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Research Article
Sugarcane Genotypes Assessment Under Drought Condition Using Amplified Fragment Length Polymorphism

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Abstract

Background and Objective: Improving tolerance of crop plants to different types of environmental stress is an important key to crop production sustainability. Sugarcane productivity and geographical distribution are affected with drought stress. This study was focused on evaluation the performance of eight sugarcane (*Saccharum officinarum* L.) genotypes under drought stress. **Materials and Methods:** Eight sugarcane genotypes were assessed for drought tolerant using following yield-related traits:stalk height, stalk diameter, stalk weight, leaf area and number of stalks/plant. Eight AFLP combination were used to detect the genotype specific marker. **Results:** The eight sugarcane genotypes were assessed for their water stress tolerance in sand culture experiment. Analysis of variance showed significant differences for these traits among the eight genotypes under control and drought treatments. The results indicated that genotypes Co. 285, Co. 997 and Bo.19 were the most tolerant and genotypes Co. 775, F.141 and Co. 396 were the most sensitive ones. However, genotypes G2003-47 and G2007-61 were moderate drought tolerant genotypes. Among the sensitive genotypes, the Co. 775 was the most sensitive one that recorded the highest reduction (%) with all traits except stalk diameter which increased for all genotypes. The Co. 775 and Co. 997 genotypes were used in AFLP analysis. Eight AFLP primer combinations were used to estimate the level of polymorphism among drought tolerant sugarcane genotype Co. 997 and drought susceptible genotype Co. 775. The eight AFLP primer combinations amplified a total number of 886 amplicons, where 55 were polymorphic representing 6.2% polymorphism. **Conclusion:** The eight genotypes genetically different in their response to drought tolerance. The AFLP marker can be used as genetic marker to assess the sugarcane genotypes and Co. 775 was more sensitive and Co. 997 was most tolerant genotype.

Key words: Sugarcane genotype, *Saccharum officinarum*, environmental stress, drought tolerance, crop plants

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a main crop for sugar production worldwide and is considered an important feedstock to produce second-generation bioethanol biomass. Sugarcane is a comparatively high water-demanding crop and its growth is affected by water deficit[1] and this is a major abiotic stress affecting sugarcane productivity. As a consequence of this drought sensitivity the major sugarcane producing countries recognize the need to develop drought tolerant cultivars[2-4]. In sugarcane, the formative phase (60-150 days) has been identified as the critical water demand period[5] and any amount of water stress during this early growth phase has a direct influence on growth, dry matter accumulation, cane yield and juice quality. Experiments that impose drought at this stage could therefore be useful in identifying drought tolerant cultivars[6].

The main cultivars of sugarcane in general production are interspecific hybrids between the domesticated species *Saccharum officinarum* and the wild relative *S. spontaneum* and include polyploid and aneuploid cultivars[7] and chromosome number ranges from 100-130. Many sugarcane cultivars have unknown ancestors with an apparent narrow genetic base and with no accurate or even non-existing pedigree records. For this reason, plant improvement is difficult and it has become very important to assess sugarcane cultivars using different genetic markers to estimate genetic distances and to facilitate future cultivar improvements[8]. Sugarcane is generally considered to be one of the most genetically complex crops that researchers have attempted to map its genome.

Various molecular marker such as random amplified polymorphic DNA (RAPD)[9,10] inter simple sequence repeat (ISSR)[11] and amplified fragment length polymorphism (AFLP) were used to investigate sugarcane genetic variability[12,13]. These techniques have many advantages including their relative simplicity of use and the ability to create relatively high numbers of polymorphic DNA markers. More generally the analysis of genetic diversity and QTL mapping frequently use RAPD, ISSR and AFLP markers[13,14]. The AFLP is one of the best currently available molecular markers and shows distinct advantages including reproducibility and the high number of markers and the duration of the assay[12]. The AFLP has been efficiently used to estimate genetic diversity[15,16] to analyze quantitative traits[17,18] and to construct various genetic maps[19,20]. The AFLP markers have been used in sugarcane to tag genes for smut resistance and to establish close linkage with rust resistance genes[21] to assess genetic diversity among the Brazilian cultivars[22] and to map QTLs for yield components[23]. This current investigation aimed to evaluate the yield-related traits of eight sugarcane genotypes under drought stress conditions and to detect molecular markers associated with drought tolerance in sugarcane using AFLPs-PCR analysis.

