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Field Performance and Molecular Profile of Commercial Egyptian Clover (*Trifolium alexandrinum* L.) Cultivars under High Temperature Conditions

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Abstract: Heat stress due to high ambient temperatures is a serious threat to crop production worldwide. In Egypt, heat stress Not yet been determined the effect of temperature and height of its role in yield and germination in Egyptian clover. This investigation determined field performance, genetic parameters and variation in molecular level,using RAPD-PCR and protein SDS-PAGE analysis, for six cultivars from Egyptian clover varieties, i.e. Helaly, Serw 1, Gemmiza 1, Giza 6, Sakha 4 and Fahl under Shandweel Agricultural Station Farm as high temperature location relative to some cultivars. The seeds were spread planted in a randomized complete block design (RCBD), fresh forage yield (t ha^{-1}),dry forage yield (t ha^{-1}), crude protein %, crude fiber % and ash % were recorded for each cut and for the whole season under experiment conditions. The results showed that Giza 6 had the best total fresh (78.35 t ha^{-1}) followed by Sakha 4 (74.7 t ha^{-1}) . In dry yield the cultivars Giza 6 (multy cut) and Helaly (multy cut) had highest values 11.28 and 11.57 t ha^{-1}, respectively and significant differences among cultivars were recorded. The values of crude protein and crude fiber and ash % were differing from cut one to cut three and Fahl Egyptian clover cutting is the only non-existent vulnerability of both air temperature, soil temperature and air humidity. SDS-PAGE analysis for the water soluble proteins in the six Egyptian clover revealed a total number of 19 bands with molecular weights (MW) ranging from about 12.24 to 121.2 KDa and this six Egyptian clover cultivars can not be uniquely identified (fingerprinted) with cultivar protein markers. Results obtained by using RAPD-PCR marker, referring to the six Egyptian clover varieties differing in the number of unique marker and it gave adequate distinctions among all the six tested cultivars. The genetic similarity matrix based on all possible pairs of cultivars ranged from 0.0% to 100%. The lowest genetic similarity value was between cultivar Giza 6 and Sakha 4 followed by Sakha 4 and Gemmiza 1(25.0%). While, the highest genetic similarity values was between Serw1 and Fahl (100 %) followed by (93.0%) between Helaly and Sakha 4. Combined analysis based on PAGE protein electrophoresis and RAPD analyses revealed highest similarity matrix was 100% occurred between Giza 6 and Sakha 4, while the lowest similarity 0% occurred between Serw 1 and Fahl. The results indicted that RAPD-PCR marker gave more reliable marker compared with protein SDS-PAGE and it is important for the development of Egyptian clover cultivars to adapt to a wide rang of climate stress.

Key words: Egyptian clover · Heat tolerance · Soil temperature · Randomly Amplified Polymorphic DNA · Protein analysis · Genetic similarity and cluster analysis

INTRODUCTION

Egyptian clover (*Trifolium alexandrinum* L.) is considered the main winter forage crop in Egypt. It is cultivated as an animal feed and for soil improved. Heat is defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause damage to plant growth and development. Heat stress due to high ambient temperatures is a serious threat to crop production worldwide [1]. In nature, plants are often subjected to environment fluctuations among those salt stress, drought and heat stress. Temperature stresses experienced by plants can be classified into three types: those occurring at (*a*) temperatures below freezing, (*b*) low temperatures above freezing and (*c*) high temperatures. This review outlines how biological substances that are

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deeply related to these stresses, such as heat-shock proteins, glycinebetaine as a compatible solute, membrane lipid, etc. and also detoxifiers of active oxygen species, contribute to temperature stress tolerance in plants [2]. Essemine *et al.* [3] reviewed that both germination and early development in higher plants seem to be much affected by temperature fluctuations and this cause harmful effect to the yield and productivity of plants by affecting all the physiological, biochemical and molecular processes in the plant cells.

