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DEVELOPMENT OF HARDY SORGHUM CULTIVARS FOR THE ARID AND SEMI ARID REGIONS

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ABSTRACT

Tissue culture is the starting point of many techniques in biotechnology aimed at genetic modification of plant cells and whole plants. Plant tissue and cell culture was used to induce and maintain embryogenic calli established from young embryogenic explants of three sorghum bicolor cultivars (Mtama 1, El Gardam and Seredo) selected basing on tannin content. Embryogenic calli were obtained by culturing young embryos on Linsmaier and Skoog's (LS) medium containing 2 mg/12, 4- dichlorophenoxyacetic acid (2,4-D) and 0.5 mg l^{-1} Kinetin. Calli were subjected to various NaCl concentrations to screen for salinity tolerance. Calli subjected to 0.0 mM NacCl served as controls. Calli with tolerance to salinity stress had a higher activity of succinate dehydrogenase, which reduced trimethyl tetrazoliumn chloride (TTC) to formazan. From the TTC viability test, 1000 mM NaCl was selected as the optimum concentration, which was incorporated into LS media to select salt tolerant calli. Random amplified polymorphic DNA (RAPDs) technique showed that variation existed between NaCl treated plants and the controls in the individual and pooled DNA samples. This may indicate that somaclonal intra-cultivar variation had resulted in some of the cell lines becoming tolerant to salinity. A measure of CO_2 assimilation rate, synchoronised with Photosynthetic Active Radiation (PAR), stomata conductance (SC) and Transpiration Rate (TR) using infrared gas analyser (IRGA), along with titratable acidity and malate content were used to assess productivity under stress. The treated plants significantly ($P<0.05$) had higher CO_2 assimilation rate, higher levels of titratable acidity and malate than the controls in the 3 cultivars. The three parameters had a day pattern of increasing starting from morning, reached a peak at noon and decreased as the afternoon progressed in parallel with PAR. High positive regression correlations ($r^2 \geq 0.7$) existed between titratable acidity and malate content; malate and CO_2 assimilation rate ($r^2 \geq 0.6$) illustrated that these parameters were interdependent.

Key Words: RAPDS, salinity, somaclonal variation, *Sorghum bicolor*

RÉSUMÉ

La cullture tissulaire constitue le point initial de plusieurs techniques de biotechnologie destinées à réaliser la modification génétique de cellules de plantes et des plantes entières. Le tissu de plantes et la culture cellulaire étaient utilisés en vue de générer et de maintenir les cals embryonnaires établis à partir de jeunes explants embryogéniquesde cultivars bicolores de sorgho (Mtama 1, El Gardan et Seredo) séléctionnés en fonction de leurs contenus en tannins. Des cals embryogéniques étaient obtenus par culture de jeunes embryons sur milieu Linsmaier & Skoog's (LS) contenant 2 mg/12,4 d'acide dichlorophénoxyacétique (2,4-D) et 0,5 mg l^{-1} de

Kinetine. Les cals étaient soumis à diverses concentrations de NaCl dans le but d'établir leur tolérance à la salinité. Des cals soumis à 0,0 mM de NaCl servaient de contrôles. Les cals qui étaient tolérants au stress salin présentaient une activité élevée au succinate deshydrogénase qui réduisait le chlorure triméthyl tetrazolium (TTC) en formazan. Partant du test de viabilité TTC, 1000 mM de NaCl était séléctionné comme étant la concentration optimale qui était incorporé dans le milieu LS en vue de pouvoir réaliser le choix de cals tolérants au sel. La technique d'amplification aléatoire à ADN polymorphique (RAPDs) a montré qu'il existait une certaine variation entre les plants traités au NaCl et les contrôles tant en ce qui concerne les échantillons ADN individuels que ceux groupés. Ceci pourrait montrer qu'une variation intracultivars par clonage somatique s'était produite au sein de certaines lignées cellulaires, les rendant tolérantes face à la salinité. Une mesure du taux d'assimilation de CO_2, synchronisée à la Radiation Photosynthétique Active (PAR), conductance stomatologique (SC) et au Taux de Tansplantation (TR) obtenus au moyen d'un analyseur à infrarouges pour gaz (IRGA), avec l'acidité titrable et contenu de malate étaient utilisés dans le but de déterminer la productivité dans des conditions de contraintes. Les plants traités présentaient un taux d'assimilation de CO_2 significativement ($P<0.05$) plus élevé, des niveaux élevés d'acidité titrable et de malate par rapport aux contrôles au sein des 3 cultivars. Les 3 paramètres avaient un motif journalier ascendant, débutant le matin, ils atteignaient un sommet à midi puis déclinaient progressivement dans le courant de l'après-midi en parallèle avec la PAR. Des regressions positives élevées ($r^2=0,7$) existaient entre l'acidité titrable et contenu de malate, les taux d'assimilation de CO2 ($r^2=0,6$) illustraient l'interdépendance de ces paramètres.

