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Morphological Diversity and Molecular RAPD Markers of Sugarcane Mutane (*Saccharum officinarum* L.) in Inundation Tolerance

Sholeh Avivi [1,2*], Eko Nur Suliswanto [1], Didik Pudji Restanto [1,2], Miswar [1], Anang Syamsunihar [1], Sigit Soeparjono [1] and Sri Hartatik [1]

[1] Department of Agronomy, Faculty of Agriculture, Jember University, East Java, Indonesia
[2] Center for Development Advance Sciences and Technology (CDAST), Jember University, East Java, Indonesia

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*) Corresponding author:
E-mail: savivi.faperta@unej.ac.id

ABSTRACT

The study aimed to identify variations in morphological and molecular character of sugarcane mutants from the mutation of Ethyl Methane Sulphonate (EMS). It used 21 sugarcane mutants and two non-mutant PS865 plants as controls. The treatments with inundation were carried out on 1-17 mutants and non-mutants, while the treatments without inundation were carried out on 18-21 mutant plants and non-mutants. The tolerance characteristics base on the agronomic characters. The RAPD molecular character was observed to detect changes in genotypes and kinship relationships of the plants tested. The results showed that the characteristics of tolerance to the best inundation were found in mutants 1, mutants 3 and mutants 6 which were characterized by the root volume (cm3), fresh root weight (g), sucrose content and brix value (%). Mutant plants treated with inundation showed higher levels of sucrose and brix values. Whereas the control plants in fats, showed lower levels of sucrose and brix. Changes in sugarcane mutant genotypes from non-mutants based on RAPD markers ranged from 14.7 - 56.7 % which resulted in an average polymorphic band of 35.1 % from 37 DNA bands and produced four main groups based on dendrogram analysis.

INTRODUCTION

The efforts to increase sugar production can be performed by increasing the production of the main sugar raw material, namely sugar cane. One of the efforts carried out is by utilizing marginal land that can potentially be used as cultivation land such as inundated land or tidal land. The flooding land conditions caused trouble to the growth and productivity of plants (Pezeshki & DeLaune, 2012). In the case, it is needed excellent varieties which have tolerance to inundation.

Mutation is one way to create tolerant sugarcane varieties that can be planted in inundated land. It is expected to produce genetic diversity and produce a mutagen genotype that has resistance to inundation. The morphological diversity of mutants is a result of genetic diversity expression. Identification of genetic diversity from mutants can be identified using RAPD (Random Amplified Polymerase DNA) molecular markers. Some researchers have tested genetic diversity of mutants by molecular method markers on some plants including rice plants (Tripathy et al., 2016), *Jatropha curcas* (Dhakshanamoorthy, Selvaraj, & Chidambaram, 2015), *Zea mays* (Erturk, Nardemir, Hilal, Arslan, & Agar, 2015), Banana (Nettyani, Miftahudin, & Sobir, 2016), *Desmodium gangeticum* (Linn.) DC (Cheruvathur, Abraham, & Thomas, 2013), and on Orchid plants *Spathoglottis plicata* Blume (Romeida, Sutjahjo, Purwito, Sukma, & Rustikawati, 2012).

Sugar cane mutants resistant to inundation are needed to overcome the problem of sugarcane cultivation in flooded land. In this study, sugarcane planting material used is sugar cane which has been given mutation treatment with EMS chemicals and has been tested for its tolerance to inundation at the plantlet level. EMS (Ethyl Methane Sulfonate) is the most common chemical mutation material

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used as a chemical mutagen. EMS causes guanine to be changed with thymine instead of cyanin, so the transition that occurs is G / C to - A / T (Talebi, Talebi, & Shahrokhifar, 2012).

The results of research on sugarcane mutants resistant to plantlet level inundation by Avivi, Sigit, Slameto & Rizki (2016) showed that PS 865 sugarcane mutants had the highest tolerance level compared to mutants PS863 and PS864 based on observations of plantlet height, number of shoots, number of leaves, number of roots, root length, and percentage live planlet. Therefore, further research is needed on PS865 sugarcane mutants from in vitro studies to identify inundation stress responses in the greenhouse.