Pivotal role played by peroxidase under heat stress were signalled that the total soluble peroxidase activity, even decreases as function of time at 5 and 45°C, plays a crucial role in adaptation to stress. Thereby, peroxidase was implicated in growth and development processes [4]. Their activities often varied with prevailing conditions [5]. Petruzzelli and Taranto [6] showed a decrease in the rate of reserve mobilization and metabolites of hard wheat seeds under heat stress. This drop was associated with a loss of the seed viability [6, 7]. Lin *et al.* [8] found the cool-season grasses showed more shade tolerance when grown during the summer-fall than when grown during the spring early summer. Seven of the selected cool-season grasses grown during the summer fall did not display significant reductions in mean dry weight (MDW) under 50% shade as compared to full sun. Iba [9] studied protein after exposure to high temperatures and perception of signals, changes occur at the molecular level altering the expression of genes and accumulation of transcripts, thereby leading to the synthesis of stress-related proteins as a stress-tolerance strategy. Saruyama and Tanida [10] have shown the activation of oxygen-scavenging enzymes when seedlings of rice (*Oryza sativa* L.) cultivar K-sen4 were exposed, at the germination and leaf stages, to 5°C for 7 days. This molecular mechanism is implicated in the processes of adaptation to by triggering the synthesis of a specific range of protein involved in the tolerance to heat or other abiotic stress and increasing the synthesis of normal structural and functionally proteins [11]. The synthesis of the so-calledheat shock proteins (HSPs) allows the plants to adapt to biotic and abiotic stress. Setimela *et al.* [12] found the additive and dominance effects contributed to coleoptile elongation under normal conditions, but only additive effects were significant in recovery growth under heat condition. Epistatic effects were present in both conditions. General combining ability (GCA) effects for heat tolerance index (HTI) were highly significant in both conditions, but specific combining ability effects were negligible. These results indicate that it is possible to improve seedling heat tolerance and, thus, improve sorghum variety and hybrid plant populations in tropical areas where hot soil temperatures occur. Tarrad and Zayed [13] studied fresh and dry yield for some clover variety under Agricultural Research Farm, Giza. The highest total dry matter yield of 15.86 t ha^{-1} was recorded for Gemmiza 1 compared to the other varieties. Both of Gemmiza 1 and Serw 1 varieties were the superior in forage production with non significant difference.

In Egypt, heat stress not yet been determined the effect of temperature and height of its role in yield and germination of Egyptian clover, this investigation studied field performance, genetic parameters and variation in molecular level for six varieties from Egyptian clover under Shandweel farm as high temperature location relative to some cultivars.

MATERIALS AND METHODS

Plant Materials: Six cultivars of Egyptian clover (*Trifolium alexandrinum* L.) were used in the present study as shown in Table 1.

Field Trials: A field experiment was conduct at Shandweel Agricultural Research Station Farm during winter growing season of 2007-2008. Seeds of six Egyptian clover varieties were cultivated on 20th of Nov, 2007 in a randomized complete block design (RCBD), replicated four times in 4 m^2 plots. Recommended agronomical practices were followed. Three consecutive cuts were obtained at 60, 93 and 127 days after sowing. At each harvest the fresh herbage from each plot was weighed in the field and a sample of 1 kg was randomly taken and stored in a polythene bag. In the laboratory a 500 g sub sample was weighed out and dried to constant weight in an oven controlled at 105°C and amount of dry matter (DM) harvested was determined. From plot and experimental field average samples of all cuts were made to determine content of crude protein, crude fibre and ash, according to AOAC [14]. Analysis of variance (ANOVA) was done to find out the least significant differences among clover cultivars as well as genetic parameters following Gomez and Gomez [15]. Regression was estimated according to Sysovevs and Markovskaya [16].

