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R-ISSR As Marker Assisted Selection For Drought Tolerance In Sugarcane

Khaled, K. A. M. [1*]; Sheren E. E. El sherbeny[2] and A. A. Abdelhadi [3]

1-Genetics department, Faculty of Agriculture, Beni-Suef University

2-Department of breeding & genetics, Sugar Crops Research Institute (SCRI), Agricultural Research Center(ARC), Giza, Egypt.

3-Department of Genetics; Faculty of Agriculture, Cairo University; Giza Egypt.

***Corresponding author (khaled.adly@agr.bsu.edu.eg)**

ABSTRACT

Sugarcane is one of the most important crops in the world for its syrup and by-products. Eight sugarcane genotypes (i.e.G.T.54-9, G84-47, Sp 80-32-80, F.153, Co.997, BOT-41, Co.775, and F.161) were kindly provided by Sugar Crops Research Institute (SCRI), they were selected depending on previous screening of SCRI germplasm for studying the efficiency of technique that combined Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) as marker to assist selection for drought tolerance in sugarcane and compared it with RAPD and ISSR each separately. This technique is called R-ISSR.The performances of studied genotypes revealed that RAPD, ISSR, and R-ISSR techniques are useful as marker assisted selection for drought tolerance in sugarcane. 26 positive and negative markers were obtained from used techniques (RAPD, ISSR and R-ISSR), which would be used as marker to assist selection for drought tolerance. The study revealed that R-ISSR technique was more efficiency in this case. The study encourages breeder to use R-ISSR technique in the early selection for drought tolerance through sugarcane breeding programs.

Keywords: Sugarcane, RAPD, ISSR, R-ISSR, drought, tolerance

INTRODUCTION

Sugarcane (*Saccharum* spp.) is an important crop in the world for its sugar production and by-products. Sugarcane genotypes (*Saccharum* spp. hybrids) are complex polyploid resulting in chromosome mosaicism 2n=100-130 (D'Hont *et al*., 2001). Molecular marker technique was used in sugarcane breeding programs to identify and develop hybrid genotypes with improved characters. PCR based molecular marker techniques such as RAPD (Random amplified polymorphic DNA) (Williams *et al*., 1990) and ISSR (Inter Simple Sequence Repeat) (Shrivastava and Gupta, 2008) were used in many studies among different accessions of sugarcane. RAPDs used short primers with arbitrary sequence to generate fingerprints of multiple amplification products (Welsh & McClelland, 1990 and Williams *et al*., 1990). ISSRs are semi-arbitrary markers amplified by PCR using a single primer composed of a microsatellite repeated sequences (Shrivastava and Gupta, 2008).

Environmental or abiotic stresses are limiting sugarcane production worldwide, where drought is a major and one of the most important abiotic stresses (Jain *et al.*, 2005). Drought decreased sugarcane productivity through some morpho-physiological effects (Andrade *et al.* 2015), it reduced stalk height, leaf area and stalk diameter (Cia, M. C *et al.* 2012 and Jangpromma *et al.* 2012). Molecular markers were used as a marker-assisted selection (MAS) of sugarcane (Costa *et al.,*2011, Khaled *et al.* 2011, 2015 and 2016). Khaled *et al*. 2011 used RAPD, ISSR and R-ISSR techniques for detecting markers associated to sugar content. The performances of studied clones revealed that RAPD, ISSR and R-ISSR techniques are useful as marker assisted selection for sugar content in sugarcane clones. Khaled *et al*. 2015 used RAPD and ISSR for genetic diversity in sugarcane.

This study is aiming to answer the question "Is R-ISSR useful as marker assisted selection associated with drought tolerance in sugarcane?"

MATERIALS AND METHODS

This study was carried out during 2011-2015 at the farm, greenhouse and laboratories of Breeding & Genetics department, Sugar Crops Research Institute (SCRI), Agricultural Research Center (ARC), Giza, Egypt. Eight sugarcane genotypes (i.e.G.T.54-9, G84-47, Sp 80-32-80, F.153, Co.997, BOT-41, Co.775, and F.161) were kindly provided by Sugar Crops Research Institute (SCRI), they were selected to study R-ISSR combination technique as Marker Assisted Selection for stress tolerance (mainly drought stress) in sugarcane. The origin and pedigree of these genotypes were presented in Table 1.