The sugarcane mutants need to be identified for their diversity of morphological characteristics and molecular characteristics with RAPD molecular markers in inundation conditions. The purpose of this study was to identify differences in morphological responses to inundation treatments of mutant plants, identify changes in sugarcane mutant tolerant genotypes with inundation with PCR-RAPD (Random Amplified Polymerase DNA) markers and choose inundated sugarcane mutants that produce high sucrose.

MATERIAL AND METHODS

The experiment was carried out at University of Jember from December 2015 to June 2016. The experimental materials consisted of polybags, sugarcane mutants from the PS865 variety which was given EMS treatment and had been tested in vitro prior to inundation, non-mutant PS865, timba, manure, sand, and soil. The experimental device consisted of a digital refractometer, Chlorophyll meter SPAD-502 Minolta, and equipment for sugarcane cultivation. DNA banding pattern analysis using a set of PCR and electrophoresis tools. Primers used to obtain RAPD molecular markers are OPA 19, OPC 19, OPE 02, OPF 04, OPN 11. This experiment used 21 sugarcane mutant genotypes and two non-mutant plants. The inundation treatment was carried out with a water level of 5 cm below the surface of the planting medium on the 1-17 mutants and non-mutants, while the inundation treatment was carried out on the 18-21 and non-mutant mutants.

This inundation treatment was carried out for 3 months and started when the plant was 3 months old. The tolerance characteristics of sugarcane plants were based on observational characters observed and consisted of plant height, number of segments, stem diameter, number of leaves, number of tillers, root volume (ml), fresh root weight, stem aerenkhim, chlorophyll content, brix and sucrose content. The molecular identification stages consisted of DNA isolation (tissue separation, lysis solving, genomic DNA binding, washing, DNA release), PCR amplification and electrophoresis. The PCR amplification stage consisted of initial denaturation at 94°C for 2 minutes followed by 40 amplification cycles, where each cycle begins with a temperature of 94°C for 15 seconds followed by annealing temperature (Ta) 36°C for 30 seconds and then 72°C for 1 minute. The final extension stage was carried out at 72°C for 10 minutes. The 7 µl PCR product was electrophoresed on 1.2 % agarose gel with a voltage of 100 volts. The distance measure of the genetic similarity of sugarcane plants was observed based on the similarity coefficient using the Unweight Pair Group Method Arithmetic (UPGMA) method.

RESULST AND DISCUSSION

The effect of inundation treatment on 23 sugarcane plant genotypes based on morphological characters on the parameters of plant height, number of internodes, number of leaves, stem diameter, number of tillers, chlorophyll content, sucrose content of sugarcane juice and brix are presented in Table 1.

Based on Table 1, the mutant diversity occurs in all characters observed. If it is compared between plants treated with standing water and untreated inundation treatment, sugarcane mutant plants produced higher yields in submerged treatment on characters of plant height, number of leaves, number of segments, level of sucrose and brix. This shows that the inundation treatment of mutant plants has the potential to raise some production characters including the main character of sugar production which is shown in sucrose and brix content. The caharaterisitics of mutant plants 1, mutants 3 and mutants 6 in inundation conditions showed plant resistance characteristics characterized by the character of plant height, number of internodes, number of leaves, sucrose content and brix value (%) which was better than non-inundated conditions. The percentage of sucrose and brix content in mutant plants is found in mutants 1, 3 and 6. Thus the three mutant plants have the potential to be tolerantly inundated and can produce high in inundated conditions.

Table 1. Morphological characters of 23 sugarcane plant genotypes in inundated and not inundated conditions

