SOMACLONAL VARIATION IN SUGARCANE THROUGH TISSUE CULTURE AND SUBSEQUENT SCREENING FOR MOLECULAR POLYMORPHISMS

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Sugarcane (*Saccharum* sp.) is globally the main source of raw material for the production of sugar, followed by sugar beets (*Beta vulgaris* L.). In Egypt, sugarcane crop is one of the important crops and the main source of sugar production. Egypt ranked the first grade in the unit area production according to the absolute growing season (12 month). The sugar production in Egypt reached 1,497 million ton sugar from cane and beet in 2006. Developing sugarcane varieties as industrial crop has been considered among the most important objectives of the Egyptian Agricultural Policy; to face the gap between sugar production and sugar consumption. Since the cultivated area of sugarcane is too limited in Egypt and there is no way to increase it horizontally; improving agricultural practices, in addition to developing new promising varieties become the possible way to raise sugarcane production (Sugar Crops Council, 2006).

The use of tissue culture for creating somaclonal variation in plants for development of new varieties offers advantage over all other conventional methods. This method is very efficient and has tremendous potential for producing novel and useful varieties (Larkin and Scowcroft, 1981), they coined the term somaclonal variation to describe the occurrence of genetic variants derived from *in vitro* procedures. Factors such as explant source, time of culture, number of subcultures, phytohormones, genotype, media composition, the level of ploidy and genetic mosaicism are capable of inducing *in vitro* variability (Silvarolla, 1992). An alternative method for the production of *in vitro* plantlets of sugar cane from such mother plants, using leaf discs as explants were used by Mulleegadoo and Dookun-Saumtally (2006), where contamination and oxidation were minimal and more than 70% of the leaf discs cultured formed shoots. Regenerated plants that produced by culturing tissue sections lacking a preformed meristem (adventitious origin) (Karp, 1995) or derived from callus and cell cultures (*de novo* origin) (Damasco *et al*., 1996) is more susceptible to somaclonal variation. Khan *et al*. (2004)

used tissue culture techniques to develop somaclones in sugarcane to screen salt tolerant clones subsequently.

Molecular markers are widely used to detect and characterize somaclonal variation at the DNA level (Barrett *et al.,* 1997). Of the available techniques, RAPD is among the most useful ones (Rani *et al.,* 1995). Changes in the RAPD pattern may result from the loss/gain of a primer annealing, caused by point mutations or by the insertion or deletion of sequences or transposition elements (Peschke *et al*., 1991). Zucchi *et al.* (2002) used the random amplified polymorphic DNA (RAPD) to detect tissue culture-induced variations in sugarcane. Approaches such as molecular markers have permitted significant advances in the establishment of the evolutionary origin and genome structure of sugarcane, an important polyploid crop (Grivet and Arruda, 2003).

The present study aimed to induce somaclonal variation in some sugarcane varieties by using different combinations of 2,4-D and coconut water and to use RAPD-PCR to investigate the variation in regenerated sugarcane plants derived from leaf cultures at the molecular level.

MATERIALS AND METHODS

Plant material

Four varieties of sugarcane under study were supplied from Sugar Crop Research Institute (SCRI); the commercial GT54-9 and three promising varieties i.e. Phil8013, G98-28 and G98-24, their pedigrees and source are shown in Table (1). The vegetative and technological traits of the parental varieties were determined in randomized complete block design with three replications. A sample of ten stalks from each sugarcane variety was used after harvest for the analysis. The primary juice was extracted by electric pilotmill, screened and mixed thoroughly. One liter juice was taken to determine juice quality traits according to the formula described by methods of Sugar and Integrated Industries Company (SIIC), Chemical Control. The technological traits studied were: Brix percentage that was measured using brix hydrometer standard, Sucrose percentage which was determined using saccharemeter according to A.O.A.C. (1990). Purity percentage was calculated according to

$$\text{Purity \%} = \frac{\text{Sucrose \%}}{\text{Brix \%}} \text{ X } 100$$

Sugar recovery percentage was calculated according to the following formula:

Sugar recovery % = Richness % X Purity %,

Where; Richness = (sucrose in 100 grams X factor)/100.

Factor=100-(Fiber% + physical impurities % + water free sugar %). Total Soluble Solids percentage (T.S.S %) was determined by hand refractometer.

Construction of the dendrogram tree according to the vegetative traits and technological traits of the donor plants

was performed using SPSS Program Version 10.

Biological materials and Ex-plant

Spindle sections were taken from the four varieties at the age of 6-8 months. The outer leaves were removed to expose the six inner leaves which were cut into small transverse sections (2-3 mm) and used as explants according to Khan *et al.* (2005) who used leaf sheath as an ex-plant.

Culture media

The explants were cultured on initiating medium containing MS (Murashige and Skoog, 1962) supplemented with 500 mg/l casein hydrolyzate, 30 g/l sucrose and 2.8/l phytagel. Six concentrations of 2,4-D were used i.e. 0, 1, 2, 3, 4 and 5 mg/l in combination with three concentrations of coconut water 0, 5 and 10%. For each treatment three replications were used containing 30 explants each. The cultured explants were incubated at 28°C±2 for four weeks in the dark. Number of responded ex-plants%, number of embryonic calli induced% and number of germinated embryos% were recorded.

Five concentrations of NAA 0, 1, 3, 5 and 7 mg/l were used in combination with five concentrations of sucrose 2, 3, 4, 5 and 6% (w/v) for rooting and plantlets elongation. For each treatment three replications were used each containing seven to ten embryogenic calli ex-plants each. The cultured explants were incubated at 28°C ±2 for two to three weeks under diffuse cool white fluorescent lamps (4000 Lux) with 16 h day-length (Chengalrayan and Gallo-Meagher, 2001). The cultures were evaluated for rooting percentage, root length, leaves number, shoot number/ex-plant and shoot length/ex-plant.

Acclimatization of the regenerated sugarcane plants was carried out in the greenhouse facilities according to Khalil (2002). The plantlets were separately transferred to 250 ml glass flasks containing 30 ml of water and maintained for a week in a naturally illuminated acclimatization room at 28°C before transferring to soil. The plantlets were soaked in a fungicide solution (1 g/l of Benlate) for 3-5 min and transplanted to a peat moss and sand 1:1 (v/v) mixture in plastic pots 15 cm in diameter. Each pot was enclosed within a transparent plastic bag. After ten days the plastic bag was opened gradually and then removed after four days. The acclimatized sugarcane plants were maintained under green house conditions until transferred to the open field.

Statistical analysis

The collected data were statistically analyzed using Mstat C program. The differences among means were compared using Duncan's new multiple range test (Waller and Dancan, 1969)

Molecular analysis

The tests and analysis were carried out at the laboratories of the Cell Res.

Dept., Field Crops Res. Institute (FCRI) and Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt.

DNA isolation

DNA was extracted from 100 mg of young leaves of four sugarcane varieties and three regenerated genotypes according to Junghans and Metzlatt (1990). The quantity of the DNA was assessed by agarose mini-gel electrophoresis and adjusted to between 20 and 40 ng/ml.

