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Evaluation of Genetic Variation in Mutants of *Senna occidentalis* using Protein Pattern and RAPD Markers

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Abstract: Seeds of *Senna occidentalis* were soaked in EMS at 0, 1000, 2000, 3000, 4000 and 5000 ppm and DMS at 200, 400, 800, 1600 and 3200 ppm, to produce new patterns of vegetative and flowering growth. Nine mutants were found, then analysis of protein electrophoresis pattern and RAPD markers were used to evaluate the obtained-genetic variations. Total protein in the wild type (control) and all mutant lines gave band patterns ranged from 17 to 135 kD. The 48 kD band was produced by the mutants EMST3 (2000 ppm), EMST6 (5000 ppm), DMST3 (200 ppm) and DMST4 (400). Approximately 30 kD band was produced with the mutant EMST5 (4000 ppm). Protein of molecular weight 95 and 26 kD were disappeared in the mutant EMST5 (4000 ppm). Polymorphic bands observed using RAPD markers. These polymorphic bands could be very valuable for DNA fingerprinting and identification of mutant lines and the wild strain control plant. The phylogenetic tree grouped the mutants into two main clusters (A, B). The mutants obtained after EMS treatment and control were scored in cluster A. While others obtained after DMS treatment were scored in the cluster B.

Key words: *Senna occidentalis*, protein pattern, RAPD markers, genetic variations, EMS, DMS

INTRODUCTION

Senna occidentalis (L.) Link (Syn. *Cassia occidentalis* L.) is a shrub belonging to the family Fabaceae. It grows throughout the tropics and subtropics. It reaches heights of about two meters and produces yellow flowers in the leaf axils. It is used for landscape purposes as medium size- flowering shrubs and as a source of colour during the warm months of the year (Helmy, 2004). *Senna occidentalis* has an enormous potential for use as an ornamental shrub, medicinal plant and for pest control (Caceues *et al*., 1991).

Induced mutation using physical and chemical mutagen is a method to create genetic variation resulting in new varieties with better characteristics. The most powerful chemical mutagens are found among alkylating agents. Alkylating agents as Ethyl Methane Sulphonate (EMS), Diethyl Sulphate (DES) and Sodium Azide (SA) are very reactive compounds which can add an alkyl group (such as ethyl or methyl) at various positions on DNA bases and thereby alter base pairing properties. These mutagens cause both transitions and transversions in replicating or non-replicating DNA. Alkylating agents were found to be very effective in inducing morphological mutations in plants as found by El-Nashar (2006), Floria (2006), Al-Gawwad and Makka (2009) and Mostafa (2009, 2010).

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments generated by the amplification of genomic DNA through PCR reaction using a single primer or arbitrary nucleotide sequences in each reaction. The most important advantage is that no previous knowledge of the genome is required for its application, which makes this method suitable for the analysis of non- studied species. RAPD markers are simple, consistent and can be analyzed at any stage of plant growth (Ling *et al*., 1997). RAPD also has profoundly increased the potential to easily, quickly and inexpensively detect genetic polymorphism among organisms (Nebaure *et al*., 1999). This method uses genomic DNA as a template and does not employ radioisotopes, DNA probes or prior sequence information but only requires nanograms of DNA per reaction (William *et al*., 1990; Erdem and Oldacay, 2004).

The aims of this investigation were to study the effect of Ethyl Methan Sulphonate (EMS) and Dimethyl Sulphate (DMS) treatments on producing new patterns of vegetative and flowering growth of *Senna occidentalis*.

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Also to evaluate the obtained-genetic variations along with parent (control, wild type) using protein patterns and RAPD profile analyses.