Treatment	Code	Height of plant (cm)	No. of leaves	No.of segments	Diameter of stems (cm)	No. of tillers	Chlorophyl (µmol/m²)	Sucrose contens (%)	Brix (%)
Flooding	mutant 1	309	12	10	1.5	5	340.3	19.6	18.2
	mutant 2	300	11	11	1.5	3	324.7	18.3	13.4
	mutant 3	314	12	8	1.8	4	345.1	19.7	18.2
	mutant 4	274	9	8	1.3	8	280.2	11.5	16.6
	mutant 5	318	11	10	1.6	5	315.6	13.5	15.4
	mutant 6	294	10	10	1.5	8	314	17.2	17.1
	mutant 7	267	10	8	1.4	4	348.3	10.4	12.8
	mutant 8	270	10	8	1.4	4	309.5	16.4	15.4
	mutant 9	304	10	9	1.6	4	309.5	11.7	15.5
	mutant 10	282	11	9	1.7	5	305	10.5	16
	mutant 11	276	10	9	1.6	6	302.1	16.4	14
	mutant 12	283	9	8	1.3	6	312.5	7.6	17.7
	mutant 13	301	11	9	1.9	4	290.3	14.9	17.2
	mutant 14	274	11	8	1.5	6	293.2	11.7	16.2
	mutant 15	283	10	8	1.8	4	288.8	14.6	13.7
	mutant 16	272	10	10	1.3	4	289.7	8.7	13
	mutant 17	231	11	11	1.8	3	298.2	8.4	13.1
	Mutant Average	285.4	10.5	9.1	1.6	4.9	309.8	13.6	15.5
	Non mutant	327	12	11	2.1	4	424.2	11.1	13.9
Without flooding	mutant 18	230	10	8	1.8	8	368.6	8.9	10.6
	mutant 19	218	9	6	1.5	6	380.8	8.1	10.2
	mutant 20	290	11	9	1.7	6	410.1	13.2	10.4
	mutant 21	288	10	8	1.7	5	366.1	11.5	15.6
	Mutant Average	256.5	10	7.8	1.7	6.3	381.4	10.4	11.7
	Non mutant	315	13	11	1.9	6	494.6	18.1	14.9

In some characters, the sugarcane mutant produces a lower average value on stem diameter, number of tillers and leaf chlorophyll content after flooding treatment. The quantitative reduction in morphological characters is likely due to the influence of EMS chemical compounds in acting as active ingredients for mutations. Pratiwi, Pharmawati, & Astarini (2013) and Hakin & Arumingtyas (2014) stated that mutant plants originating from the treatment of chemical compounds showed a quantitative decrease in most observed morphological observations.

While the characteristics of non-mutant plants, in inundation conditions showed a decrease in stem diameter, number of tillers, chlorophyll content of leaves, sucrose and brix content. The inundation treatment resulted in the emergence of stem aerenkhim and adventitious roots in mutant and non-mutant plants. Mickelbart, Hasegawa, & Bailey-Serres (2015) stated that tolerant plants in puddle conditions showed resistance characteristics characterized by faster growth as a avoidance mechanism and showed aerenkhim appearance, whereas in intolerant plants showed stopping growth. The appearance of adventitious roots in puddle conditions causes the volume of stagnant roots to be greater compared to the treatment without puddles. (Fig. 1.)

Fig. 1 shows the conditions of flooding in mutant and non-mutant plants concerning the appearance of roots with volume and a greater amount than the roots of plants in conditions without flooding. The addition of root volume and number

of roots in higher inundation conditions showed an increase in the growth of adventitious roots and the resistance response of sugarcane plants to inundation conditions in both mutants and non-mutants. The results of volume and fresh weight of roots can be seen in Fig. 2.

Fig. 2 shows the highest fresh root weight was found in the combination of non-mutant and inundation treatment with 1150 cm^3 and 1110 g respectively. Overall, the inundation condition results in a higher response to volume and fresh weight both mutants and non-mutants. The addition of volume and weight of the roots is likely due to adventitious root formation as a form of plant response to inundation conditions compared to conditions without flooding. The oxygen deprivation in inundation conditions allows the root plant to respond in the process of looking for oxygen, resulting in elongation and addition of root volume. Striker (2012) also states that adventitious roots that have high porosity will help the plant to continue uptake of water and minerals and oxygen in stagnant conditions, to replace the function of the main root system as a form of response to inundation stresses. The tolerant plants will be more resistant to inundation stresses when forming many aerenchymal tissues on adventitious roots (Bellini, Pacurar, & Perrone, 2014).

