THE USE OF BIOTECHNOLOGY IN THE SEARCH OF HARDY *SORGHUM BICOLOR* (L) MOENCH CULTIVARS

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ABSTRACT

Sorghum bicolor (L) Moench is generally sensitive to salt and acid (high aluminium) soil stresses. As with any stress phenomenon, intra-specific variability exists within the genus. *In vitro* cell selection and somaclonal variation offer an alternative to traditional breeding methodology for generating improved breeding lines for hybrid development. Tissue culture method was developed for induction and maintenance of embryogenic calli established from young embryo explants of three *Sorghum bicolor* cultivars, namely, Mtama 1, El Gardam and Seredo selected basing on tannin content, which in turn determines palatability. Embryogenic calli were obtained by culturing young embryos on Linsmaier and Skoog's (LS) medium containing 2 mg/l 2, 4 dichlorophenoxyacetic acid (2,4D) and 0.5 mg/l kinetin. Calli were subjected to 50 mM, 100 mM, 150 mM and 200 mM NaCl to screen them for salinity tolerance. Calli subjected to 0.0 mM NaCl served as controls. Calli with a tolerance to salinity stress had a higher activity of succinate dehydrogenase, which reduced trimethyl tetrazolium chloride (TTC) to formazan. The amount of formazan was measured spectrophotometrically and a graph of NaCl concentration against absorbance plotted. From the TTC test (viability test) 100 mM NaCl was selected as the optimum concentration, which was incorporated into LS media to initiate tolerance in sorghum calli.

Sorghum shoots were regenerated from embryogenic calli of both NaCl treated and the controls cultured on LS medium supplemented with 1.0 mg/l Indole Acetic Acid (IAA) and 0.5 mg/l benzyl adenine (BA). Rooting was achieved by supplementing LS medium with 3 mg/l Indole Butyric Acid (IBA).

By using random amplified polymorphic DNA (RAPDs) technique, it was established that there was variation between NaCl treated plants and the controls. This was both in the individual and pooled DNA samples. A genetic distant matrix was calculated and used to construct a dendrograme as a measure of relatedness, which led to the conclusion that some somaclonal intra cultivar variation had occured.

Key words: Sorghum cutivars, tissue culture, somaclonal variation, RAPDs, salt tolerance

1.0 INTRODUCTION

The need to improve and select local varieties of crop plants for cultivation in the vast arid and semi-arid regions of the world has already been identified. Sorghum as a crop was selected for this study because it has not yet received full attention in the scientific community, and by governments. Therefore, much of its production is on a small scale compared to maize, yet it fills a unique and highly significant position in drought and salinity tolerance in arid zones, in coparison to maize, wheat and rice.

The idea of repeated backcrossing to incorporate a desired gene into crops makes traditional breeding a slow means of producing a suitable crop variety to withstand various stress factors. The breeding of crop varieties by means of *in vitro* tissues and somatic hybridisation followed by genetic screening has the following advantages over traditional breeding methods:

(i) The culture often consists of millions of cell lines, each a potential plant which can be screened in a less amount of culture medium, an easier task compared to screening whole plants.

(ii) Through somatic hybridisation, advantageous genes can be transferred to crop plants from completely unrelated species, something that cannot be easily accomplished by conventional plant breeding methods (Vasil and Vasil, 1981).

Plant cell culture offers breeders an alternative strategy to conventional methodology for plant improvement. As plant cells in culture may be generally variable, *in vitro* induced spontaneous mutations can be selected for a specific trait (Larkin and Scowcroft 1981; Maliga, 1984). Somaclonal variants may result from cell culture exposure to mutagenic agents or as a result of pre-existing genetic mutations in the somatic cells of the explants (Smith *et al.*, 1993). The utility of somaclonal variation as a form of undirected mutation breeding and a random process generally provides few useful variants (Baillie *et al.*, 1992). Considerable somaclonal variations have been documented for phenotypic traits (Ulrich, 1991). Cells do not have to be exposed to *in vitro* selective agents to develop new somaclonal variants with useful agronomic traits, but at callus level in tissue culture, somaclonal variation has been observed to occur. (Evans and Sharp, 1986).

Tissue culture is the starting point of many techniques in biotechnology aimed at genetic modification of plant cells and whole plants. The adaptation of tissue culture technique to cereal and grasses, the major food and livestock feed plants, has been slow (Vasil, and Vasil 1981; Strange, 1990). This has been attributed to difficulties in efficient and reproducible regeneration of cereal and grass plants from isolated cells and prototypes. The property of cereal callus cells to form somatic embryos seems to be lost early in development of the explant source (Vasili and Vasil, 1981). Immature embryos are often the explants of choice in cereals to ensure the production of embryogenic callus with high efficiency for plant regeneration (Mackinnon *et al.*, 1987).

Somaclonal variation / cell selection methods are similar to mutation breeding (Sanford *et al.*, 1984). Several mechanisms governing somaclonal variation induced during cell culture include gene amplification, single gene nucleotide base change, transposon migration, altered methylation states, chromosome instability, chromosome inversions, translocations, cytoplasmic genetic change, ploidy changes, rearrangements and partial chromosome deletion (Altman *et al.*, 1991; Cassels *et al.*, 1991; Shoemaker *et al.*, 1981). Useful variations must be stable, durable and inherited in Mendelian fashion, while not altering other agronomic or economically-important traits of the donor parent (Cassels *et al.*, 1991; Smith *et al.*, 1993). Plants can be asexually propagated via somatic embryos obtained through tissue culture. Proper field selection can result in near-isogenic lines for subsequent genome mapping or marker-assisted identification of specific genes. A quick method which can be used to detect variation in such studies is RAPDs (Smith et al, 1993). At field level, the quick methods used in selection of salinity tolerant plants include: measurement of shoot length (Al-Khatib, 1993); yield index (Steppuhn *et al.*, 2005); glycine-betain accumulation (Mickelbart, 2003); water potential (An-ching, 2002). The objective of this study was to produce *in vitro* selected regenerates with ability to tolerate salinity at field level.