Random amplified polymorphic DNA (RAPD)

The extracted DNA was used to perform polymerase chain reaction (PCR) using RAPD primers according to Williams *et al.* (1990). The available primer set consists of 6 primers (10-mer) with the following arbitrary sequences: primer 1=5`-(GGTGCGGGAA)-3`; primer 2=5`-(GTTTCGCTCC)-3`; primer 3=5`-(GTAGACCCGT)-3`; primer 4=5`-(AAGAGCCCG T)-3`; primer 5=5`-(AACGCGCAAC)-3`and primer 6=5`-(CCCGTCAGCA)-3` and obtained from BioNeer (Operon Technologies, Inc).

RAPD-PCR reactions

RAPD-PCR reactions were optimized and mixtures (25 µl total volume) were composed of dNTPs (200 µ M), Mg Cl_2 (1.5 mM), 1x buffer, primer (0.2 µM), DNA (50 ng), Taq DNA polymerase (2 units). Amplification was carried out in a Thermo Cycler (MWG-BIO TECH Primuse) programmed for 94°C for 3 min (one cycle), followed by 94°C for 30 sec, 36°C for 1.5 min and 72°C for 30 sec (40 cycle), 72°C for 5 min (one cycle), then 4°C (infinitive). Amplification products (15 µl) were mixed with 3 µl loading buffer and separated on 1.2% agarose gel and stained with 0.5 µg/ ml ethidium bromide with a constant electric current (100 volts) for 25 minutes at room temperature. Bands were visualized, photographed and scored using gel documentation system (UV transilluminator) Bio-Rad video densitometer Model 620, at a wave length of 577, Software data analysis for Bio-Rad Model 620 densitometer and computer were used as illustrated by the manufacturer. DNA fragment sizes were determined using the 100 bp DNA Ladder marker.

Data analysis

The data of RAPD-PCR analyses were entered in a computer file as binary matrices. Similarity coefficients were calculated according to Dice matrix (Nei and Li, 1979). Construction of the dendrogram tree was performed using the unweighted pair group method based on arithmetic mean (UPGMA) as implemented in the SPSS Program Version 10.

RESULTS AND DISCUSSION

Vegetative traits and technological traits of varieties

The influence of the varieties behavior of the examined sugarcane varieties on some yield and its attribute

(vegetative traits) is presented in Table (2). Data revealed that there were significant differences between varieties under study with respect to cane yield and its components; stalk (length, number of internodes and number of plant/feddan), however the differences between varieties for stalk diameter did not reach the level of significance. The significant differences between varieties in the above mentioned traits mainly due to gene make up effect (Gandonou *et al*., 2005). It could be noticed that 'except for variety G98-28' the differences between the commercial variety (GT54-9) and the other promising varieties were insignificant with respect to stalk length. However, it is distinctly shown that the commercial variety is still characterized by stability and responses in the different studied parameters i.e. internodes numbers, millable cane number and stalk yield/fed. compared with the other varieties. This finding may throw some light on the relative importance between the environments and gene makeup effect.

Concerning the technological traits, data in Table (2) clear the occurrence of relative differences between the examined sugarcane varieties with respect to juice quality. All the studied varieties relatively and significantly surpassed sugarcane variety G98-28 with respect to the various juice quality parameters in terms of total soluble solids (TSS), sucrose, purity, richness and sugar recovery%. However, the highest values of these parameters were recorded for variety G98-24. This finding assured that there is an inverse relationship between the vegetative parameters in terms of cane yield and the qualitative one in terms of juice quality. This observation shows the heavy burden on the breeder to select the high yielding varieties coupled with high quality.

Similarity index as percentage based on the vegetative traits and juice quality using UPGMA computer analysis was shown in (Table 3). The highest similarity index recorded was 85%, which was observed between the two varieties G. 98-28 and G. 98-24, while the lowest similarity index recorded was 78%, which was observed between Phil. 8013 and GT 54-9 varieties. The dendrogram (cluster analysis) for the genetic relationships among the four sugarcane varieties is presented in Fig. (1). The varieties were separated into two clusters; cluster one included Phil. 8013, while cluster 2 included all the other three varieties. Within cluster 2, one subcluster contained one sugarcane cultivar GT 54-9, while the second subcluster contained G. 98-28 and G. 98-24, since they were produced from the same mother C34-33 as shown in Table (1).

Callus induction, embryonic callus induction and regeneration

Effect of the different concentrations of 2, 4-D were significant on callus induction percentages overall studied varieties as shown in Table (4). The absence of 2, 4-D from media composi-

tion significantly decreased callus induction percentage. However using 2, 4-D at level of 5 mg/l gave the highest callus induction percentage i.e. 88.1%. 5mg/l of 2, 4-D with the two varieties Phil8013 and G98-28 gave 91.4 and 89.7% response, respectively. On the other hand, the best callus induction percentage for the two varieties GT54-9 (91.8%) and G98-24 (85.1%) was obtained with the application of 2,4-D at the level of 4 mg/l. Callus induction percentage of Phil8013 variety is illustrated by Fig. (2).

The above mentioned results agreed with those of Nand and Lal (2003) who mentioned that the highest callus induction was obtained at the level of 5 mg/l 2,4-D. Moreover, Gill *et al*. (2002) reported that callus formation was highest with 2,4-D at the level of 4 mg/l. On the contrary, Alam *et al.* (2003) achieved the highest callus induction at the level of 3 mg/l 2,4-D.

These variations could be due to the genetic differences of varieties. The obtained results are in agreement with Gandonou *et al*. (2005) who studied nine sugarcane genotypes and found different responses percentage. They confirmed that callus induction ability was greatly influenced by the genotype.

Concerning the callus induction of varieties, significant effects were recorded between varieties. Variety Phil8013 was significantly superior over the other varieties. It`s callus induction responses% was 84.7% followed by GT54-9 (71.5%), G98-28 (67.3%) and G98-24 (66.5%). These results are in agreement with those of Ranju *et al*. (2000). They noticed that callus initiation showed some varietals differences. Gill *et al*. (2002) found that the mean percent callus induction was highest for Co.J.86 (94.02) followed by Co.J.64 (93.2 %) and was least for Co.J.8 (90.24%).

It could be noticed from the obtained results that the effect of different concentrations of coconut water (CW) on the callus induction percentage were insignificant. Using CW at the level of 10 % gave the highest callus induction percentage for the four varieties under study i.e. Phil8013 (99.8%), GT54-9 (75.5%), G98-28 (71.9%) and G98-24 (71.8%). These results agreed partially Karim *et al*. (2002) who obtained 90 and 100 % callus production with the two studied cultivars using 10 % coconut water with 3 mg/l 2,4-D. Also, Virupakshi *et al.* (2002) mentioned the comparable results with cultivar CoC671.

Significant effects of all studied factors were noticed. The influence of applying 2,4-D at level of 4 mg/l significantly increase embryonic callus induction percentage. It recorded 93.8, 91.8 and 88.3 % for Phil8013, G98-24 and G98-28, respectively. On the other hand, GT54-9 recorded the highest embryonic callus induction percentage (88.5 %) with 2 mg/l of 2, 4-D. G98-28 gave a significant decrement in comparison with the other varieties in this trait. G98-28 recorded

46.4%. Meanwhile, the differences between G98-24, Phil8013 and GT54-9 were not significant. They recorded 53.9, 52.6 and 50.4 %, respectively.

Regarding the interactions, all the studied interactions were significant. Data in Table (4) indicate that using 4 mg/l of 2,4-D in the combination with either 5 or 10 ml/l of CW gave the highest value i.e. 83.3 %. In the meantime, the three way interaction also was significant. Applying 2,4-D at the level of 4 mg/l in the combination with either 5 or 10 ml/l of CW gave the highest values i.e. 100, 98.8 and 94.4 % with Phil8013, G98-28 and G98-24, respectively. GT54-9 recorded the highest percentage with 2 mg/l of 2, 4-D and 10 ml/l of CW.