Mots Clés: RAPDs, salinité, variation par clonage somatique, *Sorghum bicolor*

INTRODUCTION

Tissue culture is the starting point of many techniques in biotechnology aimed at genetic modification of plant cells and whole plants. The adaptation of cell tissue culture to cereal and grasses in general has been slow. This has been attributed to difficulties in efficient and reproducible regeneration protocols in isolated cells and prototypes (Saharan *et al.*, 2004). The property of cereal callus cells to form somatic embryos seems to be lost early during development of the explant plant. Immature embryos have been successfully used as explants in the production of embryogenic calli for plant regeneration in wheat and sorghum (Duncan *et al.*, 1995). Also cytodifferentiation and transformation of embryogenic callus line derived from anther culture of wheat has been achieved (Ebiamadon *et al.*, 2000). The advantage of tissue culture is that somaclonal variation occurs at callus level that has occasionally lead to isolation of mutant cell lines with beneficial traits to environmental stresses. Evaluation of tissue culture derived somaclones has resulted in variants with increased or decreased levels of tannins (Cai *et al.*, 1995). Sorghum somaclonal variants resistant to head smut (*Sphacelothe careliana*) have been established (Wang *et al.*, 1997) and somaclonal sorghum and wheat variants have been isolated and grown in acid ($AICI_3$) soil (Duncal *et al.*, 1995).

Somaclonal variation/cell selection methods are similar to mutation breeding. Several mechanisms governing somaclonal variation induced during cell culture include gene amplification, single gene nucleotide base change, transponson migration, altered methylation states, chromosome instability, chromosome inversions, single mutations, translocations, cutoplasmic genetic change, ploidy changes, rearrangements and partial chromosome deletion (Duncal *et al.*, 1995). Useful variations must be stable, durable and inherited in Mendelian fashion, while not altering other agronomic or economically important traits of the donor parent. Other factors contributing to somaclonal variation in cultured cells included time in culture, explant source, pathway of regeneration, genotype of donor plant, environmental conditions during culture, concentration and type of hormones in the culture media and presence or absence of in vitro selective agents (Amzallag *et al.*, 1998; Breatrz *et al.*, 2005). Therefore, plant cell culture offers breeders an alternative strategy of convectional methodology for plant improvement.

Somaclonal variants in *Sorghum bicolor* for growth in saline soils has not yet been reported.

Therefore, the main goal of this study was to assess whether somaclonal variation during callus formation could result in salinity and drought tolerant sorghum cultivars. From the study, embryonic calli were obtained in all the 3 cultivars from young embryos sliced away from young seed still in the milk latex producing stage as explants. Treatment of calli with 100 mM NaCl and performance of TTC viability test were useful in selection of salinity tolerant calli from non-tolerant calli. Carbon dioxide assimilation rate, as a measure of instantaneous productivity under stress conditions, showed that plants generated from NaCl treated calli had superior performance as compared to the controls in all the 3 cultivars under same field conditions of salinity temperature and PAR.

MATERIALS AND METHODS

Source of explants and callus initiations. Three cultivars of *Sorghum bicolor* (L.) Moench were obained from Katumani research station, 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). These are representatives of all sorghum cultivars according to the tannin levels. 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 25 min 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/1 sucrose, 2 mg/12,4-dichloophenoxy acetic acid (2,4-D) supplemented with 0.5 mg/1 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/12, 4D and 0.1, 0.2, 0.3, 0.4, 0.5 mg/I kinetin. The hormonal concentrations were selected basing on previous work in same areas as guidelines.

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 kg cm^{-1} pressure at 121°C. The embryos were grown in culture chamber in continuous light at 26°C to initiate calli formation. Calli were passed t o fresh media every week due to formation of a brownish substance, which retarded calli g rowth. At the end of the 4th passage time, some of the calli were transferred straight to regeneration media (controls) and some were subjected to NaCl.

***In vitro* selection.** For salt screening 0, 50, 100 and 200 mM (selected basing on salinity ranges in various soils in Kenya where wild sorghum grows), NaCl was added to the culture media to autoclaving. Calli from the growth chamber weighing 0.5 g were transferred to sterilised bottles (8 x 2) 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 continuos 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 without killing calli. From the viability test, 100 mM NaCl was selected as optimum concentration to which calli were subjected for selection of salinity tolerant from non-tolerant ones. After selection of the optimum NaCl concentration, calli from the growth chamber weighing 0.5 g were inoculated in bottles (8 x 2) cm containing 10 ml LS culture media to which a 100 mM NaCl had been added prior to outclaving. Each cultivar had 100 replicates. The calli were subjected to this treatment for one week then transferred to culture media containing no NaCl to enable further growth/ multiplication of the treated calli.

Regeneration and rooting. After four subcultures, 7 day embryogenic calli were visually selected and inoculated in 10 ml of LS regeneration medium, with 1.0 mg/indole acetic acid (AAA) and 0.5 mg/1 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 has 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 green house and potted in plastic bags containing 500 g vermiculite for acclimatisation. They were watered twice a week and after 2 weeks they were transferred to field conditions at Jomo Kenyatta University of Agriculture and Technology (JKUAT) experiment plot.