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

Variety name	Pedigree			Source of seed
	Male		Female	
G.T.54-9	NCO310	X	F 37-925	Seed fuzz from Taiwan
G84-47	NCO310	X	?	Local seed fuzz Official cross
Sp 80-32-80	SP71-1088	**X**	H575028	Sao Paulo, Brazil
F.153	NCo 310	X	P341-36	Taiwan
Co.997	Co. 683	X	P63-32	India
BOT-41	Wild sugarcane *Saccharum spontaneum*			a grass from Indonesian
Co.775	POJ 2878	X	Co.371	India
F.161	F.146	X	F.149	Taiwan

? Unknown parent

Sand Culture Experiment

A sandy culture experiment was carried out to study the performance of the eight sugarcane genotypes against drought stress. The eight genotypes were sown according to Heakel *et al*. (1981) and Modified-Hoagland solution was used as the base nutrient solution (Johnson *et al*. 1957). Sugarcane genotypes were distributed in a completely randomized with three replications. Drought treatment initiated at 21 days after emergence of seedling. Control plants were irrigated with the base nutrient solution every three days while drought stresses plants were irrigated with the base nutrient solution every ten days. Samples were taken and data were recorded after 90 days for the following yield-related traits: stalk height, stalk diameter, stalk weight, leaf area and number of stalks /m^2 for identifying the most tolerant genotype and the most sensitive one, then molecular analysis was carried out to compare markers obtained from R-ISSR technique and that obtained from RAPD and ISSR techniques.

Molecular genetic studies

DNA isolation

Genomic DNA was extracted from sugarcane seedlings according to CTAB method described by Doyle *et al.* (1987) and modified by Khaled and Esh (2008). DNA quantification measured using spectrophotometric and DNA checked by agarose gel electrophoresis. The DNA was diluted in TE buffer to a working concentration of ~10 ng/µL

Polymerase Chain Reactions (PCR)

Randomly Amplified Polymorphic DNA (RAPD) Analysis

The reaction was conducted using twenty-three arbitrary 10 mer primers only eight primers give results, their names and sequences are shown in Table 2. The reaction conditions were optimized and the amplification was performed for 32 cycles as follows; initial denaturation at 94ºC for 4 min, one cycle, denaturing at 94ºC for 1min, annealing at 37ºC for 30 secs, extension at 72ºC for 2 min (32 cycle) and final extension at 72ºC for 10 min (one cycle), then hold at 4ºC (infinite). The product was fractionated on agarose (1.2 %) in TAE buffer and was stained with ethidium bromide. Thermo Scientific Gene Ruler 100 bp plus DNA ladder was used as a DNA marker.

Table 2. Random primers names and their sequences for RAPD-PCR analysis

Primer names	Primer sequence (5'- 3')	Primer names	Primer sequence (5'- 3')	Primer names	Primer sequence (5'- 3')
OP-A01	CAGGCCCTTC	OP-AO15	GAAGGCTCCC	OP-K20	GTGTCGCGAG
OP-A03	AGTCAGCCAC	OP-B06	TGCTCTGCCC	OP-M05	GAAGGCTCCC
OP-A04	AATCGGGCTG	OP-B07	GGTGACGCAG	OP-M12	GGGACGTTGG
OP-A07	GAAACGGGTG	OP-B08	GTCCACACGG	OP-O10	TCAGAGCGCC
OP-A18	AGGTGACCGT	OP-B10	CTGCTGGGAC	OP-O13	GTC AGA GTCC
OP-A19	CAAACGTCGG	OP-B18	CCACAGCAGT	OP-O19	GGTGCACGTT
OP-AA14	AACGGGCCAA	OP-G10	AGGGCCGTCT	OP-O14	AGCATGGCTC
OP-AM18	ACGGGACTCT	OP-K19	CACAGGCGGA		

Inter Simple Sequence Repeats (ISSRs) Analysis

ISSRs-PCR, has been used to obtain molecular markers for drought tolerance. The reactions were conducted using fifteen primers as shown in Table 3. The amplification was performed for 35 cycles as follows; initial denaturation at 94ºC for 5 min, one cycle, denaturing at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 2 min (35 cycle) and final extension at 72ºC for 10 min (one cycle). Then hold at 4ºC (infinite). The product was fractionated on agarose (1.8 %) in TAE buffer and was stained with ethidium bromide. Thermo Scientific Gene Ruler 100 bp plus DNA ladder was used as a DNA marker.

Table 3. Primers names and their sequences for ISSR -PCR analysis.

