Detected molecular markers for Alfalfa (*Medicago sativa*) using ISSR and SSR under Egyptian conditions.

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***Abstract*: Alfalfa (*Medicago sativa* L.) is worldwide forage and grown in different environmental conditions. This high geographical adaptation stimulates the genetic variation and gives breeders the possibility of using highly diverse gene pools. In this work five of Egyptian alfalfa (*Medicago sativa L*) cultivars of different geographic origin (Ismailia 1, Ismailia 2, Nubaria 1, Ramah and Sewi) in addition to the Australian cultivar (*Medicago truncatula*) to complete a set of six cultivars represent six genotypes were evaluated for their responses to water regime on the level of some morphological characters such as flag leaf, stem diameter, plant height, leaf length and weight of 1000 grains then undergone a test for their genetic diversity with two selected molecular markers SSR and ISSR . The ISSR marker is more discriminating, provides more informative data than SSR marker. The five used ISSR primers succeeded in identifying four positive markers, the A1 & A2 primers generated positive bands at molecular size 650 & 510 bp in Nubaria 1 & Australian respectively which positively mark the width of flag leaf, stem diameter plant height and the pods productivity when correlates the obtained ISSR results with some of yield traits. The Sewi, Ramah and Australian cultivars which scored the highest plant and larger leaves under water regime are close relatives and genes can flow smoothly among them to produce new Alfalfa genotype with high tolerance to water deficit. Also, Ismailia 1 & 2 and Nubaria 1 are close relatives can exchange genes in between smoothly.**

***Key words*: Alfalfa morphological traits, Water regime, ISSR and SSR markers.**

I. INTRODUCTION

Maximum efforts from almost all countries to improve their nature gift resources to maximize benefits from it, plants are the first ring in the energy chain and so undergoes many scientific researches worldwide to develop new techniques and methods to increase plant production. The classical breeding methods depend on the breeder's innovative experiences from mass selection to the use of a small set of selected families to obtain high yielding modified varieties, and to hybrid production by the use of a cytoplasmic male sterility system (**Desprez et al. 1994**). Although the success of this traditional way, it still has the undeniable disadvantage of the try and error method which is very time consuming. In alternative to classical methods, plant breeding based on their genome is very effective to get enough quantity production with high quality in shorter time and less effort to cover the growing population needs. Enlargement plants production accompanied with successful and advanced breeding for more cultivars in well harmony accommodation to the environment; depend mainly on the well understanding the genetic diversity among the already existing cultivar ancestors. This understanding assures the gene easy exchange and flow between relatives, save huge efforts, money and time. More recently, molecular tools such as QTL analysis (**Paterson *et al.* 1988**) and the molecular marker such as: ISSR and SSR markers for analysis of genetic diversity in the seagrass *Posidonia oceanica***(Serra et al., 2007),** ISSR and SCoT markers for assessment the biodiversity among some Fennel genotypes **(Ramadan et al., 2019),** ISSR and Srap Markers for assessment of biodiversity among some sesame Genotypes **(Aboelnaga et al., 2020)** assisted selection have facilitated these endeavors.

Forage quality traits such as digestibility, nutritional quality and palatability present the molecular biologist with interesting new targets for gene discovery. Genetic modification of these traits should enhance economics and animal health.

Medicago sativa L. (alfalfa) one of the most important legume forage crops, consist of crud protein 16-25%), fibers (20-30%), vitamins (A, B, D, K and E) in addition to minerals (**Patra & Paul, 2019)**. It is characterized by wide range of soil types can grow on despite its texture or alkalinity (**El-Ramady et al., 2020**). Alfalfa plant exists in Egypt in many forms of germplasm under different cultivar names (ex. Ismailia1&2, Nubaria1, Ramah and Sewi). Best irrigation interval 7-10 days, irrigation water in needs is about 7500m^3/ha per season. The annual production of Alfalfa in Egypt reaches 400 tons and about 90%of this amount is exported to the gulf countries in exchange for hard currency. Egyptian alfalfa is cultivated in autumn season (late September).

Medicago truncatula (barrel medic) is a high-quality forage legume commonly grown in Australia and introduced to Egypt many years ago, it is very closely related to the world's major forage legume, common in Egypt; alfalfa (*Medicago sativa*) (**Cook 1999; Oldroyd and Geurts 2001**).

DNA sequence information alone is only one part of an integrated genomics program. Having a "unigene set" of all the expressed genes in a plant allows the researcher to analyze responses to biotic and abiotic stresses, and developmental programs, on a global level using DNA array techniques (**Wu *et al.* 2001**). This has been successfully done in Arabidopsis (**Azpiroz-Leehan and Feldmann 1997**). Development of such resources, which might also include alternative mutational approaches such as transposon tagging (**Fitzmaurice *et al.* 1992 and Baulcombe 1999**), will be a rate limiting factor for the full exploitation of genomics approaches to forage crops.

Recently, DNA-based molecular markers have been counted for assessment of the genetic diversity between germplasm in many plant species. Those markers are characterized by their neutrality and feasibility, despite the age, tissue type and environmental conditions. (**Zietkiewicz *et al*., 1994**) stated that Inter Simple Sequence Repeat (ISSR) markers detect the polymorphism in the non-coding region have to identify and determine relationships in between species. ISSR protocol is widely applicable because it is rapid, inexpensive, require small amounts of template DNA. ISSR markers used earlier to discriminate polymorphism among Egyptian population and varieties of fennel aromatic plant and even shows it superiority and accuracy over the SCoT marker (**Ramadan et al., 2019**).ISSR markers have been efficiently used for study of genetic diversity of different medicinal plant species and crops such as *Phaseolus vulgaris* L. (**Galvan *et al*., 2003**), barley (**Yong-Cui *et al*, 2005**), *Artemisia capillaries* (**Shafie *et al*, 2009**), *Lippia alba* Mill. (**Manica-Cattani *et al*, 2009**) and *Achillea millefolium* (**Farajpour *et al*, 2012**).