MATERIALS AND METHODS

Plant material and treatment application: Seeds of *Senna occidentalis* were obtained from Antoniadis Botanical Garden, Alexandria. The seeds were soaked in boiled water on May 2008 for 18 h during which the water temperature decreased gradually up to room temperature. Then seeds were soaked in (EMS) solution (1000, 2000, 3000, 4000, 5000 ppm) and (DMS) solution (200, 400, 800, 1600, 3200 ppm) for 6 h while the control seeds were soaked in water for the same period of soaking. Seeds were washed with distilled water and sown in 25 cm. plastic pots containing a soil mixture of clay and sand (1:1 v/v). One hundred and fifty seeds were sown for each treatment. After 45 days from sowing, seedlings were transplanted into 25 cm diameter plastic pots containing a soil mixture of sand and clay (3:1, v/v). This work was carried out at the nursery of Ornamental Plants, Faculty of Agriculture, South Valley University, Qena, Egypt during the two successive seasons of 2008/2009 and 2009/2010.

All plants of the different treatments were examined daily to search for morphological variation in the vegetative and inflorescences growth of each treatment. Selfings were carried out in all changed plants to produce M2 generation and investigate the genetic bases of these changes. The procedure of germination and planting were done as in M1 generation. The mutants were identified using protein electrophoresis pattern and RAPD marker analysis.

Total protein and SDS-PAGE: For extraction of protein, 2 g fresh weight of plant leaves and 10 mL of extraction buffer (0.5 M Tris-HCl (pH 6.8), 10% sucrose, 2% SDS and 5% 2-mercaptoethanol) were grinded together in mortar. The slurry was centrifuged at 5000 rpm for 20 min. Three milliliter of ammonium sulphate solution were added 1 mL^{-1} of the supernatant to precipitate the proteins then kept over night in a refrigerator. It was then centrifuged at 5000 rpm for 20 min and the pellet was washed two or three times in 70% acetone. SDS-PAGE was performed by the method described by Laemmli (1970). Protein were analyzed on 1.5 mm thick and 15 cm long gels run in a dual vertical slab unit (Hoefer Scientific Instruments, San Francisco, CA, USA, MODEL SE 600 Series Hoefer, Pharmacia Biotech). From each sample, 50 μL of extract was loaded into a polyacrylamide gel. The separation gel (12%) and staking (4%) were prepared from an acrylamide monomer solution. Electrophoresis was carried out at constant current of 35 mA through the stacking gel and at 90 mA through the separation gel at 4°C After electrophoresis, the gel was stained by Coomassie Brilliant Blue R-250 and the Molecular Weight (MW) of protein corresponding to each band was calculated by protein marker.

DNA extraction and RAPD analysis: Genomic DNA from young leaves was extracted using DNeasy mini plant kit according to manufacturer's instructions (QIAGEN). RAPD analysis of DNA was performed using eight primers (Table 1). The amplification was done in volume 25 μL consists of 10x Taq polymerase buffer, 50 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP, dGTP, 25.0 p moles of RAPD primers, 10 ng of genomic DNA and 0.5 U Taq DNA polymerase (Promega). The amplification was performed by including the reaction mixture for 45 cycles in a thermocycler Gene Amp 9700. Each cycle consisted of denaturation at 95°C for 1 min followed by annealing at 30°C for 1 min and extension at 72°C for 1 min with initial delay for 5 min at 95°C at the beginning of the first cycle and post extension step for 10 min at 72°C after the end of the last cycle (Ting and Manos, 1990). PCR products were then separated on agarose gel electrophoresis using 1.5% (w/v) agarose in 0.5x TBE buffer. The size of each band was estimated by using DNA molecular weight marker. Finally, the gel was photographed by using gel documentation system.

Table 1: Primers' sequences employed in the RAPD-PCR

Primers	Sequence 5′ → 3′
OPA 01	CAGGCCCTTC
OPA 07	GAAACGGGTG
OPA 14	CCAGCCGAAC
P1	CGCTGTCGCC
NS1	GTAGTCATATGCTTGTCTC
NAH1	TTCGACAATGGCGTAGGTCCAGACCTCGGT
A9B7	GGT GAC GCA GGG GTA ACG CC
A7A10	GAA ACG GGT GGT GAT CGC AG

RAPD band pattern analysis: Polymorphism bands were scored as present or absent in all treatments, bands in each treatment were analyzed for clustering using the formula of Nei and Li (1979). A similarity coefficient was used for cluster analysis following the UPGMA (unweighted pair grouping method of averages). The resulting clustering was presented as a dendrograme.

