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RESEARCH ARTICLE

Evaluating heat stress tolerance and molecular relationship among inbred lines of maize during early generations

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Abstract

Heat stress is one of abiotic stress that has deleterious effects on crops yield. Therefore, this study aims are to evaluate heat-tolerant maize lines and characterizing them by DNA molecular markers. Inbred lines were generated for two generations by selfing pollination. They were evaluated in each generation for the performance of some agro-morphological traits under normal and heat stress conditions. All the traits of the S_1 and S_2 inbred lines varied significantly among them under both conditions, except plant height was not significant under heat stress for the S_1 lines. The L6 inbred line had the highest yield under the stress conditions in both S_1 and S_2 generations. Moreover, the heat susceptible index showed that the lines, L6 and L40 were the highest tolerant in the both generations. Furthermore, cluster analysis based on morphological traits for the 5 selected S_2 inbred lines could be able to isolate the worst S_2 inbred line under heat stress conditions in an independent cluster. In addition, they were characterized by ISSR and SRAP molecular markers. The ISSR detected higher polymorphism (79.79%) than SRAP marker (58.46%). The ISSR clustering patterns managed to classify the highest yield line (L6) under the heat stress in a separated cluster, but both the SRAP and combined isolated the worst line (L32) in one cluster. The Mantel's test showed a positive correlation among all the studied markers. Additionally, the correlation was significant and highly strong (r=0.915) between morphological traits under normal conditions and SRAP marker. However, the identified S_2 inbred lines with resistance to heat tolerance could be a beneficial source in the development of heat-tolerant maize hybrids.

Keywords: HSI; ISSR; maize; molecular markers; SRAP.

1. Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops all over the world. It plays one of the main suppliers of food, feed, fuel, and fodder for millions of poor farmers, also in animal feed, and many industrial targets (Osti, 2019). The world production is about 967 million tons; about 35% of them are produced only by the United States of America (USA). In Egypt, the cultivated area is around 994818 ha with an annual production of 7450000 tones (FAO, 2019). Heat stress is one of several abiotic stress factors that affect worldwide maize productivity (Rowhani *et al*., 2011). Whereas, the exposure of maize for heat stress (>30 °C) for an extended period the grain output drops dramatically, while 20-22 °C is the average ideal temperature for the entire growth season (Schauberger *et al*., 2017). Generally, temperature fluctuations disrupt the photosynthetic process, damage biological membranes, reduce nutrient uptake, and limit the action of numerous enzymes. Moreover, its effect during the reproductive period that produces dried silks, low seed germination and sterility of pollens resulting in a sever drop in yield (Sánchez *et al*., 2014). To face the heat damaging effects and the ever-increasing demand, must improve genotypes that have both high yield and heat tolerance characteristics. Moreover, increasing maize production is one of the major efficient approaches for food security in developing countries. DNA molecular markers are the accurate techniques for the determination of genetic diversity among different genotypes. They are preferable than morphological characterization in that they are not affected by environmental changes. In addition, they are screening along with all genomic sequences of the organism (Prasad *et al*., 2009). Each of Inter Simple Sequence Repeat (ISSR) and Sequence Related Amplified Polymorphism (SRAP) markers are

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dominant PCR based molecular markers and have widely been applied in plants (Liu *et al*., 2008; Shao *et al*., 2010; Liu *et al*., 2012; Bashandy and El-Shaieny, 2016; Luo *et al.*, 2020; Mahmoud and Abd El-Fatah, 2020). The ISSR uses two simple sequence repeat to amplify the DNA fragments inter them without the need for any prior sequence information (Zietkiewicz *et al*., 1994). On the other hand, the SRAP marker technique amplifies the coding regions or Open Reading Frames (ORFs). Its action is based on using a forward primer to amplify the exon sequences and reverse primer specific to the intron and promoter regions (Li and Quiros, 2001).The present study is aimed to develop heat-tolerant maize inbred lines and identify them by DNA molecular markers.

2. Materials and Methods

2.1. Plant materials and field experiment

The field experiment was carried out at the experimental farm of Agriculture Faculty, New Valley University, Egypt for three years 2017, 2018 and 2019. In the first year, S_1 generation was generated by the cultivation of one Maize population Corn Belt Composite E.T.O (was provided by the National Maize Research program) on the 14th of March. Then, 200 strong and healthy plants were chosen before silking, and were self-pollinated. After the harvest, 60 selfed ears (S_1 seeds) that had enough seeds were selected and were individually shelled. In the second year, the S_1 generation was evaluated for the heat stress by growing the 60 S_1 inbred lines at the two planting dates, 15 March 2018 as the heat stress time and 15 July 2018 as the normal planting time. The date of the normal planting time was chosen to avoid excessive heat during the pollination and grain-filling period. Furthermore, selfing pollination was performed for each S_1 inbred line to produce the S_2 seeds. In the third year, the S_2 seeds of the most tolerant S_1 lines (29 lines that had HSI value less than 1.00) and the worst line (L32, as a negative control) were selected and grown under the same conditions as the S_1 inbred lines cultivation. The experimental layout was a Randomized Complete Block Design with two replicates. Each plot consisted of one row 0.70 m apart, three meters in length and 13 hills. The weather conditions of the cultivation seasons are shown in Fig. 1.

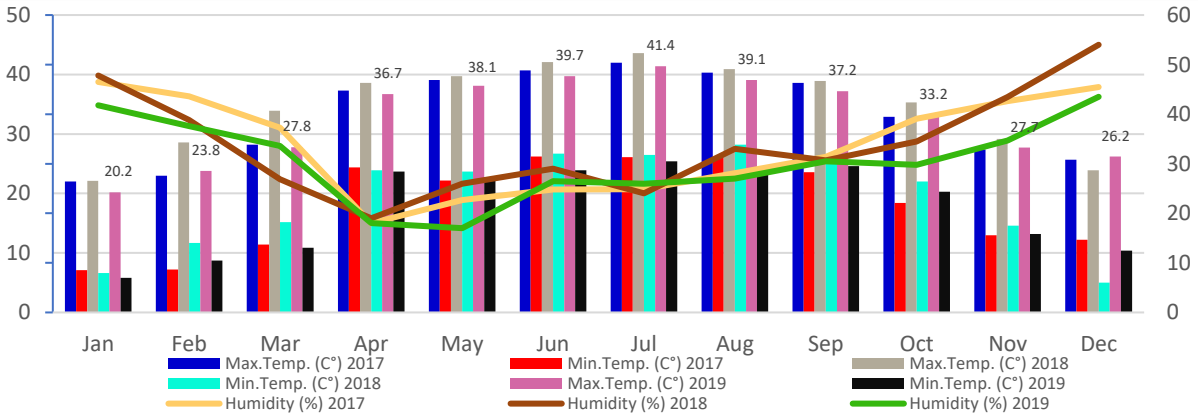

Fig. 1. Weather data of research farm during 2017, 2018 and 2019 (Meteorological station, El-Kharga, New Valley, Egypt).