SSRs are co-dominantly inherited, allowing the heterozygote in diploid genomes to be distinguished (**Goldstein & Schlotterer 1999**). Despite the relatively high coast of SSRs markers; the method protocol itself is fast and easy to employed (**Goulão & Oliveira 2001**) SSR analysis detect the variation in DNA at pre-determined sequence sites while the ISSR analysis is more efficiency to scan the whole genome, counting on those two types of marker analysis can configure the polymorphism more than either marker dose alone due to the nature of genetic variation detected by each marker category.

Increased growing population in Egypt need for more and more demands from animals wealth which using alfalfa as common forage. In this research aim to study the effect of water regime to 50% on some morphological parameters on six of Alfalfa cultivars, five of which collected from different regions in Egypt and the sixth is with Australian origin, to point to the most tolerant genotypes to such biotic stress. Then we work on these cultivars to detect the distance relationships between these cultivars with ISSR and SSR markers, aiming to help the breeders to improve the production of alfalfa under stressful conditions.

II. MATERIALS AND METHODS

Five of Egyptian Alfalfa (*Medicago sativa L*) cultivars of different geographic origin (Ismailia 1, Ismailia 2, Nubaria 1, Ramah and Sewi) in addition to the Australian cultivar (*Medicago truncatula*) to complete a set of six cultivars represent six genotypes were used in the conducted experiment, they were obtained from Agriculture Research Center, Giza, Egypt.

Field experiment:

The genotypes were grown in Ismailia governorate, they were undergoing to cut slicing in two successive seasons, 2019-2020 and 2020- 2021. The studied genotypes were cultivated in lines by winter. The field experiment was randomized complete block design in three replicates. Two cuts were performed in (January and May). The cultivated plants were watered with normal amount of water with interval time 20 days (first season 2019-2020, first cut), after then plants were supplied with only 50% of their water needs till second cut (second season 2020-2021, the second cut). The morphological characters recorded just before each cut. These characters were; the flag leaf length, the flag leaf width in cm, the plant height measure from the soil surface to the tip of the tallest tiller in meter, the stem diameter in cm, the leaf length and the weight of 1000 grains.

Lab experiment:

This work took place in bench top of Genetics and Cytology lab, National Research Centre during fourteen months between 2020 and 2021.

ISSR-PCR analysis:

PCR reactions will be carried out using ISSR primers according to **(Zietkiewciz *et al*., 1994)**. The isolated DNA will be performed for ISSR-PCR reaction of different samples. The reaction mixture in 25µl will be contain 2µl of genomic DNA, 1µl of the primer, 2.5µl of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase and 200mM of each dNTP. Amplifications will be performed in DNA amplification Thermocycler (PTC-100 PCR version 9.0 from M J Research-USA). The apparatus is programmed to execute the following conditions, denaturation step of 5 min at 94°C, followed by 35 cycles composed of 30 s at 94°C, 90 s at the annealing temperature (Table 1: Primer sequences and codes used according to each primer) and 90 s at 72°C. The amplifications will be performed at least twice and only reproducible products will be taken into account for further data analysis.

Table 1: The primer codes and sequences of the selected ISSR primers used to characterize six Alfalfa genotypes.

NO.	Primer codes	Sequence (5` to 3`)
1	A1	AGAGAGAGAGAGAGC
2	Am2	TCTCTCTCTCTCTCC
3	UBC890	(AGC)(ACT)(AGC)(GT)7
4	A12	(GA) 6 CC
5	UBC818	(CA)7G

Ssr-Pcr Analysis:

PCR reactions will be optimized and mixtures (25 µl total volume) will be composed of dNTPs (200 µM), Mg Cl2 (1.5 mM), 1x buffer, primer (0.2 µM), DNA (50ng), Taq DNA polymerase (2 units). Amplification will be carried out in a Thermo Cycler programmed for 94 ˚C for 3 min (one cycle); followed by 94 ˚C for 30 sec, 60 ˚C for 1 min and 72 ˚C for 2 min (36 cycle); 72 ˚C for 10 min (one cycle), then 4 ˚C (infinitive). Amplification products (15 µl) will be mixed with 3 µl loading buffer and separate on 1.3% agarose gel and stain with 0.5 µg/ml ethidium bromide, and visualize under ultraviolet light and photographed. DNA fragment sizes will be determined by comparisons with the DNA marker.

SSR primers

In the SSR experimental technique, five primer pairs were used for genotyping the alfalfa (Table 2). DNA was extracted from fresh plant material (leaf tissue).

Table 2: Sequences of five selected SSR primers used to characterize six of Alfalfa genotypes.

Primer	Size	SSR motif	Forward primer (5`-3`)	Reverse primer (3`-5`)
RCS084	156	AAG	CCTCATCATCAAAT TCATTCT	AGCCAGAA
RCS125	200	ATC	TGCAAACTCCGCTT TATGC	CTCGCTGA
RCS022	153	ATC	GGTAGTTTCTGACTT TCCCGT	TACAAAAG
RCS048	175	ATC	GAATGCCAAGACAC CTGTGA	TCTCATCA
RCS073	199	AAG	CGCAATCTTTCTTCT CATTTCA	TTCAACAT

Gel electrophoresis: Amplification products of ISSR and SSR will be separated on 1.5% agarose gels in 1X TAE buffer with DNA ladder (1Kb for ISSR analysis) and 100bp DNA ladder for SSR analysis. Then, the gels will be detected by staining with ethidium bromide according to **Sambrook *et al.*, (1989).** The PCR products will be visualized by UV-transilluminator and photographed by gel documentation system, Biometra - Bio Doc. Analyze.