2.0 MATERIALS AND METHODS

2.1 Source of Explants and Callus Initiations

Three cultivars of *Sorghum bicolor* (L) Moench namely, Mtama 1 (creamish in colour with no testa and no tannins) El Gardam (chalky white with testa and low tannins) and Seredo (brown with testa and high tannins), were obtained from Katumani Research Station. The three were selected basing on the tannin level because sorghum cultivars fall in these three categories. The tannin level also determines palatability of sorghum - low tannin cultivars are better for human consumption.

The explants were immature sorghum seeds still in the milk oil latex producing stage. They were disinfected briefly for 1.0 min in 70% ethanol followed by 25min wash in 20% sodium hypochlorite and three drops of tween-20 added as a wetting agent to sterilise them. The seeds were rinsed 6 times with double distilled sterile water and embryos sliced away from endosperm using a sterilised scalpel. The embryos were inoculated on Linsmaier and Skoog (LS) medium containing 2 g/l agar, 8 g/l sucrose, 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4D) supplemented with 0.5 mg/l kinetin. The hormone concentrations were arrived at after performing trial experiments involving use of 0.5, 1.0, 1.5, 2.0, 2.5 mg/l 2,4D and 0.1, 0.2, 0.3, 0.4, 0.5 mg/l kinetin. Each trial experiment had 10 replicates per hormone concentration. The pH of the media was set at 5.5. The media was autoclaved for 15 min at 1.5 $kgcm^{-1}$ pressure at 121^{0}C. The embryos were grown in culture chamber in continuous light at 26°C to initiate calli formation. Calli were passed to fresh media every week due to formation of a brownish substance, which retarded calli growth.

At the end of the 4th subculture time, some of the calli were transferred straight to regeneration media (controls), and some subjected to NaCl (Mackinnon *et al.*, 1987).

2.2 *In vitro* Selection

For salt screening 0 mM, 50 mM, 100 mM, 150 mM, 200 mM NaCl was added to the culture media prior to autoclaving. Calli from the growth chamber weighing 0.5 g were transferred to sterilised bottles (8x2)cm containing 10 ml of culture media. Each treatment had 50 replicates per cultivar. The treatments were maintained in sealed bottles for one week in a growth chamber in continuous light at 26° C. Triphenyl tetrazolium chloride (TTC) viability test was done to determine the optimum NaCl concentration for treatment of sorghum calli to induce salinity tolerance. From the viability test, 100 mM NaCl was selected as optimum concentration to which calli were subjected for selection of salinity tolerant variants. After selection of the optimum NaCl concentration, calli from the growth chamber weighing 0.5 g were inoculated in bottles (8x2) cm containing 10 ml LS culture media to which a 100 mM NaCl had been added prior to outoclaving. Each cultivar had 100 replicates. The calli were subjected to this treatment for one week. The calli were then transferred to culture media containing no NaCl to enable further growth / multiplication of the treated calli (De Ronde *et al.*, 1993).

2.3 Regeneration and Rooting

After four subcultures of 7 days each, embryogenic calli were visually selected and inoculated in 10 ml of LS regeneration medium with 1.0 mg/l indole acetic acid (IAA) and 0.5 mg /l benzyl adenine (BA) to stimulate regeneration. The concentration of IAA and BA were arrived at after trial experiments involving use of (0.2, 0.4, 0.6, 0.8, 1.0 mg/l) IAA and (0.1, 0.2, 0.3, 0.4, 0.5 mg/l) BA. Each hormone concentration had 10 replicates. Shoots 2-3 cm long were transferred to LS media containing 3 mg/l indole butyric acid (IBA) to enhance root formation. The IBA concentration was arrived at after performing trial experiments involving use of (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) IBA. Each hormone concentration had 10 replicates. Regenerates with established root systems were washed thoroughly with tap water to remove growth media. They were transferred to the greenhouse and potted in plastic bags containing 500 g vermiculite for acclimatisation. They were watered twice a week and after 2 weeks, transferred to field conditions at JKUAT experimantal plots (Macknnon *et al.*,1987).

2.4 Extraction Procedure

Sorghum fresh leaves weighing 0.125 g obtained from 10 NaCl treated plants and 10 control plants per each of the three cultivars were put into a 1.5 ml Eppendorf tubes and liquid nitrogen added to degrade the cell walls. Plastic disposable grinders

were used to grind the leaves thoroughly into powder form. This was followed by addition of 500 ml of Cetyl trimethyl ammonium bromide (CTAB) extraction buffer pre-warmed to 60°C to remove polysaccharides and dispersal of lumps was done by inverting the tubes ten times. The CTAB extraction buffer contained 4M sodium chloride, 1M Tris HCl, 0.5M EDTA, 2% Mercaptoethanol and 10% CTAB. The pH of the Tris buffer was adjusted to 8.0 with HCl. All the reagents were outoclaved at 121°C for 15 min before use. Unautoclaved 2 mercaptoethanol was added just before use. The tissues were incubated at 60°C for 15 min with occasional mixing by version.