RESULTS AND DISCUSSION

Induction of variations: The concentration of 1000 ppm EMS produced two plants having flowers with six and seven petals while the control plants having five petals as shown in Fig. 1. The treatments of 2000 and 3000 ppm produced plants taller than the control. Plants with highly

Fig. 1: Photograph showing flowers of *Senna occidentalis* which having six and seven petals produced by the treatments of EMS at 1000 ppm compared to the control (at the left)

Fig. 2: Photograph showing taller and early flowering plants of *Senna occidentalis* produced by the treatments of EMS at 3000 and 4000 ppm

Fig. 3: Photograph showing taller and early flowering plant of *Senna occidentalis* produced by the treatment of EMS at 5000 ppm

rapid growth (tall and early flowering) were produced using the concentrations of 3000, 4000 and 5000 ppm EMS, 200 and 400 ppm DMS as shown in Fig. 2-4.

The stimulating effect might be attributed to physiological activation of plant metabolism which took place as a result of using the mutagen. Selfings for these plants were made to obtain M2 generation. These variations were transmitted to the next generation. These results indicated that, these changes were combined with inheritance changes. So, traditional vegetative growth and tissue culture technique were still needed to propagate them as new cultivars.

Detection of protein profile: Considerable phenotypic variations were observed among the plants after treatment (Table 2), these changes were combined with inheritance changes. Protein profile could be used as indicator for these changes, which showed differences between treated plants (mutants) and control. As shown in Fig. 5, total protein in control and all mutants gave band patterns ranged from 17 to 135 kD. Approximately, 48 kD band was produced by the mutant EMST3, EMST6, DMST3 and DMST4. At the same time, approximately 30 kD band was present in the mutant EMST5. This might be related to the improvement of these mutants' traits comparable to control. The mutagenesis treatments seemed to activate expression of some genes which resulted in the appearance of some new bands. These results are almost in agreement with those of Osama (2002), Mahmoud and Nada (2006) and Tripathy *et al.* (2010) who found variations in number, intensity and or density of SDS electrophoretic bands of proteins from wheat, maize and

Fig. 4: Photograph showing taller and early flowering plants of *Senna occidentalis* produced by the treatments of DMS at 200 and 400 ppm

Table 2: The characteristics of the mutants of *Senna occidentalis* identified by protein pattern and RAPD analysis

Treatment (mutagene and concentration)	Characteristics of the mutant
T_1 (0 ppm)	Normal plant
EMS T_2a (1000 ppm)	Plant with inflorescences having six and seven petals
EMST$_2$b (1000 ppm)	Plant with inflorescences having six and seven petals
EMS T_3 (2000 ppm)	Bottom branching and taller plant
EMS T_4a (3000 ppm)	Early flowering and taller
EMS T_4b (3000 ppm)	Taller plant
EMS T_5 (4000 ppm)	Taller plant, early flowering and bushy type
EMS T_6 (5000 ppm)	Taller plant and early flowering
DMS T_3 (200 ppm)	Taller plant and early flowering
DMS T_4 (400 ppm)	Taller plant and early flowering

Vigna after mutagenesis treatments. On the other hand, some protein of molecular weight 95 and 26 kD were disappeared in the mutant EMST5.

The similarities observed among the control and all treatment may be heritable (Skirvin *et al*., 1994). However, some proteins are less densely and others are more densely and this may be due to the mutagenic effects of the EMS and DMS (Aly and Elsayed, 2006).