DNA profile of salinity hardened and the control plants. DNA extraction was done according to the method outline by Pammi *et al.* (1994) with minimum modifications; whereby 0.125 g leaf tissue obtained from 10 NaCI treated plants and 10 control plants per each of the three cultivars were used. The gels were photographed using UV trnasilluminator camera (uvp inc. san Gabriel 91778 U.S.A. model).

Screening of primers. The reaction mixture consisted of: PCR buffer 2.5µl, 0.5µl, 2.5mM dNTPs 2.0 µl, Primer 5.0 µl, Taq 1.0 µl, DNA 2.0 µl, water 12.0 µl. These concentrations were arrived at after preliminary experiments. Aliquots 18 µl of the reaction mixture were added to 20 microcentrifuge 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 microcentrifuge tubes were fitted in a PCR machine and run in the desired programme for 5 hr (MJ Research Inc. Watertown, USA). 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).

RAPDs process. 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-aplified and showed clear presence or absence of polymorphisms among individuals were scored and used in the data analysis. Mizing equal quantities of DNA from 10 individuals of the NaCl treated and 10 of the control plants of each cultivar made pools.

Genetic finger printing involved comparison of genetic markers among individuals and required pair-wise comparison of amplified bands. The similarity of individuals was estimated using a binary system where the presence of a band was entered into the computer as "1", the absence of a band as "0". The proportion of matches (*M*) was estimated suing 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 indicated that two individuals had identical patterns; a value of 0 indicated that two individuals shared no band in common. *M* values were used to construct a symmetrical distance matrix, which was used to construct a dendrogram using UPGMA with FORTRAN program RAPDplot.

Field evaluation. The regenerates after acclimatisation in the green house, in 500 g vermiculite for two weeks (watered twice a week), were transferred to the demonstration plots with saline soil of average pH = 8.0, EC = 0.2 ds/M, CEC = 19.6 and grown for 3 months.

Leaf sampling. The samples were analysed every 2 weeks for 3 months to eliminate the senecence factor. Leaves used in the determination of CO_2 assimilation, Titratable acidity and malate content were samples at 9.00 h, 12.00 h and 15:00 h at the demonstration plots by the infrated gas analyser (ADDC-UK). For each investigation 5 leaves from 5 plant replicates selected at random from a sample population of over 100 plants in each treatment and each cultivar. A mean value of 5 samples (one from each plan) was obtained. All the experiments were carried out using relatively mature leaves from the 3rd to 4th pair of leaves from the apex to ensure uniform physiological age of the samples.

Measurements of CO_2 assimilation. In this study, CO_2 assimilation rate was used as instantaneous measure of productivity under stress (salinity). The technique used in the measurement of CO_2 assimilation rate was adapted from Wall *et*

al. (2001) with modifications outlined involving IRGA in open gas flow system rather than closed gas flow system. The diurnal patterns of CO_2 assimilation were monitored at the demonstration plots by the infrared gas analyser (ADC-UK). Infrared gas analyser (IRGA) is a null point instrument that allowed the flow of CO_2 into the system at a rate equivalent to the rate of uptake of the leaf. Outside air was drawn into the system by a pump, then passed onto an air conditioning system to minimise changes in gas concentrations and through silica gel to control air humidity. Air was passed through a flow meter/regulator and into the leaf chamber in which a leaf was enclosed. The air from the lead chamber was passed into the IRGA that measured the difference in the CO_2 mole fraction before and after entering the chamber. Sorghum leaves from 5 plants out of the NaCI treated and controls in each cultivar were enclosed in the leaf chamber (Leaf chamber area = 2.5 cm^2) one at a time. The airflow rate through the chamber remained fixed 200 - 300 ml/min. The CO_2 assimilation was monitored for 1 min for each leaf by the IRGA connected in an open gas flow system. The amount of CO_2 assimilated by the leaf was read directly from IRGA. During the CO_2 assimilation measurements, the chamber air temperature under light varied between 26° - 30° C. During the CO_2 assimilation measurements, the photosynthetic active radiation (PAR) stomatal conductance (SC) and transpiration rate (TR) were also recorded directly from IRGA.

Determination of titratable acidity and malate content. This was an indirect measure of adaptation to stress where malate had been reported to play a major role in osmo-regulation in C_4 plants (Lacerda *et al.*, 2003). The technique used in determination of titratable acidity was ordinary titration, where 2.5 g of leaf discs were crushed and aliquots of 20 ml were used in the titration against 0.02 M NaOH using phenolphthalein as in indicator. Malate determination was carried out by the assay described by Moriano *et al.* (2005), with modification where 2.5 g tissue was homogenised in distilled water with a mortar and pestle and centrifuged at 4000 g for 20 min (Martin Christ Osterode/Hase - model Type UJIA). The assay was carried out at 25° C following decrease in absorbance of nucleotide (NADH) at 340 nm at 30 s interval (3 min) using spectrophotometer (Beckman DV 640B).