MATERIALS AND METHODS

Plant material: Eight sugarcane genotypes with known pedigrees were selected from sugarcane germplasm of the Sugar Crops Research Institute (SCRI), Giza, Egypt (Table 1).

Sand culture experiment: The eight genotypes were sown in a sand culture experiment maintained outside the breeding greenhouses of Sugar Crops Research Institute, in plastic dishes 45 cm in height, 50 cm in diameter and with a capacity of 50 kg sand obtained from the field station. The plastic dishes were loaded to 7 cm from the top with washed sand. Three single bud cuttings were planted in each dish. Modified Hoagland and Arnon[24] solution was used as the base nutrient solution. All sugarcane genotypes were arranged in a fully randomized design with three replications. Drought treatment was initiated after 21 days from germination. The Hoagland solution was used under two irrigation regimes (drought stressed and non-drought stressed [control]). Regular irrigation was adopted every 5 days and drought stress treatment (initiated 21 days after germination) while control plants continued to receive regular irrigation. Samples were taken and data recorded after 90 days for the following yield-related traits: stalk height, stalk diameter, stalk weight, leaf area and number of stalks/plant[25]. The study was conducted at the Genetic Department experimental station at the Faculty of Agriculture, Cairo University and Sugar Crop Research Institute, Agriculture Research Centre, Giza, Egypt during the period from March, 2017-2018.

Amplified fragment length polymorphisms (AFLPs) analysis: Genomic DNA was isolated from sugarcane tissues using a modified CTAB method[26]. The AFLP analysis was conducted

Table 1: Names, pedigrees and origins of eight sugarcane genotypes

Genotypes name	Pedigree			
	Male		Female	Source of seed
Co. 396	Co. 243	X	Co. 244	India
Co. 775	POJ 2878	X	Co. 371	India
Co. 997	Co. 683	X	P63-32	India
Bo.19	POJ 2878	X	?	India
Co. 285	Co. 243	X	Co. 244	India
F.141	NCO310	X	PT 48-21	Taiwan
G.2003-47	CP 55-30	X	85-1697	Local seed fuzz
G.2007-61	SP 71-1406	X	CO 842	Local seed fuzz

using the AFLP® Analysis System II (Invitrogen, USA) (Cat. No.10483-022), according to the manufacturer's protocol with minor modifications. Approximately 400 ng DNA was digested with a mixture of *Eco*RI and *Mse*I restriction enzymes overnight at 37°C and then the samples were incubated to inactivate the enzyme at 70°C for 15 min. For generating the template DNA for amplification, *Eco*RI and *Mse*I adapters were ligated to the digested DNA samples. Then 24 µL adapter/ligation solution and 1 µL of T4 DNA ligase were mixed with the digested DNA samples and incubated overnight at 20±2°C. The TE buffer was used to dilute the ligation products tenfold. The PCR reaction mixture (25 µL) was composed of the following: 75 ng of each of the primers (*Eco*RI-core and *Mse*I-core); 0.2 mM dNTPs (Boehringer Mannheim); 1.0 µL *Taq*DNA polymerase (HT Biotechnologies); 1x reaction buffer (HT Biotechnologies) and 300 ng of digested ligated DNA. The PCR amplification was programmed as follows: one cycle at 94°C for 30 sec, 65°C for 30 sec and 72°C for 60 sec; followed by 12 cycles of touchdown PCR in which the annealing temperature was decreased by 0.7°C every cycle until a 'touchdown' annealing temperature of 56°C was reached. Once reached, another 23 cycles were conducted as described above for pre-amplification. Denaturing polyacrylamide (6% w/v) gels were used to analyze the PCR products. The gel was silver stained according to the protocol described by the manufacturer (Promega Corp., USA, Silver Sequence DNA Staining Reagents, Lot. No. 171120). The PCR products were fractionated on a sequencing system (BIO-RAD Sequi-Gen Sequencing gel system).