RAPD analysis: The eight arbitrary primers used for PCR amplification of the genomic DNA showed band numbers ranging from 3 to 15 (Fig. 6-13). The results revealed that the mutant EMST2a and control gave the same band pattern with all primers except by using the primers NAH1 and P1 where the 1000 bp were present. The amplification profiles with the primer OPA14 and OPA01 showed a highly polymorphic profile in the mutant DMST3 (Fig. 6 and 7). Moreover, it was found that the primer p1 showed polymorphic bands in the mutant EMST4a in comparison to another mutant (Fig. 8). On the other hand the amplified products of mutants by each primer OPA07,

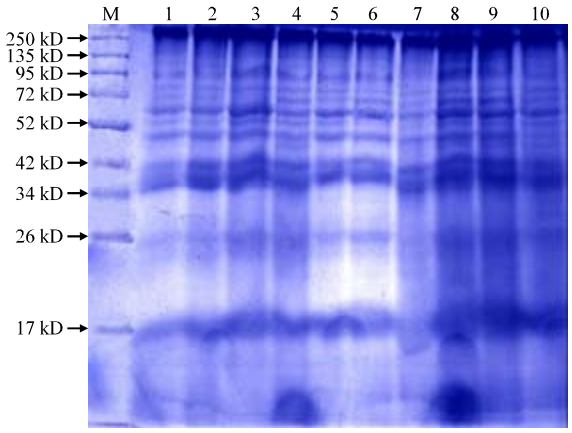

Fig. 5: SDS-electrophoresis of soluble proteins of mutant plants and control of *Cassia occidentalis*; Lane 1-10: T1(control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants, respectively

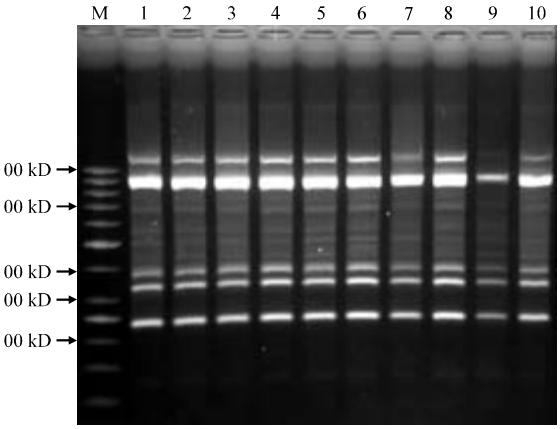

Fig. 6: RAPD-PCR using the primer OPA14; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

NAH1 and A7A10 were highly similar to control (Fig. 9-11). The amplification profiles produced using each primer NS1 and A9B7 showed polymorphic profile in the mutant EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST5 compared to control (Fig. 12, 13). These polymorphic bands could be very valuable for DNA fingerprinting and differentiation between mutants and the control plant (Hwang *et al*., 2002). The analysis of the data with scored amplified data is presented in Fig. 14 and similarity matrix in Table 3. The phylogenetic tree grouped the mutants and control into two main clusters

(A and B). The mutants obtained after EMS treatment and control were scored in cluster A. While others obtained after DMS treatment were scored in the cluster B. These variations came from mutagenic effects of the EMS or DMS treatment. Changes in DNA are caused by mutagens results in genetic variation detected by RAPD analysis. It has to be noted that direct acting alkylating agents such as EMS primarily cause point mutations, which are the results of single base deletion, addition or substitution (Schy and Plewa, 1989). DMS is monofunctional alkylating agents that have been shown to induce mutations, chromosomal aberrations and other genetic alterations in a diversity of organisms (Hoffmann, 1980). Appearance of new bands can be explained as results of different DNA structure (breaks, transpositions, deletion, etc.) (Danylchenko and Sorochinsky, 2005). The polymorphism revealed by RAPD due to deletion and/or addition may be caused by variation in DNA binding pattern by gamma rays and EMS (Arulbalachandran *et al*., 2009). It can be concluded that RAPD markers can be used for the identification of *Cassia occidentalis* mutants. This result agreed with the findings of Erdem and Oldacay (2004) on *Helianthus annuus* and (Gowda and Bhat, 2009) on groundnut.

