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***In vitro* Selection and Characterization of Drought Tolerant Somaclones of Tropical Maize (*Zea mays* L.)**

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Abstract: Somaclonal variation for drought tolerances of two Kenyan maize genotypes, KAT and PH01, were obtained from *in vitro* selection. Three-month-old calli derived from immature embryos were grown on N₆ medium supplemented with mannitol or polyethylene glycol (PEG-6000) to find-out the appropriate concentrations of selection pressure. Calli were sequentially transferred to 3.28 and 5.28% mannitol or 18 and 20% PEG and maintained for 9 weeks. Survival percentage and regeneration capacities of selected and unselected calli were determined. It was observed that selected calli had lower survival and regeneration capacities than unselected calli. The result showed that survival percentages of maize calli grown in mannitol and PEG were 8.4 and 4.2%, respectively. Six plantlets were regenerated from mannitol-tolerant calli, while, only two plantlets were regenerated from the PEG tolerant calli. Plantlets were regenerated from selected calli at lower frequencies than those from unselected calli. To investigate the putative genetic basis of water stress tolerance, the mannitol tolerant PH01 somaclones designated MT JM1-01, MT JM2-01, MT JM3-01 and MT JM4-01 and the PEG tolerant clone, PT JM1-01, were further evaluated using RAPD. The assay with the decamers OP-07 and OPD-08 revealed that the water stress tolerant somaclones were genetically different from the controlled, unselected plants.

Key words: Maize, somaclonal variation, drought tolerance, PEG, mannitol, RAPD

INTRODUCTION

Maize is a top five carbohydrate source with high nutritional, economic and social value in the world. In Kenya, maize is the most important food crop and constitutes the staple food for over 95% of the population (Muhammad and Underwood, 2004). Drought continues to be a major set back to the production of food crops such as maize. This is particularly so for a country like Kenya where, drought is an endemic problem, characterising over 85% of the Land, thus classified as arid and semi arid lands (ASAL). Increasing food production in stressful environments may be achieved through breeding of crops that are more tolerant to the stress. However, since conventional breeding efforts are time, cost and labour intensive, biotechnologies such as somaclonal variation (SCV) obtained by tissue culture techniques offer a rapid and reliable alternative in crop improvement. Desirable qualitative and quantitative

agronomic traits have been achieved in crops through the SCV approach (Machuka, 2004). For instance, mutants with resistance to disease pathotoxins or tolerance to environmental and soil factors have been produced through *in vitro* selection (Jain, 2001). These somaclonal variants can then be released for direct use as new lines or used in breeding programmes. Since November 2005, research work has been ongoing at the Plant Transformation Laboratory (PTL) in Kenyatta University (KU), to investigate the efficacy of *in vitro* selection in developing maize lines that are tolerant to drought. This report is a part of the initial work.

Successful *in vitro* selection for drought tolerance has been reported for wheat (Almansouri *et al.*, 2001), maize (El-Aref, 2002), sugarcane (Yadav *et al.*, 2006) and Tagetes (Mohamed *et al.*, 2000). These investigators used non-penetrative osmotica as selective agents in the culture media to induce osmotic stress. Purified mannitol and polyethylene glycol (PEG) have been found to have

no injurious or toxic effects on the plant (Ober and Sharp, 2003), but inhibit growth by lowering the water potential of the medium so that cultured explants are unable to take up water. Somaclonal variation studies in maize have previously focussed on temperate maize genotypes (Dolgykh *et al.*, 1992; Lupotto *et al.*, 1988). However, there are few reports on *in vitro* selection of tropical maize for drought tolerance purpose and the subsequent characterisation using RAPD markers.

Somaclonal variation was first described by Larkin and Scowcroft (1981) as genetic changes present among cultured cells, plants derived from such cells or progeny of such plants. These changes may manifest as cytological abnormalities or as single-gene nuclear mutations in the form of translocations, inversions and deletions/insertions (Aguiar-Perecin *et al.*, 2000; Kaeppler *et al.*, 1998). Although identification of genetic variations in regenerated plants due to SCV has been difficult (Vidal and Garcia, 2000), the RAPD-PCR analysis, using arbitrary primers, has been used widely to characterise clones by their genetic changes. For example, RAPD was used to characterise somaclonal variants tolerant to the yellow sigatoka disease in banana (Vidal and Garcia, 2000), resistance to freezing in eucalyptus (Fernandez *et al.*, 2006), tolerance to Fe chlorosis in wild Pear (Palombi *et al.*, 2007) and to identify specific markers for pear mutants (Schiliro *et al.*, 2001) as well as drought tolerance in sugarcane (Yadav *et al.*, 2006) and cowpea (Badiane *et al.*, 2004).

In this study, we used a stepwise procedure to isolate water stress tolerant calli of Kenyan maize using mannitol and PEG as selective agents. RAPD-PCR was subsequently used to screen plants regenerated from the tolerant calli to confirm the genetic variations in the clones.