Our results are in disagreement with Gandonou *et al.* (2005) who obtained high embryonic callus percentages about 95 % but no significant differences among them were showed and they found that callus induction ability, embryonic callus induction and plant regeneration capacity in sugarcane were significantly affected by genotype.

Our results indicated different responses of studied cultivars within the different treatments of 2,4-D and coconut water. Varieties gave the highest callus induction percentage with different concentrations of 2,4-D at range between 2-4 mg/l. Lowering 2,4-D to 1 mg/l enhanced the embryogenesis. On the other hand, all the varieties responded to 7 mg/l NAA but differed with the sucrose concentrations, they ranged between 40, 50 and 60 g/l (El-Geddawy, 2006).

Varieties showed no significant differences for the regeneration percentage, they recorded 26.1, 25.4 and 24.4% for G98-24, Phil8013, G98-28 and GT54-9, respectively.

On the other hand, the effect of using 2, 4-D was clearly significant on the regeneration percentage. Sub culturing of the embryonic calli on a media with low concentration of 2,4-D enhanced the regeneration. Phil8013 and G98-24 regeneration percentages were 94 and 82.2 %, respectively, at 2, 4-D level of 1 mg/l. While the varieties G98-28 and GT54-9 preferred 2 mg/l 2, 4-D and recorded 87.7 and 84.2 %, respectively.

The interactions between the different treatments recorded significant effect on regeneration percentage. Decreasing the concentrations of 2, 4-D caused a significant increment in the obtained values. Variety G98-24 gave the highest value (100%) using 2,4-D by 1 mg/l combined with 10% coconut water, while Phil8013 recorded 97.7 % using 2,4-D by 1mg/l combined with either (0 or 5%) coconut water. On the other hand, G98-28 gave the highest regeneration percentages (75.5 and 72.1) at 2, 4-D by 1 mg/l combined with (5 or 10%) coconut water, respectively, with no significant differences between CW applications.

Attree and Fowke (1993) demonstrated that conditions favoring embryo

maturation also favor the recovery of plants.

Rooting percentages and root length

Rooting percentage was estimated by counting number of ex-plants rooted on the different concentrations of NAA and sucrose in order to decide the best combination that aids in the root elongation (Table 5).

The results reveal that using NAA in combination with different concentrations of sucrose had significant effect on rooting percentage. As for the influence of NAA on rooting percentage, data indicated that using NAA at the level of 7 mg/l significantly affected the rooting percentage in all varieties. The rooting percentages were 96.2, 92.8, 91.5 and 89.9% for the varieties G98-24, G98-28, GT54-9 and Phil8013, respectively.

Regarding the effect of sucrose concentrations, applying sucrose at 60 g/l significantly surpassed the other concentrations in rooting percentage. The rooting percentage recorded 78.3, 69.7, 69.4 and 65.5 % in G98-28, G98-24, GT54-9 and Phil8013, respectively. This result is in agreement with that of Gandonou *et al*. (2005).

The rooting% of varieties is also shown in Table (5), data revealed that there were significant effect between the examined varieties with respect to their rooting percentages. It could be noticed that G98-28, GT54-9, G98-24 significantly surpassed variety Phil 8013, as they recorded 66.2, 62, 60.9 and 58.4%, respectively.

The interactions between factors of the 1st and 2nd order were significantly affected rooting percentage. The highest rooting percentage was obtained by the combination of 7 mg/l NAA and 50 g/l sucrose with variety G98-24, and 7 mg/l NAA with 40 g/l sucrose with G98-28and Phil8013. On the other hand, GT54-9 recorded the highest rooting percentage with 7 mg/l NAA in combination with 60 g/l sucrose. Meanwhile the differences between 40, 50 and 60 g/l sucrose and 5 and 7 mg/l NAA did not reach the level of significance. These results are in agreement with Khalil (2002) who achieved the highest percentage (93.3%) of root formation by using NAA at the level of 7 mg/l with 60 g/l sucrose.

Using NAA at the level of 7 mg/l is significantly affected root length. The highest root lengths recorded were 4.5, 3.4, 2.8 and 2.7 cm for the varieties G98-24, G98-28, GT54-9 and Phil8013, respectively, as shown in Table (5).

Using sucrose with 60 g/l significantly surpassed the other concentrations with variety G98-24, as it gave the highest root length i.e. 2.7 cm. On the other hand, using sucrose at 50 and 60 g/l with the other studied varieties significantly affects this trait in comparison with the other studied concentrations. Also, the rooting % of the varieties under study was significant. Variety G98-24 significantly surpassed the other varieties followed by

G98-28, GT54-9 and Phil8013. They recorded 2.1, 1.7, 1.4 and 1.3, respectively.

Significant effect for the interaction between the studied factors was found. Using 7 mg/l NAA was superior with all varieties. While using 40, 50 and 60 g/l sucrose did not show any significant difference between them. This fact was true with all studied varieties except variety G98-24 which gave a highly significant value with 40 g/l sucrose. These results disagreed with Karim *et al.* (2002), who obtained the highest root length with 3mg/l NAA for the two studied varieties. On the contrary, our results are in agreement with those of Mamun *et al.* (2004), who used a combination of BA and NAA (2.5+0.5 mg/l) with two varieties and obtained 1.8 and 1.5 cm root length.

Number of leaves, number of shoots and length of shoots

Table (6) show the effect of NAA, sucrose applications and their interaction effect on the number of leaves on the main shoot. Regarding the effect of NAA application, it indicated that it had a significant effect on this trait. Also, it showed a different trends as the highest value was recorded with GT54-9 (5 leaves) at 7 mg/l NAA, while variety G98-24 gave the highest significant value with 5 mg/l NAA (4.2 leaves). The other two varieties i.e. Phil8013 and G98-28 did not show any significant difference between the 3, 5 and 7 mg/l NAA concentrations.

As for the influence of sucrose application, the result indicated that sucrose significantly affected leaves number of sugarcane varieties under study. Increasing sucrose percentage in media up to 60 g/l resulted in the highest leaves number. The differences between applying sucrose with 50 and 60 g/l were not significant with the varieties Phil8013 and GT54-9.

Variety Phil8013 showed a significant decrease in its number of leaves with respect to the other varieties. It gave 2.2 leaves on the main shoot, while the other varieties ranged between 3 and 3.5 leaves. However, the differences between the other varieties did not reach the level of significance.

The interactions between the studied factors of the 1st and 2nd order were significant. The lower dose of both NAA and sucrose significantly decreased the leaves number. Meanwhile there were no significant differences between the higher doses from both treatments i.e. 5 and 7 mg/l (NAA) and (40, 50 and 60) g/l sucrose.

The results in Table (6) showed the effect of different concentrations of NAA and sucrose and the interactions between them with respect to number of shoots.

Concerning number of shoots, the data indicated highly significant differences for NAA high concentration i.e. 7 mg/l on all studied varieties. Shoot numbers per explant ranged between 9.4-20.1

shoot/ex-plant for Phil8013 and G98-24, respectively.

The effect of sucrose application showed significant increment in shoots number as the applied concentration increased. However, there were no significant differences between the high concentrations i.e. 40, 50 and 60 g/l. This finding was true for all studied varieties except GT54-9 which gave the highest significant shoot number with 60 g/l.