*Data analysis***:** Only clear, unambiguous and reproducible bands will be considered for data analysis. Each band will be considered a single locus. Data will be scored as (1) for presence and (0) for absence for each cultivar according to (**Nouh Gel analyser, 2004**) to detect positive and negative markers. The similarity coefficients will be generated by SPSS program version-20 **(Microsoft office)** to construct a dendrogram by the unweight pair group method with arithmetical average (UPGMA).

Statistical Analyses:

All the data were expressed as means ±S.E. The statistical significance was evaluated by T-test. Values were considered statistically significant when $p < 0.05$.

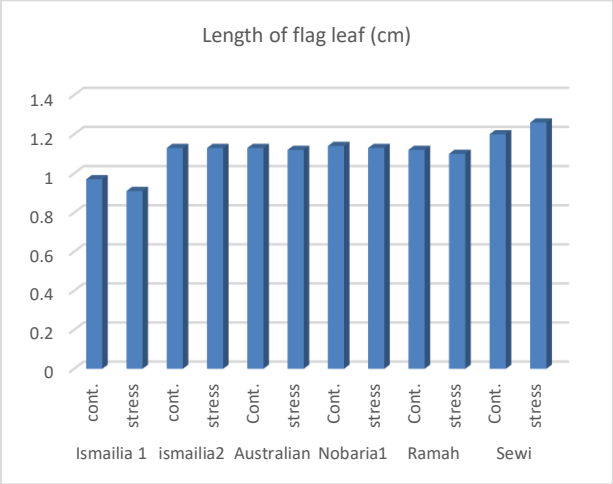
III. RESULTS

In two seasons at period of 2019 to 2021 studied some morphological characters in means recorded under drought stress with 50% of water irrigating (Table 3) & (chart 1,2 and 3).

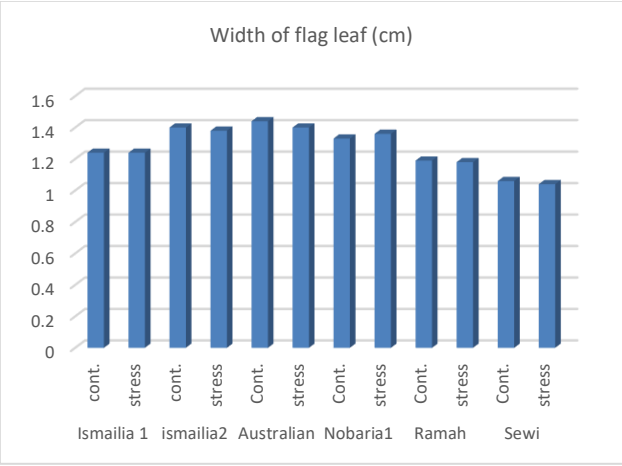
Table 3: The mean performance for some morphological character of six alfalfa genotypes in two successive: 2019-2020 as control (first slicing) and 2020- 2021 after 50% of irrigation water regime (second slicing) as stress.

Cultivar name	**Means± SE of morphological characters**											
	Ismailia 1		**ismailia2**		**Australian**		**Nobaria1**		**Ramah**		**Sewi**	
	cont. ±S.E.	**Stress ±S.E.**	**cont. ±S.E.**	**Stress ±S.E.**	**Cont. ±S.E.**	**Stress ±S.E.**	**Cont. ±S.E.**	**Stress ±S.E.**	**Cont. ±S.E.**	**Stress ±S.E.**	**Cont. ±S.E.**	**Stress ±S.E.**
Length of flag leaf (cm)	0.97± 0.01	0.91± 0.01*	1.13±0 .00	1.13± 0.01	1.13 ± 0.01	1.12± 0.01	1.14± 0.00	1.13± 0.00*	1.12±0 .00	1.1± 0.01*	1.2± 0.01	1.26± 0.03
Width of flag leaf (cm)	1.24± 0.02	1.24± 0.01	1.40±0 .01	1.38± 0.02	1.44± 0.02	1.40± 0.03	1.33± 0.02	1.36± 0.02	1.19±0 .04	1.18± 0.03	1.06±0 .01	1.04± 0.02
Flag leaf area (cm^2)	1.20	1.12	1.58	1.56	1.63	1.57	1.52	1.53	1.33	1.30	1.27	1.31
Stem diameter (cm)	0.76± 0.02	0.74± 0.01	0.72±0 .01	0.73± 0.03	0.85± 0.01	0.82± 0.01	0.57± 0.01	0.59± 0.00	0.86±0 .03	0.89± 0.02	0.65±0 .01	0.61± 0.01
Plant height (m)	1.42± 0.01	1.42± 0.01	1.76±0 .01	1.72± 0.01*	1.95± 0.01	1.92± 0.02	1.52± 0.01	1.53± 0.02	1.84±0 .02	1.79± 0.01	1.75±0 .01	1.71± 0.01*
Leaf length (cm)	1.84± 0.02	1.83± 0.01	1.9± 0.01	1.88± 0.02	1.86± 0.01	1.86± 0.01	1.73± 0.01	1.71± 0.02	1.63±0 .01	1.64± 0.01	1.83±0 .01	1.82± 0.01
1000 grain weight (g)	27.3± 1.2	26± 0.58	32.3±0 .88	32.66±1 .45	41.6± 0.88	43.67±0 .88	30± 0.57	33.33± 0.88*	27± 0.58	24± 0.58*	31.7±0 .88	32.33± 0.88