Primer names	Primer sequence (5`- 3`)	Primer names	Primer sequence (5`- 3`)	Primer names	Primer sequence (5`- 3`)
17898A	$(CA)_6AG$	814	$(CT)_8TG$	HB11	$(GT)_6GG$
17898B	$(CA)_6GT$	844A	$(CT)_8AC$	HB12	$(CAC)_3GC$
17899A	$(CA)_6AC$	844B	$(CT)_8GC$	HB13	$(GAG)_3GC$
17899B	$(CA)_6GG$	HB10	$(GA)_6CC$	HB14	$(CTC)_3GC$
				HB15	$(GTG)_3GC$

RAPD -ISSR combination (R-ISSR) Analysis

Combination between two ISSR primers and two RAPD primers were constructed, Table 4 showed primers name and their sequences for R-ISSR-PCR analysis. The conditions were optimized according to YE *et al*. (2005). The amplification was performed for 35cycles, as follows; initial denaturation at 94ºC for 2 min, one cycle, denaturing at 94ºC for 30 secs, annealing at 38ºC for 30 secs, extension at 72ºC for 2 min (35 cycle) and final extension at 72ºC for 5 min (one cycle). Then hold at 4ºC (infinite). The product was fractionated on agarose (1.5 %) in TAE buffer and was stained with ethidium bromide

Table 4. Combination between ISSR primers and RAPD primers ~~names~~ and their sequences used for R-ISSR-PCR analysis.

ISSR primer names	Sequence (5'- 3')	RAPD primers names	Sequence (5'- 3')
17898 B	$(CA)_6GG$	OPK20	GTGTCGTGAG
		OPK19	CACAGGCGGA
844B	$(CT)_8GC$	OPK20	GTGTCGTGAG
		OPK19	CACAGGCGGA

Statistical analysis

The collected data were statistically analyzed according to Bernardo (2002). Differences between means were compared using Duncan's Multiple Range Test (Duncan, 1955) and declared significant at P≤0.05.

RESULTS AND DISCUSSION

Eight sugarcane genotypes (i.e.G.T.54-9, G84-47, Sp 80-32-80, F.153, Co.997, BOT-41, Co.775, F.161**)** were selected to study the performance of sugarcane genotypes against drought stress and investigate efficiency of the combinations between Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) (referred as R-ISSR technique) as marker to assist selection for drought tolerance in sugarcane.

Sand Culture Experiment

The eight sugarcane genotypes were screened for drought tolerance depending on five traits (i.e. stalk height, stalk diameter, stalk weight, leaf area and number of stalks /m2). The results were presented in Table 5. All studied genotypes were significantly affected by drought. The most affected trait was stalk weight, which had the highest mean of reduction percentage (28.93%), while genotype Co775 had the highest reduction percentage (recorded by stalk weight) (64.44 %). The results in Table 5 revealed that the drought tolerant genotypes were Sp8032-80, Co997 and BOT41 (Sp8032-80 was the most one), while the sensitive genotypes were Co775, F153 and F161 (Co775 was the most one). However, genotypes G84-47 and G.T.54-9 were moderate drought tolerant genotypes. These results agreed with that of Hemaprabha and Simon (2012), Ribeiro *et al. (*2013) and Vantini *et al*. (2015) who found that drought is an abiotic stress that limits the productivity and geographical distribution of sugarcane. Drought is the major abiotic stress that affect morphological parameters such as stalk length, stalk diameter, leaf area and number of stalks.

Table 5. Means of five yield-related traits under drought stress for eight sugarcane genotypes compared with the control.