MATERIALS AND METHODS

Plant materials and general methodology: Plants from two Kenyan maize genotypes, Katumani composite B (KAT) and Pwani hybrid 01 (PH01), were established on the research field of PTL at KU on November 2005 as sources of immature embryos. Immature embryos were aseptically obtained 14-16 days after pollination and calli initiated and maintained as reported by Oduor *et al.* (2006). Friable embryogenic calli were isolated and transferred to fresh callus initiation media (CIM) at 2-3 week intervals depending on growth rate. For *in vitro* treatment, calli were cultured on 25-30 mL of CIM enriched with the selective agent. Regeneration frequency was computed on 15 randomly selected 1 g

pieces of calli as the percent number of shoots per regenerating callus.

For random amplified polymorphic DNA (RAPD) analysis, four clones (designated MT JM1-01, MT JM2-01, MT JM3-01 and MT JM4-01) from 5 different calli of PH01 that survived 5.28% mannitol-induced water stress were chosen. One clone (designated PT JM1-01) tolerant to 20% PEG was also analysed. PH01 plants that had not undergone selection, but cultured for a similar time period, were used as controls.

Determination of optimum selection concentrations of mannitol and PEG:

Preliminary experiments were performed to determine the optimum concentrations of PEG and mannitol used as initial selection levels and for water stress exertion. This was done by determining the reduction in the fresh weight of one-month-old calli induced from immature embryos of KAT. The calli were grown on CIM containing various levels of PEG (0, 6, 12, 18, 24 and 27%) and mannitol (0, 1.28, 3.28, 5.28 and 7.28%). Five callus pieces weighing 200±50 mg were used for every treatment level and replicated twice. The fresh weight of the calli was determined by weighing each callus piece separately under sterile conditions. Measurements were done at a seven-days interval for four weeks. The optimum mannitol and PEG levels selected were those that decreased the fresh weight of the selected calli by about 50% relative to untreated calli (controls) after the fourth week.

***In vitro* selection for water stress tolerance:** After induction, calli were grown on CIM for 3 months to generate somaclonal variation. They were then subjected to a step-up selection scheme modified from Lupotto *et al.* (1988) (Fig. 1) to isolate the water stress tolerant clones. Water stress was exerted using two levels of the selection agent: PEG at 18 and 20% or mannitol at 3.28 and 5.28%. A total of 204 KAT and 200 PH01 embryogenic calli were screened for tolerance to mannitol, while, another 283 KAT and 219 PH01 calli were screened for tolerance to PEG. The calli (diameter ranging between 6-10 mm) of each genotype were grown on 30 ml CIM containing either 3.28% mannitol or 18% PEG (level 1) for 3 passages (Fig. 1, stage 1-3), each lasting 21 days (Fig. 1, step 2). At every passage stage, embryogenic outgrowths from surviving calli were excised and transferred to the same media conditions. After the third subculture (stage 3), a pool of the tolerant somaclones was transferred on maturation media (Oduor *et al.*, 2006) for three weeks and subsequently to shoot induction media (Oduor *et al.*, 2006) (Fig. 1, step 6). Calli that performed

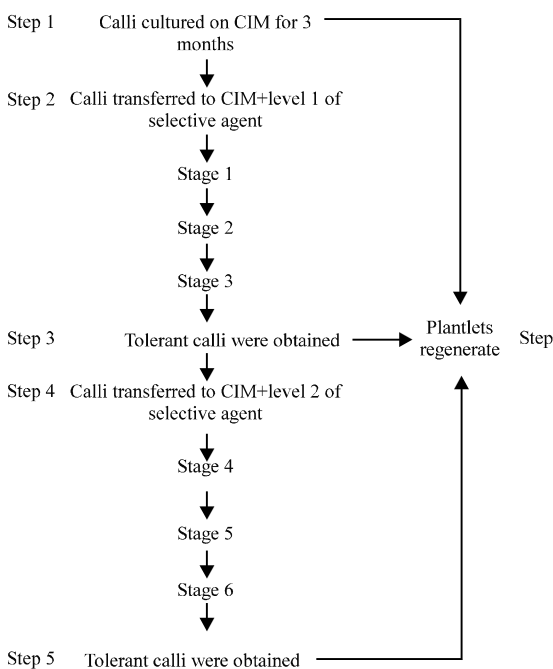


Fig. 1: Schematic flow chart for *in vitro* selection for PEG and mannitol tolerance in clones of *Zea mays* L. CIM, callus induction media = N_6 (Chu *et al.*, 1975)+3% sucrose+2 mg L⁻¹ 2, 4-Dichlorophenoxyacetic acid: Each stage is 21 days at the end of which embryogenic outgrowths from surviving callus lines were subcultured on fresh CIM supplemented with 0, 18, or 20% PEG or 0, 3.28 or 5.28% mannitol

particularly well and were perfectly embryogenic were transferred on CIM supplemented with 5.28% mannitol or 20% PEG (level 2) (Fig. 1, step 4) to assess their ability to tolerate higher levels of water stress. These calli were subcultured three times at a three-week interval on the level 2 (Fig. 1, stages 4-6). The entire selection period lasted a total of 18 weeks. At the end of stage 3 and 6 (Fig. 1), calli survival capacity was calculated as a percentage of surviving calli over the total number evaluated.