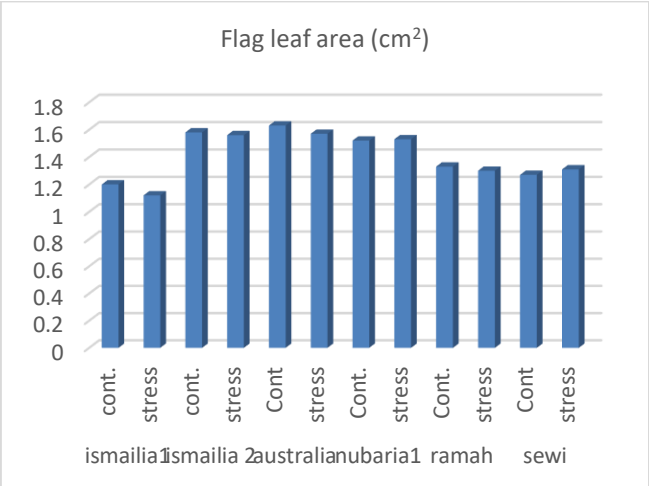
*: The data is significant (the significant value at P< 0.05) means ±S.E.



(A)

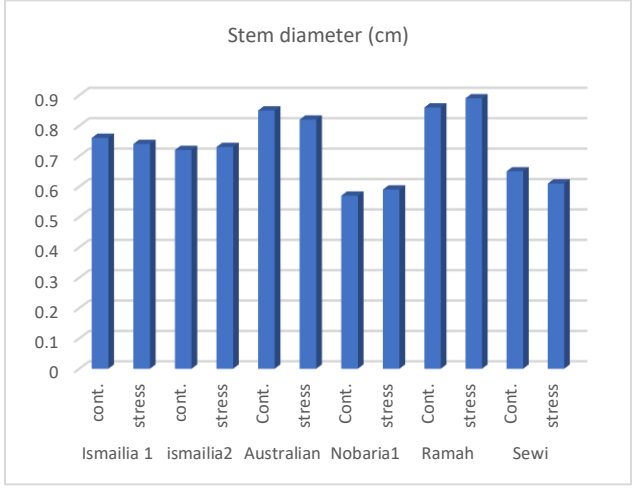


(B)

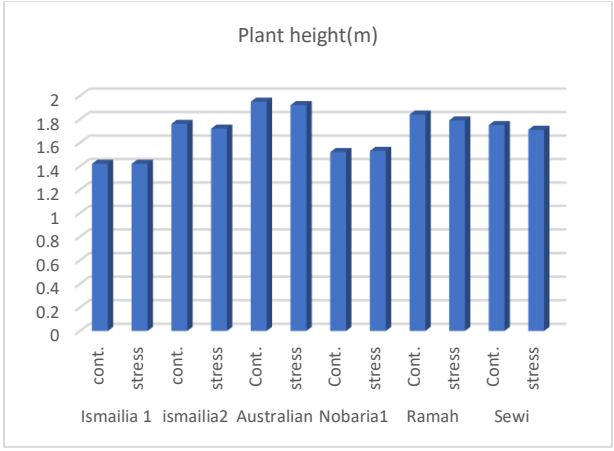


(C)

Chart (1): The response in means for the six alfalfa cultivars to 50% of water regime as a stress relative to the normally watered as a control on the level of flag leaf length (a), flag leaf area (b) and flag leaf area(c).

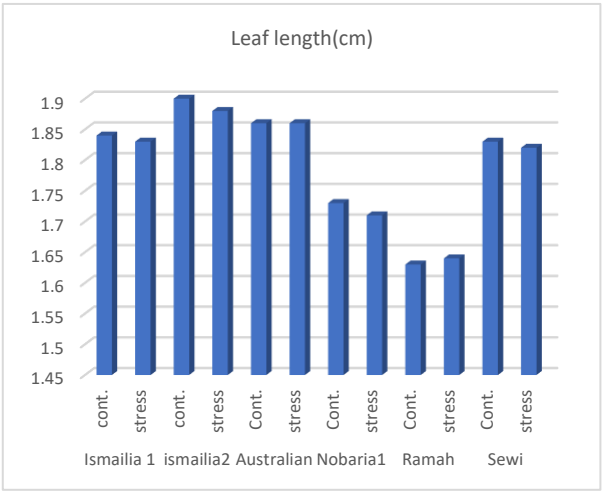


(A)

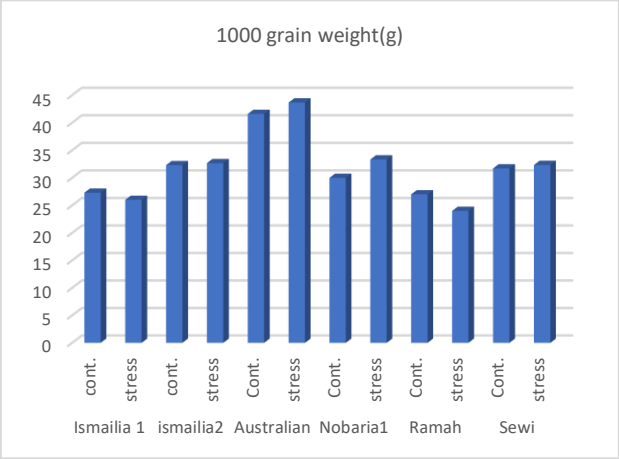


(B)

Chart (2): The response in means for the six alfalfa cultivars to 50% of water regime as a stress relative to the normally watered as a control on the level of stem diameter in centimeter (a) and plant height in meter(b).