To remove the lipid bilayer, chloroform was added and mixed by inverting until an emulsion was formed. The mixture was centrifuged at 13,000 rpm in a table top centrifuge for 10 min at room temperature for separation of lipid layer and aqueous layer. The clear aqueous phase was transferred to fresh 1.5 ml Eppendorf tubes and 150 ml isopropanol added and mixed by inversions to precipitate the DNA. The tissues were left for 10 min at room temperature then centrifuged at 13,000 rpm and liquid part removed without disturbing the pellet. To wash the DNA pellet, 500 ml ethanol was added and mixed by inversions then centrifuged at 13,000 rpm for 5 min at room temperature. All the liquid was removed without disturbing the pellet and vacuum dried for 15 min to get rid of all ethanol and the DNA was resuspended in 100 l of Tris-EDTA (TE) and stored in the fridge (4°C) awaiting quantification (Doyle and Doyle, 1987).

To quantify the DNA, absorbance was done at 260 nm using a spectrophotometer (Beckman DV 640B). (An absorbance of 1unit at 260 nm = 50 ng/ml. DF was 1000. Total amount of DNA was = Ab 260 nm x 50 ng x 1000. Amount of DNA used in the experiments was between 2 ml(24ng/ml) to 5 ml(21ng/ml). Preparation of 2% agarose gel was done by dissolving 3 g of agarose in 150 ml TBE buffer. The mixture was heated to boil and topped with Tris Borate-EDTA (TBE) buffer upto the original level. It was left to cool and 15 ml ethidium bromide added. 5 ml DNA and loading buffer of 5 ml forming 10 ml mixture was loaded on the 2% agarose gel and fitted in a tank to which 15ml ethidium bromide had been added. Electrophoresis was done at 80 volts for 2 hours and the gels photographed using uv transilluminator camera (uvp inc. san Gabriel 91778 U.S.A. model).

2.5 Screening of Primers

The reaction mixture consisted of PCR buffer 2.5 ml, 2.5Mm $MgCl_2$ 0.5 ml, 2.5 mM dNTPs 2.0 ml, 200nm Primer 5.0 l, 0.5units Taq 1.0 ml, DNA 2.0 ml, water 12.0 ml. These concentrations were arrived at after prior preliminary experiments. Aliquots 18 ml of the reaction mixture were added to 20 Eppendorf tubes. A total of 35 random decamer primers (Operon Technologies, Alamada, CA, USA) were screened. Only 4 primers gave polymorphic bands and were selected for subsequent

use. The Eppendorf tubes were fitted in a PCR machine (PTC - 100 programmable thermal controller) and run in the desired programme for 5 hr (A33 Programme - MJ Research Inc. Watertown, USA was used). The programmme had the following cycles:

(i) Initial denaturation at 94°C for 4 min.
(ii) Denaturation at 94°C for 1 min.
(iii) Primer annealing at 45°C for 1min.
(iv) Primer extension at 72°C for 2min.
(v) Repetition of steps (ii) - (iv) 39 times.
(vi) Final primer extension at 72°C for 10 min, and
(vii) Incubation at 4°C.

Loading buffer of 10 ml was added to the tubes after which 10 ml of the mixture was loaded on a 2% agarose gel and electrophorised in 0.5 x TBE running buffer at 80 volts for 2 hrs (10 ml of Ethidium Bromide was added to the gel and running Buffer). The gel was examined under uv light to check for DNA amplification and a photograph taken using uv transilluminator camera (Uvp Inc. Gabriel 91778 USA Model). (Innis *et al.*, 1990).

2.6 Analysis of Rapid - PCR Markers as Alleges

Ten DNA samples from the NaCl treated and ten from the control plants from each cultivar were subjected to RAPD-PCR using each of the 4 primers. The bands that were well-amplified and showed clear presence or absence of polymorphisms among individuals were scored and used in the data analysis. Pools were made by mixing 2.0 ml quantities of DNA from 10 individuals of the NaCl treated and 10 of the control plants of each cultivar.

2.7 Genetic Fingerprinting

Genetic fingerprinting involved comparison of genetic markers among individuals and required pair-wise comparison of amplified bands. The similarity of individuals was measured by scoring both the shared presence and shared absence of a band. The presence of a band was entered into the computer as "1", the absence of a band as "0" (Table 1). The proportion of matches (M) was estimated using the formular: $M = NAB/NT$. (NAB is the total number of matches [both bands present or absent] in individuals A and B, and NT is the total number of bands scored in the overall study). An M value of 1 indicates that two individuals have identical patterns; a value of 0 indicates that two individuals share no band in common. M values were used to construct a symmetrical distance matrix, which was used to construct a dendrogram using UPGMA with a FORTRAN program RAPDPLOT (Black, 1993; Nei, 1972). Genstat software package was used to analyse the data.

3.0 RESULTS

3.1 Calli Growth

Production of calli from immature embryos of the three sorghum cultivars was successful. The calli formed were compact nodular in texture and ranged in colour from whitish, creamish to brownish. Some of the calli were embryogenic and others non-embryogenic. The calli formation and growth was slow due to formation of a brown-purple colouration around the Calli, which became apparent within 3-5 days after embryo inoculation and retarded calli growth. The browning effect was controlled by transferring calli to fresh media on a weekly basis. Regenaration and rooting were successful and the growth stages were as summarised in plate 1- 3.