	C	D	Red%	C	D	Red%	C	D	Red%
	Stalk length (cm)			**Stalk diameter (cm)**			**Stalk weight (kg)**		
G.T.54-9	48.98	46.52^{E}	5.02	0.83	0.73^{FG}	12.09	0.19	0.12^{GHI}	39.39
G.84-47	54.22	49.87^{D}	8.03	0.84	0.83^{BCD}	1.15	0.21	0.14^{EFG}	32.64
Co.997	63.35	59.07^{A}	6.75	0.92	0.85^{B}	7.53	0.31	0.29^{A}	7.94
F.161	69.69	46.43^{E}	33.38	0.84	0.75^{E}	10.57	0.20	0.13^{FG}	34.02
F.153	67.57	57.73^{B}	14.56	1.23	1.01^{A}	18.21	0.38	0.26^{B}	31.81
Co. 775	68.88	49.59^{D}	28.00	0.94	0.65^{I}	30.97	0.32	0.11^{GHIJK}	**64.44**
BOT-41	64.35	56.63^{BC}	12.00	0.84	0.81^{CD}	4.08	0.27	0.23^{C}	13.35
Sp 80-32-80	46.24	43.31^{F}	6.33	0.76	0.69^{H}	8.56	0.16	0.15^{DEF}	7.87
Mean	**60.41**	**51.14**	**14.26**	**0.90**	**0.79**	**11.64**	**0.25**	**0.18**	**28.93**
	Leaf area (cm)2			No. stalks/m2			Mean of five traits		
G.T.54-9	125.95	108.90^{F}	13.54	7.51	6.60^{GH}	12.09	16.42		
G.84-47	99.13	94.85^{G}	4.33	20.74	14.21^{AB}	31.46	15.52		
Co.997	147.78	144.59^{A}	2.16	8.67	7.21^{E}	16.81	*8.24*		
F.161	132.52	126.76^{D}	4.35	9.87	8.11^{D}	17.82	20.03		
F.153	170.17	141.14^{BC}	17.06	6.79	5.16^{I}	24.05	21.14		
Co. 775	118.69	54.89^{JKL}	53.75	7.51	4.95^{J}	34.07	42.25		
BOT-41	99.39	92.20^{HI}	7.23	10.73	9.08^{C}	15.38	*10.41*		
Sp 80-32-80	125.20	120.93^{E}	3.41	7.36	6.93^{F}	5.90	*6.41*		
Mean	127.35	110.53	13.23	9.90	7.78	19.70			

C: control, D: drought treatment and Red: reduction percentage

Molecular genetic studies

This investigation aimed to study the efficiency of technique that combined Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) as marker to assist selection for drought tolerance in sugarcane and detect molecular markers associated with drought, tolerance or sensitivity, in sugarcane using RAPD, ISSRs and R-ISSRs based-PCR analysis**.**

RAPD Analysis:

Depending on screening of sugarcane genotypes in sandy culture, genotypes could be classified into two contrasting groups, the tolerant genotypes (Sp8032-80, Co997 and BOT41) and the sensitive ones (Co775, F153 and F161). Twenty-three arbitrary decamer oligonucleotide primers were used to detect molecular markers associated with drought. Seven primers (OPAO-15, OPG-10, OPK-19, OPM-12, OPAM-18, OPM-05 and OPAA-14) successfully amplified fragments for all genotypes. Data presented in Table 6 and figures 1,2 showed that primers produced a total number of 52 fragments with polymorphism ranged from 70 to 100 %. The seven primers produced 10 bands which could be used to diverse our genotypes due to their drought tolerance. Primers OPM-12, OPAM-18 and OPAA-14 produced positive markers only, while OPK-19 and OPM-05 produced negative markers only, and primers OPAO-15 and OPG-10 produced positive and negative markers.