Table 1: Six Egyptian clover names and their cultivation area and No. of cuts

Common name	Cultivation area	No. of cuts
Fahl	All Egypt	Mono
Helaly	Delta Egypt	Multy
Serw 1	North Egypt	Multy
Gemmiza 1	Middle delta Egypt	Multy
Giza 6	South Egypt	Multy
Sakha 4	Middle delta	Multy

SDS-PAGE Protein Analysis: Total seed soluble protein was extracted by a modification of the procedures of Smith and Payne [17]. Ten seeds from each variety were crushed and transferred to 350 µl of extraction buffer, which consisted of 1.7 ml extraction buffer, 0.6 ml β-mercaptoethanol. Stock solution contained 3ml of 1M Tris -HCL (pH 6.8), 6 ml distilled water, 5 ml glycerol and 1 g SDS. Sample were digested at 100°C for 10 min and centrifuged at 10000 rpm for 10 min. Extracted soluble protein were immigration in SDS-PAGE according to Laemmli [18]. Electrophoresis was conducted at a constant current 25 mA until the brome phenol blue tracking dye reached the bottom of the gel. The gel was stained overnight in Coomassie brilliant blue-R250 followed by de-staining solution methanol and acetic acid for 30 min. Gel was further de-staining in acetic acid until background is clear for scoring. Protein marker (Fermentas) from 100 to 10 Kda.

DNA Extraction and RAPD Amplification Analysis: DNA extraction was performed from seedling samples according to Dellaporta *et al.* [19]. RAPD analysis was performed using six 10-mer random primers (Operon Technologies). RAPD amplification reaction was done in a final volume of 25 µl containing 10X PCR buffer (10 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, pH 9.0), 100 mM dNTPs, 10 mM primer, 50 ng of template DNA and 0.5 U of *Taq* polymerase (Fermentas GmbH).

Reactions were performed in a thermocycler (Techno 512, Mastercycler® Thermal Cyclers). RAPD-PCR was performed as one cycle of 94°C for 4 min (denaturation), 35 cycles of {94°C for 1 min, 36°C for 1 min and 72°C for 2 min (annealing)} and a final extension of 10 min at 72°C. PCR products were analyzed using 1.5% agarose gel electrophoresis and visualized with 0.0005 µg/µl ethidium bromide staining and photographed by the gel documentation analysis system (Syngene, UK). The sizes of the fragments were estimated based on a tandem DNA ladder of size 2kbp-100bp (Lonza). Genetic similarities between all varieties were estimated by simple matching co-efficient according to Sokal and Michener [20] SPSS software version 17.

RESLUTS AND DISCUSSION

Egyptian clover is a winter crop in Egypt, where it requires a low temperature, the Egyptian clover cultivars are sensitive to high temperatures in Lower Egypt and it leads to flowering early, reduction in number of cuttings and redness of the stem (unpublished data). Evaluation and selection of cultivars tolerant to high temperatures are a means to increased yield of Egyptian clover in those areas. From this observations, this research aims to re-evaluate field performance and molecular profile of some Egyptian clover varieties, taking into account the environmental conditions surrounding agriculture, such as soil temperature on the average depths of 5 cm, 10 cm, 20 cm and the average air temperature and average humidity, taking the maximum value and minimum value for this grade. Sowing date were also signed to, cutting No. 1, No. 2, as well as the third cutting on the ground temperature, air temperature, humidity curves and air to record these scores in each case as is evident from Figs 1, 2, 3 and 4.