2.2. Traits measured

Both the S_1 and S_2 generations were evaluated and the data were collected on the different characters i.e., plant height, ear height, leaf rolling, days to 50% anthesis, days to 50% silking, and grain yield (g/plot). When half of the plants had shed days to anthesis were recorded pollen, as well the days to silking when 50% of the plants had silks. At physiological maturity, plant height was measured on five guarded plants per plot and then all plants were harvested, and grain yield was measured. Grain weights were adjusted to 15.5% moisture content. All data were subjected to statistical analysis using SAS 9.2 (SAS Institute, Cary, NC, USA), according to Gomez and Gomez, 1984. The grain yield was used to assess the heat susceptibility index (HSI) according to Fischer and Maurer, 1978.

The morphological data of the two most tolerant S_2 lines (L6 and L40), the two highest yield production S_2 lines (L27 and L46) under normal conditions and the worst S_2 line (L32, as a negative control) under normal and stressed conditions were standardized, then the genetic similarity and the Euclidean

distance were calculated among them. Clustering analysis was done using Minitab 18 (Minitab Ltd., Coventry, UK).

2.3. Molecular analysis

The molecular analyses were applied at the Department of Genetics, Faculty of Agriculture, New Valley University, Egypt.

DNA isolation

DNA isolation kit (Favorgenv Biotech Corp. Cat. No. FAPGK001) was used to extract genomic DNA from young leaves of each S_2 inbred line. The extraction procedures were performed as mentioned in the manufacturer manual. DNAs were quantified and qualified by spectrophotometer and then were adjusted to a final concentration of 50 ng/µl.

2.3.1. PCR amplification procedures

The reactions of ISSR and SRAP markers were applied in a total volume of 25 µl containing: green PCR Master Mix (12.5µl) including 2X (4 dNTPs mixture (400 µM), 50 units/ml Taq DNA polymerase, and 3 mM $MgCl_2$), 2 µl of ISSR primer (10 µM) or 1 µl for each forward and reverse SRAP primers (10 µM) and 2 µl of DNA (50 ng) and 8.5 µl of ddH_2o. The amplifications were applied in a thermal cycler (Labocon, U.K.) PCR. Nine ISSR and seven SRAP primers (by metabion) were applied (Table 1).

Table 1. Names and sequences of the ISSR and SRAP primers used in this study.

ISSR primers	Sequence (5´ to 3´)		SRAP primers	Sequence (5´ to 3´)
UBC 807	(AG)8T	Forward	Me 1	TGAGTCCAAACCGGATA
UBC 808	(AG)8C		Me 4	TGAGTCCAAACCGGACC
UBC 810	(GA)8T		Me 5	TGAGTCCAAACCGGAAG
UBC 811	(GA)8C		Me 8	TGAGTCCTTTCCGGTGC
UBC 812	(GA)8A		Em 1	GACTGCGTACGAATTAAT
UBC 816	(CA)8T	Reverse	Em 2	GACTGCGTACGAATTTGC
UBC 817	(CA)8A		Em 3	GACTGCGTACGAATTGAC
UBC 818	(CA)8G		Em 8	GACTGCGTACGAATTCTG
UBC 823	(TC)8C		E m10	GACTGCGTACGAATTCAG

The PCR program for ISSR marker started with initial denaturation for 5 min at 94°C, then 38 cycles were applied including denaturation at 94°C for 45 sec, annealing for 1 min at 48°C, extension at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR program for SRAP marker was managed as following: initial denaturation for 5 min at 94°C, five cycles included three steps: denaturation for 1 min at 94°C, 1 min annealing at 35 °C and elongation for 1 min at 72 °C, then followed by 35 cycles (1 min at 94°C, 1 min annealing at 50°C and 2 min elongation at 72°C) and a final extension at 72°C for 7 min. The products of PCR amplifications were separated on 1.5% and 2.5% agarose gels for ISSR and SRAP, respectively. The separations were performed in 1×TBE (Tris-Borate-EDTA) ruining buffer at 5 Volt/cm.

Data analysis

The present and absent bands were scored as 1 and 0, respectively. Dendrograms were constructed using the unweighted pair group method with arithmetic average (UPGMA) based on similarity matrix data (Jaccard, 1908). The computational package MVSP 3.1. software program was applied to assess the cluster analysis. Some indices were calculated (polymorphic information contents (PIC, Anderson *et al.*, 1993), resolving power (Rp, Prevost and Wilkinson,1999) and marker index (MI, Powell *et al*., 1996). To calculate the correlation among all the used markers for evaluating the 5 selected S_2 inbred lines (morphological traits under normal and stressed conditions, ISSR, SRAP and combined), the correspondence-coefficient was assessed based on their genetic similarity matrices by using the Mantel test (Mantel, 1967).