RESULTS

Calli growth. Production of calli from mature embryos of the three sorghum cultivars was successful. The calli formed were compact nodular in texture, and ranged in colour from whitish to creamish. 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 retarded calli growth. The browning effect was controlled by transferring calli to fresh media on a weekly basis. Regeneration and rooting were successful and the growth stages were as summarised in Plates 1 - 3.

Triphenyl tetrazolium chloride (TTC) test (viability test) results. Subjection of calli to various NaCl concentrations to screen them for salinity tolerance had the effect of killing some calli. Therefore triphenyl tetrazolium (TTC) test was a quick test of establishing viability of calli upon salinity hardening. 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) that 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 are as shown in Figure 1. The absorbance approached 100% in the controls and it decreased with increase in NaCl concentration in all 3 cultivars. The magnitude of decrease in absrobance at each concentration of NaCl was not significant (as shown by standard error bars). The absorbance of 50% linked with 100 Mm NaCl hence, was chosen as the optimum concentration that induced salinity tolerance in the calli. Beyond 150 mM NaCl, absorbance approached 0%, indicating that succinic dehydrogenase was no longer active, hence, too strong for calli survival.

RAPDs results. With DNA analysis, a greater and more direct detection of genetic diversity was

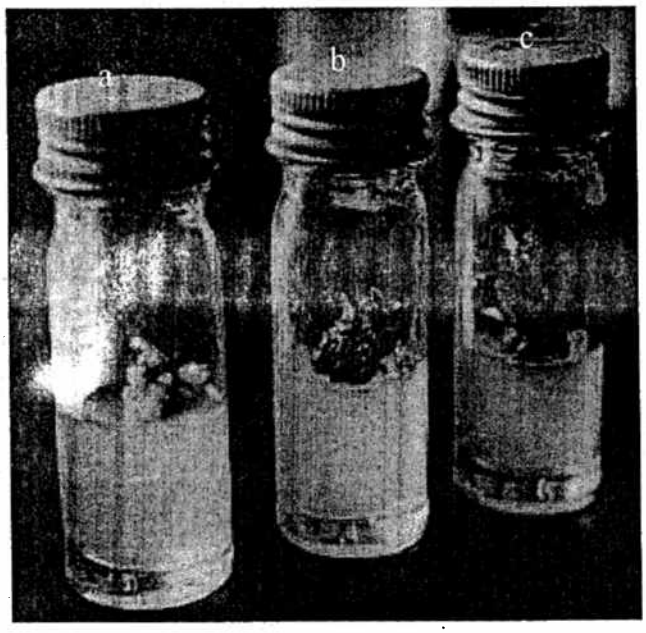

Plate 1. (a) Callus initiation (b) Organogenesis (c) Advanced regeneration.

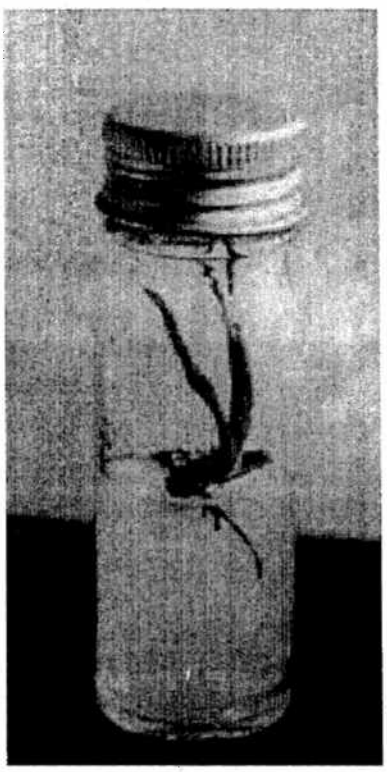

Plate 2. Rooting of sorghum regenerates.

achieved. Out of a total of 35 random decamer primers (Operon Technologies, Alamada, CA, USA) that were screened, only 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 primers resulted in a series of discrete fragments, amounting to 12 plates for individual samples of which only one been show as Plate 4. The plates not shown have their results summarised in the pooled DNA. Ten DNA samples from individual treated plants, were in Lanes 1 - 10 and ten from the control plants were in Lanes 11 - 20. Polymorphism was identified within the cultivars which might have been as a result of somaclonal variation that occurred at callus level. In EI Gardam, primer OPO - 4 (Plate 4) amplified Molecular weight (Mw) bands ranging from 450 bp to 2500 bp. Lanes 17 and 18 were not amplified.

Plate 3. Regenerated sorghum plants growing in the experimental plot.