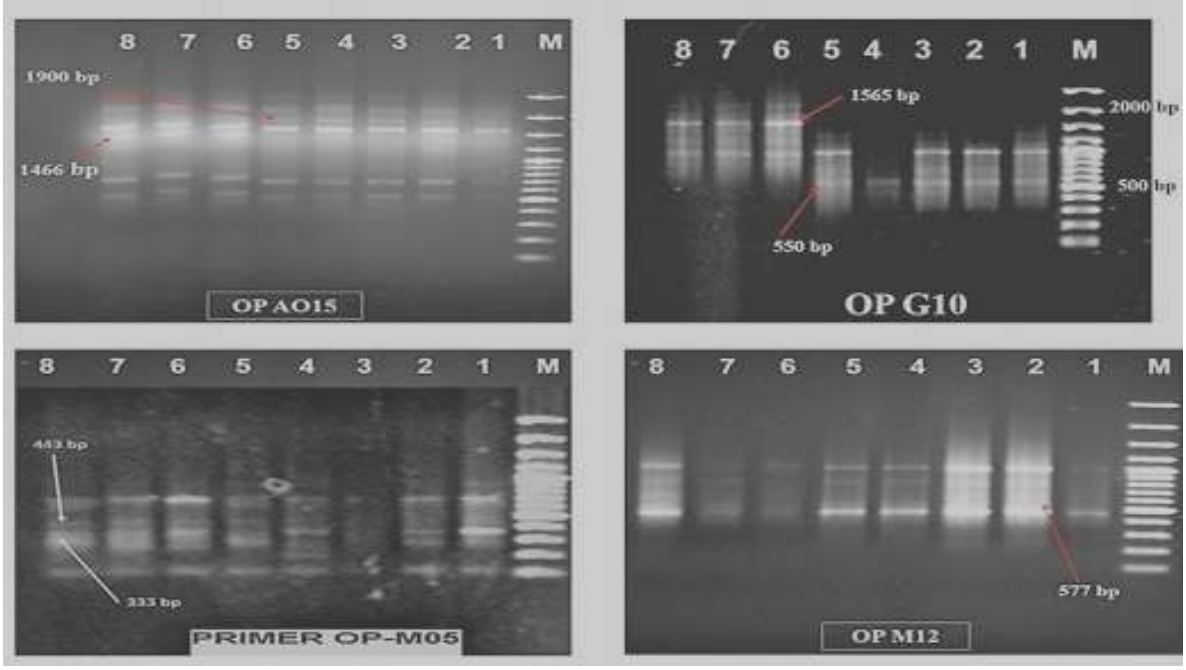

Figure 1: RAPD-PCR polymorphism pattern of drought tolerant and drought sensitive genotypes against primers OP AO15, OP G10, OP M05 and OP M12; M: 100 bp plus DNA ladder, Lane1: Sp 80-32-80, Lane2: Co 997, Lane3: BOT-41, Lane4: G.T. 54-9, Lane5: G. 84-47, Lane6: F. 153, Lane7: F. 161 and Lane8: Co 775

The number and size of bands for the seven primers were presented in Table 6. Imtiaz Khan *et al.* (2013) found that drought tolerance is polygenic and complex trait interplay with the environment makes phenotypic evaluation difficult. Hence, the use of DNA markers can help breeders in improving the speed as well as reliability of the process. RAPD markers were used to assess the genetic diversity for drought tolerance with high cane/sugar yield. Khaled *et al*. (2011) reported that 173 RAPD bands exhibited 95.95% polymorphism in 26 sugarcane clones, while Khaled *et al*. (2015) used RAPD for genetic diversity between nine sugarcane genotypes and found some unique bands specific for each genotype.

Table 6. Total number of amplified and polymorphic fragments, polymorphism % and the specific markers for drought stress in sugarcane using RAPD analysis.

Primer name	Primer sequence (5'- 3')	TAF	PF	P%	SM	
					-ve	+ve
OPAO-15	GAAGGCTCCC	8	6	75	1 (1466 bp)	1 (1900 bp)
OPG-10	AGGGCCGTCT	8	8	100	1 (1565 bp)	1 (550 bp)
OPK-19	CACAGGCGGA	6	6	100	1 (415 bp)	
OPM-12	GGGACGTTGG	6	5	83.33		1 (517 bp)
OPAM-18	ACGGGACTCT	4	3	75		1 (679 bp)
OPM-05	GAAGGCTCCC	10	8	80	2 (333 bp & 443 bp)	
OPAA-14	AACGGGCCAA	10	7	70		1 (920 bp)
Total		**52**	**43**		**5**	**5**

3.2.3 ISSR Analysis:

The data presented in Table 7 and figure 3 revealed that out of 4 ISSR primers produced a total of 57 fragment, 49 out of them were polymorphic fragment. The four primers produced 8 bands which could be used to diverse our genotypes due to their drought tolerance. Primer 17899B produced both negative and positive markers while, the other three primers produced positive only (Table 7). Costa, *et al*. 2011 and Khaled *et al*. 2015 found that some of ISSR primers produced polymorphic bands specific to set of genotypes. They reported that ISSRs amplification proved to be a valuable method for determining genetic variability among sugarcane varieties and for identification of the genotypes.

Table 7. The total number of amplified and polymorphic fragments, polymorphism % and the specific markers for drought stress in sugarcane using ISSR analysis.