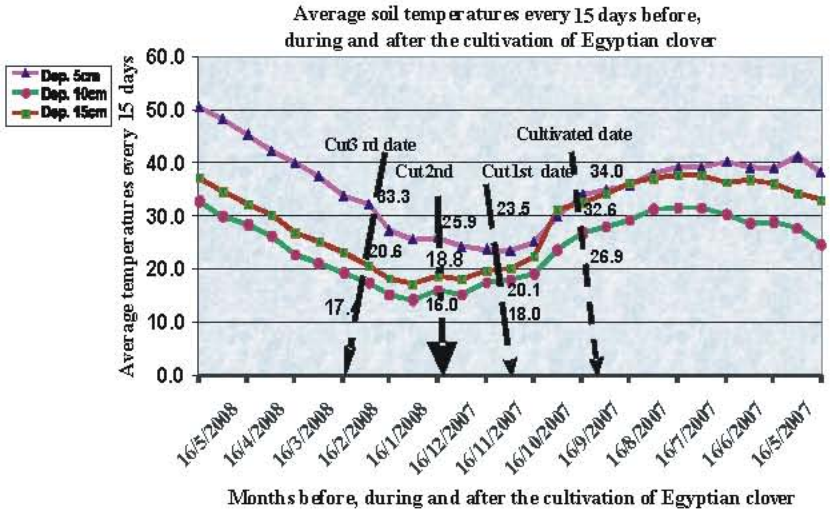

Fig. 1: Maximum Minimum mean soil temperatures at experimental site during Egyptian clover growth season (5 Nov 2007 – 17 Apr 2008) as recorded in local station (the arrow indicates sowing time).

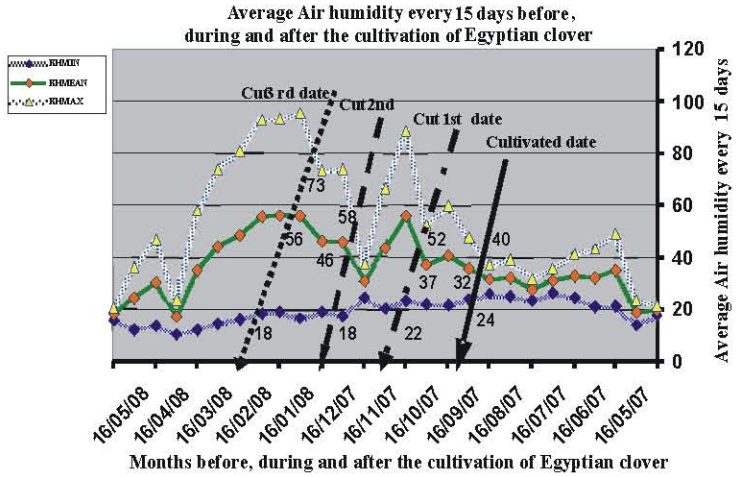

Fig. 2: Maximum Minimum and mean air humidity at experimental site during Egyptian clover growth season (5 Nov 2007 – 17 Apr 2008) as recorded in local station (the arrow indicates sowing time).

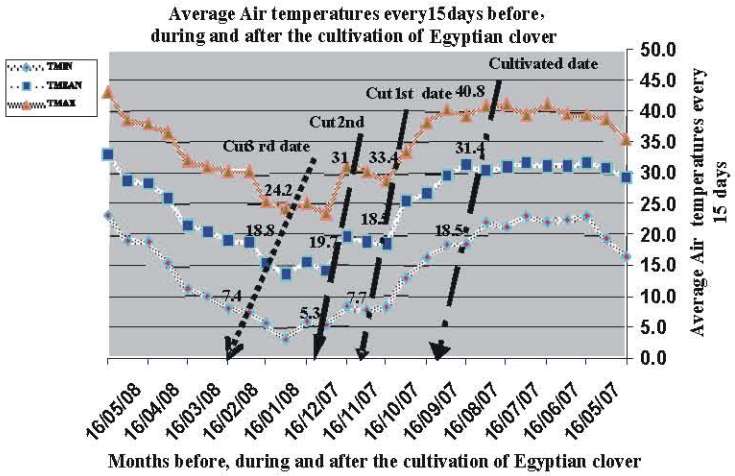

Fig. 3: Maximum Minimum and mean air temprature at experimental site during Egyptian clover growth season (5 Nov 2007 – 17 Apr 2008) as recorded in local station (the arrow indicates sowing time).