Regeneration of plants: The regeneration of putative tolerant plants was done as described previously by Oduor *et al.* (2006). Tolerant callus lines obtained at stage 3 and stage 6 (Fig. 1) were evaluated for their ability to regenerate shoots. Briefly, tolerant somaclones were matured on callus maturation media before shoot induction. Shoots were induced from the mature calli on Shoot Induction Medium (SIM). The shoots obtained simultaneously formed roots but those that did not were transferred to rooting medium (Oduor *et al.*, 2006). Shoots

Table 1: The No. of fragments and polymorphic bands generated by the 17 primers on the 6 DNA samples from the tolerant and control plants

| Primer | Sequence | Total No. of Fragments | No. of polymorphic bands |
|---------|---------------------|------------------------|--------------------------|
| OPA-02 | 5'-TGCCGAGCTG-3' | - | - |
| OPA-20 | 5'-GTTGCGATCC-3' | - | - |
| OPB-12 | 5'-CCTTGACGCA-3' | - | - |
| OPB-15 | 5'-GGAGGGTGT-3' | - | - |
| OPC-02 | 5'-GTGAGGCCTC-3' | 4 | 4 |
| OPC-03 | 5'-GGGGTCTTT-3' | - | - |
| OPC-18 | 5'-TGAGTGGGTG-3' | - | - |
| OPD-08 | 5'-GTGTGCCCA-3' | 4 | 2 |
| OPD-20 | 5'-ACCCGGTAC-3' | 6 | 0 |
| OPE-18 | 5'-GGACTGCAGA-3' | - | - |
| OPE-20 | 5'-AACGGTGACC-3' | - | - |
| OPH-10 | 5'-CCTACGTCAG-3' | - | - |
| OPT-17 | 5'-CCAAACGTCG'-T-3' | - | - |
| OPU-19 | 5'-GTCAGTGCGG-3' | 6 | 4 |
| OPU-20 | 5'-ACAGCCCCA-3' | 6 | 2 |
| OPAH-18 | 5'-GGGCTAGTCA-3' | - | - |
| OP-07 | 5'-ACCACCCGCT-3' | 8 | 3 |
| Total | | 34 | 15 |

-: Denotes the absence of detectable amplification

were maintained on rooting media until the roots were properly developed before being acclimatized. The shoots regenerated were counted after attaining a length of 2 cm. The regeneration capacity of the calli was computed as calli that regenerated at least a shoot over the total calli attempted for regeneration.

Data analysis: Differences in survival percentage and regeneration capacity between genotypes were analysed by analysis of variance (ANOVA) using Genstat for Windows (Discovery Edition). All percentage data were standardized [$x - \text{mean}(x) / \sqrt{\text{var}(X)}$] before analysis. Means were separated at 5% significance using the Least Significant Difference (LSD).

RAPD analysis: DNA extraction was carried out according to Saghai-Marooof *et al.* (1984). Genomic DNA was isolated from 200 mg of leaf from five somaclones designated MT JM1-01, MT JM2-01, MT JM3-01, MT JM4-01 and PT JM1-01 and one control plant. Agarose (0.8%) gel electrophoresis was used to determine the quality of the DNA extracts. The protocol of Chin and Smith (1993) was first modified for optimum RAPD-PCR reaction conditions. To achieve this, a range of concentrations of MgCl₂ (2.7, 4.7 and 6.7 mM), dNTPs (200, 400 and 600 μM) and Taq polymerase (0.5, 0.8 and 1.1 U) were investigated for band clarity. All optimisation reactions were carried out using a DNA sample from one clone and one primer (OPU-20). Seventeen RAPD decamer primers having a high G+C content of 60-70% and lacking inverted repeated sequences at their termini were randomly selected (Table 1). They were first tested for

their ability to amplify a DNA sample from one of the somaclone using the method of Chin and Smith (1993). Primers that produced at least one amplification product were used in screening the DNA samples isolated from the tolerant somaclones. All primers were from Operon Technologies Inc., USA.