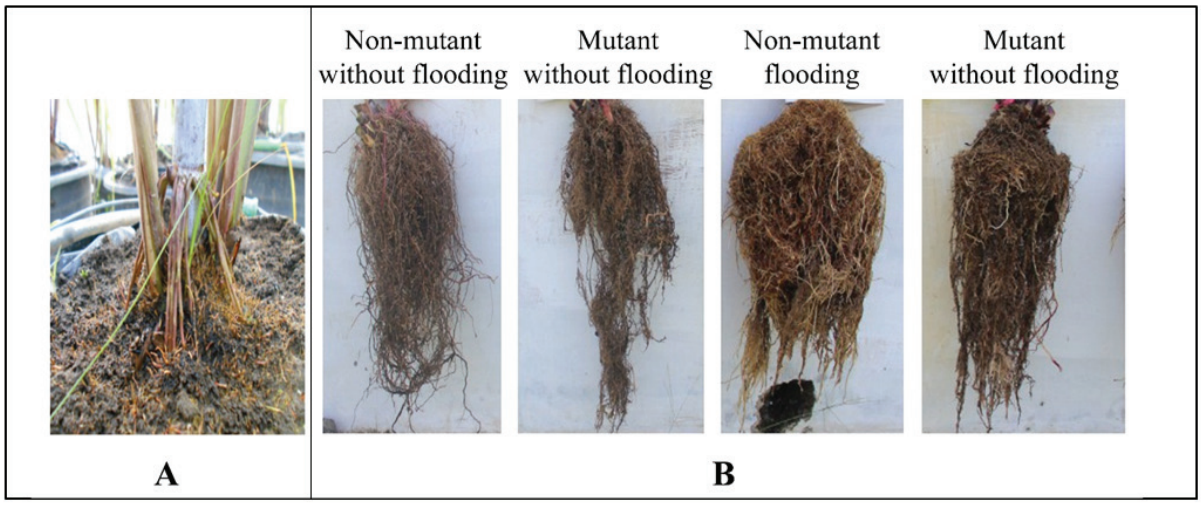

Fig. 1. (a) Appearance of adventitious root response in flooding conditions; (b) Appearance of roots in each treatment combination

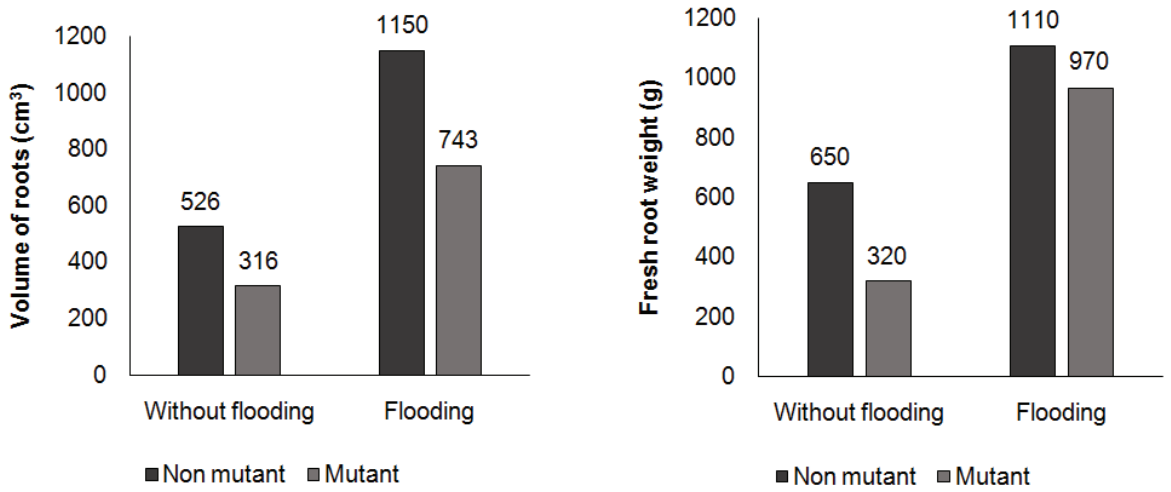

Fig. 2. Volume of roots and fresh and fresh root weight of sugarcane mutant and non-mutants under flooding stress and without flooding

The inundation stress results in energy reduction due to the decreased of photosynthesis activity and oxygen availability, as well as storage and release of energy as a form of mechanism in dealing with inundation stresses. The character of tolerant sugarcane plants in the face of inundation stress is characterized by an increase in plant height, stem diameter, number of tillers, chlorophyll content, and brix value and appearance of aerenkhim (Gomathi & Chandran, 2012; Gomathi, Gururaja Rao, Chandran, & Selvi, 2015; Morris & Tai, 2004; Tetsushi & Karim, 2007).