Varieties responded in a highly significant manner with different media composition. G98-24 was the best followed by G98-28, GT54-9 and finally Phil8013, with 8.8, 6.4, 5.5 and 4.7 shoots, respectively.

Also, the interactions between all studied factors revealed a significant effect on this trait. It could be noticed that using NAA by 7 mg/l recorded the highest result. However, there was no significant effect between high sucrose concentrations i.e. 40, 50 and 60 g/l. This finding was true with all varieties, except GT54-9 which preferred the maximum sucrose concentrations. Our results are in agreement with Mamun *et al.* (2004) who used a combination of BA and NAA (2.5+0.5 mg/l) with two varieties and obtained 6.20 and 7 shoot/ex-plant, respectively.

It could be noticed from the data presented in Table (6) that shoot length trait was significantly affected by the different treatments. Data indicated significant superiority of NAA at high concentration i.e. 7 mg/l on all studied varieties. The shoot length ranged between 7-11 cm with Phil8013 and G98-28, respectively.

Concerning the effect of sucrose application, data showed significant increases in shoots length as the applied concentration increased. However, there were no significant differences between high concentrations i.e. 40, 50 and 60 g/l. The results ranged between 4-6.5 cm for GT54-9 and G98-28.

Both G98-28 and G28-24 showed a significant increment in this trait in comparison with the other two varieties. Both of them recorded 5.4 cm for the shoot length, while, both GT54-9 and Phil8013 gave 4 cm.

The influence of the interactions varied with the different varieties. However, they were all significant. Using sucrose by 40 g/l significantly increased the shoot length with G98-28 and G98-24. It reached 13.5 and 12.5 cm, respectively. On the other hand, there were no significant differences between 50 and 60 g/l with Phil8013 and GT54-9, respectively.

Some researchers obtained similar results but with different media composition for shoot elongation. Mohatkar *et al.* (1993) obtained a shoots of 6-7cm on culturing sterilized explants of sugarcane variety Co740 on semisolid MS medium containing 100 mg myo-inositol and 10% v/v coconut milk after 22 days. On the other hand, Patel *et al.* (2001) obtained the highest shoot length (7.36 and 6.33) for the studied varieties using 1 mg/l

kinetin+ 1mg/l BA+ 20% CW. Meanwhile, Mamun *et al.* (2004) used a combination of BA and NAA (2.5+0.5 mg/l) with two varieties and obtained 6.8 and 4.12 cm.

Figure (3) illustrated the establishment of regeneration protocol from callus to mature sugarcane plants

All acclimatized sugarcane plants were maintained under green house conditions and then were transferred to the open field and developed to mature plants, except GT54-9 variety. Young leaves were collected randomly as bulk from the four varieties and three regenerated genotypes i.e. Phil8013, G98-28 and G98-24, which were used to extract DNA and molecular analyses.

Identification of somaclonal variation induced through tissue culture based on RAPD-PCR

Six oligonucleotides (1, 2, 3, 4, 5 and 6) were selected based on the repeatability of their amplification profiles. Five of the six primers successfully amplified DNA fragments for all genotypes as shown in Table (7) and Fig. (4). Fragments ranged in size from 158 bp to 1550 bp. The original pattern of each variety was partially similar to their somaclones i.e. Phil 8013 R, G98-28R and G98-24 R. Twenty eight out of 46 loci were found to be polymorphic in the regenerated genotypes, as detected by primer 2=5`-(GTTTCGCTCC)-3` (one locus) for Phil 8013R, four loci for G98-28R and two loci for G98-24R, while primer 3=5`-(GTAGACCCGT)-3` (three loci) for Phil 8013R, one locus for G98-28R and three loci for G98-24R, whereas primer 4=5`-(AAGAGCCCG T)-3`(four loci) for Phil 8013R, three loci for G98-28R and two loci for G98-24R, while primer 5=5`-(AACGCGCAAC)-3`(two loci) for Phil 8013R, one locus for G98-24R and primer 6=5`-(CCCGTCAGCA)-3` (one locus) for Phil 8013R and one locus for G98-28R, corresponding to a 23.9% ,19.6% and 17.4% rate of polymorphism for Phil 8013R, G98-28R and G98-24R, respectively.

Tissue culture was thus responsible for the generation of new variability, since an increase in the rate of molecular polymorphism was observed. Heinz and Mee (1971), working with callus-derived cultures from sugarcane variety H50-7209, detected clones with chromosomal numbers ranging from 2n = 94 to 120. In contrast, chromosome stability was described for varieties NA56-79 (2n = 114) and Co419 (2n = 213) by Silvarolla and Aguiar-Perecin (1994), who developed a technique to obtain intact somatic metaphase sugarcane cells. Together, these observations suggest either that some genotypes are more susceptible to somaclonal variation, or that the *in vitro* instability is actually a consequence of a genotype *versus* culture medium interaction.

The RAPD technique reveals DNA polymorphisms as differences in the amplification patterns, and uses primers of random sequences that search for

complementarity in the genome. It is suggested that RAPD bands possibly represent mainly repetitive DNA (Grattapaglia and Sederoff, 1994). Polymorphism in repetitive DNA sequences has frequently been observed during plant propagation by tissue culture (Smulders *et al*., 1995) and undergoes more alterations than the coding sequences. In vitro stress may provoke changes at preferential sites, such as repetitive DNA, thereby activating transposable elements.

Similarity index (as percentage) based on RAPD-PCR analysis using UPGMA computer analysis (Table 8) between the regenerated varieties and their parental lines Phil 8013, G98-28 and G98-24 were 84%, 85% and 88% respectively, which indicated that there were induction of mutation *via* somaclonal variation through tissue culture.

A dendrogram for the genetic relationships among the four sugarcane varieties and their three regenerated genotypes was developed based on the banding patterns as in Fig. (5). The four sugarcane varieties and their three regenerated genotypes were separated into two clusters; cluster one included Phil8013 and R. Phil8013 (regenerated genotype derived from Phil8013 *via* tissue culture), cluster 2 included the other three varieties and their regenerated ones. Within cluster 2, one subcluster contained one sugarcane variety G98-28, while the second subcluster contained two sub-sub clusters, the first one, contained R. G98-28 and G98-24, while, the other one contained R. G98-24 and GT 54-9.

The polymorphism in the amplification products may be either from changes in the sequence of the primer in binding site (e.g. point mutations) or changes which alter the size or prevent successful amplification of a target DNA (e.g. insertions, deletions or inversion) as suggested by Rani *et al.* (1995). Also, Larkin and Scowcroft (1981) reported that *in vitro* culture environment is mutagenic. The molecular mechanism underlying somaclonal variations have been attributed to chromosome breakage, single base changes, and changes in copy number of repeated sequences and alternation in DNA methylation patterns (Munthali *et al*., 1996).

Our results are in agreement with Attree *et al*. (2000) who reported that somatic embryogenesis provides a regeneration system suitable for producing genetically modified plants. The genetic changes detected here may be due to the interaction between culture conditions, including growth regulators.

The results confirmed that *in vitro* culture of the sugarcane is very efficient and has tremendous potential for producing novel and useful varieties.