(A)



(B)

Chart (3): The response in means for the six alfalfa cultivars to 50% of water regime as a stress relative to the normally watered as a control on the level of leaf length in centimeter (a), weight of 1000grain in grams (b).

*On the level of flag leaf***:** The six cultivars accommodate differently under water regime, the flag leaf length Ismailia 1 showed the maximum decrease in the length by 6.1%, followed by Ramah by 1.7% decrease, Australia and Ismailia 2 by 0.8% decrease. Only the Sewi cultivar showed a 5% increase in the leaf length after water regime. Sewi cultivar scored the maximum flag leaf length among the all-tested cultivars before and after water regime.

Australian cultivar scored the maximum percentage of lowering the flag leaf width (2.7%) followed by Sewi scored 1.8%. only Nubaria1 showed an increase in the flag leaf width by 2.25%.

Leaf area is: the ratio of total one-sided area of leaf tissue to ground surface area (Ghimire, 2017), it is calculated as the following formula: Area = length× width× k where k is a multiplying constant factor. Relaying on that, Australian cultivar scored the maximum leaf area before water stress (1.6 cm^2) and after water regime (1.57 cm^2), Sewi cultivar followed by Nubaria 1 cultivar scored an increase in the flag leaf area after the water stress 1.3 cm^2 with percentage of increase (3.02%) and 1.5 cm^2 with percentage (1.35%) respectively. While Ismailia 1 cultivars responded to water regime by decreasing the flag leaf area with the highest minimization percentage (6.18%).

On the level of stem diameter: Ramah scored the highest stem diameter in normal irrigation conditions (0.86 cm) and even more after the water regime conditions (0.89 cm) by increasing percentage 3.3%, Australian cultivar came in the second place in normal watering (0.85 cm), then showed a little decrease in stem diameter as response to water regime to reach (0.82 cm) with variation reached 3.5%.

Ismailia 2, Nubaria1 and Ramah responded to water deficit by increasing the stem diameter while the other genotypes lowered the stem diameter after regime

On the level of plant height: The variation in the plant height in normal watering condition reached 27 % as the highest plant was the Australian genotype scored 1.95 m and the shortest plant was the Ismailia genotype scored 1.42 m.

The water regime scored variation in plant height reached 26% between Australian (1.92 m) and Ismailia 1 (1.42 m) genotypes.

Ismailia 1 did not response to water regime on the level of plant height, Ismailia 2, Australian, Ramah and Sewi responded to water deficit by shortening in their plant heights. This shortening was statistically significant only after Ismailia 2 and Sewi genotype. Worth to mention that, only the Nubaria 1 genotype scored an increase in the plant heights after water deficit.

*On the level of leaf length***:** Most of the cultivars showed non-significant leaf shortening as a response to water regime except for Ramah. It showed an increase the mean of leaf length after such stress by variation reached 0.6%. on the other side, the Australian genotype did not react to the stress and did not show any difference in the leaf length.

Change in the leaf morphology including the leaf length refers to the plant survival attempts to increase the leaf protein content to increase the photosynthesis per unit of leaf volume **(Marcelis et al. 1998)**.

*On the level of 1000 grains weight***:** Regarding the grain weight as quality trait for the forage, among the six tested cultivars Australian scored the highest grains weight in normal watering condition (41.6 g) and even more after the water regime (43.67g) with variation reached 4.7%. Nubaria 1 responded to water regime significantly and positively by induction of significant increase in the grains weight from 30 g to 33.33 g by variation percentage reached 10%. On the other side the Ramah, it significantly responded to water deficit by lowering the grains weight from 27 g to 24 g by percentage reached 11%.

On collecting the morphological data, the Australian cultivar showed well accommodation to the water regime by keeping its character with minimum difference. Also, Sewi scored the maximum increase in the flag leaf area, Ramah scored the maximum increase in the stem diameter which are favorable characters.

ISSR analysis:

Five semi-specialized ISSR primers (A1, Am2, UBC890, A12and UBC 818) were used to assessment the genetic diversity among six alfalfa cultivars grown under water shortage condition. The obtained molecular markers were seven, four of them were positive markers, and three negative molecular markers (Table 4).

Table 4: Positive and negative markers using five ISSR primers with six Alfalfa varieties under drought stress.

Primers name	MS	Alfalfa Ismailia1	Alfalfa Ismailia2	Alfalfa Australian	Alfalfa Nobaria1	Alfalfa Ramah	Alfalfa Sewi	Positive(P)and Negative(N) markers
A1	650	0	0	0	1	0	0	P
Am2	510	0	0	1	0	0	0	P
	330	1	1	0	1	1	1	N
UBC890	570	1	1	1	1	1	0	N
A12	710	1	0	0	0	0	0	P
	430	1	0	0	0	0	0	P
UBC818	625	1	1	0	1	1	1	N

The generated monomorphic /polymorphic, unique bands, the percentage and the average of polymorphism in between the six tested alfalfa cultivars are presented in table (5) and figure (1).

Table 5: List of the selected ISSR primers and the polymorphisms obtained total number of fragments detected by each primer, %P and fragments sizes of six alfalfa populations.