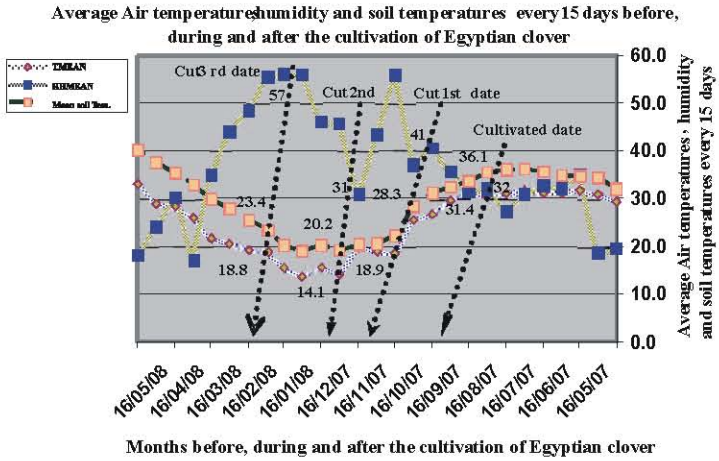

Fig. 4: Maximum Minimum and mean air temprature,soil temprature and air humidity at experimental site during Egyptian clover growth season (5 Nov 2007– 17 Apr. 2008) as recorded in local station (the arrow indicates sowing time).

Table 2: Statistical analysis of air temperature, air humidity and soil temperature during Egyptian clover growth season (2007-2008).

Condition	Status	Cultivation	1st cut	2nd cut	3rd cut
Air Temperature(AT)	Min.	18.5	7.7	5.3	7.4
	Max	40.8	33.4	31.0	24.2
	Mean	31.4	18.5	19.7	18.8
Air Humidity(AH) %	Min.	24.0	22.0	18.0	18.0
	Max	40.0	52.0	56.0	73.0
	Mean	32.0	37.0	46.0	56.0
Soil Temperature(ST)	5 cm.	34.0	23.5	25.9	33.0
	10 cm.	26.9	20.1	18.8	17.4
	20 cm.	32.6	21.0	19.0	20.6
Mean	AT	32.03	18.9	14.1	18.8
	AH%	31.4	41.0	31.0	57.0
	ST	36.1	23.8	20.2	23.4

Table 3: Mean value of fresh, dry and total forage yield of clover cultivars at each cut

	Fresh yield (t ha^{-1})				Dry yield (t ha^{-1})			
Varieties	1st cut	2nd cut	3rd cut	Total	1st cut	2nd cut	3rd cut	Total
Fahl	48.61	0.00	0.00	48.61	8.77	0.00	0.00	8.77
Helaly	23.40	26.40	26.80	76.60	3.04	3.69	4.55	11.28
Serw 1	19.53	23.90	23.61	67.04	2.53	3.34	4.06	9.93
Gemmiza 1	22.42	24.36	25.32	72.03	2.91	3.41	4.30	10.62
Giza 6	24.16	26.09	28.10	78.35	3.14	3.66	4.77	11.57
Sakha 4	22.90	25.91	25.90	74.70	2.97	3.62	4.40	10.99
CV%	16.1	16.10	15.89	17.36	14.00	17.84	9.64	7.21
$LSD_{0.05}$	6.2	6.28	3.74	2.53	4.30	1.75	ns	0.12

Climate and Soil Condition to Field Performance: The origin releasing growing locations of varieties were a significant source of variation induction for fresh, dry matter yield, crude protein, crude fiber and ash. From Figs 1- 4 and Table 2, it is clear that the temperatures of air and soil at the cultivation were 18.5 and 40.8°C with mean 31.4°C at humidity percentage 24, 40 and 32 respectively. As well as, the soil temperatures were 34, 26.9 and 32.6°C in 5, 10 and 20 cm, respectively, while the mean degrees in this stage 32, 31.4 and 36.1°C respectively. Moreover, the cut number three was had different degrees in this stage. The first and second cuts had the low degrees and percentages in soil, air temperature and humidity. The cuts number two and three, were very important to Egyptian clover, which was the basis of production and a pointer to it, especially the second cutting. It is well known behavior of Egyptian clover production after first cut would rise in the next cutting while only low in the third cutting and extremely low in the first cut as a result of high humidity in the green crop, which refers to the impact of high temperature on Egyptian clover yield Tarrad and Zayed [13]. This agree with Harbans *et al.* [21] studied the white lupine forage crops under different location have different degrees from temperatures and found differences were attributed to differences in temperatures during lupine growth and soil types at different locations.