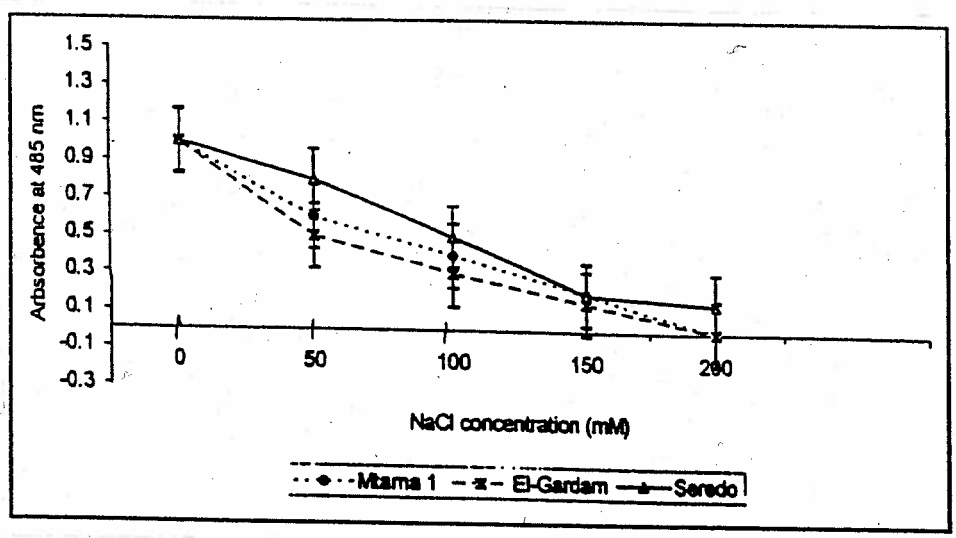

Figure 1. TTC Test outcome on amount of fomozan produced by Calli treated with NaCl.

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 17 and 18 were not amplified.

The pooled DNA is as shown in Plate 5. The Mw bands amplified by primer OPO-4 ranged from 500 - 900 bp. Those unique to NaCI treated plants were Mw 800 bp lane 4 (Mtama 1) Mw 400 bp lane 5 and 6 (EI Gardam and Seredo, respectively). Mw bands amplified by primer OPO-18 ranged from 600 bp to 400 bp. Those unique to the NaCI treated plants were Mw 800 bp lane 11 (El Gardam) Mw 700 bp lane 12 (Seredo). The Mw bands amplified by primer OPO-19 ranged from 350 - 1500 bp. Primer OPO-20 amplified Mw bands ranging from 200 bp to 1000 bp. All the bands were common to both the NaCl treated and the control plants.

The data obtained were used to show the relationship amongst the sorghum amongst the cultivars used in the study. The genetic distant

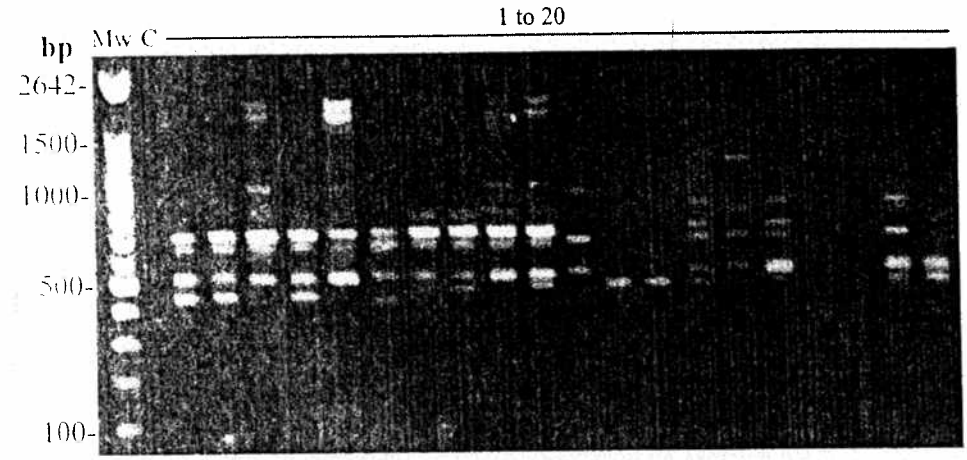

Plate 4. Primer OPO - 04 4l Gardam molecular weight banding. Lanes 1 - 10 treated. Lanes 11 - 20 control.