3. Results and Discussion

3.1. Mean performance of inbred lines

The results in Table 2, show significant differences among the 60 S_1 and S_2 inbred lines for the traits, plant height, ear height, leaf rolling, days to 50% anthesis, days to 50% silking and grain yield (g/plot) under normal and heat stress conditions. except the plant height was not significant under heat stress (Tables 2 and 3, respectively). The mean performances for the evaluated traits of the S_1 and S_2 inbred lines are displayed in Tables 2 and 3, respectively. Concerning the plant height, the mean values of S_1 lines under normal conditions were ranged from 75.00 cm for L35 to 213.50 cm for L42 line with an average of 138.00 cm. These values in S_2 generation ranged from 103 cm for L33 to 185 cm for L42. On the other hand, under high temperature the values of S_1 lines ranged

from 120.00 to 165 cm for the L4 and L38, respectively, with an average of 146.59 cm. Moreover, the values of the S_2 lines ranged from 117.67 to 177.67 cm for L4 and L46, respectively. Plant height varied among the S_1 and as well among S_2 inbred lines in both normal and the stress conditions according to their genetic makeup, this is consistent with many previous studies (Chen *et al*., 2012; Surender *et al*., 2017; Tripathi *et al*., 2019). These variations were more significant under normal conditions, but their average was higher under heat stress conditions, indicating the exposed temperature during stress time was more optimal for vegetative growth than during normal time. For the ear height, the mean values significantly varied among the inbred lines in both generations under both conditions. Under normal conditions, the values of S_1 lines ranged from 33.50 cm for L35 to 86.50 cm for L42 with an average of 64.46 cm. Among the S_2 lines, the L42 had the highest ear height (82.88 cm), while L32 had the lowest value (47.00 cm). Whereas, under heat stress conditions the values of S_1 lines ranged from 49.50 for L32 to 90 cm for L59 with an average of 74.83 cm. In S_2 generation these values ranged from 57.90 cm for L4 to 86.70 cm for L6. Like the plant height, the ear height trait was not affected by the heat stress due to may be the heat stress planting date was not limited to plant growth, this ties well with the previous findings that showed a positive correlation between the two traits (Tripathi *et al*., 2019). Moreover, increasing the ear height leads to more photosynthates loss from the leaves which affect the crop yield. Thus, this trait is one of the potential traits in improving maize yield (Khayatnezhad *et al*., 2010). For the leaf rolling, the performance of the S_1 inbred lines under normal conditions varied from 1.50 for L9 to 5.00 for L49, while under heat stress ranged from 1.50 for L15 and L27 to 5.00 for L3, L45, L49 and L55. However, in the S_2 generation the values under normal conditions ranged from 1.20 to 3.30 for L46 and L20, respectively. Under stress conditions, the L32 had the highest value (4.33), while L27 had the lowest value (1.50). On the other hand, days to 50% anthesis differed significantly among the evaluated inbred lines at the two conditions. Under the normal conditions, the values of S_1 inbreed lines ranged from 63.00 days for L15 to 79.00 days for L37 with an average of 72.19 days. Moreover, in the S_2 generation, L40 had the shortest period (64 days), but L59 had the longest period (76 days). Under high temperature stress, in the S_1 generation the L9 had the shortest period (63.5 days), while the L39 had the longest period (77 days) for 50% anthesis. In the S_2 generation the shortest period (64.65 days) was recorded by L14, while the longest period (76.50 days) was recorded by L7. Regarding the days to 50% silking, in the S_1 generation, the L15 was the earliest line (70 days) under normal conditions, while under stress temperature was L42 (73 days). On the other hand, L3, L37 and L58 were the latest (86 days) lines under normal conditions, while under heat stress were L18 and L39 (85 days) S_1 inbreed lines. Moreover, in the S_2 generation L16 (69 days) and L6 (73.80 days) were the earliest under the normal and stressed conditions, respectively while, L59 (83.67 days) and L32 (85.90 days) were the latest lines under the normal and stressed conditions, respectively. Under the heat stress conditions, the anthesis silking interval increased causing a reduction in grain yield. However, the most tolerant genotypes had the shortest anthesis siliking interval. Thus, shorter anthesis siliking interval is a better characteristic to determine more tolerant genotypes. These results corroborate the earlier findings of Kandel *et al.* (2017). The grain yield trait significantly fluctuated among all the S_1 or S_2 inbred lines under the normal and the stress conditions. Under the favorable conditions, the values of S_1 inbred lines varied from 169.00 to 975.00 g for L32 and L27, respectively. In S_2 generation these values ranged from 199.77 to 980.22 g for also L32 and L27, respectively. The heat stress conditions highly reduced the grain yield of all the S_1 inbred lines. The reduction varied from 25.83% for L40 to 100% for 23 lines. On the other hand, the L6 line had the highest grain yield value (396.50 g). Also, the yield of S_2 inbred lines was affected by the heat stress conditions and the L6 line had the highest grain yield value (560.45 g). The reduction in grain yield may be due to that the temperature above 38°C during grain filling may affect the photosynthetic rate, pollen viability and fertilization (Rowhani *et al*., 2011; TAO *et al.,* 2016).

3.2. *Heat stress tolerance assessment*

The heat susceptible index (HSI) was estimated to discriminate among the S_1 or/and S_2 inbred lines for their heat stress tolerance capacity (Table 2 and 3, respectively). The estimation was done as the result of variation in yield performance between normal and

stress environments. Based on the HSI value and according to Khanna-Chopra and Viswanathan (1999), all the S_1 lines were classified into three groups, one of them (H) contained the highest heat stress-tolerant lines (2 lines: L6 and L40) which had HSI value less than 0.5. The second group (M) included the most moderate heat-stress tolerant lines (27 lines) which had an HSI values from 0.5 to less than 1. The third group (L) gathered the least heat-stress tolerant lines (31 lines) which had HSI values equal to or more than 1. Moreover, as well as the S_2 lines were also divided into three groups, the highest heat stress-tolerant group contained L6 and L40, the moderate heat-stress tolerant group included 24 lines and the heat-stress sensitive group contained L7, L16, L32 and L33.

Table 2. Mean performance of the 60 S_1 inbred lines for the studied traits under normal (N) and heat stress environments (HS), heat susceptibility index (HSI) and the tolerance (Tol).