Primer name	Primer sequence (5'-3')	TAF	PF	P%	SM	
					-ve	+ve
HB 11	$(GT)_6GG$	9	7	77.8		2 (567 bp & 630 bp)
844B	$(CT)_8GC$	17	15	88.24		1 (1015 bp)
17899 B	$(CA)_6GG$	17	15	88.24	1 (731 bp)	1 (808 bp)
HB 14	$(CTC)_3GC$	14	12	85.71		3 (337 bp, 590 bp and 767 bp)
Total		**57**	**49**		**1**	**7**

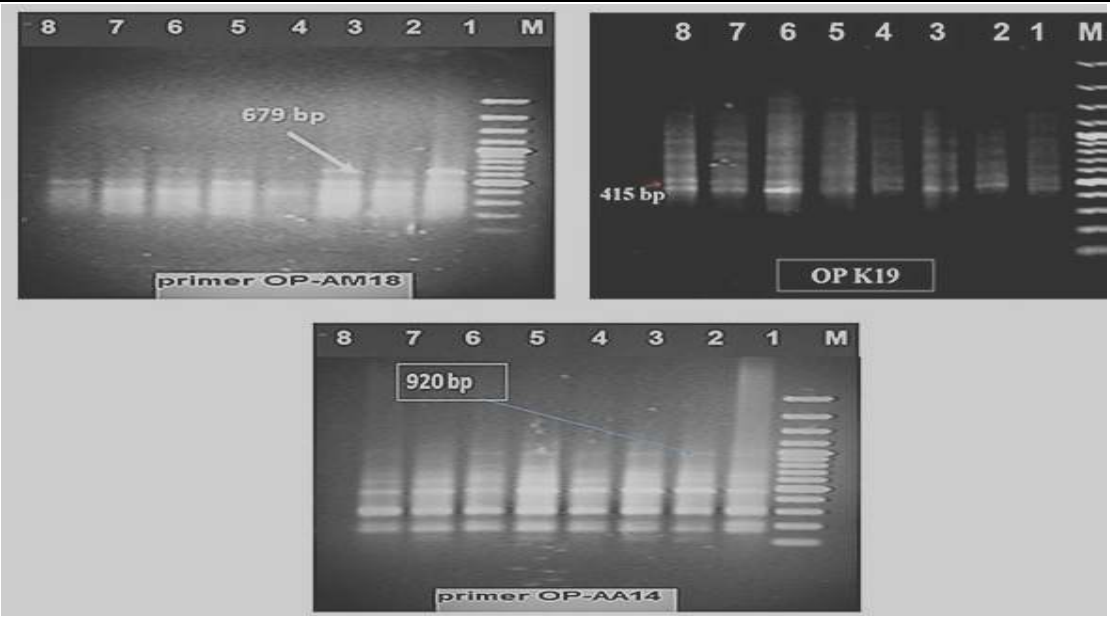

Figure 2. RAPD-PCR polymorphism pattern of drought tolerant and drought sensitive genotypes against primers OP AA14, OP AM18 and OP K19; M: 100 bp plus DNA ladder, Lane1: Sp 80-32-80, Lane2: Co 997, Lane3: BOT-41, Lane4: G.T. 54-9, Lane5: G. 84-47, Lane6: F. 153, Lane7: F. 161 and Lane8: Co 775