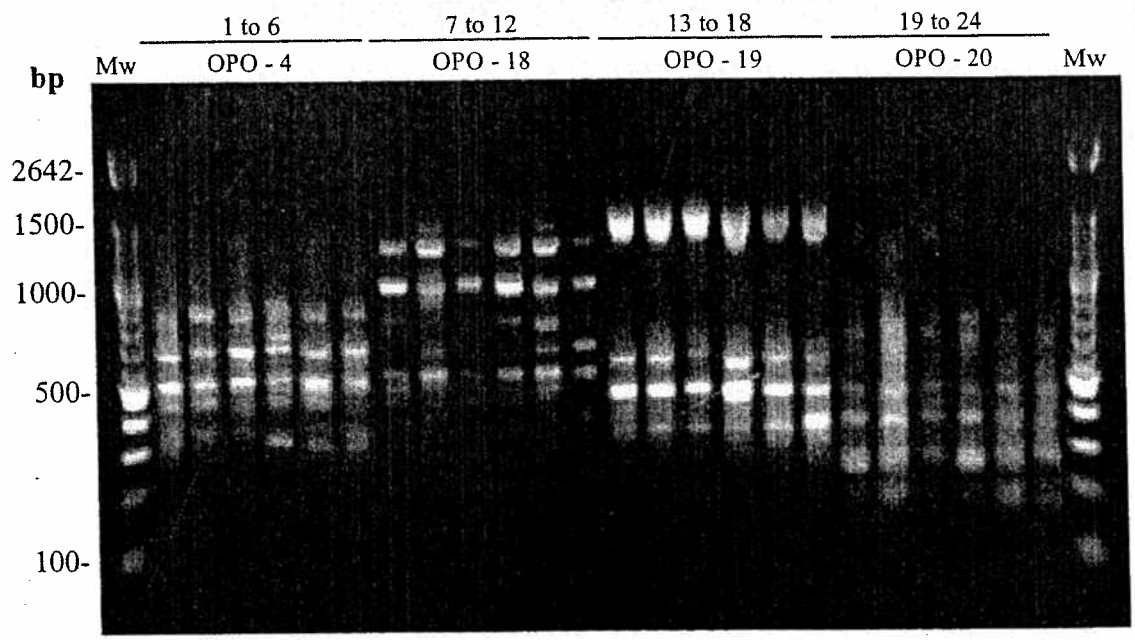

Plate 5. Salt tolerant cultivars of *Sorghum bicolor.*

matrix (Table 1) showed that intra cultivar genetic distance between the NaCl treated and the controls although low (0.030 - 0.032) indicated a low percentage of somaclonal variation. The inter-cultivar genetic distance was high (0.136 - 0.341) indicating that genetic differences exist between the three cultivars. Cluster analyses of the genetic distance values were performed and generated a dendrogram (Fig. 2), where the NaCl treated plants were separated from the controls. However, RAPIDS results were highly inconsistent, hence, the source of variation being somaclonal may not be conclusive. Bootsrap analysis will be used in part II of this study for further establishment.

During field evaluation, the rate of CO_2 assimilation in the 3 cultivars and incident PAR showed a direct relationship (Fig. 3a, b, c). The results showed a daylight pattern in which CO_2 assimilation rate was synchronised with stomata conductance (SC) and transpiration rate (TR). (Fig. 3d, e, f.). As the magnitude of CO_2 assimilation rate increased in the morning following increase in PAR and stomatal conductance the rate of transpiration also increased. They reached a peak value at noon corresponding to peak PAR values. As the afternoon progressed, the CO_2 assimilation rate, SC and TR also decreased following decrease in PAR values.

The CO_2 assimilation rate in the control plants followed the same daylight pattern as in treated plants except their values were much lower. The

TABLE 1. Genetic distance matrix

	IT	2R	3T	4R	5T	6R
IT	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 Gardama T, 4 = El Gardama R, 5 = Seredo T, 6 = Seredo R. Number of loci compared = 31, Seed = 67, Ninapo = 8)

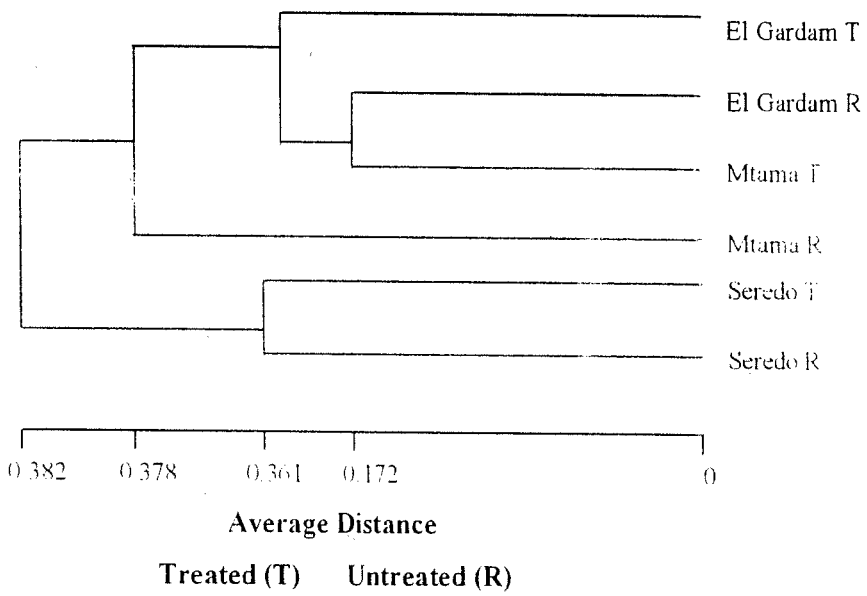

Figure 2. Dendrogram generated by clustering computed from pairwise comaprisons of NaCl treated and control Sorghum bicolor plants.