SUMMARY

Sugarcane plants of cv. Ph8013, G98/28, G98/24 and GT54-C9 were used for somaclonal variation induction using

the spindle leaves as ex-plants. The used varieties differed according to the vegetative traits, juice quality and their similarity index. The effect of five concentrations from 2,4-D and three concentrations of coconut water were examined for the somatic embryogenesis induction. The 2, 4-D was applied at 0, 1, 2, 3, 4 and 5 mg/l in combination with 0, 5 and 10 g/l coconut water. Also, the effect of using NAA at five concentrations i.e. 0, 1, 3, 5 and 7 mg/l in combination with five concentrations of sucrose i.e. 20, 30, 40 50 and 60 g/l were examined to obtain the highest root and shoot elongation. The obtained results indicated the different responses of studied varieties with the different treatments of 2,4-D and coconut water. The varieties gave the highest callus induction percentage and somatic embryogenesis callus induction percentage with different concentrations of 2, 4-D in range between 2 - 4 mg/l. Lowering 2, 4-D to 1 mg/l enhanced the embryos development. On the other hand, all the varieties responded to 7 mg/l NAA but differ with the sucrose concentrations, they ranged between 40, 50 and 60 g/l. Molecular genetic studies were done to assess the somaclonal variation induced through tissue culture compared to the parental varieties. Five out of six primers succeeded in amplifying DNA fragments. Tissue culture was thus responsible for the generation of new variability, since an increase in the rate of molecular polymorphism was observed. Twenty eight out of 46 loci were found to be polymorphic in the regenerated genotypes. Similarity index (as percentage) based on RAPD-PCR analysis using UPGMA computer analysis between the regenerated varieties and their parental lines Phil. 8013, G98-28 and G98-24 were 84%, 85% and 88% respectively, which indicated the induction of mutation *via* somaclonal variation through tissue culture. The consensus tree was developed based on the banding patterns of the four varieties and their three regenerated genotypes using SPSS statistical analysis program to study the genetic relationships among these genotypes at the molecular level. The results confirmed that *in vitro* culture of the sugarcane is very efficient and has tremendous potential for producing novel and useful varieties.

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Table (1): Pedigree and origin of the studied sugarcane varieties.

Cultivar	Pedidegree	Source
Phil8013	CAC71-312 X Ph 642227	Philippine
G98-28	C34-33 x polycross	Egypt
G98-24	C34-33 x polycross	Egypt
GT54-9	NC0310 X f37/925	Taiwan

Table (2): Vegetative traits and the technological traits for the four varieties used as mother plants.

Varieties	Vegetative traits				
	Stalk diameter (cm)	Stalk length (cm)	Number of internodes	Stalk yield (ton)	Number of plants/fed. (thousands)
Phil8013	2.9	309.3	18.3	54.93	51.6
G98-28	2.8	307.3	16.6	59.00	44.7
G98-24	2.7	290.0	18.0	52.10	49.0
GT54-C9	3.1	308.0	22.0	55.10	52.5
LSD at 0.05	ns	5.6	1.5	2.70	2.5

Varieties	Juice Quality (technological traits)					
	TSS % (W)	TSS % (V)	Sucrose %	Purity %	Richness %	Sugar recovery %
Phil8013	21.4	23.55	19.70	79.4	14.6	12.3
G98-28	20.4	22.00	18.01	73.3	13.3	10.7
G98-24	21.8	23.80	20.20	84.1	14.6	12.4
GT54-C9	21.4	23.30	19.16	74.2	14.5	12.3
LSD at 0.05	0.5	0.70	0.70	ns	0.7	0.5

Table (3): Similarity index as percentage among the four sugarcane varieties based on their vegetative traits and technological traits.

	Phil 8013	G98-28	G98-24
G98-28	81		
G98-24	80	**85**	
GT 54-9	78	80	82

Table (4): Callus induction percentage, embryonic callus induction percentage and regeneration for the four varieties under the different concentrations of 2, 4-D and coconut water applications.

Varieties	CW v/v	Callus induction % (CI)							Embryonic callus induction% (ECI)							Regeneration% (Reg.)						
		2,4-D mgl¯1																				
		0	1	2	3	4	5	Mean	0	١	٢	3	4	5	Mean	0	1	2	3	4	5	Mean
Phil8013	0	33.5	67.5	91.6	79.4	83.9	89.6	74.2	0.0	75.4	53.3	63.9	81.6	27.2	50.3	0.0	97.7	58.7	0.0	0.0	0.0	27.0
	5	25.2	94.4	93.3	88.3	88.3	90.0	79.9	0.0	94.4	76.6	77.5	100	0.0	58.1	0.0	97.7	53.3	0.0	0.0	0.0	26.0
	10	26.6	68.8	70.0	100	88.6	95.0	99.8	0.0	62.2	66.7	67.6	100	0.0	49.4	0.0	86.6	58.8	0.0	0.0	0.0	23.3
Mean		28.4	77.0	85.0	89.2	86.2	91.4	84.7	0.0	0.0	65.5	69.7	93.8	9.2	52.6	0.0	0.0	56.6	0.0	0.0	0.0	0.0
98-28	0	0.20	63.6	65.3	63.3	78.1	94.3	60.8	0.0	38.6	66.0	65.5	71.7	50.0	48.6	0.0	62.2	50.0	0.0	0.1	0.0	19.8
	5	21.0	77.5	63.5	80.0	90.0	83.1	69.1	0.0	38.6	62.0	65.4	98.8	0.0	44.1	0.0	75.5	44.4	0.0	38.8	0.0	27.4
	10	37.7	67.7	62.7	74.3	97.6	91.6	71.9	0.0	60.6	63.3	60.0	94.4	0.0	46.4	0.0	72.1	50.3	0.0	39.3	0.0	25.9
Mean		19.6	69.6	63.8	72.5	88.5	89.7	67.3	0.0	0.0	63.7	63.6	88.3	16.6	46.4	0.0	0.0	87.7	0.0	0.0	0.0	0.0
G98-24	0	5.54	78.8	75.5	50.0	90.0	87.7	64.6	0.0	60.0	72.2	59.7	92.1	44.4	54.7	0.0	71.0	68.8	0.0	0.0	0.0	26.4
	5	6.53	80.0	66.6	71.1	77.7	76.6	63.1	0.0	63.2	69.9	61.0	88.8	19.9	50.5	0.0	75.5	68.6	0.0	0.0	0.0	24.0
	10	8.83	95.0	75.5	90.0	87.7	73.3	71.8	0.0	100.0	65.5	78.8	94.4	0.0	56.4	0.0	100	67.7	0.0	0.0	0.0	27.9
Mean		6.9	84.8	72.5	70.3	85.1	79.2	66.5	0.0	0.0	69.2	66.5	91.8	21.4	53.9	0.0	0.0	74.7	0.0	0.0	0.0	0.0
GT54-9	0	0.0	70.3	67.7	74.0	90.0	86.6	64.8	0.0	78.8	74.4	52.2	64.4	55.5	54.2	0.0	47.7	83.8	0.0	0.0	0.0	21.8
	5	17.7	67.3	92.2	88.8	97.7	81.1	74.2	0.0	76.6	91.1	56.6	65.5	0.0	48.3	0.0	56.6	88.8	0.0	0.0	0.0	24.2
	10	35.3	55.5	94.4	92.2	87.6	88.8	75.7	0.0	66.6	100.0	62.2	64.4	0.0	48.8	0.0	83.3	81.1	0.0	0.0	0.0	27.4
Mean		17.7	64.4	84.8	85.1	91.8	85.5	71.5	0.0	0.0	88.5	57.0	64.7	18.5	50.4	0.0	0.0	84.4	0.0	0.0	0.0	0.0
Mean CW	0	9.8	70	75.0	66.7	8.5	89.5	66.1	0.0	63.3	66.5	60.3	77.5	44.4	52.0	0.0	12.5	14.6	1.3	3.7	0.4	15.3
	5	17.6	79.9	78.9	82.0	88.4	82.7	71.6	0.0	68.3	75.0	65.1	88.3	4.9	50.3	0.0	12.7	14.0	1.2	8.6	0.0	17.4
	10	64.9	72	75.6	89.1	90.4	87.2	79.8	0.0	72.3	73.9	67.2	88.3	0.0	50.3	0.0	14.9	11.8	1.7	7.4	0.0	16.9
General Mean		30.7	73.9	76.6	79.3	88.1	86.5		0.0	0.0	71.8	64.2	84.7	16.5		0.0	0.0	30.8	12	27.9	3.1	