PCR reactions were performed on duplicate samples of DNA from each somaclone and control. The reaction constituents were 8 pM of the primer, 200 μM of a mixture of the four dNTPs, 2.7 mM MgCl₂, 20 ng of genomic DNA, 1x Taq polymerase reaction buffer and 0.8 units of Taq polymerase (PeQLab Biotechnologie GmbH). RAPD amplification was carried out in an advanced primers 25 thermal cycler (PeQLab Biotechnologie GmbH) using the following reaction conditions: an initial denaturation of 30 sec at 94°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min, with a terminal elongation at 72°C for 6 min. For each primer, a tube containing all the reaction components but the DNA template was included as negative control. The PCR products were resolved on a 1.5% agarose gel. O'GeneRuler 1 kb DNA ladder (Fermentas Inc, Hanover, MD) was used as the molecular size standard. Visualization and photography of gels was done using the Genesnap image acquisitions software from Syngene (Synoptics LTD, UK) while sizing of bands was done using the Genetools software from Syngene (Synoptics LTD, UK).

RESULTS

Optimum mannitol and PEG selection concentrations: It was observed that the fresh weight of KAT calli decreased as the level of stress increased in the culture media (Fig. 2). Calli growing on 18% PEG and 3.28%

mannitol had fresh weights of 45.92 and 50.94% of the unstressed callus, respectively (Fig. 2A). It is noteworthy that the embryogenic capacity of the callus was drastically reduced and growth seriously affected at concentrations above these levels. Therefore screening for water stress tolerance was carried out using 18% PEG or 3.28% mannitol as initial selection concentrations (Fig. 2B).

In vitro selection of calli for water stress tolerance

Effect of water stress on callus survival: Most KAT and PH01 calli exposed to 3.28% mannitol for nine weeks showed an even brown coloration indicating death (Fig. 3A). Calli surviving on 3.28% (Fig. 3A) and 5.28% mannitol were comparable to unselected calli in embryogenicity, growth and colour. Analysis by ANOVA showed that the survival capacities of KAT and PH01 calli did not differ significantly ($p = 0.05$), though KAT had a lower average survival capacity after selection on 3.28 and 5.28% mannitol (Table 2). On both mannitol levels, the average survival capacities of mannitol-selected PH01 and KAT calli were significantly lower ($p < 0.05$) than their respective controls (Table 2). Five callus lines (independent events) surviving selection on 3.28% mannitol grew remarkably well under selection. They were more embryogenic and persistent in growth than the rest of the surviving calli. Moreover their colour and growth was similar to that of their respective controls. Clones derived from these calli survived and grew on 5.28% mannitol-induced water stress.

Both KAT and PH01 Calli exposed to 18% PEG showed progressive necrosis and growth inhibition with total death occurring within 50 days of selection. A total of 49 calli survived this treatment and were observed to comprise of both dead somatic embryos that were brown in color and live cream or white-colored ones. Upon

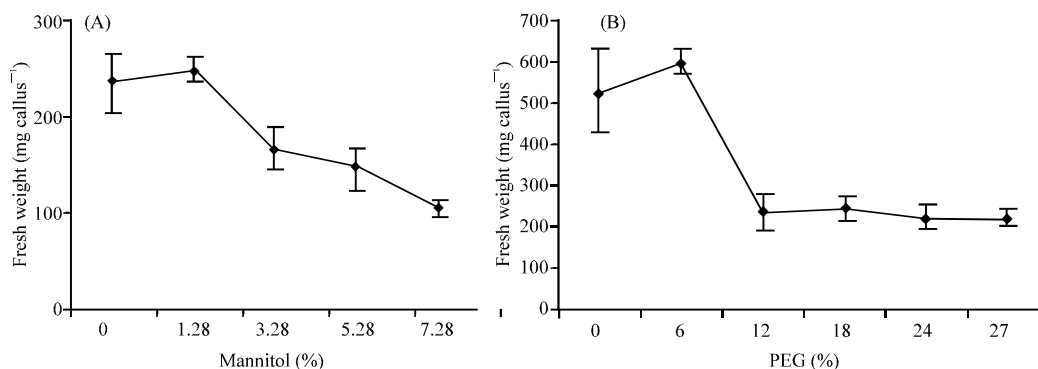


Fig. 2: Fresh weight changes of KAT calli over 28 days of exposure to various concentrations of (A) mannitol and (B) PEG in CIM. Calli derived from immature embryos of KAT were cultured on CIM supplemented with mannitol (between 0 and 5.28%) and PEG (between 0 and 27%). Values are the means of 5 replicates and vertical bars are SE