S_1 lines	Plant height (cm)		Ear height (cm)		Leaf rolling		Days to 50% anthesis		Days to 50% silking		Grain yield (g/plot)		HSI	Tol
	N	HS	N	HS	N	HS	N	HS	N	HS	N	HS		
L1	130.50	146.00	61.00	74.20	3.500	3.50	71.00	71.00	79.00	79.00	271.00	86.00	0.86	M
L2	126.00	145.00	60.50	74.50	3.50	4.50	76.00	73.00	83.00	84.00	384.00	0.00	1.27	L
L3	86.50	152.00	43.50	84.50	2.50	5.00	78.00	70.00	86.00	80.00	542.00	0.00	1.27	L
L4	145.00	120.00	77.50	60.50	4.00	3.50	73.50	71.00	81.00	78.00	409.00	125.50	0.88	M
L5	125.00	162.50	55.00	87.00	2.50	3.00	75.50	69.00	84.00	76.50	215.00	95.00	0.71	M
L6	136.50	164.50	69.00	87.10	2.00	2.00	71.50	69.50	79.00	74.00	638.00	396.50	0.48	H
L7	126.00	150.50	57.50	82.50	2.50	4.00	73.50	74.50	81.00	82.50	421.00	123.00	0.90	M
L8	157.50	162.50	77.00	78.00	4.00	3.50	72.50	73.00	80.50	80.00	225.50	86.50	0.78	M
L9	103.00	140.00	53.50	66.00	1.50	4.00	73.50	63.50	81.00	75.50	535.00	0.00	1.27	L
L10	140.50	163.00	62.00	72.50	3.00	4.50	69.00	70.50	76.50	80.50	276.00	0.00	1.27	L
L11	150.50	161.85	68.00	76.00	4.50	3.50	74.50	76.00	82.50	83.00	245.50	129.50	0.60	M
L12	145.00	153.50	65.00	68.90	4.00	3.50	72.00	73.00	84.50	80.50	227.00	116.50	0.62	M
L13	159.00	135.50	67.50	71.00	3.00	4.00	67.50	72.00	75.00	79.00	387.50	141.50	0.80	M
L14	169.00	162.00	80.50	84.50	2.00	3.50	70.00	72.00	83.00	79.00	260.50	127.00	0.65	M
L15	168.50	155.00	71.00	71.00	3.50	1.50	63.00	71.50	70.00	80.00	607.50	94.00	1.07	L
L16	129.50	150.50	69.00	73.00	3.50	4.00	64.00	72.00	76.00	79.00	435.00	105.00	0.96	M
L17	150.00	144.50	63.50	64.30	2.50	2.50	68.00	72.00	76.00	79.00	398.50	221.50	0.56	M
L18	127.00	135.50	57.00	70.00	3.00	3.00	70.50	75.50	78.00	85.00	407.00	78.50	1.02	L
L19	161.00	144.50	86.00	80.50	2.50	3.00	67.50	70.50	74.50	77.50	353.00	206.50	0.53	M
L20	160.50	156.00	82.50	85.00	4.00	3.50	70.50	73.00	78.00	80.00	205.00	106.00	0.61	M
L21	129.00	155.00	63.00	69.00	4.00	3.50	71.00	74.50	79.00	81.50	306.00	145.00	0.67	M
L22	141.00	155.00	66.00	64.00	3.50	2.50	72.50	69.50	79.50	76.50	301.50	94.50	0.87	M
L23	141.50	145.00	71.00	73.50	2.00	3.50	72.50	73.50	80.00	80.50	244.00	131.50	0.58	M
L24	128.00	156.00	60.00	75.00	4.00	3.00	73.50	71.50	81.00	81.50	598.00	120.50	1.01	L
L25	132.00	155.00	57.50	60.00	3.00	2.50	66.00	71.00	73.00	79.50	715.00	123.00	1.05	L
L26	150.00	135.00	70.50	60.00	3.50	4.50	73.50	73.00	81.00	82.50	304.50	0.00	1.27	L
L27	169.00	155.00	85.00	77.50	2.5	1.5	69.50	73.00	77.50	80.00	975.00	250	0.94	M
L28	141.50	164.00	61.50	86.00	2.50	3.00	70.00	68.50	78.00	77.50	694.50	132.50	1.02	L
L29	111.00	164.50	51.00	70.50	2.00	3.50	72.50	69.00	80.00	78.50	195.00	0.00	1.27	L
L30	110.00	156.50	44.50	63.50	3.00	2.50	73.50	75.50	81.00	82.50	320.50	161.00	0.63	M
L31	141.50	148.50	55.50	68.00	3.00	2.00	75.00	75.50	83.00	82.50	243.00	130.50	0.59	M
L32	110.00	142.50	43.50	49.50	3.00	4.50	73.00	73.00	82.00	82.50	169.00	0.00	1.27	L
L33	103.00	149.50	65.00	79.00	3.00	4.00	71.00	69.50	78.00	76.50	371.00	110.00	0.89	M
L34	116.50	145.40	46.00	69.50	3.00	3.00	77.00	72.00	84.50	77.00	244.00	93.50	0.78	M
L35	75.00	140.00	33.50	74.00	3.00	4.50	74.00	74.50	83.00	82.00	319.00	0.00	1.27	L
L36	116.50	144.80	55.00	72.90	3.00	3.00	72.50	73.00	81.00	79.00	460.00	138.50	0.88	M
L37	162.50	146.85	61.00	72.35	2.50	3.00	79.00	75.50	86.00	83.50	351.50	0.00	1.27	L
L38	131.50	165.00	70.00	87.00	3.50	2.50	68.00	76.50	76.00	81.50	605.00	0.00	1.27	L
L39	130.50	151.65	72.00	84.50	3.00	4.50	75.00	77.00	83.00	85.00	417.00	0.00	1.27	L
L40	140.50	152.00	68.50	76.40	2.50	2.50	70.50	69.50	78.00	74.00	317.50	235.50	0.33	H
L41	139.00	158.00	67.50	81.00	2.50	3.50	68.00	71.00	75.00	74.50	719.00	119.50	1.06	L
L42	213.50	162.50	86.50	78.60	3.00	4.00	69.50	69.50	77.00	73.00	304.00	114.50	0.79	M
L43	152.50	152.00	66.50	75.50	4.00	4.50	73.00	73.00	80.50	76.50	391.50	0.00	1.27	L
L44	156.00	147.50	86.00	77.35	3.00	4.00	66.00	74.00	73.00	77.50	420.50	0.00	1.27	L
L45	162.50	150.50	70.00	80.50	4.00	5.00	72.00	70.50	79.00	74.00	238.50	0.00	1.27	L
L46	194.00	157.50	67.50	82.60	3.00	2.00	71.50	73.50	78.50	79.50	936.50	256.50	0.92	M
L47	144.00	146.00	68.00	75.50	3.00	4.50	73.50	75.00	80.50	82.50	370.00	0.00	1.27	L
L48	134.00	143.30	61.50	73.00	4.00	4.50	76.50	73.00	83.50	82.00	305.50	0.00	1.27	L
L49	130.00	137.50	64.00	62.50	5.00	5.00	71.0	69.50	75.00	78.00	225.00	0.00	1.27	L
L50	88.50	127.50	43.00	76.50	3.00	3.50	76.50	74.00	84.00	81.50	349.00	0.00	1.27	L
L51	132.50	121.30	56.50	75.50	2.50	3.00	74.00	71.50	81.50	79.00	690.50	105.00	1.07	L
L52	156.00	132.50	66.50	71.90	2.50	4.50	74.50	72.00	82.50	79.50	952.00	0.00	1.27	L
L53	90.00	135.00	47.50	73.00	4.00	4.50	77.00	71.00	85.00	74.50	281.50	0.00	1.27	L
L54	153.50	144.50	68.50	87.50	3.00	3.50	76.50	70.50	84.00	78.00	342.00	123.00	0.81	M
L55	128.00	123.00	71.00	80.00	2.00	5.00	70.00	74.00	77.50	82.50	791.50	0.00	1.27	L
L56	128.50	122.50	55.00	73.00	4.50	3.00	75.00	72.00	82.50	79.50	579.50	0.00	1.27	L
L57	143.50	120.50	78.50	74.50	3.50	3.50	71.50	69.50	83.50	75.00	326.00	145.50	0.70	M
L58	143.00	124.50	66.00	75.00	3.00	2.50	78.00	73.00	86.00	76.50	398.50	0.00	1.27	L
L59	165.50	132.00	66.00	90.00	3.00	3.00	76.00	70.50	83.00	76.50	346.00	113.50	0.85	M
L60	148.50	125.5	85.00	83.00	4.00	2.50	70.50	70.00	77.50	79.00	618.00	110.50	1.04	L
F value	**	ns	**	**	*	**	**	**	**	*	**	**	-	-
LSD 0.05	38.56	29.18	16.80	12.62	1.32	1.18	7.41	3.33	12.06	4.44	124.25	4.71	-	-