Plate 1: (a) Callus intiation (b) Organogenesis in callus (c) Advanced regeneration

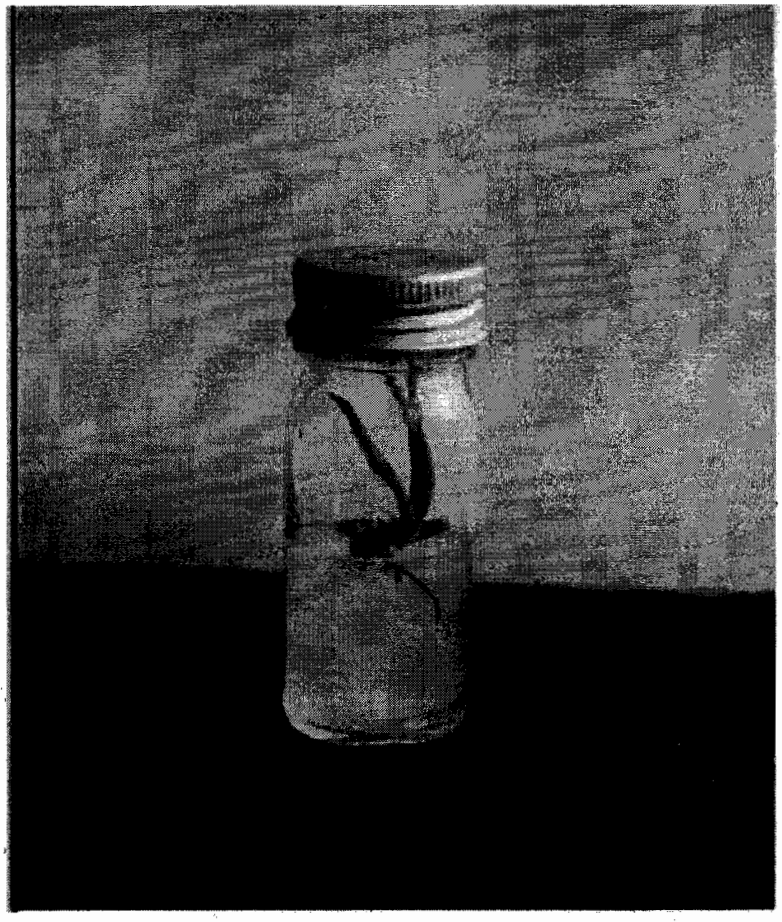

Plate 2: Rooting of sorghaum

Plate 3: Regenerated sorghum growing in the experimental plot

3.2 Results on Triphenyl Tetrazolium Chloride (TTC) Test (Viability test)

Subjection of calli to various NaCl concentrations to screen them for salinity tolerance had the effect of killing some. Therefore, triphenyl tetrazolium (TTC) test was a quick test for establishing viability of calli upon salinity hardedning. TTC test worked on the principle of assessing activity of succinic dehydrogenase enzyme present in cells. Succinic dehydrogenase had the ability to reduce TTC to formazan (red coloured substance) which accumulated in the cells. The spectrophotometric estimation of the amount of formazan accumulated in calli at each treatment was done by reading absorbance at 485 nm on DV 640 spectrophotometer. The results were as shown in Figure 1. The absorbance approached 100% in the controls and decreased with increase in NaCl concentration in all the 3 cultivars. The magnitude of decrease in absorbance at each concentration of NaCl were not significant (as shown by standard error bars). The absorbance of 50% linked with 100mM NaCl hence was chosen as the optimum concentration that induced salinity tolerance in the calli. Beyond 150 mM absorbance approached 0%, indicating that succinic dehydrogenase was no longer active hence too strong for calli survival.

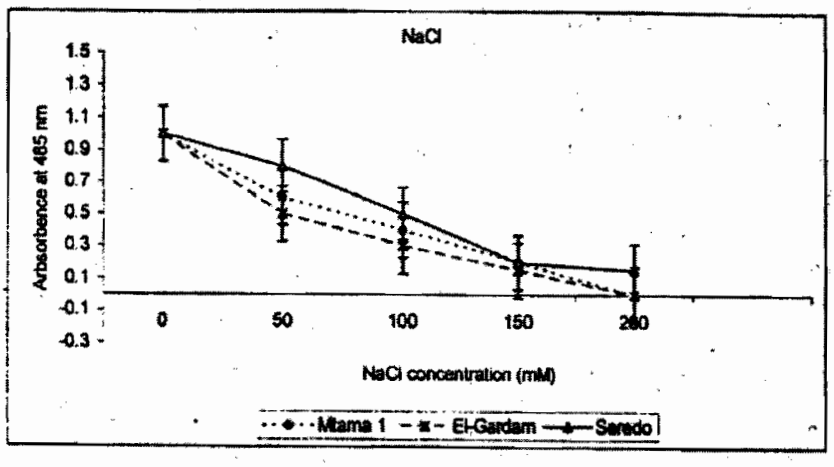

Figure 1: TTC test outcome on sorghum callus treated with sodium chloride

3.3 RAPD-PCR Results

With DNA analysis, a greater and more direct detection of genetic diversity was achieved. Out of a total of 35 random decamer primers (Operon Technologies, Alamada, CA, USA) that were screened, only 4 that gave polymorphic bands were selected for subsequent use. These were OPO - 4 (5' AAGTCCGCTC-3'), OPO-18 (5' CTCGCTATCC-3'), OPO-19 (5' -GGTGCACGTT-3'), OPO-20 (5' -ACACACGCTG-3').