Statistical analysis: The collected data were statistically analyzed by analysis of variance according to Bernardo[27]. Data was analyzed with Two-way Analysis of variance (Two-way ANOVA) using SPSS statistical program (Version 25 64x edition); homogeneity among data means were compared using Duncan's Multiple Range Test and declared significant at $p \leq 0.05$[28].

RESULTS

Effect of water stress on yield-related traits: In this study eight sugarcane genotypes were assessed for drought tolerance depending on five important yield related traits (i.e., stalk height, stalk diameter, stalk weight, leaf area and number of stalks/plant). Analysis of variance showed significant differences for these traits among the eight genotypes under control and drought treatments. All genotypes were significantly affected by drought treatment. Stalk height, stalk diameter, stalk weight, stalk number and leaf area were affected by genotypes and drought treatment (Fig. 1a-e), but the most affected trait was stalk weight, the control resulted in higher weights compared to the drought treatment. The average stalk weight for genotypes of the drought treatment was 0.2 kg compared to 0.28 kg for the control with 28.6% of reduction (Fig. 1c).

The average stalk height was 66.45 and 56.26 cm for control and for drought treatment, respectively with 14.26% of reduction, while average of stalk diameter was 0.87 cm for the control and 0.99 cm for the drought treatment (Fig. 1a and b). Results could be related to the fact that on the presence of water, sugarcane could be more elongated, so drought treatment resulted less elongation and increasing in stalk diameter.

Average of leaf area was 140.09 cm^2 for control and 121.59 cm^2 for the drought treatment (Fig. 1d). Leaf area was an indicator for extremely productive genotypes under drought treatment.

The results in Fig. 1a-e revealed that the sugarcane genotypes can be clustered into two groups, the drought tolerant genotypes (Co. 285, Co. 997 and Bo.19) and the sensitive ones (Co. 775, F.141 and Co. 396). However, genotypes G2003-47 and G2007-61 were moderate drought tolerant genotypes. Among the sensitive and tolerant genotypes, Co. 775 was the most sensitive, while, Co. 997 was the most tolerant. The average of five trait revealed the effect of drought among all studied genotypes (Table 2).

Polymorphism as detected by AFLPs: In the present investigation, a total of eight combinations of AFLP primer were used with two sugarcane genotypes, Co. 775 and Co. 997 which were respectively the most and the least sensitive to drought. The eight AFLP primer combinations produced credible PCR products, however, only seven AFLP primer

Table 2: Average of five traits of eight sugarcane genotypes as affected by drought treatment

	Average of five traits	
Genotype	Control	Drought treatment
Co. 396	46.89±1.23	40.08±1.05
Co. 775	43.19±1.13	24.24±0.63
Co. 997	48.63±1.27	46.64±1.22
Bo. 19	38.63±1.01	34.97±0.91
Co. 285	39.54±1.03	37.84±0.99
F.141	54.15±1.42	45.17±1.18
G.2003-47	38.53±1.01	35.18±0.92
G.2007-61	40.36±1.06	35.83±0.94
Mean	43.74	37.49