*, ** and ns significant, highly significant and non-significant at 0.05 and 0.01 levels of probability, respectively; Capital letters H, M and L refer to high, moderate and low tolerance degree, respectively.

Table 3. Mean performance of the 30 S_2 inbred lines for the studied traits under normal (N) and heat stress environments (HS), heat susceptibility index (HSI) and the tolerance (Tol).

S_2 lines	Plant height (cm)		Ear height (cm)		Leaf rolling		Days to 50% anthesis		Days to 50% silking		Grain yield (g/plot)		HSI	Tol
	N	HS	N	HS	N	HS	N	HS	N	HS	N	HS		
L1	145.50	154.00	62.50	75.50	3.00	3.00	70.00	69.67	76.50	77.00	265.45	145.3	0.96	M
L4	150.50	117.67	67.50	57.90	3.00	3.00	69.00	70.00	74.00	77.00	399.99	215.67	0.98	M
L5	162.57	167.69	63.67	85.00	2.67	3.00	72.00	70.00	77.67	77.50	221.56	132.67	0.85	M
L6	141.67	161.97	61.00	86.70	2.00	2.50	68.67	68.67	74.67	73.80	712.72	560.45	0.45	H
L7	151.00	158.00	57.68	80.00	2.55	3.33	70.67	76.50	75.00	84.00	499.1	120.2	1.62	L
L8	155.00	160.55	62.66	73.00	3.00	3.00	67.50	75.67	74.00	84.00	243.67	133.67	0.96	M
L11	150.43	161.70	61.00	77.69	3.00	3.00	72.00	73.67	78.00	80.00	230.5	145.55	0.78	M
L12	145.00	154.60	60.00	66.00	3.00	4.00	68.00	75.00	73.67	81.67	215.6	139.9	0.75	M
L13	156.00	155.80	67.89	69.00	2.36	3.00	67.50	71.00	72.00	78.50	389.87	217.67	0.94	M
L14	169.00	172.54	75.46	86.68	3.00	3.00	68.67	64.65	78.67	82.50	290.87	165.5	0.92	M
L16	148.00	155.77	64.00	73.00	3.00	4.00	64.00	71.00	69.00	79.67	528.89	113.7	1.67	L
L17	146.33	158.90	53.68	62.69	2.97	2.74	66.00	70.00	72.00	77.00	422.8	225.25	0.99	M
L19	161.00	162.67	64.00	75.67	2.75	3.00	67.67	70.67	73.67	78.50	368.55	224.67	0.83	M
L20	157.22	156.30	73.67	85.00	3.30	4.00	70.50	70.67	78.00	77.67	262.55	143.4	0.97	M
L21	154.00	165.90	61.00	71.67	3.00	3.65	70.00	74.00	75.33	81.67	342.7	185.1	0.98	M
L22	146.00	152.67	58.67	62.65	2.66	3.00	70.50	71.50	76.00	79.00	361.67	198.99	0.96	M
L23	146.50	161.70	56.00	64.89	2.67	4.00	68.67	72.50	74.00	80.67	234.67	125.45	0.99	M
L27	167.00	162.67	75.00	72.75	1.33	1.50	67.50	75.50	71.67	83.67	980.22	522.88	0.99	M
L30	150.00	156.97	57.67	63.67	3.00	2.87	71.00	75.00	75.00	82.67	335.66	178.98	0.99	M
L31	149.22	156.00	55.64	68.00	2.62	2.00	75.00	75.00	79.00	82.67	269.9	185.67	0.66	M
L32	139.00	157.69	47.00	60.00	2.46	4.33	70.00	74.50	76.67	85.90	199.77	78.88	1.29	L
L33	103.00	157.67	65.00	77.00	3.00	3.50	71.00	69.00	78.00	7690	485.5	105.98	1.66	L
L34	154.00	160.00	56.00	83.00	3.00	3.35	71.00	73.50	75.00	82.67	300.93	160.67	0.99	M
L36	148.00	155.00	55.00	76.66	3.00	3.00	72.50	73.00	81.00	80.00	435.9	238.67	0.96	M
L40	148.13	162.77	62.78	84.67	2.00	2.50	64.00	68.67	68.67	74.00	430.87	358.77	0.36	H
L42	185.00	168.67	82.88	78.64	3.00	3.67	69.00	69.67	75.00	74.67	335.9	188.64	0.93	M
L46	182.12	177.67	76.67	83.00	1.20	2.00	68.50	73.67	73.50	79.67	958.11	520.78	0.97	M
L54	151.46	168.64	68.25	79.98	3.00	3.00	73.00	69.50	78.60	77.00	365.64	195.74	0.99	M
L57	143.24	120.66	78.89	74.50	3.00	4.00	71.00	69.50	83.00	75.00	368.77	197.67	0.99	M
L59	165.15	152.89	66.60	80.00	3.00	3.71	76.00	70.67	83.67	76.67	380.12	203.67	0.99	M
F value	**	**	**	**	**	**	**	**	**	**	**	**	-	-
LSD 0.05	8.41	5.30	7.65	6.36	0.68	2.20	3.13	2.57	2.74	0.62	58.57	27.16	-	-