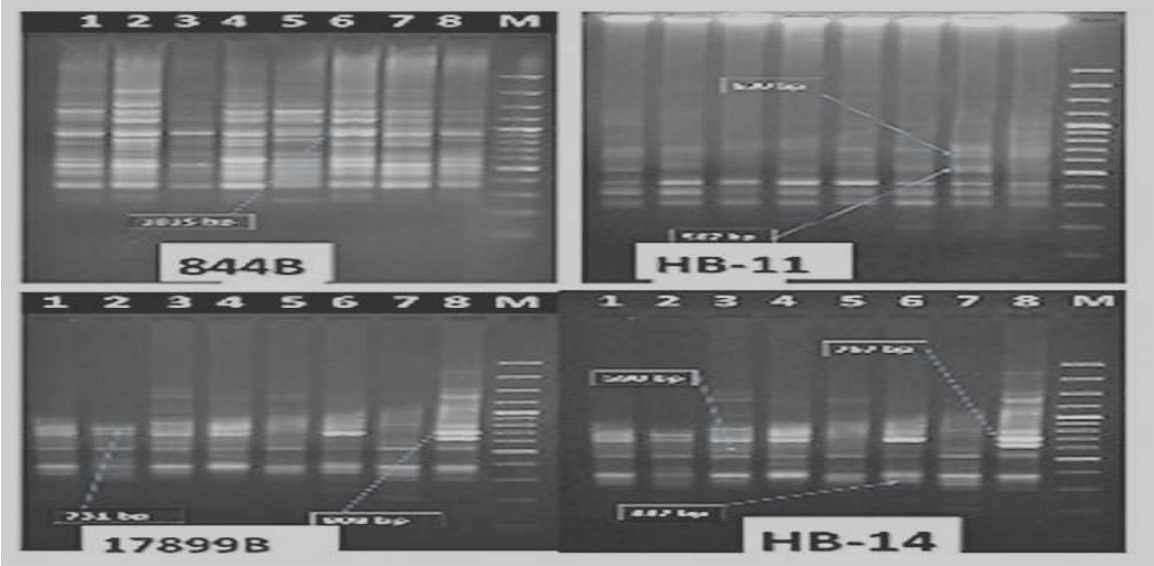

Figure3: ISSR-PCR polymorphism pattern of drought tolerant and drought sensitive genotypes against primers 844B, 17899B, HB-11 and HB-14; M: 100 bp plus DNA ladder, Lane1: Sp 80-32-80, Lane2: Co 997, Lane3: BOT-41, Lane4: G.T. 54-9, Lane5: G. 84-47, Lane6: F. 153, Lane7: F. 161 and Lane8: Co 775

R-ISSR Analysis:

As shown in Table 8 and Figure 4, OPK-19 + ISSR (844B and 17899B) combination completely differ from that revealed by either RAPD primers or ISSR primers alone. OPK-19 with ISSR primers (844B and 17899B) combination exhibited twenty-four amplified fragments with 91.67 and 83.33 polymorphism %, respectively. Among the 24

fragments, 5 fragments could be considered as markers (Table 8 and Fig 5). The number of amplified fragments were 17 when used OPK-20 with ISSR primer 844B. However, the combination OPK-20+844B exhibited 3 fragments which could be used as markers. The total number of fragments obtained from combinations of RAPD (OPK-19 and OPK-20) with ISSR (844B and 17899B) were 41 fragments with polymorphism ranged from 83.33-94.12%, while OPK-19 primer was used alone and exhibited 6 fragments, however, combing OPK-19 with 844B resulted a different pattern of 12 fragments also 12 fragments were obtained when combine OPK-19 with17899B. On the other hands, primer OPK-20 exhibited seventeen fragments when combined with 844B. Our finding was in agreement with that of Ye *et al*. (2005) who combined ISSR and RAPD primers in PCR reactions to detect new genomic loci in two maize lines (Q319 and 1145), they used sequencing gels to separate PCR products and showed good resolving ability in comparison with agarose gels, also to detect SSR loci in the genome that could not be detected by ISSR analysis only. Khaled *et al*. 2011 and 2015 used R-ISSR technique to detect molecular associated to sugar content and genetic diversity in sugarcane. They found that R-ISSR was more reliable and more accuracy than RAPD or ISSR alone.

Table 8. The total number of amplified and polymorphic fragments, polymorphism % and the specific markers for drought stress in sugarcane using R-ISSR analysis.

RAPD	ISSR	TAF	PF	P%	SM	
					-ve	+ve
OPK-19	844B	12	11	91.67	1 731 bp	1 1732 bp
OPK-19	17899B	12	10	83.33	1 573 bp	2 540 bp 677 bp
OPK-20	844B	17	16	94.12	1 321 bp	2 537 bp 1000 bp
Total					**3**	**5**