Mean Value and Genetic Parameters: From Table 3 the data revealed that, Fahl (mono cut) gave the lowest values for dry and fresh yield 8.77 and 48.61 t ha^{-1}, respectively, while Giza 6 and Sakha 4 (multy cut) recorded the highest value 78.35 and 74.7 t ha $^{-1}$ for fresh yield, respectively. In dry yield the cultivars Giza 6 and Helaly (multy cut) had highest values 11.28 and 11.57 t ha^{-1}, respectively. Gemmiza 1 gave the dry and fresh forage yield 10.62 and 72.03 t ha^{-1} in 2ndC, respectively. This nearly agree with Tarrad and Zayed [13] where they found Gemmiza 1 had the best total fresh (136.91 t ha^{-1}) as well as dry yield (15.86 t ha^{-1}) over the studied cultivars and total dry yield of Serw 1 was significantly differed from that of Gemmiza 1 in fresh and dry forage yield. Furthermore, the cut number three was non significant in dry yield among cultivars. Crowley [22] improved yield and quality of forage maize and presented early maturing varieties and reported that using photodegradable polythene gives quicker germination, faster growth and earlier flowering. It also increases both dry matter yield and starch content and crops under polythene will reach harvest maturity up to three weeks earlier than conventional maize.

Data in Table 4 revealed that genetic variance exceeded the environmental variance and heritability was high for dry and fresh yield dry yield. The expected genetic advance reached from 63.23 and 34.46 for dry and fresh yield, respectively. Bakheit [23] found that both

Table 4: Genetic parameters of dry and fresh yield of clover cultivars

	Genetic parameters							
Traits	σ 2 g	σ 2 e	σ 2 p	GCV %	PCV %	h^2 b %	Gs	Gs %
Dray yield	987.20	47.28	1034.48	73.57	75.32	95.43	63.23	148.06
Fresh yield	354.87	95.18	450.05	17.60	19.82	78.85	34.46	32.19

Genotypic(ó2g),environment (ó2e) and phenotypic(ó2p) variances, genotypic(GCV %) and phenotypic(PCV %) coefficient variance, heritability(h^2b%), genetic advance(Gs) and genetic advance percentage(Gs %)

Table 5: Dry and fresh yields of the six cultivars and their formula of factor effect in yields.

Varieties	Yield	F value	Formula
Fahl	Dry	**	= 0 AT+ 0 AH+ 0 ST +13.3
	Fresh	**	= 0 AT+ 0 AH+ 0 ST +110
Helaly	Dry	**	= -13.6 AT+ 6.2 AH+ 0 ST +17.6
	Fresh	**	= -5.1 AT + 1.7 AH+ 0 ST +140.5
Serw-1	Dry	**	= -12.9 AT + 6.3 AH+ 0 ST +3.2
	Fresh	**	= -0.7AT + 1.3 AH+ 0 ST +77.9
Gemmiza 1	Dry	**	= -13.9 AT + 6.2 AH+ 0 ST +24.2
	Fresh	**	= -16.8 AT + 3.7 AH+ 0 ST +246.1
Giza 6	Dry	**	= -13.5 AT + 6.1 AH+ 0 ST +20.6
	Fresh	**	= -15.5 AT + 3.3 AH+ 0 ST +237.1
Sakha 4	Dry	**	= -13.5 AT + 6.1 AH+ 0 ST +20.6
	Fresh	**	= -10 AT + 4.5 AH+ 0 ST +20

AT =Air temperature, AH =air humidity %, ST =soil temperature and constant.

Table 6: Forage quality traits for six Egyptian cultivars.