Primer name		Amplified bands				%P	Fragments' size	
	Bands number	polymorphic bands	Monomorphic bands	Unique bands	absent bands		Larger size	Smaller size
A1	15	11	4	1	-	73.3	710	140
Am2	9	7	2	1	1	77.7	540	240
UBC890	7	5	2	-	1	71.4	630	170
A12	8	6	2	2	-	75	1260	250
UBC818	6	2	4	-	1	33.3	910	330
Total	45	31	14	4	3	68.8%	1260	140
Average	9	6.2	2.8	-	-			

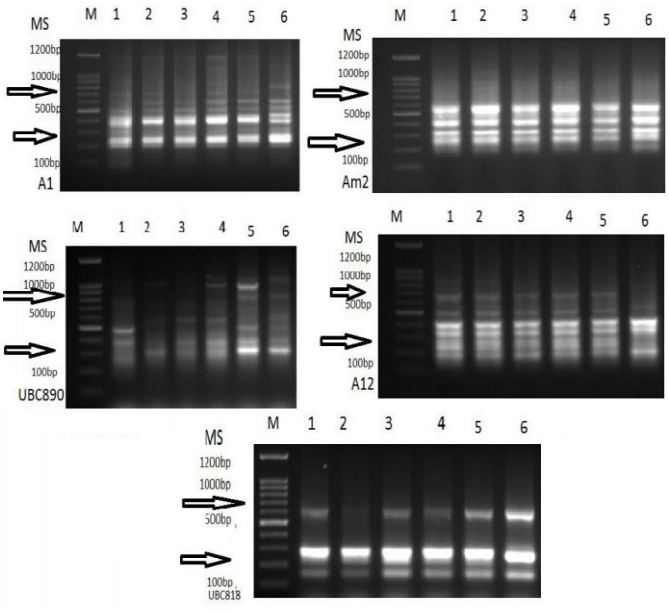


Figure (1): Amplify DNA using five ISSR primers for five Egyptian alfalfa cultivars (Medicago sativa L) and an Australian cultivar (Medicago truncatula). M: marker, 1: Ismailia 1, 2: Ismailia 2, 3: Australian, 4: Nubaria 1, 5: Ramah and 6: Sewi cultivar

The amplified bands size ranged from 140bp after A1 primer to 260 bp after A12. The total bands number ranged from 6 bands after UBC 818 to 15 bands after A1 with average 9 amplicons per primer, all the used primers detected polymorphism and revealed 62 amplicons with 68.8%.

The highest percentage of polymorphism was 77.7% after Am2 and the lowest percentage of polymorphism was 33.3% after UBC 818. The number of monomorphic amplicons varied from four after A1 ana UBC 818 to two after Am2, UBC 890 and A12, with average 2.8 monomorphic bands per primer.

The number of polymorphic bands varied from two after UBC 818 to eleven after A1 with average 6.2 polymorphic amplicons per primer. The fragments with molecular size: 650bp (after A1 primer characterizes the alfalfa Nubaria cultivar), 510 bp (after Am2 primer characterizes the Alfalfa Australian cultivar) and with molecular size 710& 430bp (after primer A12 characterizes the Ismailia 1) were detected and considered as positive markers.

Three common bands between all cultivars and absent from only one cultivar; considered as negative markers were detected at molecular band sizes: 330bp (missed after Australian), 570 (missed after Sewi) and 625 bp (missed after Australian), these negative markers were distinguished by AM2, UBC 890 and UBC 818 primers respectively (Table 4).

The results of ISSR analysis were translated and analyzed to generate the dendrogram and similarity index (Fig 1 and table 6).

Table 6: Proximity matrix for six alfalfa varieties using five ISSR primers.

Case	Matrix File Input					
	Ismailia1	Ismailia2	Nobaria1	Sewi	Ramah	Australian
Ismailia1	1					
Ismailia2	0.910	1				
Nobaria1	0.867	0.883	1			
Sewi	0.366	0.194	0.222	1		
Ramah	0.422	0.249	0.276	0.979	1	
Australian	0.387	0.194	0.522	0.898	0.624	1

PIC, MI and fragments sizes

SSR analysis:

Five specialized SSR primers (RCSO 84, RCS 125, RCS022, RCS048 and RCS073) were used to assessment the genetic diversity among six alfalfa cultivars grown under water shortage condition. The amplified bands sizes ranged from 180 bp after RCS084 to 890 bp after RCS 125.

Total bands number was 23 bands, ranged from 4 bands after RCS048 &RCS073 to five bands after RCS084, RCS 125 and RCS022 with average 4.6 amplicons per primer. All the used primers detected polymorphism with average 52%. The highest percentage of polymorphism was 60% after RCS125& RCS022, and the lowest percentage of polymorphism was 40% after RCS084 primer (Table 7).

Table 7: List of the selected SSR primers and the polymorphisms obtained, total number of fragments detected by each primer, %P and fragments sizes of six alfalfa populations.

SSR primer	Amplified bands				%P	Fragments size	
	Total number	monomorphic bands	polymorphic bands	unique bands		Larger bands	Smaller bands
RCS084	5	3	2	0	40	640	180
RCS125	5	2	3	0	60	890	450
RCS022	5	2	2	1	60	712	245
RCS048	4	2	1	1	50	870	430
RCS073	4	2	1	1	50	510	220
Total	23	11	9	3	52%	890	180
erage	4.6	2.2	1.8	0.6			

The total number of monomorphic amplicons was 11 varied from 2 amplicons after RCS125, RCS022, RCS048 and RCS073 to 3 amplicons after RCS084 with average of 2.2 monomorphic band per primer. The total number of polymorphic bands was 9 bands varied from 1 after RCS 048 and RCS073 to 3 bands after RCS125 with average 1.8. The five SSR primers successfully defined two positive marker and three negative markers (Table 8).