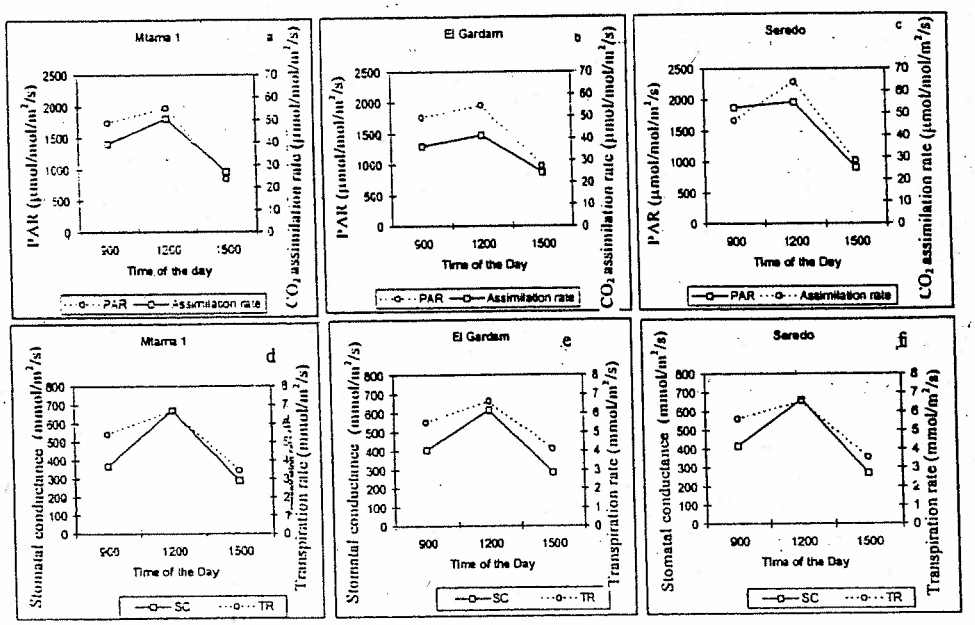

Figure 3. (a-c) Changes in CO_2 assimilation rate and PAR during the day (d-f) changes in stomatol conductance and transpiration rate during month 2.

differences in CO_2 assimilation rates between the control plants and the treated in all 3 cultivars in the 3 months were significantly different ($P \leq 0.05$). These significant differences might have been an indication that the treatment improved the CO_2 assimilation rate in the 3 cultivars. Comparisons between CO_2 assimilation rate in the 3 cultivars versus age in months were all significant ($P \leq 0.05$) with the higher mean values recorded in month 2. This might have been an indication that CO_2 assimilation rate increased with age, hence, improvement in productivity as the plants grew. The comparison of CO_2 assimilation rate among the 3 cultivars across the 3 months was insignificant. This might have indicated that the efficiency in CO_2 assimilation in the 3 cultivars was the same.

Figure 4 shows correlations between titratable acidity and malate content in the 3 cultivars. The treated plants accumulated significantly ($P \leq 0.05$) higher levels of titratable acidity and malate than the controls in all the 3 cultivars. There was a general increase in the two parameters with age of sorghum plants. The changes in titratable acidity and malate content with age in the 3 cultivars were significantly different. This might be an indication that metabolic changes occurred favouring formation of more malate. The two parameters increased with time, reachin a peak at noon in parallel with PAR and CO_2 assimilation rate. These differences in the two parameters with time over the 3 months were not significant. This showed that variation in the two parameters with time followed the same pattern in all the 3 cultivars. The values of titratable acidity were generally higher than those of malate content in all the 3 cultivars suggesting that other acids might have been accumulating in the leaf tissues. However, these variations were not significant. The two parameters were generally higher in Mtama 1 and Seredo as compared to El Gardam but these differences between the cultivars were also significant.

Therefore, the source of variation in the levels of titratable acidity and malate content wsa due to treatment, age and time of the day. The variations in the two parameters followed the same pattern in all 3 cultivars, indicating a close relationship

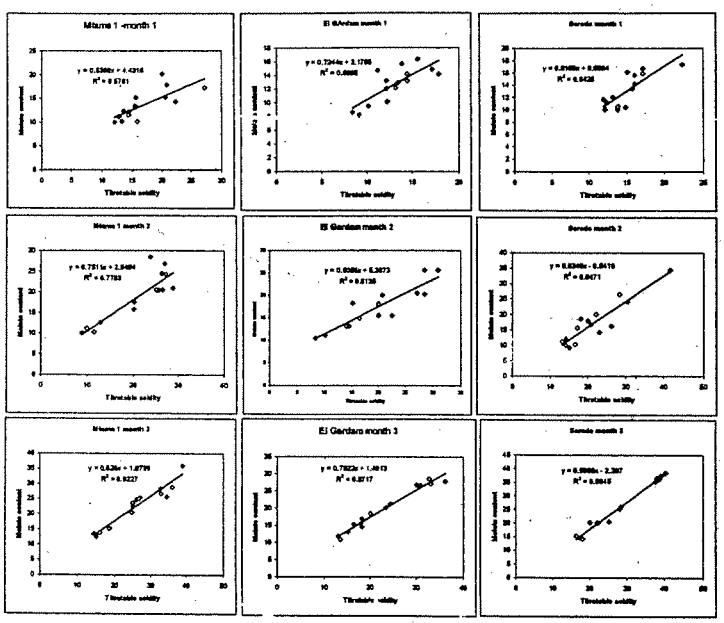

Figure 4. Regression correlations between Titratable acidity and malate content.

between them. The relationships were described by the correrelations in the 3 cultivars showed that titratable acidity and malate content were closely interdependent. There was also a relationship between malate content and CO_2 assimilation described by the correlations and linear equations shown in Figure 5.