Amplification of the DNA with the 4 primers resulted in a series of discrete fragments [Plates 4(a) and 4(b)]. Four plates for Mtama1 and four for Seredo have not been shown, but their results have been included in the pooled DNA. Ten DNA samples 10 individuals from treated plants were in Lanes 1 - 10 and ten samples from 10 individual untreated plants were in Lanes 11 - 20. Polymorphisms were identified within the cultivars which might have been as a result of the impact of NaCl treatment and somaclonal variation that occurred at callus level. The number of polymorphisms varied with each primers used.

Primer OPO-4 (Plate 4(a)) molecular weight (Mw) bands amplified ranged from 450 bp to 2500 bp. Lanes 17 and 18 were not amplified. The focus was on bands that were unique to the NaCl treated plants only. These were identified as follows: Mw 450 bp (lanes 1,2,4,6); Mw 700 bp (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9,10); Mw 2500 bp (lanes 3, 5, 9, 10). Primer OPO-18 (Plate 4(b)), Mw bands amplified ranged from 450 bp to 2000 bp. Lanes 6 and 17 were not amplified. The Mw 700 bp (lanes 13, 14, 15, 18, 20) was unique only to the untreated plants; Mw 2000 bp (lanes 1, 5, 7, 8, 9) was unique to the NaCl treated only. Primer OPO-19 (Plate 4(c)) Mw bands amplified ranged from 250 bp to 1600 bp. All the bands

were common to both the treated and untreated plants. Primer OPO-20 (Plate 4(d)) Mw bands amplified ranged between 400 bp to 1300 bp. Lanes 2, 14 and 16 were not amplified. The bands unique to NaCl treated plants only were: Mw 1300 bp (lanes 1, 4, 7, 8, 10).

The pooled DNA was as shown in Plate 5, lane 1 = Mtama1, lane 2 = ElGardam, lane 3 = Seredo (untreated); lane 4 = Mtama 1, lane 5 = El Gardam, lane 6 = Seredo (NaCl treated). The Mw bands amplified by primer OPO-4 ranged from 500-900 bp. Those unique NaCl treated plants were Mw 800 bp lane 4 (Mtama 1) Mw 400 bp lane 5 and 6 (El Gardam and Seredo respectively). Primer OPO-18 lane 7 = Mtama1, lane 8 = El Gardam, lane 9 = Seredo (untreated); lane 10= Mtama1, lane 11 = El Gardam, lane 12 = Seredo (NaCl treated). Mw bands amplified ranged from 600 bp to 400 bp. Those unique to the treated plants only were: Mw 800 bp lane 11 (El Gardam) Mw 700 bp lane 12 (Seredo). Primer OPO-19 lane 13 = Mtama 1, lane 14 = El Gardam, lane 15 = Seredo (untreated). Lanes 16 = Mtama 1, lane 17 = El Gardam, lane 18 = Seredo (NaCl treated). The amplified DNA Mw bands ranged from 350-1500 bp. All the bands were common to both the treated and untreated plants. Primer OPO- 20 lane 19 = Mtama 1, lane 20 = El Gardam, lane 21 = Seredo (untreated). Lane 22 = Mtama 1, lane 23 = El Gardam, lane 24 = Seredo (NaCl treated). The amplified DNA bands ranged from 200 bp to 1000 bp. All the bands were common to both the treated and untreated plants. Amplification of pooled leaf sample DNA revealed all the bands that were monomorphic in individuals (unique to individuals) as these were absent in the pooled. As banding patterns varied with type of primer, of special mention is bp 300 (lanes 4, 5, 6) under primer OPO-4 (pooled DNA) which was unique to only the treated for all the 3 cultivars. This showed that some variation had occured between the treated and the controls.

Therefore, the data obtained was used to show the relationship amongst the sorghum cultivars used in the study. The genetic distant matrix (Table 2) showed that genetic distance between the NaCl treated and the controls were low (0.030-0.032), an indication that somaclonal variation occurred at a low level and only a few cell llines had come up that could tolerate salinity and were isolated by the NaCl treatment. The genetic differences between these cell lines and the controls were small. The inter cultivar genetic distance was a bit high (0.136-0.341), indicating that genetic differences exist between the three cultivars. Cluster analysis of the genetic distance values was performed and generated a dendrogram, see Figure 2.