Table 8: Positive and negative markers using five SSR primers with six Alfalfa varieties under drought stress.

SSR	MS	Alfalfa Ismailia1	Alfalfa Ismailia2	Alfalfa Australian	Alfalfa Nobaria1	Alfalfa Ramah	Alfalfa Sewi	Positive(P)and Negative(N) markers
RCS084	280	1	1	1	1	0	1	N
RCS125 RCS022	640	0	1	0	0	0	0	P
RCS048	430	1	0	1	1	1	1	N
RCS073 RCS084	310	0	0	1	0	0	0	P
RCS125	405	1	0	1	1	1	1	N

Primers RCS 125 &RCS 022 determine A unique band at molecular size 640 bp as positive marker to Ismailia 2 cultivar. Primers RCS073 &RCS084 positively marked Australian cultivar at molecular band 310bp. The fragment with molecular size 280bp was detected after all cultivars except Ramah, and the fragment with molecular size 340bp &405bp was detected after all cultivars except Ismailia 2. The three missing bands are considered as negative markers for these cultivars and they were distinguished by RCS 084, RCS048 and RCS125 primers. The results of SSR analysis were translated and analyzed to

generate the dendrogram and similarity index (Fig 2 and table 9).

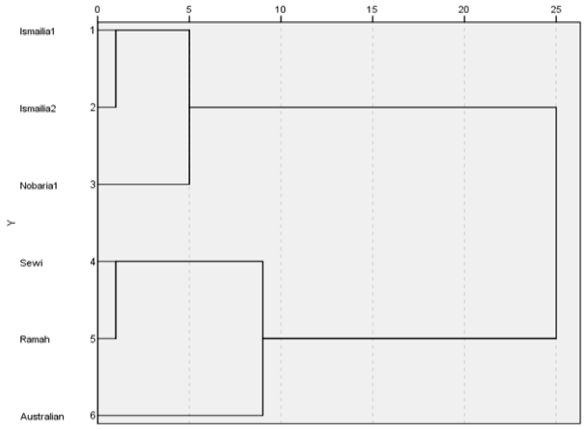


Figure (2): Dendrogram represents the average genetic relationships linkage among five Egyptian alfalfa cultivars (Medicago sativa L) and an Australian cultivar (Medicago truncatula) using the unweight pair group method with arithmetical average (UPGMA) generated by SPSS program version-10 from five ISSR markers.

Table 9: Proximity matrix for six alfalfa varieties using five SSR primers:

Case	Matrix File Input					
	Ismailia1	Ismailia2	Australian	Nobaria1	Ramah	Sewi
Ismailia1	1					
Ismailia2	0.866	1				
Australian	0.624	0.084	1			
Nobaria1	0.866	0.802	0.320	1		
Ramah	0.462	0.577	0.462	0.402	1	
Sewi	0.436	0.402	0.289	0.834	0.834	1

The combination and comparison between the ISSR and SSR were summarized in (table 10), ISSR and SSR DNA based markers given data announce that the examined six alfalfa cultivars were correlated and shared in their genetic pools despite their geographical distribution origin. Among the two used molecular markers the ISSR marker is more discriminating, provides more informative data than SSR marker.

Table 10: Comparison and combination of genetic parameter between ISSR and SSR marker analysis for the six alfalfa genotypes.

Molecular parameter	Amplified bands					%P	Fragments size	
	Total number	monomorphic bands	polymorphic bands	Defined + ve marker	Defined - ve marker		Larger bands	Smaller bands
ISSR	45	14	31	4	3	68.8%	1260	140
SSR	23	11	9	2	3	52%	890	180
Combined average	34	12.5	20	3	3	60%	1260	140

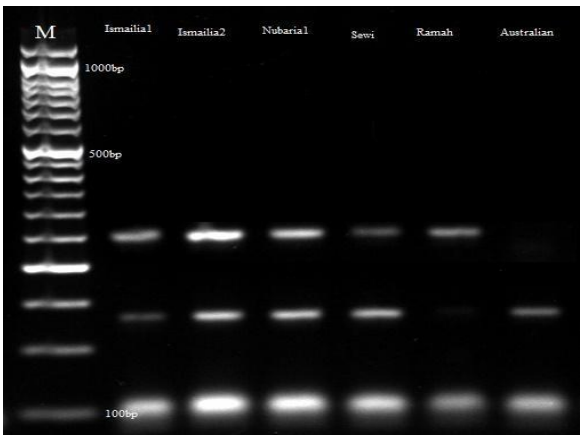


Figure (3): Amplify DNA using five SSR primers for five Egyptian alfalfa cultivars (*Medicago sativa L*) and an Australian cultivar (*Medicago truncatula*). M: marker, 1: Ismailia 1, 2: Ismailia 2, 3: Nubaria 1, 4: Sewi, 5: Ramah and 6: Australian cultivar.

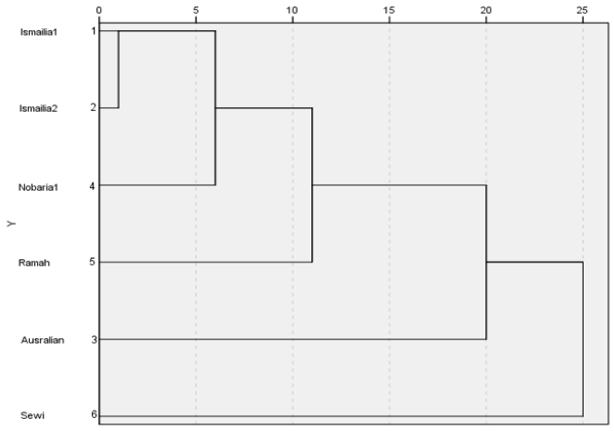


Figure (4): Dendrogram represents the average genetic relationships linkage among five Egyptian alfalfa cultivars (Medicago sativa L) and an Australian cultivar (Medicago truncatula) using the unweight pair group method with arithmetical average (UPGMA) generated by SPSS program version-10 from five SSR markers.