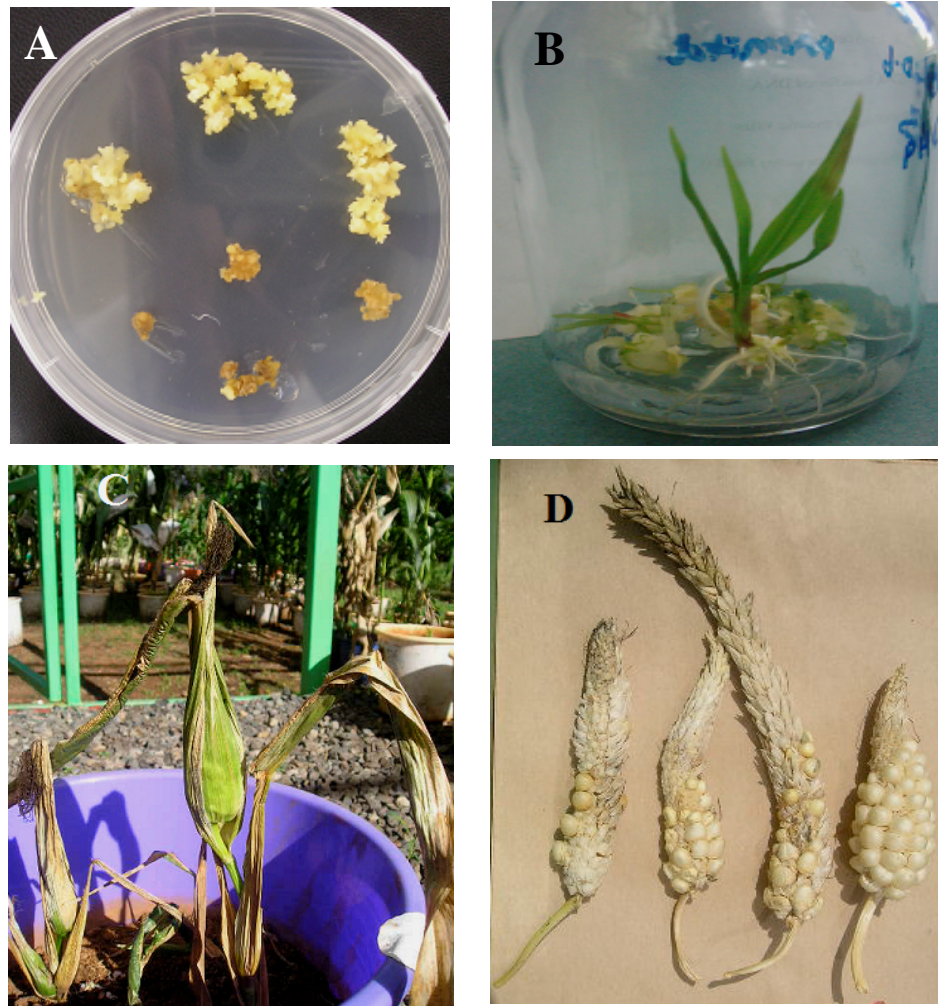


Fig. 3: *In vitro* selection for water stress tolerance and plant regeneration via somatic embryogenesis. (A) Callus cultured for 6 weeks on 3.28% mannitol-containing medium showing dead (brown) and surviving (white) calli, (B) Plantlets regenerating from mannitol selected calli showing shoots and roots formation on shoot induction medium, (C) Mature mannitol-stressed plants that are dwarfed and forming tassel ears and (D) Seeds obtained from mature water stressed plants

Table 2: Effect of PEG and mannitol on survival capacity of embryogenic KAT and PH01 callus cultures

| Selective agent | Selection level (%) | KAT | | PH01 | |
|-----------------|---------------------|-------------|--------------|-------------|--------------|
| | | Unselected | Selected | Unselected | Selected |
| PEG | 18.00 | 90.00±6.12a | 28.31±1.82b | 80.00±6.37a | 21.86±4.99b |
| | 20.00 | 70.83±8.33a | 54.55±27.79b | 83.33±4.17a | 64.31±10.85b |
| Mannitol | 3.28 | 80.00±6.37a | 23.97±5.16b | 85.00±7.29a | 41.13±9.87b |
| | 5.28 | 91.67±8.33a | 28.57±4.76b | 83.33±4.17a | 30.28±5.81b |

Survival capacity of callus without the addition of treatment to the media (unselected) and with PEG (18 and 20%) and mannitol (3.28 and 5.28%) addition to the media (selected) for a period of 9 weeks in each level. Each value represents Mean±SE of five replicates. Values in the same row which are followed by same letters are not statistically different at $p \leq 0.05$ by ANOVA

transfer of the surviving calli to 20% PEG-containing media, enhanced necrosis and marked decrease in callus embryogenicity compared to unselected calli was

observed. Twenty calli survived selection on 20% PEG, though no growth increase was apparent. PH01 and KAT calli did not differ significantly in survival capacity when

selected on 18% ($p = 0.259$) or 20% ($p = 0.544$) PEG. ANOVA also showed that PH01 and KAT had significantly lower survival capacities ($p \leq 0.05$) after selection in the two levels of PEG than their respective controls (Table 2). After 18 weeks on selection, one PH01 callus line was observed to grow as well as control calli and was closely monitored.

Effect of water stress on callus regeneration capacity:

Calli surviving mannitol and PEG selection were evaluated for their capacity to regenerate shoots after selection in each level. The statistical differences in average regeneration capacity between PEG-selected KAT and PH01 calli were found to be non-significant ($p > 0.05$) (Table 3). However, KAT and PH01 calli selected on 18% PEG exhibited significantly lower regeneration capacities ($p \leq 0.05$) compared to their respective control calli (Table 3).