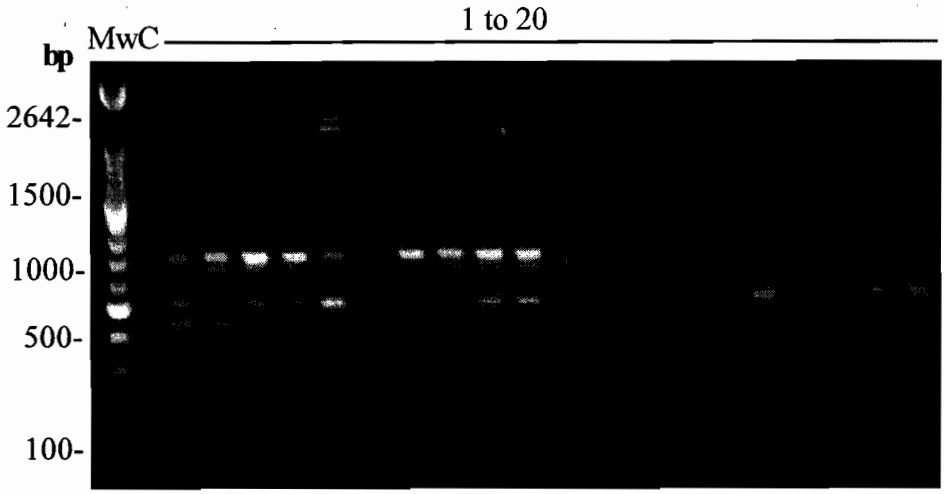

Plate 4(a): Primer OPO - 04 El Gardam molecular banding of DNA

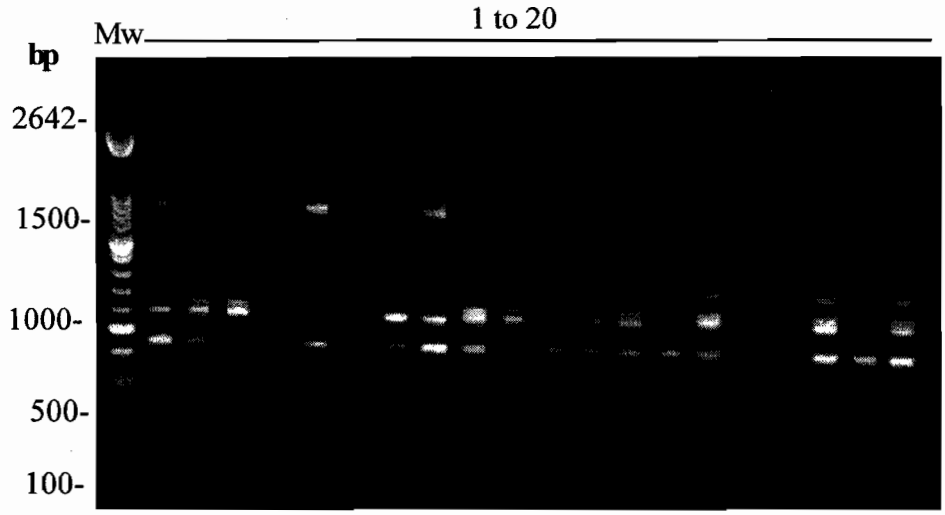

Plate 4(b): Primer OPO-18 El Gardam molecular weight banding of DNA

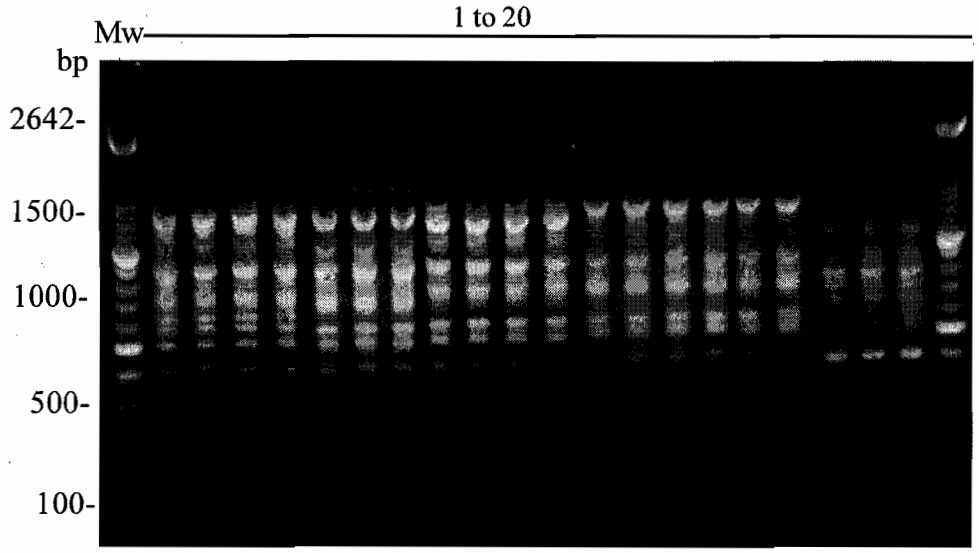

Plate 4(c): Primer OPO - 19El Gardam molecular banding of DNA

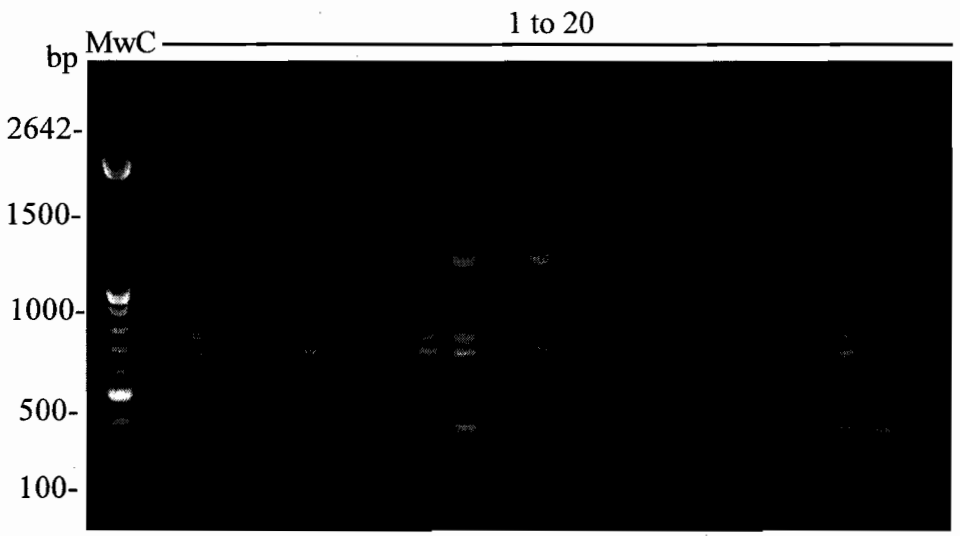

Plate 4(d): Primer OPO-20 El Gardam molecular weight banding of DNA

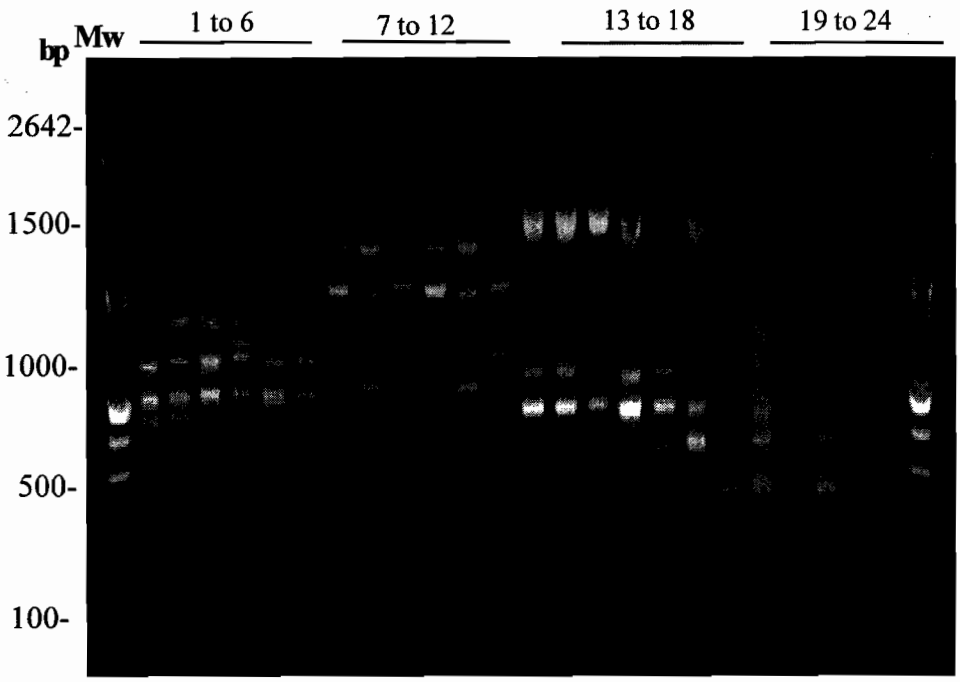

Plate 5: Molecular weight banding of pooled DNA samples in various primers Lane 1 to 6 OPO – 4; Lane 7 to 12 OPO – 18; Lane 13 to 18 OPO - 19; Lane 19 - 24 OPO - 20. (The 1st 3 in each group are untreated and the next 3 are treated)

Table 2: Genetic distance matrix

	1T	2R	3T	4R	5T	6R
1T	0.000	0.003	0.136	0.172	0.311	0.316
2R		0.000	0.163	0.177	0.319	0.321
3T			0.000	0.049	0.382	0.361
4R				0.000	0.378	0.341