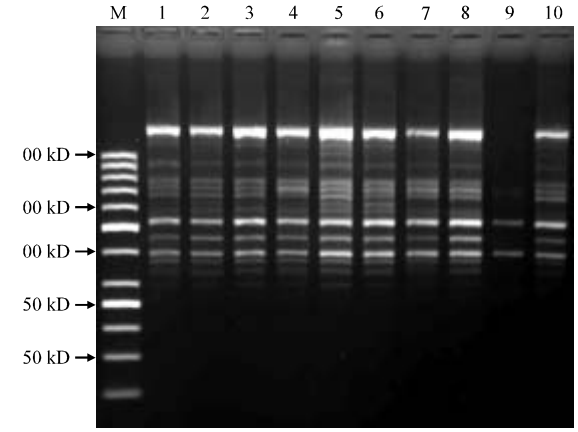

Fig. 7: RAPD-PCR using the primer OPA01; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

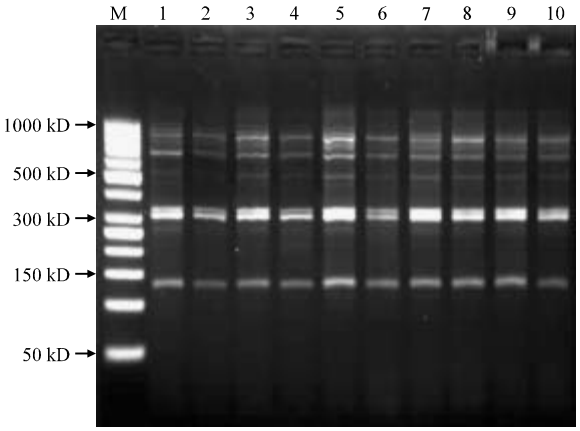

Fig. 9: RAPD-PCR using the primer OPA07; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

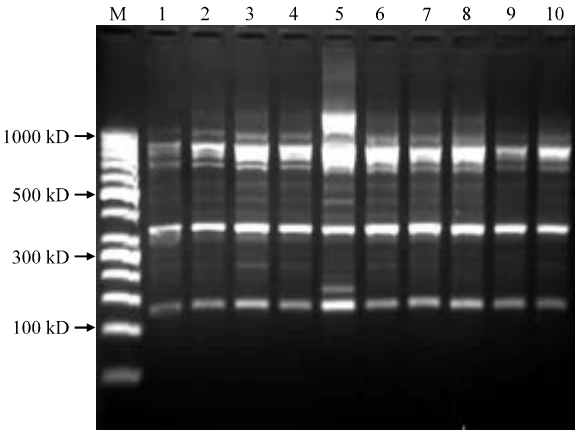

Fig. 8: RAPD-PCR using the primer P1; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

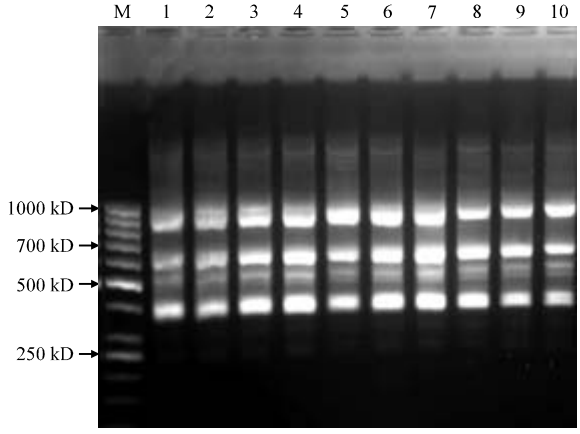

Fig. 10: RAPD-PCR using the primer NAH1; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

In conclusion, the polymorphism detected in most treatments of EMS and DMS may be due to deletion and/or insertion of DNA sequences as well as the mutagenic effects of EMS and DMS. The present study suggests that EMS and DMS could be used as effective mutagenic agents in *Cassia occidentalis*. Meanwhile, RAPD marker is a powerful technique enables detection of selected mutants in breeding of *Senna* for crop improvement.