PH01 and KAT calli selected on 3.28% mannitol had statistically significant ($p < 0.05$) lower regeneration capacities than those of their respective controls. After exposure to 5.28% mannitol, the calli exhibited significantly lower regeneration capacities compared to the control calli (Table 3). Mannitol stressed PH01 calli had higher regeneration capacity than mannitol stressed KAT, though not significant statistically ($p > 0.05$) (Table 3).

Unstressed callus started to develop green color 2-5 days after transfer to light. However, a delay in greening among PEG- and mannitol-treated calli was observed, starting 1-2 weeks after transfer to light. Shoots regeneration response was also delayed in selected calli, sometimes taking up to four weeks for a distinctive shoot to emerge.

Regeneration frequency of the two genotypes under selective and nonselective conditions is shown in Table 4. The data indicate that unselected calli regenerated shoots at higher frequency than selected calli. Additionally, the frequencies of shoot regeneration by the stressed PH01 calli were higher than those of stressed KAT calli.

The majority of water stress tolerant PH01 and KAT calli developed shoots and roots simultaneously on SIM (Fig. 3B), but roots were less abundant on this media compared to RIM. The PEG-stressed PH01 and KAT calli regenerated shoots and roots on SIM, but the majority rooted on RIM after about 2-4 weeks. Mannitol stressed calli were characterized mostly by shoot abortion, where shoots died before they were barely about 1.0 cm long. Some of the surviving shoots produced a pronounced rooting system with densely arranged hairy roots on SIM after 2-4 weeks. Well rooted plantlets were acclimatized before transplanting into soil. As transplanted plants grew to maturity, they were characterised by various abnormalities including dwarfism and tassel ear formation (Fig. 3C). Regardless of the abnormality however, plants set viable seeds most of which were established on the tassels (Fig. 3D).

RAPD analysis: The DNA extracted from plant material was of high quality with no or minimal degradation as depicted by electrophoresis on 0.8% agarose gel. The Optimum concentrations of the PCR reaction components were found to be; 0.8 units Taq, 2.7 mM MgCl₂ and 200 μM dNTPs.

Out of the 17 primers assayed, 11 primers with a 60% G+C content (except primer OPA-02) did not produce

Table 3: Effect of PEG and mannitol on regeneration capacity of embryogenic KAT and PH01 callus cultures

| Selective agent | Selection level (%) | KAT | | PH01 | |
|-----------------|---------------------|--------------|--------------|-------------|--------------|
| | | Unselected | Selected | Unselected | Selected |
| PEG | 18.00 | 86.81±6.71a | 51.02±12.88b | 82.23±9.33a | 42.91±11.03b |
| | 20.00 | 71.80±10.10a | 22.22±5.32b | 79.97±6.77a | 74.07±25.73a |
| Mannitol | 3.28 | 81.00±7.39a | 58.34±9.75b | 80.38±7.29a | 39.55±6.01b |
| | 5.28 | 89.15±10.00a | 16.67±4.21b | 84.44±5.21a | 54.29±23.79b |

Regeneration capacity of callus following exposure to media without the addition of treatment (unselected) and media with addition of PEG (18 and 20%) and mannitol (3.28 and 5.28%) for a period of 9 weeks in each level. Each value represents mean±SE of five replicates. Values in the same row which are followed by same letters are not statistically different at $p \leq 0.05$ by ANOVA

Table 4: Shoot regeneration frequency of selected and unselected KAT and PH01 calli

| Selective agent | Selection level (%) | KAT | | PH01 | |
|-----------------|---------------------|------------|----------|------------|----------|
| | | Unselected | Selected | Unselected | Selected |
| PEG | 18.00 | 79.69 | 22.67 | 38.67 | 58.22 |
| | 20.00 | 97.66 | 0.00 | 60.55 | 12.59 |
| Mannitol | 3.28 | 80.47 | 16.48 | 42.58 | 100.00 |
| | 5.28 | 135.93 | 4.00 | 55.08 | 52.50 |

Percent mean No. of shoots per regenerating callus was determined on KAT and PH01 calli on SIM after a previous exposure to various levels of PEG (0, 18 and 20%) and Mannitol (0, 3.28 and 5.28%) on CIM