The characteristic analysis of genotype diversity with DNA markers began with taking fresh leaves from 23 sugarcane plants taken as much as 0.05 g for DNA isolation and then attaching five RAPD primers to the DNA template during PCR. The DNA isolation using mini KIT DNA and continued with PCR for the use of DNA template. Then the electrophoresis results were obtained. One of the PCR results with the OPN11 primer is presented in Fig. 3. The total DNA bands produced in electrophoresis were 37 DNA bands in all primers used. The results showed that DNA amplification in 23 sugarcane plants using 5 RAPD primers where four primers produced polymorphic bands. The results of the summary of the size and number of DNA bands of 23 sugarcane samples can be seen in Table 2.

Table 2 shows that this study obtained a RAPD band measuring 250 – 2000 base pairs. The number of DNA bands of sugarcane genotypes resulting from mutations and without mutations that were successfully amplified by each primer ranged from 3 bands to 11 bands or on average produced 7.4 bands per primer. The band polymorphism produced in this study was 72.9 % (27 bands) of the 37 total DNA bands obtained. The number of polymorphism bands that occur in this study is due to the influence of chemical mutations that occur so that the base structure changes from before. Talebi, Talebi, & Shahrokhifar (2012) added that the guanine base alkylated EMS chemical mutagen was alkylated with thymine instead of cyanine, resulting in a transition that occurred G/C to - A/T.

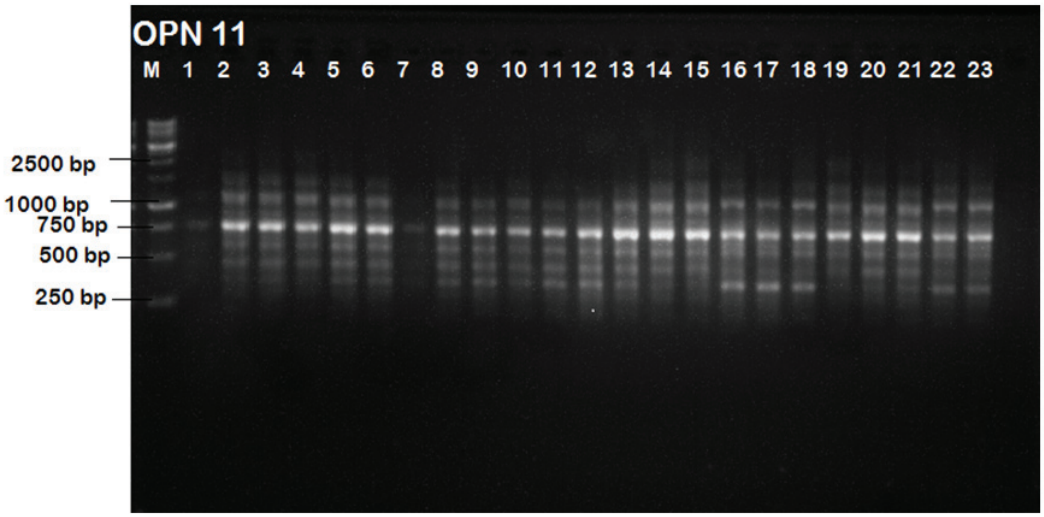

Fig. 3. Electrophoresis results using RAPD OPN11 primers

Table 2. Size and number of DNA bands 23 samples of sugarcane plants with five RAPD primers

No.	Primer code's	Sequent (5' – 3')	DNA band	Polymorphic DNA band	Percentage polymorphisms	Size (bp)
1.	OPC 19	GTTGCCAGCC	3	0	0	1000 – 2000
2.	OPN 11	TCGCCGCAAA	8	6	75	250 – 2000
3.	OPA 19	CAAACGTCGG	8	3	37.5	250 – 2000
4.	OPE 02	GGTGCGGGAA	11	11	100	250 – 1500
5.	OPF 04	GGTGATCAGG	7	7	100	250 – 1500
Total			37	27	312.5	
Mean			7.4	5.4	72.9	

The molecular markers data function in determining the level of difference and similarity of each cultivar, in this case is used as a benchmark for genotype changes due to chemical mutations so that the higher kinship can be interpreted as the effect of lower mutations. The Fragments or ribbons of RAPD amplification are assumed to be one locus. The amplification results are scaled "1" if there is a ribbon and suspension "0" if there is no amplified tape. The similarity coefficient value of 23 sugarcane plant genotypes can be seen in Table 3.