	1st cut			2nd cut			3rd cut		
Varieties	CP%	CF%	Ash%	CP%	CF%	Ash%	CP%	CF%	Ash%
Fahl	19.80	23.00	11.4						
Helaly	19.00	23.20	10.3	18.79	28.03	12.92	16.65	28.25	9.53
Serw 1	18.80	26.3	11.43	17.80	27.34	12.30	17.69	28.45	9.55
Gemmiza 1	18.89	26.04	12.30	18.88	27.04	12.90	17.07	29.02	9.35
Giza 6	18.50	24.70	10.25	17.36	25.72	13.37	16.35	28.52	10.15
Sakha 4	19.09	26.73	13.15	17.90	27.73	13.80	16.47	28.06	9.96

CP%=Crude protein percentage, CF%=crude fibre percentage

phenotypic and genotypic correlations among traits showed that mean plant height was positively correlated with each of seasonal fresh forage yield, seasonal dry forage yield, mean dry matter percentage and seasonal protein yield, but negatively correlated with mean protein percentage. Genetic variance exceeded the environmental variance for all the studied traits and heritability was high for all characters studied. As well as, data in Table 4 revealed the genetic, environment and phenotypic variation which higher in the dry yield but lowed in the fresh yield except the environment variation which had value 95.18; this means the expose the varieties to environmental effect in this study.

Regression Formula: It is clear from Table 5, there are factors of three under consideration affect the six actual cultivars of Egyptian clover under study. The effect of the temperature of the soil foster effective as treatment in the equation is always equal to zero in the varieties of Egyptian clover as different working temperature of the air from the class of the class of Egyptian clover, in terms of the rise and fall reflecting the degree of vulnerability to temperature as the air that the impact is different in the dry matter and green forage yield of Egyptian clover under Shandwel Agricultural Research Station Farm. Fahl Egyptian clover cutting is the only non-existent vulnerability of air temperature, soil temperature and air humidity, this are in agreement with those obtained by Nuray and Fahri [24].

Forage Quality Traits: Data in Table 6 revealed that Fahl mono cut had 19.8,23 and 11.4 % for crud protein, crude fiber and ash, respectively. The percentages of crud protein, crude fiber and ash as % were differed from cut

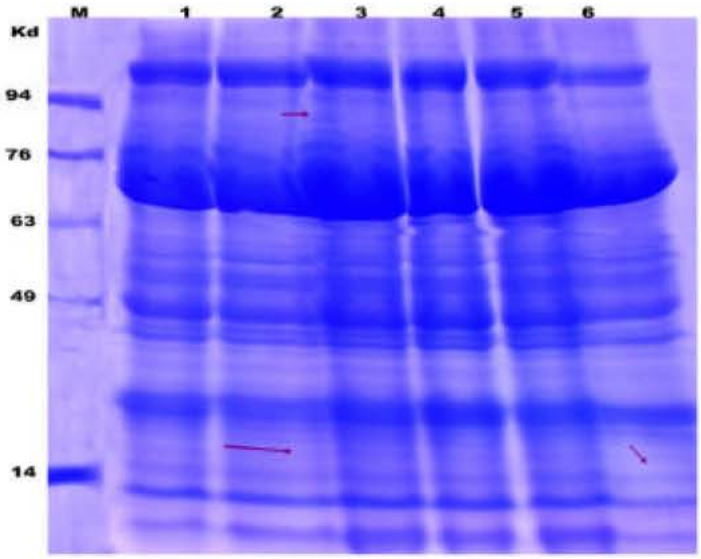

Fig. 5: Protein analysis of six Egyptian clover cultivars, Lane M: protein Marker; 1-6: 1=Fahl, 2=Sakha, 3=Gemmiza1, 4=Serw, 5=Giza 6 and 6=Helaly, the arrows indicated to the unique bands; the arrows were indicated to variation and unique bands.

one to cut three. Gemmiza 1 had values for three quality traits in first cut 18.89, 26.04 and 12.30, respectively; and they recorded in the second cut 18.8, 27.04 and 12.90, respectively. In the third cut, Gemmiza-1 gave 17.07, 29.02 and 9.35 respectively. Peri *et al*. [25] found the plant growth; morphology and nutritive value under shade can differ between temperate grasses. On the other hand, Lin *et al*. [8] reported that the shade environment produced in agroforestry practices affects the morphology, anatomy and chemical composition of intercropped forages and, therefore, may affect forage quality and acid detergent fiber (ADF) was either not affected or was slightly increased by shade.