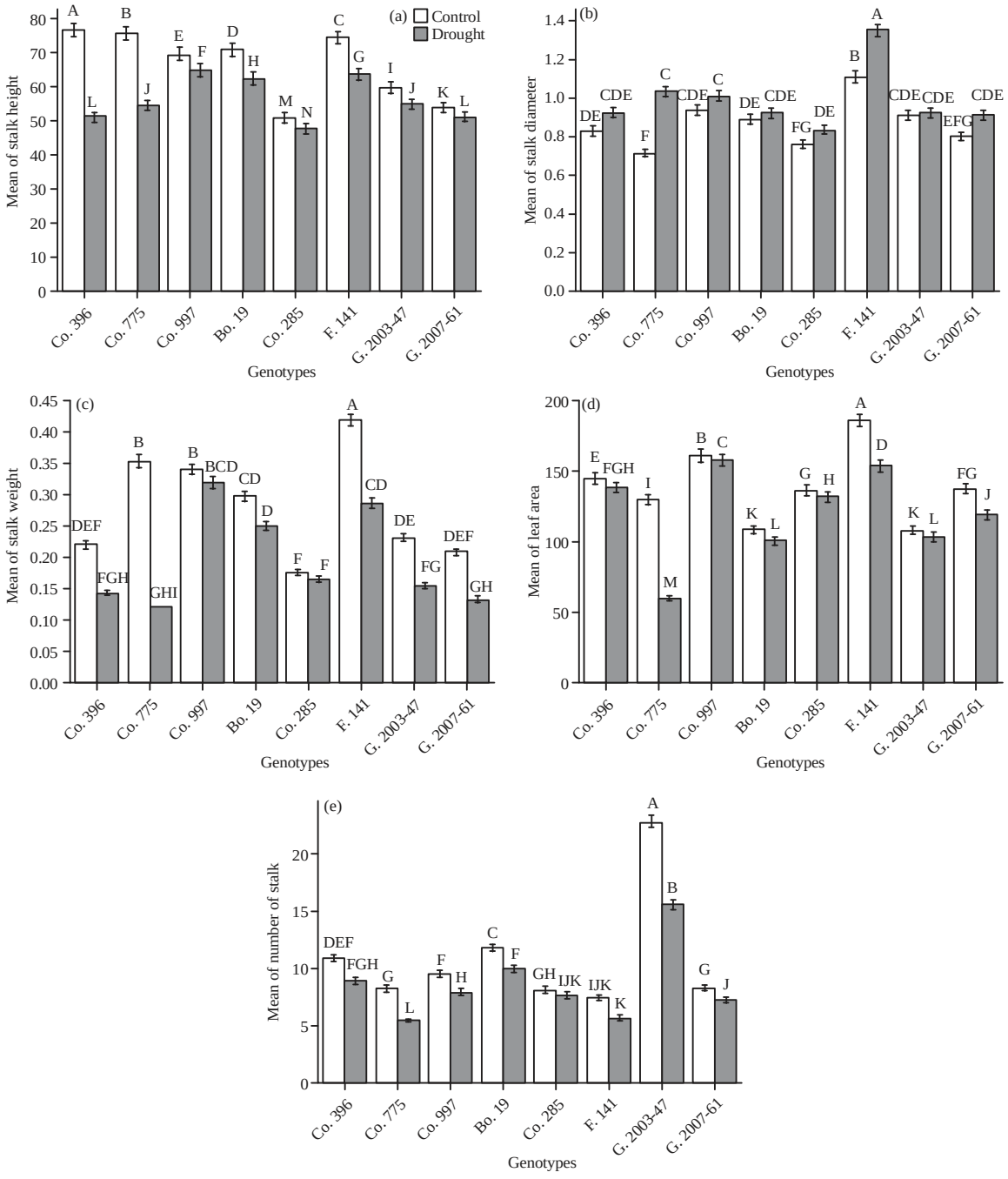

Fig. 1(a-e): Effect of drought on (a) Stalk height, (b) Diameter, (c) Weight, (d) Number and (e) Leaf area of eight sugarcane genotypes. Genotypes followed by the same capital letters are similar and do not differ significantly ($p>0.05$) between treatments, while genotypes followed by different letters are not similar and differ significantly ($p<0.05$)