LSD at 0.05 level of significance

	Varieties (V)	2,4-D	CW	V x 2,4-D	V x CW	2,4-D x CW	V x 2,4-D x CW
CI	1.35*	16.7*	NS	NS	NS	NS	NS
ECI	3.19*	3.92*	NS	7.84*	5.54*	6.77*	13.5*
Reg.	NS	3.44*	NS	6.89*	4.87*	5.96*	11.9*

Table (5): The rooting percentages and root lengths of the four varieties under the different concentrations of NAA and sucrose applications.

Varieties	NAA mg/l	Rooting percentage						Root length (cm)					
		Sucrose (g/l)						Sucrose (g/l)					
		20	30	40	50	60	Mean	20	30	40	50	60	Mean
Phil8013	0	0	0.0	4.0	5.0	12.5	4.3	0.0	0.0	0.1	0.2	0.5	0.15
	1	17.5	40.0	43.5	52.5	65.5	43.2	0.4	0.5	0.5	0.7	0.9	0.6
	3	66.5	70.5	71.0	74.0	75.0	71.5	1.0	1.1	1.5	1.4	1.5	1.28
	5	75.0	80.5	85.5	86.5	87.5	83.0	1.5	1.5	1.4	1.9	2.0	1.65
	7	88.5	89.5	91.0	90.5	90.0	89.9	2.0	2.1	3.3	3.4	3.0	2.7
Mean		49.5	56.1	59.0	61.8	65.5	58.4	1.0	1.0	1.3	1.5	1.6	1.3
G98-28	0	10.0	13.0	20.5	27.5	50.0	25.4	0.1	0.7	0.8	0.8	0.8	0.48
	1	30.0	35.0	47.5	68.0	69.0	49.9	0.4	1.0	1.0	1.2	2.0	1.1
	3	62.5	82.5	78.5	80.0	81.5	77.0	0.7	1.2	1.3	1.5	1.4	1.2
	5	72.5	77.5	92.5	96.5	95.0	86.8	1.2	1.9	2.5	3.2	2.2	2.19
	7	80.0	92.0	97.5	95.0	96.0	92.8	1.6	3.1	4.5	3.8	3.8	3.4
Mean		51.0	60.0	68.5	73.4	78.3	66.2	0.7	1.4	2.0	2.1	2.0	1.7
G98-24	0	0.0	5.0	10.0	13.5	6.0	0.9	0.0	0.1	0.4	0.4	0.4	0.25
	1	27.0	37.5	59.5	37.5	57.5	39.8	0.5	1.2	1.0	0.8	1.25	0.9
	3	52.5	72.5	75.0	75.8	77.5	70.65	1.0	1.7	1.6	1.9	2.0	1.62
	5	67.5	87.0	92.5	99.5	92.5	87.8	1.8	3.0	3.2	4.5	4.5	3.4
	7	87.5	99.5	99.0	100.0	95.0	96.2	3.5	4.0	5.1	5.0	5.3	4.5
Mean		46.9	59.4	63.2	65.2	69.7	60.9	1.4	2.0	2.2	2.5	2.7	2.1
GT54-9	0	0.0	1.0	4.5	10.0	22.5	7.6	0.0	0.2	0.5	0.7	0.9	0.5
	1	37.5	40.0	42.0	55.0	56.0	46.1	0.6	0.6	1.0	1.1	1.4	0.9
	3	75.0	80.0	80.0	75.0	84.5	78.9	1.3	1.1	1.4	1.3	1.5	1.3
	5	80.0	85.0	85.0	88.5	89.0	85.4	1.4	1.6	1.8	1.9	1.9	1.7
	7	89.5	90.7	91.0	91.5	95.0	91.5	1.9	2.0	3.0	3.5	3.5	2.8
Mean		56.3	59.3	60.5	64.0	69.4	62.0	1.0	1.0	1.5	1.7	1.8	1.4
Mean NAA	0	2.5	3.6	11.3	14.0	27.8	11.8	0.02	0.1	0.4	0.5	0.6	0.3
	1	28.0	38.1	43.1	53.3	61.2	44.8	0.5	0.8	0.8	0.9	1.4	0.9
	3	64.1	76.4	76.1	76.3	79.6	74.5	1.0	1.2	1.4	1.5	1.6	1.3
	5	73.7	82.5	88.9	92.7	91	85.8	1.4	2.0	2.2	2.9	2.6	2.2
	7	86.4	93.0	94.6	94.3	94	92.4	2.2	2.8	4.0	3.9	3.9	3.4
General Mean		50.9	58.7	62.8	66.1	70.7		1.0	1.4	1.8	1.9	2.0	

LSD at 0.05 level of significance		
Varieties	1.40*	0.10*
NAA	1.62*	0.12*
Sucrose	1.62*	0.12*
NAA x Sucrose	3.60*	0.28*
V x NAA x Sucrose	7.20*	0.55*

Table (6): Number of leaves, number of shoots and length of shoots for the four varieties under the different concentrations of NAA and sucrose applications.