IV. DISSCUSSION

Alfalfa quality can be estimated and remarked by the flag leaf area, **Rahal-Bouziane et al., 2017** stated earlier that, the flag leaf area was positively correlated with the spike weight per square meter so it is considered as quality parameter, based on that, the Sewi followed by Nubaria 1 cultivar announced their high tolerance to water stress as they both increased the leaf area after stress. In agree with (Liu et al, 2015) who stated that, the flag leaf area was positively correlated with the flag leaf's length and width in barley, the area of the alfalfa flag leaf was correlated to the leaf dimensions.

Variation in plant height is genotypic character directly related to the environmental conditions (**Arab et al., 2015**), it is an important yield component for alfalfa and used as a standard when selecting the eminent genotypes in an early stage of selection (Tuckak et al., 2008). Nubaria 1cultivar responded to water regime by shortening its height, this is considered as plant defense mechanism that works on hinder the cell elongation are primarily affected by drought. Different species responded to water deficit in different mechanisms. Two of the important mechanisms in response to drought are to minimize water use by mainly limiting leaf and shoot growth and to reduce water loss by mainly reducing transpiration. Leaf shortening reflects the leaf cell size reduction and the stomatal density decrease, as a defense mechanism to reduce the stomatal conductance (**Ghimire, 2017).**

Among the five used ISSR primers, only three primers (A1, Am2 and A12) defined four positive unique bands, when regarding the morphological differences among the genotypes on the new defined marker we can assume that, the A1primer generated a positive band at molecular size 650 bp in Nubaria 1 which positively mark the width of flag leaf, stem diameter plant height and the pods productivity. Also, Am2 primer generated a unique band at molecular size 510 bp in Australian Alfalfa which can be used as positive marker for flag leaf width, plant height and pods productivity. Thus, came in agree with (**Aboelnaga et al., 2020**) when used the ISSR marker to assess the biodiversity among some sesame genotypes and correlates the obtained results with some of yield traits.

Based on the ISSR analysis the six studied alfalfa cultivars were divided into two main groups, T group (A) contains Ismailia 1, Ismailia 2 and Nubaria 1 and group (B) contains Ramah, Sewi and Australian cultivars. Then each group was subdivided to two subgroups as follow group A is subdivided into subgroup A1 contains (Ismailia 1and Ismailia 2) and subgroup A2 contains (Nubaria). The main group B is subdivided to subgroup B1 contains (Sewi and Ramah) and subgroup B2 contains (Australian).

According to SPSS version 10 analysis program, the genetic similarities coefficient between cultivars ranged from 0.910 between Ismailia genotypes 1&2 to 0.194 between Sewi and Ismailia 2 and between the Australian and Ismailia 2. According to ISSR marker the cultivars Ismailia 1&2 and Nubaria 1 are closely distant to each other and can exchange genes in between smoothly. In agree with **Arab et al., 2015** who confirmed the maximum similarity relationship between Ismailia and Nubaria when studied the genetic diversity among forty-two of Egyptian alfalfa (*Medicago sativa* L.) landraces with recording the plant height, number of tillers per m^2, leaf to stem ratio (%), green forage weight (ton/ fed) and dry matter weight (ton/ fed). He measured and analyzed the relationship between the germplasm by calculating their Euclidean distance and complete linkage using SYSTAT version 7.0 Correlation coefficients, then ended to that, all the genotypes are divided into two groups at a distance of 9.004. The first group contains varieties Ismailia 1, Sewi and Nubaria 1. The second group contains other genotypes collected from different regions.

Among the five SSR primers, only five primers defined two unique bands which can be considered as positive markers. RCS125 &RCS022 defined a unique band at molecular size 640bp may considered as positive marker to the leaf length in Ismailia 2. And the two RCS073 &RCS084 primers defined another unique band at molecular size 310bp may considered as positive marker for the plant's height and flag leaf length in the Australian genotype. Based on SSR analysis, the six Alfalfa cultivars are divided into two main groups A contains (Ismailia 1&2, Nubaria 1, Australian and Ramah) and group B contains only Sewi cultivar. Then the group A is subdivided into two subgroups A1 contains Ismailia 1&2, Nubaria 1 and Ramah and sub group A2 contains Australian. The sub group A1 is further subdivided into two clusters A1-1 contains Ismailia 1&2 and Nubaria and cluster A1-2 contains Ramah.

According to SPSS version 10 analysis program, the genetic similarities coefficient between the six Alfalfa cultivars ranged from 0.866 between Ismailia 1 and Ismailia 2 and between Ismailia 1 and Nubaria 1 to 0.289 between the Sewi and the Australian genotype.

V. CONCLUSION

This study concluded that ISSR markers analysis is more efficient in detection of the genetic diversity among the studied cultivars of Alfalfa than SSR marker analysis. The Sewi, Ramah and Australian cultivars which scored the highest plants, biggest stem diameters and larger leaves area under water regime are close relatives and genes can flow smoothly among them to produce new Alfalfa genotype with high tolerance to water deficit. Also, Ismailia 1 & 2 and Nubaria 1 are close relatives can exchange genes in between smoothly.

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*Abbreviations***:** QTL (quantitative trait locus).
ISSR (inter simple sequence repeat).
SSR (simple sequence repeat).