combinations (6.2%) showed discernible polymorphism between the two genotypes (Table 3). The selected primer combinations, the total number of amplicons, polymorphic amplicons and polymorphism (%) are listed in Table 3. A total of 886 major AFLP bands were observed, 55 of these (6.2%) were polymorphic between the two genotypes (Fig. 2). The number of amplicons/primer combination ranged from 52 (E-AAG/M-CTA) to 144 (E-ACA/M-CAT) while, the number of polymorphic amplicons varied from 0-16. The primer combination (E-AAG/M-CTA) produced the lowest number of polymorphic products. While, the primer combination (E-ACA/M-CTA) produced the highest number

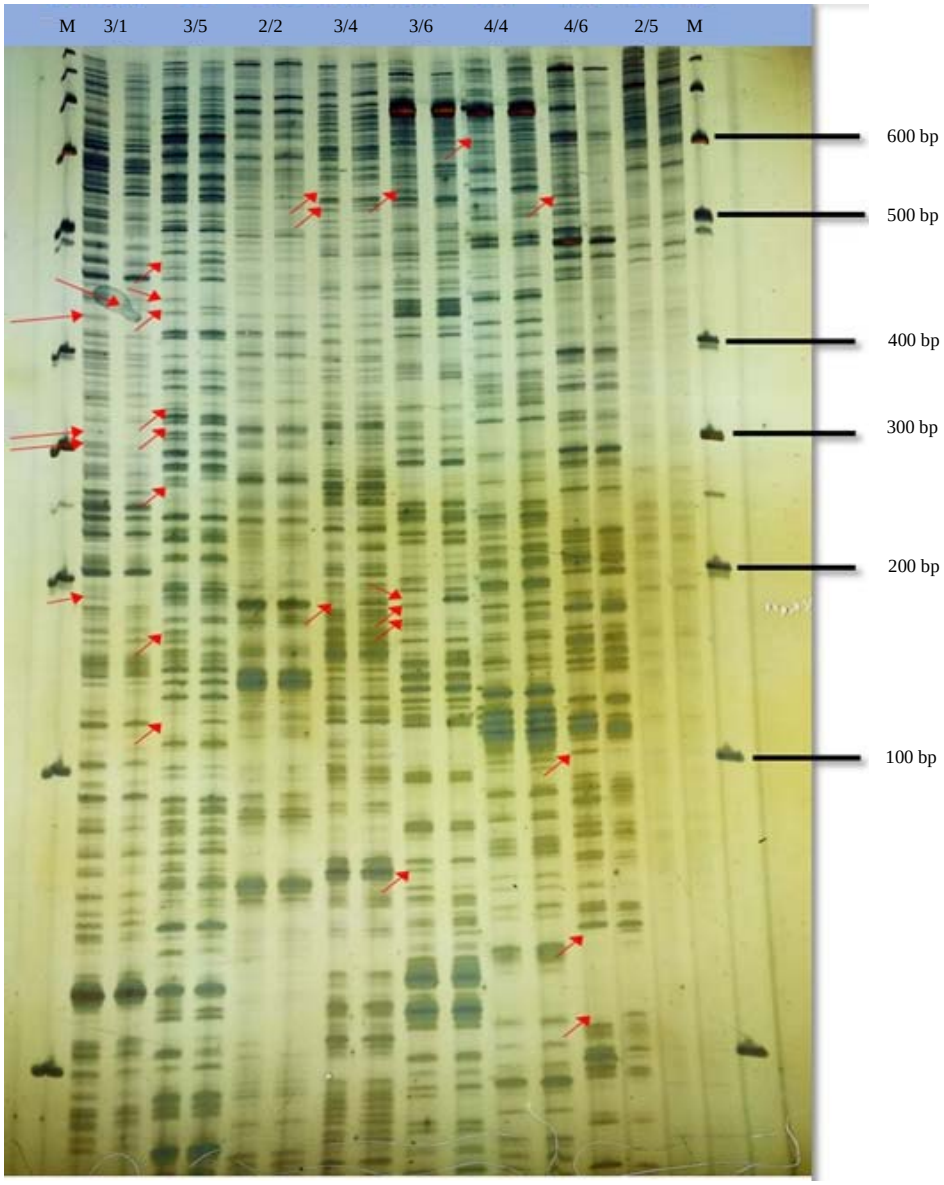

Fig. 2: AFLP polymorphism pattern with Co. 775 and Co. 997 sugarcane genotypes using combinations 3/1: E-ACA/M-CAA, 3/5: E-ACA/M-CTA, 2/2: E-AAG/M-CAC, 3/4: E-ACA/M-CAT, 3/6: E-ACA/M-CTC, 4/4: E-ACC/M-CAT, 4/6: E-ACC/M-CTC, 2/5: E-AAG/M-CTA

Table 3: Total number of amplified fragments for drought stress in sugarcane using AFLPs analysis

Primer code	Primer pair EcoRI/MseI	Total no. of band	Monomorphic band	Polymorphism (%)	Associated with Co.775	Associated with Co.997
3/1	E-ACA/M-CAA	119	11	9.2	10	1
3/5	E-ACA/M-CTA	130	16	12.3	9	7
2/2	E-AAG/M-CAC	116	5	4.3	3	2
3/4	E-ACA/M-CAT	144	5	3.5	2	3
3/6	E-ACA/M-CTC	111	7	6.3	6	1
4/4	E-ACC/M-CAT	108	3	2.7	2	1
4/6	E-ACC/M-CTC	106	8	7.4	5	3
2/5	E-AAG/M-CTA	52	0	0	0	0
	Total	886	55	6.2	37	18

of polymorphic products. Thus, the average number of polymorphic fragments per combination was 6.9.

Thirty seven out of the 55 polymorphic AFLP markers were associated with the sensitive genotype Co. 775 (180,

280, 296 bp for 3/1;155, 260 and 306 bp for 3/5;76 bp for 3/6; 576 bp for 4/4; 557 bp for 4/6) while 18 bands (40 bp for 3/5; 181 bp for 3/4; 290 bp for 3/5; 53, 58 bp for 4/6) were associated with the tolerant genotype Co. 997. Therefore, these AFLP markers can be identified as drought related markers (Table 3). The highest number of AFLP marker was detected for primers