Varieties	NAA mg/l	Number of leaves						Number of Shoots						Length of shoots (cm)					
		Sucrose (g/l)						Sucrose (g/l)						Sucrose (g/l)					
		20	30	40	50	60	Mean	20	30	40	50	60	Mean	20	30	40	50	60	Mean
Phil8013	0	1.0	1.0	1.5	1.5	1.5	1.8	1.5	1.0	2.0	2.5	2.0	1.8	0.9	1.1	1.2	1.8	2.2	1.4
	1	1.5	1.5	1.5	1.5	1.5	2.1	2.0	2.5	2.0	2.5	3.0	2.4	2.5	3.0	3.3	3.0	3.0	3.0
	3	1.0	2.0	1.5	1.5	1.5	2.5	3.5	5.0	4.0	4.0	5.0	4.3	3.3	2.9	3.1	3.3	3.1	3.0
	5	2.5	2.5	3.0	4.5	4.0	2.5	4.5	5.5	6.0	6.0	5.0	5.4	3.2	3.0	4.5	5.6	7.3	4.0
	7	3.0	3.5	5.0	3.5	3.0	2.3	7.0	8.5	11.0	10.5	10.0	9.4	6.0	6.8	7.5	7.8	7.3	7.0
Mean		1.3	11.5	1.5	3.3	3.6	2.2	3.7	2.5	5.0	5.1	5.0	4.7	3.0	3.7	4.2	4.6	4.5	4.0
G98-28	0	1.5	1.0	2.0	1.5	2.5	2.4	1.5	1.5	2.0	2.0	3.5	2.1	1.7	1.5	2.3	2.5	2.8	2.2
	1	2.0	2.0	2.0	1.5	1.5	3.0	1.5	2.0	3.0	3.0	3.0	2.5	1.7	1.8	2.3	2.0	2.3	2.0
	3	2.5	3.5	3.5	3.5	3.0	3.6	4.0	8.5	4.5	4.0	3.5	4.9	2.9	6.8	7.3	7.3	7.3	6.3
	5	2.5	3.0	5.5	5.5	5.5	3.6	4.0	6.0	10.0	11.5	5.5	7.4	2.5	3.8	7.4	8.0	6.0	5.6
	7	3.5	5.5	6.5	6.0	5.0	3.5	7.0	12.5	18.5	17.5	19.5	15.0	8.0	8.5	13.5	12.5	12.0	11.0
Mean		1.7	1.8	3.2	4.1	5.3	3.2	3.6	6.1	7.6	7.6	7.0	6.4	3.4	4.5	6.5	6.5	6.1	5.4
G98-24	0	2.0	2.0	2.0	2.5	2.5	2.7	2.0	2.0	2.0	3.0	5.0	2.3	1.9	2.0	2.1	2.3	3.5	2.3
	1	1.5	2.0	2.5	2.5	2.0	3.2	3.5	4.0	4.0	3.5	6.0	4.2	1.5	2.5	12.6	2.4	2.5	2.2
	3	1.5	3.3	3.5	4.0	3.5	3.8	4.5	6.0	5.0	7.5	6.5	5.9	3.5	3.8	3.5	4.5	5.5	4.2
	5	3.5	4.5	5.0	4.5	5.0	4.2	9.0	9.5	13.0	14.5	12.5	11.7	7.0	7.2	7.7	8.7	9.5	8.1
	7	5.0	5.5	7.0	7.5	6.0	3.8	18.5	20.5	21.0	20.0	20.5	20.1	8.5	11.5	12.2	9.5	9.5	10.3
Mean		2.2	2.1	3.0	4.2	6.2	3.5	7.5	8.4	9.0	9.5	9.8	8.8	4.5	5.4	5.6	5.5	6.1	5.4
GT54-9	0	1.0	1.0	1.0	1.5	1.5	1.2	2.0	2.0	2.0	2.0	2.5	2.1	1.8	2.0	2.0	2.0	1.9	2.0
	1	2.0	2.5	2.5	2.5	3.0	2.5	2.5	2.5	3.0	3.0	4.0	3.0	2.0	2.5	2.4	2.6	2.9	2.5
	3	2.0	2.5	2.5	2.5	2.5	2.4	3.5	4.0	4.0	4.5	5.0	4.2	2.8	2.9	3.0	3.0	2.8	2.9
	5	3.0	3.0	3.5	3.5	4.0	3.4	5.5	6.0	6.0	6.5	8.0	6.4	4.5	4.5	6.0	5.9	6.0	0.3
	7	5.0	5.0	5.0	5.0	5.0	5.0	9.5	10.0	10.0	11.5	17.5	11.7	6.4	7.0	6.9	8.5	7.0	7.3
Mean		2.6	2.8	2.9	3.0	3.2	3.0	4.6	4.9	5.0	5.5	7.4	5.5	3.5	3.8	4.0	4.4	4.2	4.0
Mean NAA	0	2.4	2.8	3.2	3.3	3.2	1.6	1.8	1.6	2.0	2.1	2.9	2.1	1.6	1.7	1.9	2.1	2.6	2.0
	1	1.4	1.3	1.6	1.8	2.0	2.0	2.4	2.8	3.0	3.0	4.0	3.0	1.9	2.5	2.6	2.6	2.7	2.5
	3	1.8	2.0	2.1	2.0	2.0	2.5	3.9	5.9	4.4	5.0	5.0	4.8	3.0	4.1	4.3	4.5	4.7	4.1
	5	1.8	2.8	2.6	2.8	2.8	3.8	5.8	6.8	8.8	9.6	7.8	7.7	4.2	5.0	6.7	7.5	7.1	6.1
	7	2.9	3.0	3.8	4.6	4.5	5.0	10.5	12.9	15.1	14.9	16.9	14.0	7.2	8.4	10.1	9.6	9.1	8.9
General Mean		4.1	4.9	5.9	5.5	4.8		4.9	6.0	6.7	7.0	7.3		3.6	4.3	5.1	5.2	5.2	
LSD at 0.05 level of significance																			
Varieties		0.31*						0.67*						0.33*					
NAA		0.35*						0.75*						0.37*					
Sucrose		0.35*						0.75*						0.37*					
NAA x Sucrose		0.8 *						1.7 *						0.86*					
V x NAA x Sucrose		1.6 *						3.36*						1.69*					

Table (7): DNA polymorphisms detected in the regenerated genotypes in comparison to the original varieties profile.

RAPD Primers	Sequence	Molecular Size (bp)		Loci generated	Polymorphic fragments (bp)		
		Max.	Min.		Phil 8013 (R)	G98-28 (R)	G98-24 (R)
2	5`-(GTTTCGCTCC)-3`	1550	200	12	793	1550 573 543 350	793 573
3	5`-(GTAGACCCGT)-3`	947	280	8	947 647 544	490	947 824 544
4	5`-(AAGAGCCCG T)-3`	1173	255	9	1173 1000 960 800	1173 800 700	800 700
5	5`-(AACGCGCAAC)-3`	1203	158	9	1000 871	-	1203
6	5`-(CCCGTCAGCA)-3`	1330	204	8	1330	1200	-

Table (8): Similarity index as percentage (pair wise comparison) among the four sugarcane varieties and three regenerated varieties based on RAPD-PCR analysis.

	Phil 8013	Phil 8013 (R)	G98-28	G98-28 (R)	G98-24	G98-24 (R)
Phil 8013 (R)	84					
G98-28	81	79				
G98-28 (R)	83	81	85			
G98-24	80	78	85	90		
G98-24 (R)	84	82	83	90	88	
GT 54-9	78	76	80	88	82	92

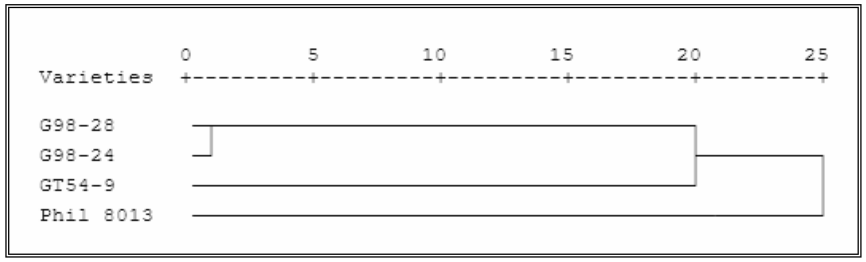


Fig. (1): Dendrogram for the genetic distances between the four sugarcane varieties based on their vegetative traits and technological traits.

8 cm spindle leaves.

2-3 mm ex-plant.

Callus initiation 10-15 days after culturing on 3 mg/l 2,4-D and 10% CW.