5T					0.000	0.032
6R						0.000

(T = Treated, R = Untreated,1 = Mtama T ,2 = Mtama R, 3 = El Gardam T, 4 = El Gardam R, 5 = Seredo T, 6 = Seredo R. Number of loci compared = 31,Seed = 67, Ninapo = 0).

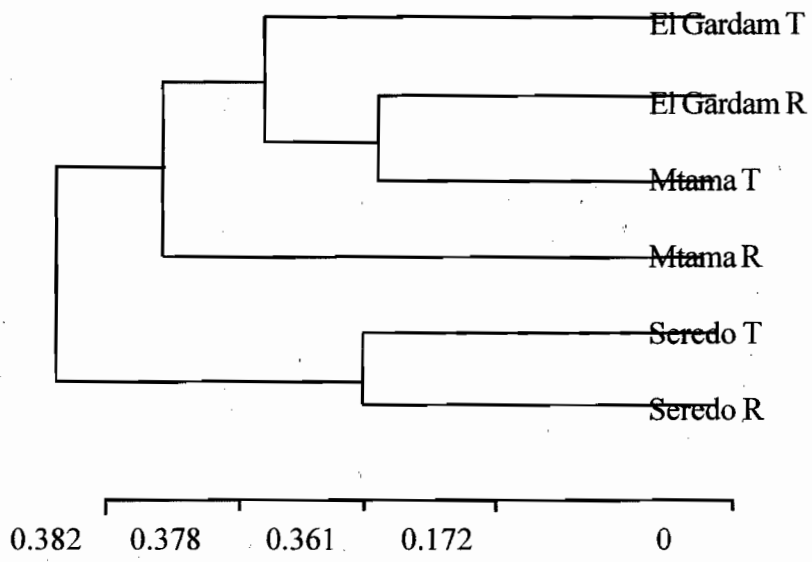

Figure 2: DNA dendrogram of the treated (T) an untreated (R) sorghum cultivars

4.0 DISCUSSION

4.1 Calli Formation

The main objective of this study was to select salinity tolerant sorghum cultivars arising from somaclonal variation by subjecting the calli to NaCl treatment. Tissue culture approach has revealed that somaclonal variation occurs at callus level, leading to development of somaclonal variants with useful agronomic traits (Vasil and Vasil, 1981). However, tissue culture manipulations in cereals which are of global importance as major food and livestock feeds has been slow and difficult (Saint-Clair, 1976; Mackinnon *et al.*, 1987).