E-ACA/M-CTA and E-ACA/M-CAA (16 and 11 bands, respectively), while the lowest was scored for E-AAG/M-CTA and E-ACC/M-CAT (0 and 3 band, respectively). The drought-tolerant genotype Co. 997 had more alleles than the sensitive one. These results suggested that the drought tolerance in Co. 997 could be due to high allelic frequency of drought tolerance genes compared to the Co. 775 genotype.

DISCUSSION

The result showed the strong effect of drought on stalk weight, this result is in accordance with Silva *et al.*[29], who recorded 1.03 kg, while Ramesh and Mahadevaswamy[30] obtained 0.66 kg and Robertson *et al.*[31] obtained 0.41 kg. The number of stalks/m^2 decreased from 10.89-8.56 representing a 21.4% reduction. The stalk number was within the expected values for number of millable canes, i.e., between 10-14 tillers per meter[32]. Sugarcane tiller formation is important due to the contribution they make to yield by acting as a storage sink[30]. Sugarcane passes during its growth with four distinguished physiological stages, named, germination, tillering, grand growth and maturity[33]. Early grand growth together with tillering are considered the critical water-demanding period and are known as the formative phase and yield is significantly affected by stress during this phase[34]. Consequently, the tillering ability and later growth effectively largely determine the yield of a given genotype[35]. Higher tiller production, irrespective of environmental conditions or genotype, generally leads to higher number of stalks at harvest, in spite of differences in tiller mortality[36]. Stalk height mightily affected under drought conditions[29,37]. Stalk diameter response to drought found to depend on the genotype[29]. Drought is the major abiotic stress that affect morphological parameters such as stalk length, stalk diameter, leaf area and number of stalks. These results agreed with Vantini *et al.*[38] who found that drought is an abiotic stress that limits the productivity of sugarcane.

