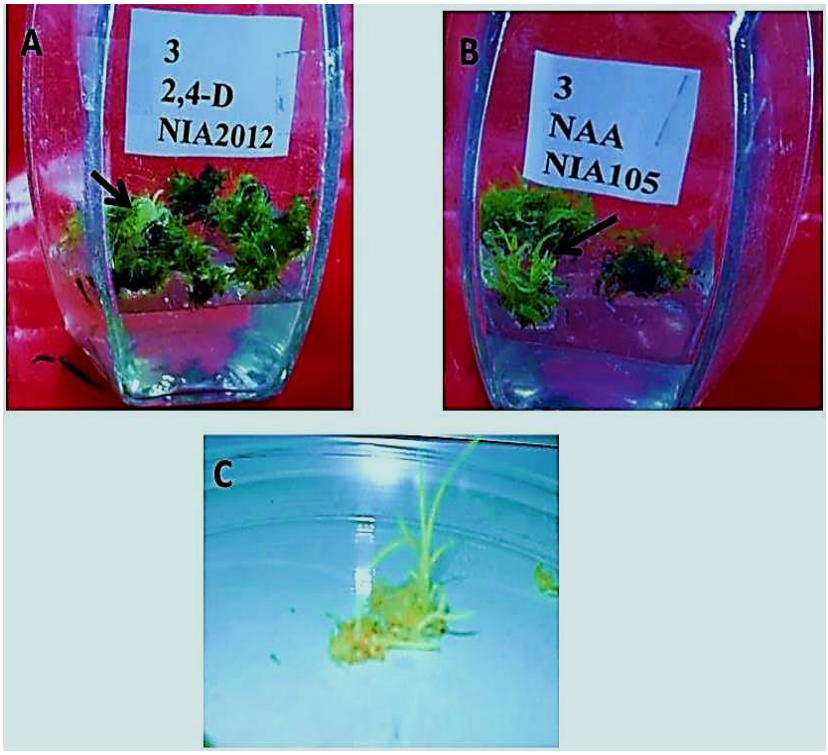

Figure 2. Maximum and minimum number of albino mutant plants/regenerated by different concentrations of cytokinin and auxin in sugarcane.

Table 5. Effect of different concentrations of auxins and cytokinin on length of shoots in regeneration of plantlets of three different genotypes in sugarcane.

Growth regulators	Concentration (mg l^{-1})	Varieties			Mean
		NIA-2012	NIA-105	Gulabi-95	
2, 4-D + ABK	0.0 + 2	2.00^{k-m}	2.33^{j-m}	3.33^{i-m}	2.44^{h}
	0.5 + 2	4.66^{g-j}	2.33^{j-m}	2.33^{j-m}	4.00^{e-f}
	1.0 + 2	2.33^{m}	1.33^{m}	3.00^{i-m}	4.44^{d-f}
	2.0 + 3	7.00^{e-g}	5.00^{g-i}	6.33^{e-h}	6.44^{b-c}
	3.0 + 3	8.33^{d-e}	8.33^{d-e}	5.33^{f-i}	7.55^{b}
Picloram + ABK	0.0 + 2	3.00^{i-m}	1.66l^{-m}	1.33^{i-m}	2.44h
	0.5 + 2	3.33^{i-m}	6.00^{e-h}	2.00^{k-m}	4.77^{d-e}
	1.0 + 2	6.00^{e-h}	11.00b¯c	3.33^{i-m}	7.66b
	2.0 + 3	7.00^{e-g}	14.66^{a}	5.33^{f-i}	11.77^{a}
	3.0 + 3	8.33^{d-e}	15.00^{a}	7.66^{e-f}	12.22^{a}
NAA + ABK	0.0 + 2	2.33^{j-m}	3.33^{i-m}	3.00^{i-m}	2.55^{g-h}
	0.5 + 2	4.00^{h-l}	6.00^{e-h}	5.00^{g-i}	3.11^{f-h}
	1.0 + 2	5.00^{g-i}	10.66^{c-d}	5.33^{f-i}	3.88^{e-g}
	2.0 + 3	5.33^{f-i}	15.66^{a}	4.00^{h-l}	5.22^{c-e}
	3.0 + 3	6.00^{e-h}	13.33^{a-b}	4.33^{h-k}	5.77^{c-d}
Mean		6.22^{a}	6.37^{a}	4.26^{b}	

In each column, means followed by common letter are not significantly different at 5% probability level. Varieties SE 0.3136; LSD 5% 0.6233; Concentrations SE 0.7013; LSD 5% 1.3937; V x C SE 1.2147; LSD 5% 2.4139.

variation which mainly depends on type and concentration of auxins and cytokinins. *In vitro* regeneration of plantlets showed increasing ability of regeneration when additive concentration of plant hormones is applied on the callus.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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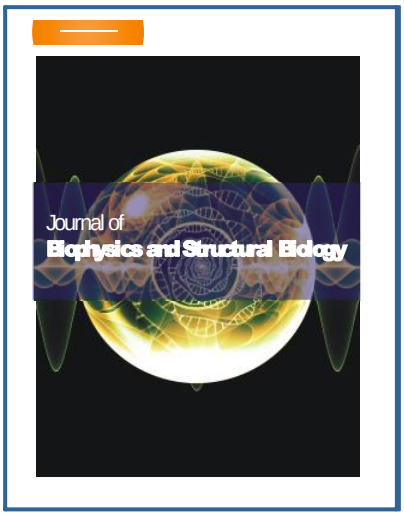

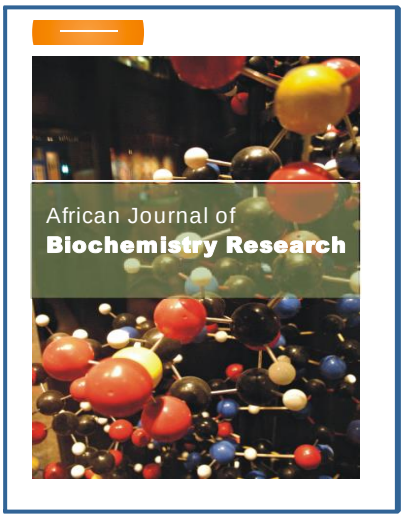

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