Fig. (2). Callus induction of Phil 8013 variety

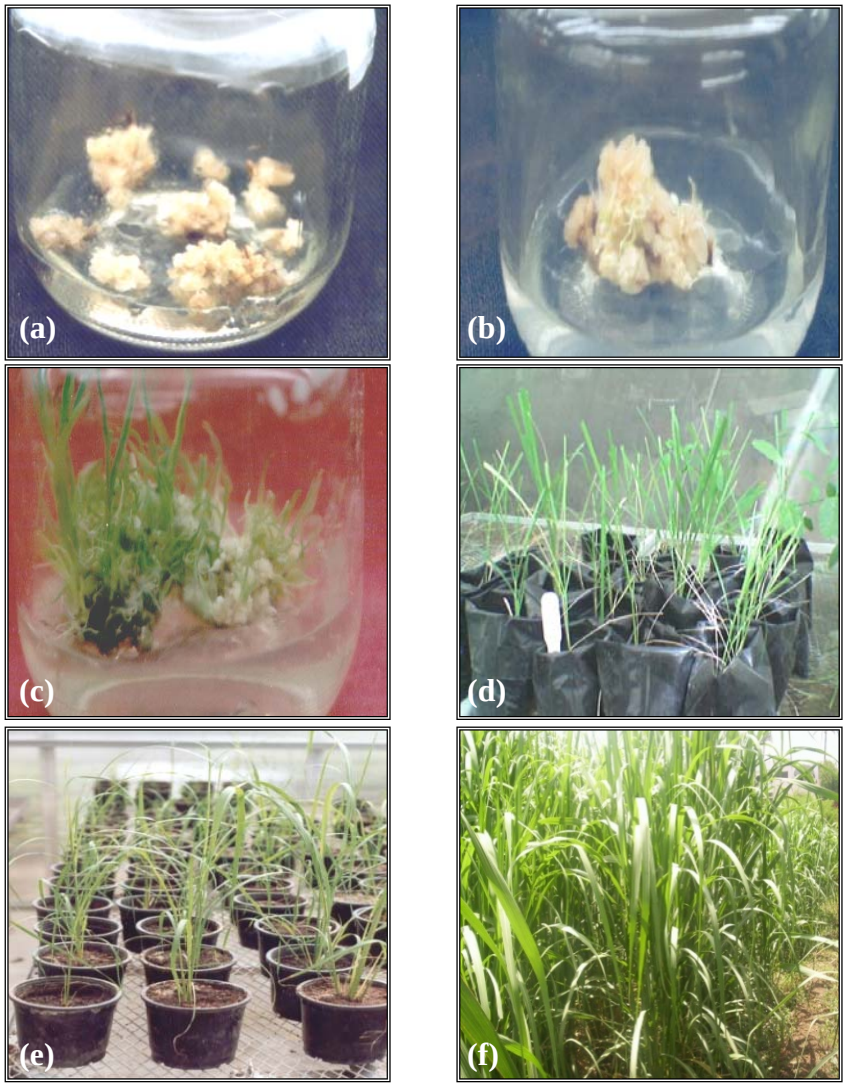
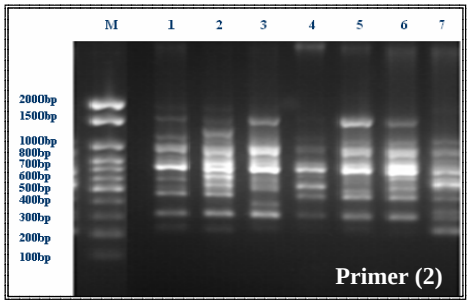
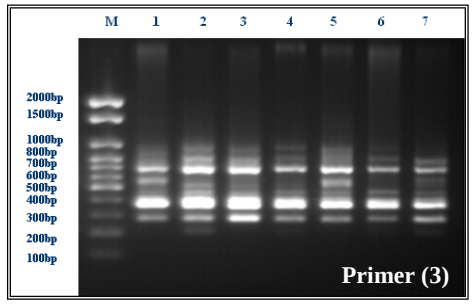
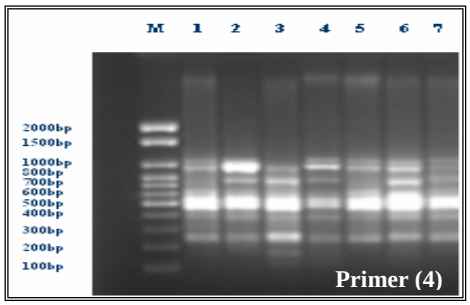


Fig. (3): The establishment of somatic embryogenesis in variety Phil8013 and the acclimatization steps under green house conditions. (a) Somatic embryogenesis callus on 4 mg/l 2, 4-D and 5% CW. (b) Differentiation stage on 1 mg/l 2,4-D and 0 or 5%CW. (c) Development and regenerated plants on 1 mg/l 2, 4-D and 0 or 5%CW. (d) Sugarcane plants after approximately 4 weeks. (e) Plants in larger pots after 6 weeks. (f) Plants in the open field 9 months after planting.







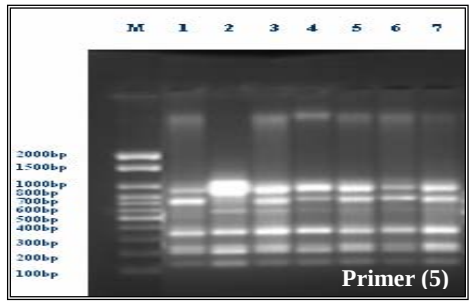
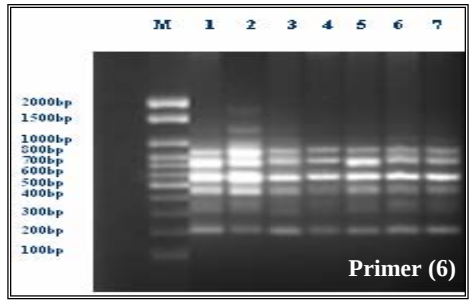


Fig. (4): DNA polymorphism of the four sugarcane varieties and their three regenerated genotypes amplified with five primers.

(1)- Phil 8013 (2)- Phil 8013 (R)
(3)- G98-28 (4)- G98-28 (R)
(5)- G98-24 (6)- G98-24 (R)
(7)- GT 54-9 (M)- DNA ladder



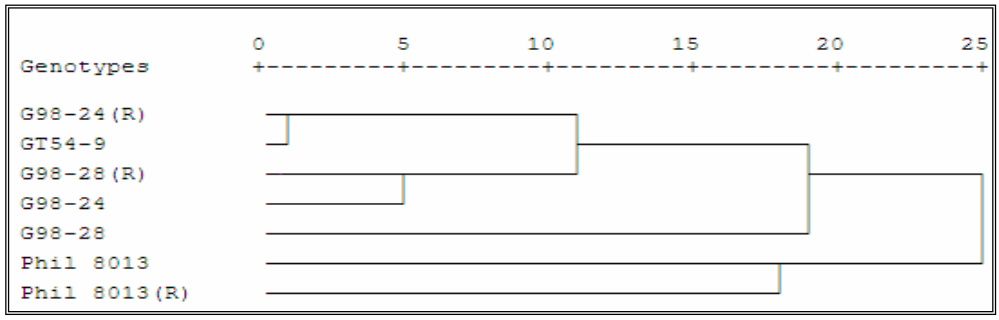


Fig. (5): Dendrogram for the genetic distances between the four sugarcane varieties and their three regenerated varieties based on RAPD-PCR analysis.