The AFLP is consider a strong DNA marker. It was developed to allow the construction of very high density genetic maps. According to Alwala *et al.*[39], AFLP markers may be more durable for polymorphisms detection among closely related genotypes, as they are more likely to sample different segments throughout the genome. In this study the AFLP was used to assess drought related DNA marker between resistance sugarcane genotype Co. 977 and sensitive genotype Co. 775. The result showed that there were 55 specific markers related to drought. The specific AFLP markers identified in this study would be useful for monitoring the drought tolerant program in sugarcane cultivars. On the other hand, AFLP markers were pointed out by Van Eck *et al.*[40] as being probably locus specific. However, the large number of fragments revealed on an AFLP gel, as in the case of the present study, maximizes the chances of fortuitous migration of two fragments of very similar size. Furthermore, the AFLP technique used extensively in sugarcane to generate genetic maps and find marker-trait associations to a number of agronomical important traits in QTL studies[41]. Bhanu *et al.*[42] studied many important traits controlled by several genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs). And detected that AFLPs can prove to be very practical for a multiplicity of purposes pertinent to crop improvement. Lima *et al.*[22] results showed that the AFLP technique allows the rapid obtaining of the necessary number of markers for this type of sugarcane genome analysis. Those markers satisfactorily assessed the genetic relationship between the 83 sugarcane cultivars. Continuation and advancing research in molecular markers as basic tools in developing biotechnology solutions to study mechanisms of sugarcane water stress tolerance are still wanted[2,43].

Constraints- genetic polymorphisms may reflect the past influences of selections, which could be different for certain specific genes. Genomic physiognomies that acclimate a sugarcane genotype to adapt certain stress phenomena (e.g., drought) could rely upon a restricted set of genes and therefore the temporal variation in such genes can probably not be an exact measure of polymorphism. For breeding purposes and assessment of economic yield phenomena, measuring the genetic diversity by molecular markers should be based on targeted genes, as these may reflect functional polymorphisms, however, it is limited for cost effectiveness and timing.

It is very important to quantify the amount of genetic variation that is presents in the germplasm of original and hybrid sugarcane cultivars, so tracking the genomic history of breeds is very important. Using different genetic marker approaches to quantify stress will provide extra information that may help in the establishment of relationships between genotypes.

CONCLUSION

This study concluded that the potential of the AFLP technique to estimate the genetic diversity in response to water deficit stress among a set of sugarcane genotypes. The polymorphism in drought tolerance genes was evaluated in eight genotypes in addition to the different quantitative yield traits of these genotypes. The results of this study are reported here. According to which, Co. 775 was more sensitive and Co. 997 was most tolerant genotype.

SIGNIFICANCE STATEMENT

We established a high-resolution amplified fragment length polymorphism (AFLP) procedures using different primer combinations to estimate the levels of genetic polymorphism among drought tolerant and susceptible sugarcane genotypes. We also evaluated performance of the tested sugarcane genotypes under drought stress through assessing the different quantitative yield traits.

The AFLP is an innovative DNA fingerprinting technique that can be applied to genomic DNAs of any origin or inconsistencies. Genetic polymorphisms can be identified by matching the absence or presence of certain DNA fragments following analysis on polyacrylamide gels. To the best of our knowledge, using AFLP in exploring stress physiology in sugarcane is a novel aspect.

Altogether, this study describes the AFLP methodology/procedures of molecular fingerprinting of DNA from sugarcane which will help researchers to uncover the critical areas of genetic variabilities in sugarcane cultivars in response to drought conditions.

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