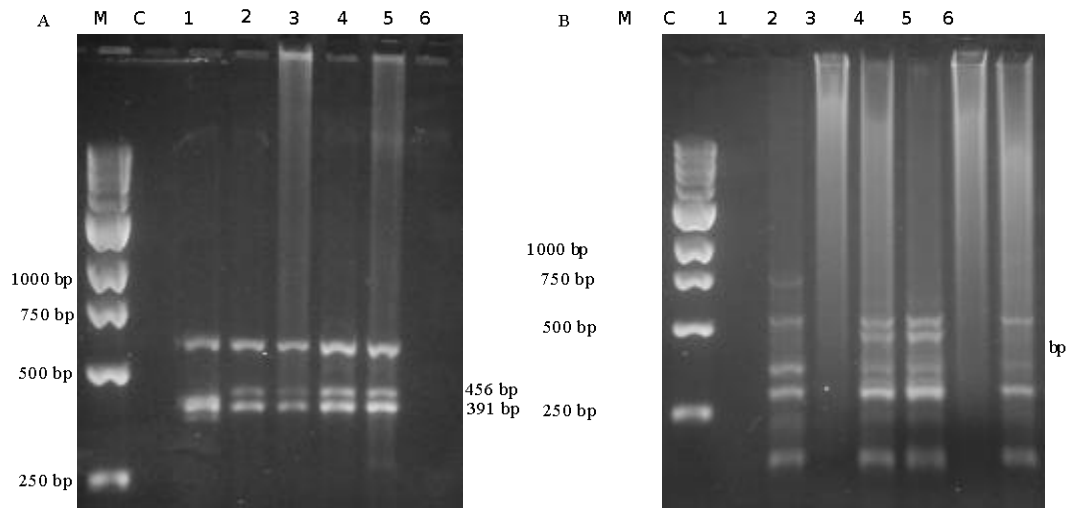


Fig. 4: Polymorphic RAPD profiles that distinguish the five tolerant somaclones from the unstressed plants were obtained after amplification with the OPD-08 (A) and OPA-07 (B) decamers. Lane M: 1 kb ladder; Lane C: Control amplification without DNA; Lane 1: control unstressed plant; Lane 2-6: Water stress tolerant variants MT JM1-01, MT JM2-01, MT JM3-01, MT JM4-01 and PT JM1-01, respectively. Arrow heads indicate polymorphic fragments

amplification of the target DNA template (Table 1). However the other six primers, all with a G+C content of 70%, amplified. The six primers produced a total of 34 fragments over all the DNA samples used. Among these fragments, 15 were found to be polymorphic across all the tolerant and control samples (Table 1). However polymorphic fragments capable of differentiating the tolerant somaclones from the control plants were generated only by primers OP-07 and OPD-08 (Fig. 4).

The marker OPD-08 was able to generate a profile that distinguished the tolerant somaclones from the control plants (Fig. 4A). Amplification with the marker revealed the presence of a 456 bp fragment in the putative drought tolerant somaclones (MT JM4-01, MT JM3-01, MT JM2-01 and MT JM1-01) but absent in the control plant. However, these somaclones showed the absence of the 391 bp band that was present in the control plants (Fig. 4A). There was no observable amplification of the somaclone PT JM1-01 DNA using the decamer OPD-08 (Fig. 4A).

Amplification products with primer OP-07 (Fig. 4B) showed the presence of a 750 bp band in the control plants. However, this band was absent in the tolerant clones MT JM2-01, MT JM3-01 and PT JM1-01. The tolerant somaclones MT JM2-01 and MT JM3-01 can be identified by the presence of a 490 bp and a 330 bp band in their profiles, while variant clone PT JM1-01 and the control plant are identifiable by the absence of the two fragments. The primer OP-07 did not produce detectable

amplification on DNA of the variant somaclones MT JM1-01 and MT JM4-01.

DISCUSSION

Results from this study show a reduction of callus growth with increasing stress concentrations (Fig. 2). However, stimulation of growth in both KAT and PH01 calli at moderate water stress was also observed. A similar trend was obtained by Liu and Staden (2001) with a water stress tolerant soybean callus line. Liu and Staden (2001) however showed that the sensitive cell line did not respond with growth stimulation at the same culture conditions. The mass of calli exposed to water stress was observed to either remain the same or become smaller after the fourth week of exposure to water stress induced by 18% PEG and 3.28% mannitol. Drastic decrease in growth of maize calli exposed to high levels of salt or drought stress has been reported (Pesqueira *et al.*, 2006; El-Aref, 2002). Callus growth reduction is probably due to reduction of cytoplasmic and vacuolar volume resulting from removal of water from cytoplasm by a lowered cellular water potential (Bartels and Sunkar, 2005). A lowered external osmotic potential is detrimental as cells are unable to take up water and nutrients from the external environment, leading to decline in growth.

It was further noted that after six to eight weeks of exposure to water stress, the calli turned brown then black and eventually died. Similar results have been reported by

others working with maize (El-Aref, 2002; Zacchini *et al.*, 1999). Tissue browning and death maybe the consequence of water loss resulting from exposure to severe osmotic stress. A high content of phenol has also been implicated in tissue browning and death (Hassanein, 1999). Some of the calli challenged with water stress were comparable to unselected ones in growth suggesting a probable rapid response to avoid severe water loss. Such a response may have enabled these calli to survive a higher level of stress.