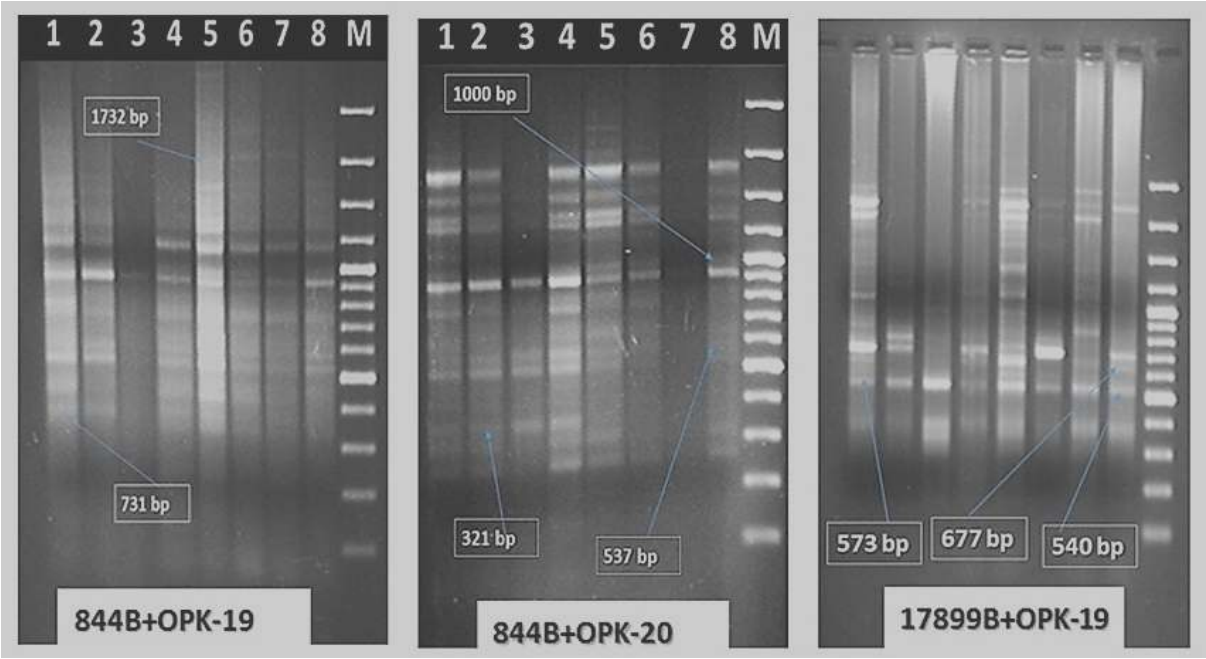

Figure 4: R-ISSR combinations polymorphism pattern of drought tolerant and drought sensitive genotypes against combinations (844B+OP K19), (844B+OP K20) and (17899B+OP K19); M: 100 bp plus DNA ladder, Lane1: Sp 80-32-80, Lane2: Co 997, Lane3: BOT-41, Lane4: G.T. 54-9, Lane5: G. 84-47, Lane6: F. 153, Lane7: F. 161 and Lane8: Co 775

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تقنية R-ISSR كمعلمات وراثية مساعدة للانتخاب لتحمل الجفاف في قصب السكر

خالد عدلى محمد خالد[1] ، شيرين السيد السعيد الشربيني[2] وعبد الهادى عبد الله عبد الهادى [3]

[1]قسم الوراثة – كلية الزراعة – جامعة بنى سويف

[2]قسم التربية والوراثة - معهد بحوث المحاصيل السكرية – مركز البحوث الزراعية

[3]قسم الوراثة – كلية الزراعة – جامعة القاهرة

تم اختيار ثمانية تراكيب وراثية من قصب السكر اعتماداً على نتائج اختبارات سابقة أجريت من خلال تقييم برنامج التربية بمعهد بحوث المحاصيل السكرية وهذه تراكيب هي G.T.54-9, G84-47, Sp 80-32-80, F.153, Co.997, BOT-41, Co.775, F.161 وقد أجريت الدراسة بهدف اختبار تقنية دمج كل من RAPD و ISSR معاً في تقنية تسمى R-ISSR وفاعليتها كمعلمات وراثية مساعدة لانتخاب تراكيب وراثية تتحمل الجفاف وقد تم زراعة التراكيب الوراثية في أصص مملوءة بالرمال وتم الرى بمحلول مغذى على فترات ودراسة مدى تحمل التراكيب الوراثية للجفاف. وقد أظهرت الدراسة أن التركيب الوراثي Sp8032-80 هو أكثر التراكيب تحملاً للجفاف في حين كانت التراكيب Co997 and BOT41 أكثرها حساسية. كما أظهرت الدراسة فاعلية تقنية R-ISSR كمعلمات وراثية مساعدة للانتخاب بالمقارنة مع تقنيات RAPD, ISSR عند استخدامها كل على حدة حيث تم الحصول على 10 معلمات (إيجابية وسلبية) في حالة استخدام تقنية RAPD بمفردها بينما تم الحصول على 8 معلمات (إيجابية وسلبية) في حالة استخدام تقنية ISSR بمفردها وعند استخدام تقنية R-ISSR كان عدد المعلمات الوراثية المتحصل عليها 8 معلمات وراثية جديدة لم تظهر في كلا التحليلين ويمكن استخدامهم في انتخاب التراكيب المتحملة للجفاف. وهذه النتائج دليل على فاعلية التقنية المستخدمة كمعلمات وراثية. وتوصي الدراسة باستخدام تقنية R-ISSR في الانتخاب المبكر لتحمل الجفاف من خلال برامج تربية قصب السكر