The results of the similarity coefficient matrix of RAPD markers between 23 sugarcane plant genotypes were based on 37 amplified loci with a range of values ranging from 33.3 % to 97.1 % (Table 3). However, to see the level of genotype changes in mutant genotypes was done by comparing non-mutant genotypes, namely PS865G and PS865NG. The magnitude of the genotype changes that occur can be obtained from the magnitude of the similarity genotype (r) coefficient of mutant plants with both non-mutant plants, where the value of the change in genotype (t) is obtained from the calculation results (t = 1 - r * 100). The results of the genotype change analysis showed that the genotype changes with the non-mutant PS865NG comparison ranged from 15.6 % to 55.2 %, where the largest genotype change (55.2 %) occurred in mutants 1. The genotype changes by comparison with PS865G ranged from 14.7 % to 56.7 %, where the greatest genotype change also lies in mutants1. The results of the kinship program 23 cane genotypes based on RAPD can be seen in Fig. 4. Hapsoro, Warganegara, Utomo, Sriyani, & Yusnita (2015), also used RAPD markers on some sugarcane genotypes from Australia, Africa, America and Asia, found genetic similarities ranging from 17-97 % with an average genetic similarity of around 57 %.

Fig. 4 shows the results of 23 sugarcane plant dendrograms based on DNA bands with the UPGMA method which produced 37 amplified loci Twenty-three sugarcane plant genotypes can be grouped into four main groups. Group I consisted of one genotype (mutants 1), group II consisted of five genotypes (mutants 10, 16, 20, PS865G and PS865NG), group III consisted of two genotypes (mutants 5 and 18) and group IV consisted of fifteen genotypes (mutants 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 15, 17, and 19). The closest kinship relationships of the plants tested were found between PS865 and PS865NG, mutants 10 and 20, mutants 5 and 18, mutants 11 and 12, mutants 4 and 6, mutants 13 and 21, mutants 14 and 15, mutants 3 and 19. Whereas the farthest relationship was found among mutants 1, 3, and 19.