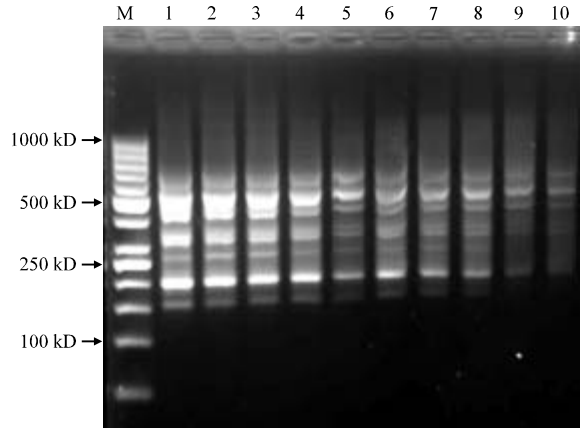

Fig. 11: RAPD-PCR using the primer A7A10; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

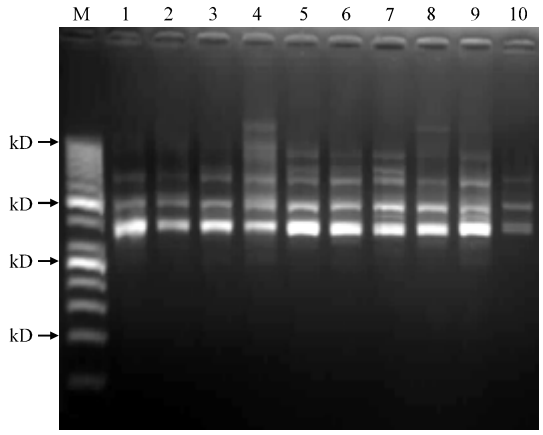

Fig. 12: RAPD-PCR using the primer NS!; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

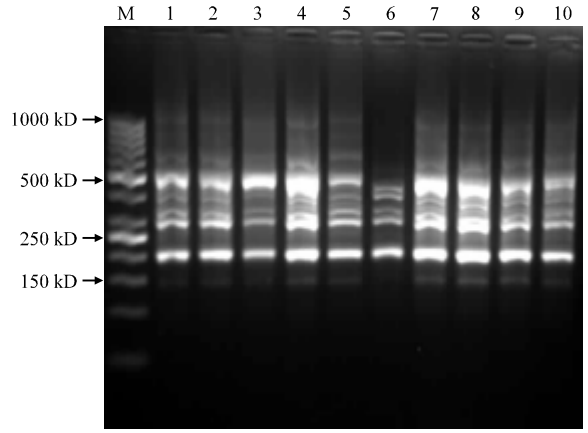

Fig. 13: RAPD-PCR using the primer A9B7; M: 50 bp marker, Lane 1-10: T1 (control), EMST2a, EMST2b, EMST3, EMST4a, EMST4b, EMST5, EMST6, DMST3 and DMST4 mutants of *Cassia occidentalis*, respectively

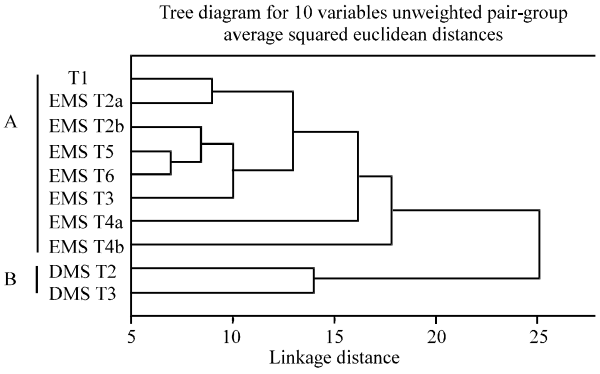

Fig. 14: Dendrograme constructed on the basis of RAPD profile for the wild type (control) and all mutants of *Cassia occidentalis* using eight RAPD primers

Table 3: Genetic similarity of DNA among all mutants and the control of *Senna occidentalis* using RAPD

	T1	EMST2a	EMST2b	EMST3	EMST4a	EMST4b	EMST5	EMST6	DMST3	DMST4
	100									
EMST2a	91	100								
EMST2b	87	90	100							
EMST3	84	91	89	100						
EMST4a	79	82	88	83	100					
EMST4b	77	84	84	85	78	100				
EMST5	85	86	92	89	86	84	100			
EMST6	84	79	91	92	85	83	93	100		
DMS T3	71	70	68	69	66	62	76	75	100	
DMS T4	70	84	80	79	72	74	84	85	86	100

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