Heat stress affected all the inbred lines in the two generations, even some of them in the S_1 generation lost their yield ability. The most heat-stress tolerant lines had not the highest yield production under normal conditions, but they had the lowest difference between the two conditions. However, the lines had HSI value less than one in both generations indicating relative resistance to heat stress reflecting stable performance over environments. In contrast, the lines which have values greater than 1 indicating relative susceptibility to heat stress. Many researchers have used the HSI and showed that it was more efficient parameter for selecting heat tolerant with high yield genotypes (Thiry *et al*., 2016; Kamrani *et al*., 2018).

3.3. *Cluster analysis using morphological characteristics*

Cluster analysis was performed for the morphological traits data of the evaluated S_2 generation to discriminate among the two most tolerant lines (L6 and L40) that have HIS values less than 0.5, the two highest yield production lines (L27 and L46) under normal conditions and the worst line (L32, as a negative control) (Fig. 2). Under normal conditions (Fig. 2a), the five lines were classified into two main clusters, the first one contained L27 and L46. The second one was divided into two sub-clusters; one included only L40 and the other contained L6 and L32. Whereas under stress conditions (Fig. 2b), the inbred lines were distributed onto two main clusters, the first one included only L32 (the worst line), while the second one branched into two sub-clusters, one of them contained L40 and the second one divided into two groups one group gathered both L46 and L27, but the other one contained only L6.

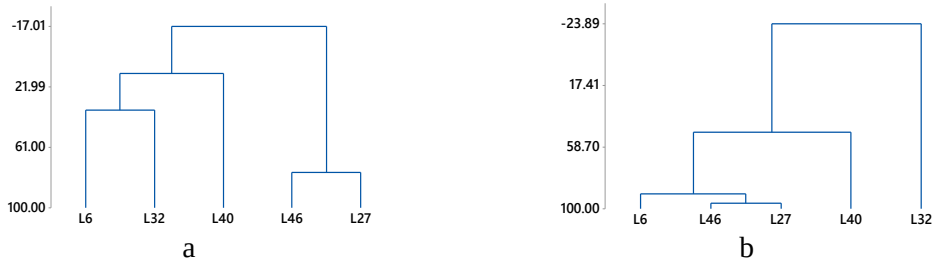

Fig.2. Clustering the 5 selected S_2 inbred lines based on the studied morphological traits. a and b are constructed diagrams under both the normal and heat stress conditions, respectively.

3.4. *Molecular analysis*

3.4.1. ISSR and SRAP markers analysis

ISSR and SRAP markers analysis were applied to verify the level of genetic variability among the five selected lines, L6, L40, L27, L46 and L32 (Figs. 3 and 4). ISSR primers amplified a total of 94 bands having sizes ranged from 145 to 1440 bp. 75 bands out of them were polymorphic with a polymorphism ratio of 79.79% (Table 4). On the other hand, the SRAP marker detected 65 bands, 38 among them were polymorphic bands. The polymorphism ratio was 58.46%. Genetic variability has successfully been determined by ISSR and SRAP markers in many plants (Liu *et al*., 2008; Shao *et al*., 2010; Liu *et al*., 2012; Bashandy and El-Shaieny, 2016; Mahmoud and Abd El-Fatah, 2020). The detected polymorphism differed in both markers due to each of them targeting different sequences of the genome. The value of detected polymorphism was higher in ISSR than SRAP, because the SRAP is designed to detect any variations in coding sequences that having low mutation rates and are more conserved among individuals (Liu *et al*., 2008). this result is consistent with the findings of Shao *et al*. (2010), Liu *et al*. (2012) and Luo *et al.* (2020). On the contrary, Mahmoud and Abd El-Fatah (2020) used molecular markers to evaluate the response of 16 faba bean genotypes to Fusarium wilt. Their results showed the SRAP marker had a higher polymorphism (82.53%) than the ISSR marker (62.24%). Moreover, the PIC value was calculated to differentiate among the two markers for their ability in polymorphism detection. This value in ISSR ranged from 0.24 to 0.4 with an average of 0.309, but in the SRAP marker it varied from 0.12 to 0.364 with an average of 0.218. Furthermore, the primer resolving power (Rp) was calculated to compare among the primers of each marker for their capacity in the detection of genetic variations. The Rp value in the ISSR marker fluctuated from 3.2 to 9.2 for UBC 807 and UBC 811 primer, respectively. For the SRAP marker, the lowest value (1.2) was recorded by Me1/Em1 primers combination, while Me1/Em10 primers combination showed the highest value (6.00). In addition, the marker index (MI) was calculated to select the preferable marker in the detection of the variability among the studied S_2 inbred lines. The ISSR marker had a higher MI value (2.56) than SRAP (1.35) indicating that the ISSR marker provided more informative data than the SRAP marker. Similar results have been found by Luo *et al*. (2020).