DISCUSSION

Calli formation. In this study 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 (Saharan *et al.*, 2004). The 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. A similar selection procedure involving TTC test has been used in selection of stress tolerant cotton cultivars (De Ronde and Van de Meschet, 1993), Oryza sativa (Saharan *et al.*, 2004).

In this study RAPDs was a useful tool in detecting variation at inter and intra-cultivar levels. The dendrogram constructed showed separation of plants derived from NaCl treated calli and t he control plants in three sorghum cultivars. This might have been due to somaclonal variation and presence of NaCl in the culture medium helped in the isolation of salinity tolerant variants. Despite the uncertainty regarding the exact nature of polymophism, RAPDs has been widely adapted. This is due to its simplicity low imput DNA, sensitivity on agarose gels and its immediate applicability to a wide range of species contributing to detection of variation even at intra-vultivar variants including *Brassica juncea* (Jain *et al.*, 1994) *Sorghum bicolor* (Tao *et al.*, 1993; Amsalu *et al.*, 2000; Hesanuzzaman *et al.*, 2002, Anas and Yoshida, 2004; Zhang *et al.*, 2006). A study involving *in-vitro* screening for aluminium chloride ($AICI_3$) 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).

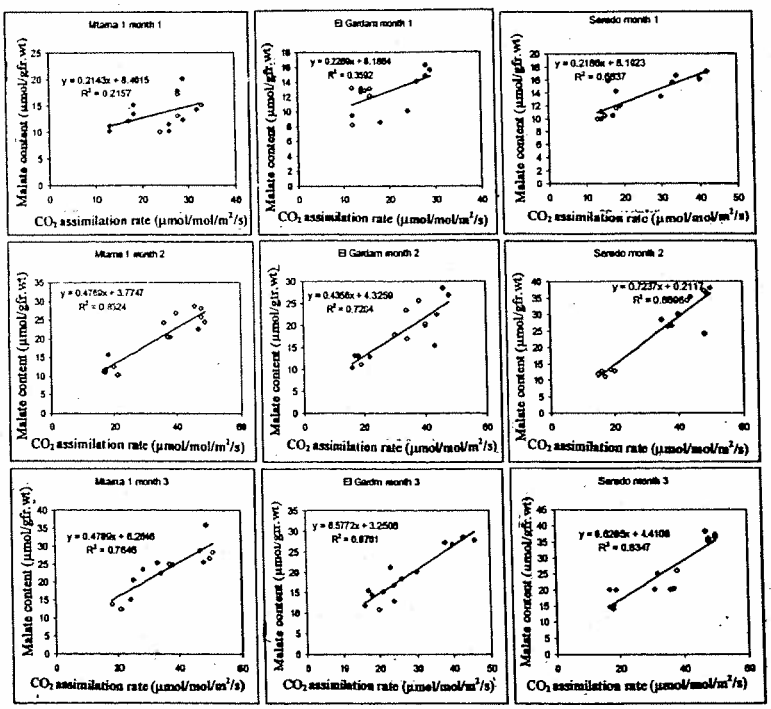

Figure 5. Regression correlations between CO_2 assimilation rate and malate content.

Similar results were obtained in this study where 100 mM NaCl treated calli regenerated into plants that had superior performance in significantly accumulating titratable acidity, malate content, CO_2 assimilation rate ($P \leq 0.05$) accompanied by higher SC and TR than the controls although, they were growing in the same field experiencing similar conditions of PAR, temperature and water potential. These results are supported by the findings of Wall *et al.* (2001) on sorghum, who by ameliorating the adverse effects of drought and salinity by elevated atmospheric CO_2, improved plant water status that indirectly caused increase in carbon gain. Stomatal conductance increased from 32 to 37%, while net CO_2, assimilation rate (M-2S-1) increased from 9 to 23%; an indication of superior performance under stress.

In this study, it was established that plants regenerated from NaCl treated calli, in the 3 sorghun cultivars, had superior performance under saline conditions than the controls. This was an indication that through tissue culture, variants with unique traits can be produced. The high positive correlations ($r^2 = 0.7$) between titratable acidity and malate content in the 3 cultivars, suggested that titratable acidity was the source of malate. The lower levels of malate than titratable acidity, suggest that there were other acids being produced during stress, or malate was being converted to other uses, i.e., serving the osmotica purpose. The relationship between malate and CO_2 assimilation was average ($r^2 = 0.6$), which suggest that although CO_2 assimilation is the source of malate, it could be getting diverted to other functions apart from photosynthesis, i.e., serving the osmotic purpose as has been observed in other C4 plants under salinity stress (Lacerda *et al.*, 2003).

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