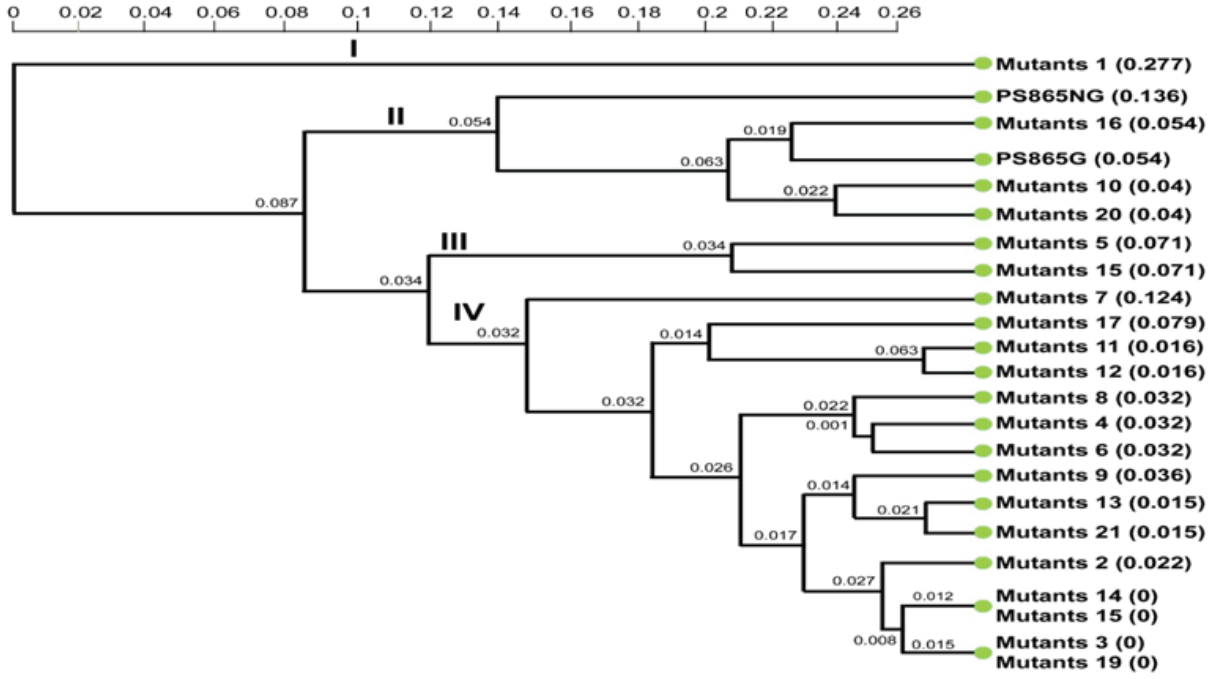

Fig. 4. 23 dendrograms of sugarcane plant genotypes resulting from mutations and without mutations based on RAPD markers analyzed by UPGMA method

Table 3. Similarity coefficients of sugarcane 23 genotypes based on 5 RAPD primers

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	PS865G	PS865NG
M1	1.000																						
M2	0.406	1.000																					
M3	0.394	0.970	1.000																				
M4	0.433	0.938	0.909	1.000																			
M5	0.464	0.818	0.848	0.839	1.000																		
M6	0.433	0.938	0.909	0.935	0.813	1.000																	
M7	0.464	0.867	0.794	0.706	0.611	0.758	1.000																
M8	0.464	0.875	0.848	0.933	0.867	0.933	0.697	1.000															
M9	0.371	0.889	0.889	0.806	0.800	0.857	0.800	0.800	1.000														
M10	0.565	0.719	0.697	0.767	0.700	0.767	0.545	0.821	0.657	1.000													
M11	0.324	0.806	0.857	0.771	0.765	0.824	0.765	0.765	0.914	0.618	1.000												
M12	0.333	0.800	0.829	0.794	0.788	0.794	0.735	0.788	0.886	0.636	0.969	1.000											
M13	0.394	0.912	0.941	0.853	0.848	0.909	0.848	0.848	0.943	0.697	0.912	0.882	1.000										
M14	0.382	0.941	0.971	0.882	0.824	0.882	0.824	0.824	0.917	0.676	0.886	0.857	0.971	1.000									
M15	0.382	0.941	0.971	0.882	0.824	0.882	0.824	0.824	0.917	0.676	0.886	0.857	0.971	1.000	1.000								
M16	0.481	0.686	0.667	0.676	0.571	0.727	0.571	0.667	0.772	0.786	0.686	0.657	0.714	0.694	0.694	1.000							
M17	0.394	0.806	0.833	0.800	0.743	0.800	0.743	0.743	0.889	0.647	0.857	0.829	0.886	0.861	0.861	0.750	1.000						
M18	0.542	0.697	0.727	0.688	0.857	0.688	0.576	0.677	0.686	0.621	0.647	0.667	0.727	0.667	0.667	0.545	0.727	1.000					
M19	0.394	0.970	1.000	0.909	0.848	0.909	0.794	0.906	0.889	0.697	0.857	0.829	0.941	0.971	0.971	0.667	0.833	0.727	1.000				
M20	0.520	0.781	0.758	0.774	0.955	0.833	0.606	0.828	0.714	0.920	0.676	0.647	0.758	0.735	0.735	0.857	0.706	0.581	0.758	1.000			
M21	0.406	0.882	0.912	0.879	0.818	0.938	0.818	0.875	0.914	0.719	0.882	0.853	0.970	0.941	0.941	0.735	0.857	0.697	0.912	0.781	1.000		
PS865NG	0.448	0.794	0.771	0.844	0.727	0.844	0.629	0.839	0.778	0.793	0.743	0.765	0.771	0.750	0.750	0.806	0.824	0.656	0.771	0.800	0.794	1.000	
PS865G	0.443	0.824	0.800	0.818	0.706	0.875	0.657	0.812	0.806	0.767	0.771	0.743	0.800	0.778	0.778	0.839	0.853	0.636	0.800	0.833	0.824	0.967	1.000

CONCLUSION

There were 3 mutants tolerant to inundation conditions; they are mutant 1, mutant 3 and mutant 6. Changes in non-mutant sugarcane mutant genotypes based on PCR-RAPD method ranged from 14.7 % to 56.7 % which resulted in an average polymorphic band of 35.1 % from 37 DNA bands that appeared on five RAPD primers and produced four main groups based on the RAPD dendrogram.

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