3.4.2. Molecular relationship assessment

Based on ISSR and SRAP data, the level of similarity among the five S_2 inbred lines was calculated according to Jaccard's similarity coefficient (Table 5). the ISSR marker showed the highest similarity (0.60) between L46 and L27, also between L27 and L32, while the lowest similarity (0.37) was between L6 and L32. Concerning the SRAP marker, the L6 and L40 displayed the highest similarity (0.82), but the lowest similarity (0.48) was between L46 and L32. The ISSR and SRAP combined data revealed that the L46 and L27 had the highest similarity (0.65), while the L6 and L32 had the lowest similarity (0.47). Furthermore, the dendrograms of genetic similarity using ISSR, SRAP markers and combined were constructed (Fig. 5). All the dendrograms divided the S_2 inbred lines into two main clusters. The ISSR dendrogram separated the L6 in one cluster and combined the rest S_2 inbred lines in the second cluster. The second one was divided into two sub-clusters, one of them contained only L40, while the second one was divided into two groups. The first group included only the L32, while the second one combined both the L46 and L27. Concerning the SRAP dendrogram classified only the worst line (L32) in one cluster and the second cluster was divided into two sub-clusters, one of them contained only the L27. The second sub-cluster branched into two groups, the L46 was classified into one group and the second one gathered both the L6 and L40. The combined results of both markers separated only the L32 in one cluster, while the second main cluster was divided into two sub-clusters one of them included only the L6 and the second one sub-divided into two groups. One group contained the L40, but the other group gathered both the L46 and L27.

The two markers did not construct similar dendrograms, because each of them detecting different sequences in the genome. Many researchers have applied different types of DNA markers to study the genetic variability and showed different dendrograms (Chen *et al*., 2013; Shahlaei *et al*., 2014; Bashandy *et al*., 2020). On the contrary, Liu *et al*. (2012) studied the genetic relationship among five species of *Pinellia* via ISSR and SRAP markers, they showed the two markers exhibited similar clustering patterns. The ISSR marker was able to isolate the line which had the highest yield under the stress conditions in one cluster, but both the SRAP and combined classified the worst line in one cluster as it also observed in the dendrogram of phenotypic traits under heat stress conditions. On the other hand, the combined ISSR and SRAP markers data constructed a closer similar

dendrogram to the dendrogram of phenotypic traits under stress conditions. Moreover, the ISSR marker cannot detect all the transcription regions, while the SRAP amplifying ORF regions, thus combining their data showed an extra result for detecting the variability among the genotypes.

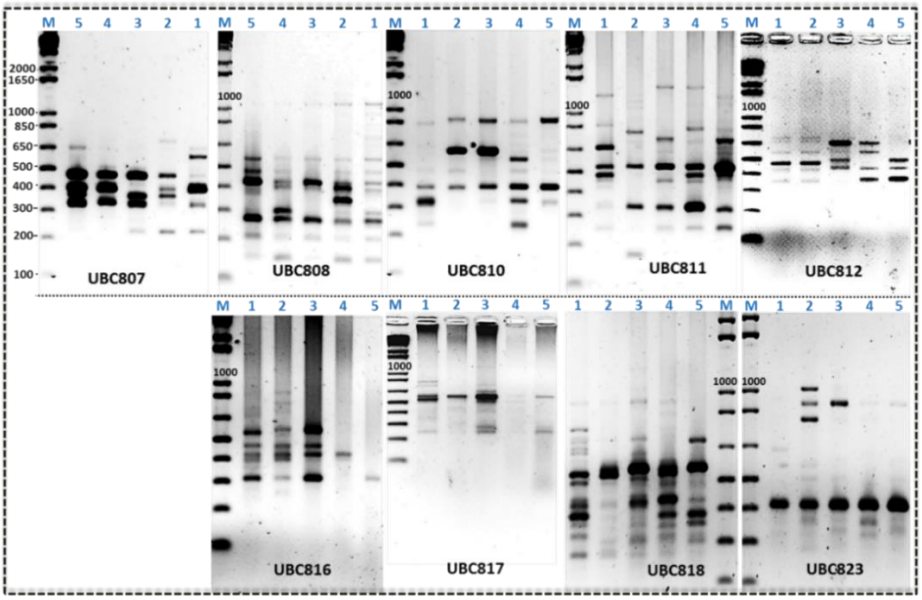

Fig.3. Banding pattern of ISSR marker of the 5 selected S_2 inbred lines. M, kbp DNA ladder; 1, L6; 2, L46; 3, L40; 4, L27; 5, L32 inbred line.

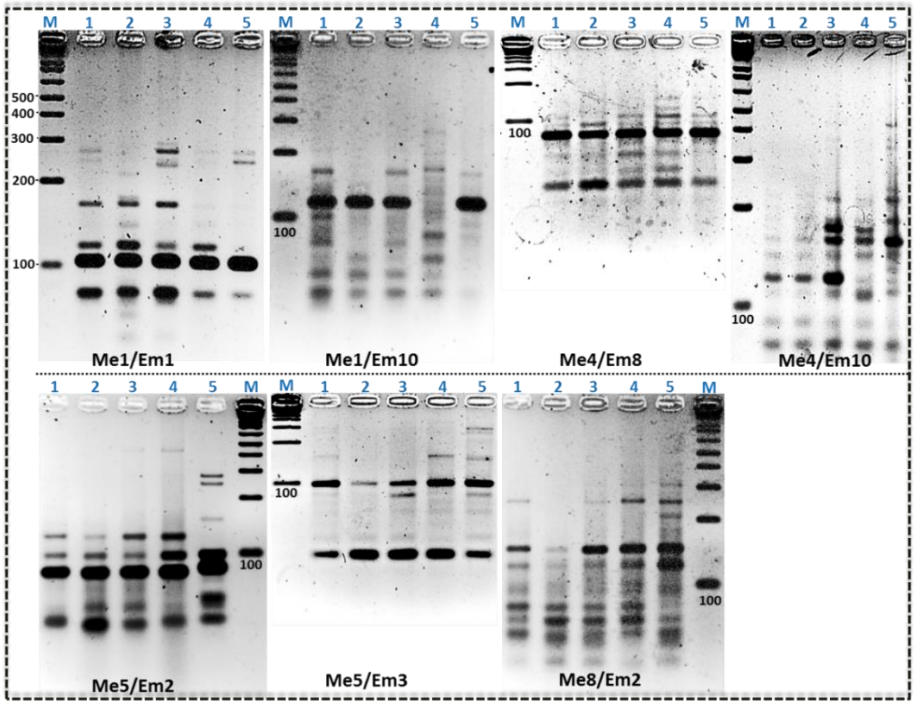

Fig.4. Banding pattern of SRAP marker of the 5 selected S_2 inbred lines. M, kbp DNA ladder; 1, L6; 2, L46; 3, L40; 4, L27; 5, L32 inbred line.