Calli cultured on medium containing the two levels of mannitol (3.28 and 5.28%) and PEG (18 and 20%) had their growth drastically inhibited. They became necrotic and died after 6 to 8 weeks on selection (Fig. 3). This notwithstanding, 21% PH01 and 28% KAT calli survived selection on 18% PEG, while, 24% PH01 and 41% KAT survived a 3.28% mannitol exposure. A reduction in cell growth due to 25% PEG-induced water stress has been previously reported in maize (El-Aref, 2002). Unselected calli had significantly higher survival capacities compared to selected ones, an indication of a negative influence of water stress on callus survival. Variations in survival to water stress response could not be attributed to the genotypes. However other studies have demonstrated a genotype-dependent response to water stress induced by PEG or ABA (Lutts *et al.*, 2004; Lu *et al.*, 2003).

Two interesting observations were made on PEG- or mannitol-tolerant callus lines: delayed regeneration compared to their respective controls and presence of water droplets on somatic embryo surface. Delayed plantlet regeneration after ABA and PEG treatments has been reported (Lutts *et al.*, 1999; Tereso *et al.*, 2007). ABA, a plant hormone produced following osmotic stress (Zhu, 2002), has been shown to delay germination of zygotic and somatic embryos (Tereso *et al.*, 2007). The presence of water droplets on the surface of callus embryos is attributable to removal of water from the cytoplasm to the extracellular space due to water stress-induced cellular dehydration (Bartels and Sunkar, 2005).

PH01 and KAT calli selected on the two levels of mannitol and PEG were different from the unstressed calli in regeneration capacity. This may be attributed to the detrimental effects of selection on regeneration. The capacity of calli cultures to regenerate plants has been proposed to be very sensitive to selection (Phillips *et al.*, 1988). After stressing, KAT and PH01 calli were not found to differ in regeneration capacity (Table 3). However other reports have shown a strong influence of genotype on regenerability under osmotic stress conditions (Lutts *et al.*, 1999; Zair *et al.*, 2003). The finding that the number of shoots per regenerating PEG-selected callus was reduced compared to unselected callus is similar to

that reported by Lutts *et al.* (1999) and El-Aref (2002). PEG causes the reduction in yield of somatic embryos as well as causing incomplete development and anatomical abnormalities of the somatic embryos (Tereso *et al.*, 2007). The shoot regeneration frequency of KAT calli was affected more than that of PH01, since only 2 shoots could regenerate from two calli out of the 31 PEG tolerant calli. In comparison, 11 shoots were derived from the 11 individual regenerating PH01 calli out of 49 tolerant ones.

PH01 calli tolerant to 3.28% mannitol-induced water stress germinated shoots particularly well, with frequencies of over 100%. One gram pieces of these tolerant lines sequentially cultured on 5.28% mannitol exhibited similar regeneration frequencies. On the other hand, the regeneration of KAT appeared to have been more affected by 5.28% mannitol as only three out of 26 calli regenerated five shoots. This is in comparison to 11 out of 30 PH01 calli that regenerated 27 shoots. Despite these variations, no genotype dependent regenerability response was observed.

The RAPD patterns obtained with primers OP-07 and OPD-08 show that the somaclones MT JM1-01, MT JM2-01, MT JM3-01 and MT JM4-01 have the same origin. This is because their RAPD profiles are identical to each other. It is possible that they may have originated from the same callus or explant and hence share identical genotypes. The clone PT JM1-01 on the other hand has a different origin altogether since it shows a RAPD profile different from the other putative tolerant somaclones.

Primers OP-07 and OPD-08 generated marker bands that were identical over all the tolerant somaclones but different for the control plants. The presence of these polymorphisms indicates presence of genetic differences between the tolerant somaclones and the control plants. This suggests a possible involvement of specific genes controlling tolerance to water stress in these somaclones.

The above observation can be explained by the fact that passage of materials through a callus phase causes genetic alterations observable in cultured cells or plants derived from the cells or progenies of these plants. In maize, SCV have been observed as cytological abnormalities or as single gene nuclear mutations in form of nucleotide base changes (Aguilar-Perecin *et al.*, 2000). Mutations occurring within the RAPD primer binding site will lead to RAPD polymorphisms such as the ones observed in this study. However polymorphisms occurring within the amplified fragments but not in the binding site will go undetected (Hofmann *et al.*, 2004). This implies that the RAPD technique may underestimate the amount of polymorphism present in an individual.

In conclusion, *in vitro* selection was successfully used to isolate water stress tolerant cells of tropical maize.

RAPD-PCR results suggest that tolerance of the cells to water stress has a genetic basis. Sequencing of the polymorphic marker bands in the variants MT JM1-01, MT JM2-01, MT JM3-01, MT JM4-01 and PT JM1-01 can help in firmly establishing the genetic mechanism responsible for tolerance to drought in these variants.

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