In this study, difficulty in tissue culture of sorghum arose from failure to obtain calli from mature tissues like leaves, stems and roots. Success in production of embrogenic and non-embryogenic calli involved use of immature embryo's still in the milk latex producing stage as explants. The brown-purple colouration that retarded calli growth in the initial stages was linked to action of copper containing enzymes such as polyphenoloxidases and tyrosinase released due to wounding during excision of the embryos (Anderson *et al.*, 1982).

Drought and high salinity stresses are the major factors limiting productivity and adaptation of crops in arid regions (Boyer, 1982). In this study, TTC viability test was a handy tool in measuring salinity tolerance in the calli of the three sorghum cultivars subjected to various NaCl treatments. Percentage killing was expressed as the amount of TTC reduced to formazan after the zero concentration of NaCl. TTC viability test revealed that 100mM NaCl corresponded with 50% formazan. An assumption was made that it had caused 50% killing of the calli, hence was selected as the optimal NaCl concentration which induced salinity tolerance to the surviving calli. Similar selection procedure involving TTC test has been used in selection of stress tolerant cotton cultivars (De Ronde and Van de Meschet, 1993), Basmati rice calli (Cushman *et al.*, 1990). Decline in growth was observed in sorghum calli subjected to NaCl treatment. This phenomenon was also observed in Basmati rice (Cushman *et al.*, 1990). The retardation of growth might be due to the fact that certain amounts of the total energy available for tissue metabolism is channeled to resist the stress (Towell, 1974). The energy is channeled to synthesis of stress oriented compounds like prolin, betain which accumulate in stressed grasses (Flowers *et al.*, 1977). Acclimatisation of the regenerated sorghum plants in vermiculite was necessary to enable them develop root hairs because Murashige (1974) observed that plants grown in culture media have poor root hair development.

The aim of carrying out RAPDs was to detect inter-cultivar variation and any intra cultivar variation which might have occurred at callus level and isolated by NaCl treatment as compared to sorghum regenerates of the untreated. RAPD markers have been useful tools to discriminate among cultivars in several crops such as *Brassica oleracea* (Kresovich *et al.*, 1992), *Lycopersicon esculentum* (Williams and Clair, 1993), *Brassica juncea* (Jain *et al.*, 1994) *Sorghum bicolor* (Tao *et al.*, 1993;

Amsalu *et al.*, 2000). Based on RAPD-PCR technique, this study found that polymorphism was evident in sorghum plants regenerated from Calli treated with NaCl and the plants regenerated from untreated calli. Failure to amplify or unexplained amplified DNAs have been attributed to PCR cycles exceeding 35 and use of low concentration of dCTP (Fukuoka *et al.*, 1992). The dendrogram revealed that low level of somaclonal variation occurred at calli level and resulted in selection of some salinity tolerant variants that had slight genetic differences from the controls. Somaclonal variations are governed by mechanisms like, altered methylation states, chromosome instability, chromosome inversions, single gene mutations, translocations, cytoplasmic genetic changes, ploidy changes and partial chromosome deletion (Altman *et al.*, 1991; Cassels *et al.*, 1991; Shoemaker *et al.*, 1991; Smith *et al.*, 1993). It has been found that somaclonal variation is a form of undirected spontaneous mutation breeding and as a random process which provides useful variants with specific traits that can be selected for. (Baillie *et al.*, 1992; Larkin and Scowcrofti, 1981; Maliga, 1984). Somaclonal variations/ cell selection methods have been linked to mutation breeding (Evan and Sharp, 1986; Sanford *et al.*, 1984). *In vitro* screening for aluminium chloride ($AlCl_3$) tolerance among monocots for growth in acid soils showed that only flax and sorghum variants from somaclonal variation had some useful agronomic traits not expressed by donor parents. They had superior performance in that acid soil-stress root plasticity was improved (Duncan *et al.*, 1995). The sorghum variants from somaclonal variation obtained from this study were tested under field conditions at JKUAT plots virgin soil (Agroclimatic zone 5 – semi-arid). The parameters used to assess salinity tolerance were water potential of leaves, CO_2 assimilation rate, rate of transpiration and malic acid accumulation. They had superior performance over the controls (Makobe *et al.*, 2007).

Evaluation of tissue culture derived somaclones for variations in biochemical (polyphenolic) reactions leading to tannin production has resulted in obtaining variants with decreased levels of tannins in sorghum (Cai *et al.*, 1995; Woodruff *et al.*, 1982). Sorghum variants resistant to sorghum midge *Contarina sorghicola* and sugarcane resistant to aphid, *Melanaphis sacchari* (Hagio, 1999), had been developed. Sorghum variants resistant to head smut (*Sphacelotheca reliana*) have also been developed from somaclonal variation (Wang *et al.*, 1997). However, it should be noted that the population of bands obtained when a pooled (ten) leaf sample is subjected to RAPD analysis is not always a summation of all the bands present in all of the individuals, presumably because of competition effects for the primer within the reaction tube (Black, 1993).

In this study, RAPDs was a useful tool in proving that somaclonal variation occurred at callus level and this was detected in the amount of polymorphism obtained in NaCl treated and the control plants. Despite uncertainity regarding the exact nature of polymophism (Black, 1993), RAPDs has been widely adapted because of the simplicity of the assay, low input DNA, sensitivity on polyacrylamide gels and its

immediate applicability to a wide range of species contributing to detection of variation even at intra cultivar level.

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