3.5. *Correlation among the used marker types*

The correlation coefficient for the similarity matrices of the used markers (morphological, ISSR, SRAP and combined (ISSR + SRAP) markers) was estimated to study the relationship among them in the evaluation of the 5 selected S_2 inbred lines (Table 6). A positive correlation was detected among all the markers. Moreover, the correlation was significant and strong (0.723) and highly strong (0.915) between combined and ISSR marker and morphological traits under normal conditions and SRAP marker, respectively. It was low and not significant between ISSR marker and morphological traits under heat stress and between morphological traits under normal conditions and combined (0.015, 0.270, respectively). On the other hand, the correlation between the rest markers was moderate and not significant. The morphological traits were highly correlated with SRAP marker than

with ISSR marker which is consistent with the finding of Shao *et al*. (2010). Also, they detected a poor correlation between SRAP and ISSR marker as like our results. The conflict between ISSR and SRAP in correlation with the morphological traits may be because they detect different DNA sequences. ISSR marker aimed to micro-satellite regions, while SRAP amplifies the open reading frames region.

Table 4. Polymorphism (P%), polymorphism information content (PIC), resolving power (Rp) and marker index (MI) obtained by ISSR and SRAP markers in the 5 selected inbred lines in S_2 generation.

Primer name	Range of fragment size bp	Total No. of fragments	Monomorphic fragments	Polymorphic fragments	P %	PIC	R p	MI
ISSR								
UBC 807	210-700	9	3	6	66.7	0.249	3.2	1.49
UBC 808	145-1070	14	5	9	64.3	0.24	4.8	2.16
UBC 810	230-850	9	1	8	88.9	0.284	3.6	2.27
UBC 811	168-1440	18	3	15	83.3	0.338	9.2	5.07
UBC 812	355-701	7	1	6	85.7	0.343	3.6	2.06
UBC 816	310-560	6	0	6	100	0.4	3.6	2.4
UBC 817	240-917	9	2	7	77.8	0.284	3.6	1.99
UBC 818	250-900	14	3	11	78.6	0.263	4.8	2.89
UBC 823	325-1015	8	1	7	87.5	0.38	4.4	2.66
Total	-	94	19	75	-	-	-	-
Average	-	-	-	-	-	0.309	-	2.56
SRAP								
Me1/Em1	75-270	8	5	3	37.5	0.12	1.2	0.36
Me1/Em10	45-262	11	1	10	90.9	0.364	6	3.64
Me4/Em8	31-154	8	3	5	62.5	0.2	2	1
Me4/Em10	77-425	12	8	4	33.3	0.133	2.4	0.53
Me5/Em2	52-455	10	2	8	80	0.272	3.6	2.18
Me5/Em3	30-307	7	3	4	57.1	0.274	3.2	1.1
Me8/Em2	65-310	9	5	4	44.4	0.16	2	0.64
Total	-	65	27	38	-	-	-	-
Average	-	-	-	-	-	0.218	-	1.35

Table 5. The similarity index among the 5 selected inbred lines in S_2 generation based on ISSR, SRAP and combined.

Genotypes	L6	L46	L40	L27	Marker type
	0.49				ISSR
L46	0.75				SRAP
	0.59				Combined
	0.40	0.54			ISSR
L40	0.82	0.73			SRAP
	0.56	0.61			Combined
	0.46	0.60	0.54		ISSR
L27	0.70	0.71	0.75		SRAP
	0.56	0.65	0.63		Combined
	0.37	0.52	0.55	0.60	ISSR
L32	0.62	0.48	0.63	0.54	SRAP
	0.47	0.50	0.59	0.57	Combined

4. Conclusion

In the current study, some derived inbred lines were evaluated in S_1 and S_2 generations for their performance to tolerate heat stress. The evaluation included some agro-morphological traits and DNA molecular marker analysis. Their response to heat stress was significantly varied among them. The L6 inbred line had the highest yield under the stress conditions in S_1 and S_2 generations. Moreover, heat susceptibility index measurement identified both L6 and L40 as the most heat stress-tolerant inbred lines. Furthermore, ISSR and SRAP molecular analysis were efficient tools in the detection of genetic variability among them. However, The L6 and L40 inbred lines can be used in breeding program in developing new heat-tolerant maize varieties.

Acknowledgements

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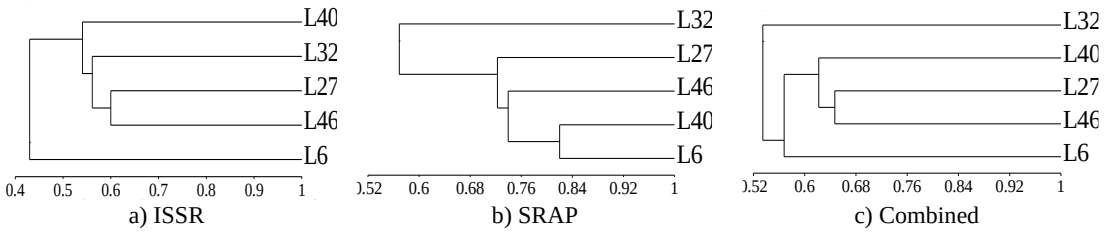

a) ISSR b) SRAP c) Combined

Fig.5. Jaccard's similarity dendrograms constructed by UPGMA based on ISSR, SRAP and combined

Table 6. Correlation analysis among all the used markers.

Marker type	Morphological traits under normal conditions	Morphological traits under heat stress	ISSR marker	SRAP marker
Morphological traits under heat stress	0.702^{ns}			
ISSR marker	0.420^{ns}	0.015^{ns}		
SRAP marker	0.915*	0.717^{ns}	0.383^{ns}	
Combined (ISSR + SRAP)	0.270^{ns}	0.543^{ns}	0.723*	0.336^{ns}

$^{*, ns}$ Correlation is significant and nonsignificant at the 0.05 level, respectively.

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