Protein Profile SDS-PAGE: SDS-PAGE analysis of water-soluble proteins in the six Egyptian clover revealed a total number of 19 protein bands with molecular weights ranging from 12 to 121 KDa (Fig. 5). The six Egyptian clover cultivars cannot be uniquely identified bands (a unique genetic fingerprint) with protein markers as shown in (Fig. 5). The genotypes Gemmiza 1 and Serw1 revealed a total number of 3 polymorphic bands. In the Serw 1 genotype three bands were found at 28.5, 26 and 14 KDa. Sakha 4 gave two bands at 24 and 14 KDa. Helaly gave one positive band (present band) at 28.5 KDa. Gemmiza 1 and Sakha 4 genotypes possessed specific bands. These results disagreed with Tarred and Zayed [13], where they found a total number of 9 bands with molecular weights (MW) ranging from about 14 to 100 KDa under Giza temperature condition.

RAPD Fingerprint: Six random primers were used to differentiate between six Egyptian clover cultivars by RAPD. The primers produced multiple bands with a number of amplified DNA fragments ranging from 6 (primer OP- C05) to 11 (primer OP-Z01 and OP-L12). Total number of the reproducible fragment amplified by the six primers reached to 255 bands; from which 110 bands were polymorphic, which indicated to level of polymorphism (43.14%) (Table 7). The highest number of unique markers at 794, 614, 543, 489 and 367 bp of primers OP-L12. As well as, 545 bp and 243 bp of primer OP-A12. The cultivar Serw 1 scored four unique markers, bands no. 10 of primer OP-C13 at 200 bp, band no.5 of primer OP-F09 at 337 bp and bands no 1, 4 of primer OP-L12 at 794 bp and 543 bp. Also, cultivar Gemmiza 1 showed three unique markers at bands no. 8 (243 bp) of primer OP-A12 and band no. 3, 5 had size respectively 614 and 489 bp of primer OP-L12. Whereas, the cultivars Sakha 4, Helaly and Fahl give one unique marker at band no. 3 had size 545 bp of primer OP-A12, no.8 had size 281 bp of primer OP-Z01 and no.7 had size 367 bp of primer OP-L12, respectively (Fig. 5).

Data in markers of RAPD-PCR system indicated that RAPD analysis seemed to be one of the effective tools for detecting polymorphism and could discriminate between all six cultivars. Abdel-Twab *et al*. [26] who used RAPD-PCR and identified nine cultivars using SDS-PAGE protein, isozymes and RAPD-PCR and found that RAPD-PCR gave more reliable markers. Kuan-Hung *et al*. [27] who found the identification of RAPD markers for heat-tolerance traits is important for the development of

Table 7: Level of polymorphism and unique cultivar-specific bands based on RAPD-PCR analysis from six primers

Primer name	Total bands	Polymorphic bands	Monomorphic Bands	Polymorphism %	Unique bands		
					Variety	Status	MW
OP-A12	40	16	24	49.8	Sakha 4	absent	545
					Gemmiza 1	absent	243
OP- C05	34	4	30	11.8	-	-	-
OP-C13	52	10	42	19.2	Serw 1	absent	200
OP-F09	36	18	18	50	Serw 1	absent	337
OP-L12	58	28	30	48.3	Serw 1	absent	794
					Gemmiza 1	absent	614
					Serw 1	absent	543
					Gemmiza 1	absent	489
					Fahl	absent	367
OP-Z01	35	34	1	97.1	Helaly	absent	281
Total	255	110	145	43.14	4.00	absent	10
Protein	112	4	108	3.2	-	-	-
Total	367	114	253	31.1	4.00	absent	10

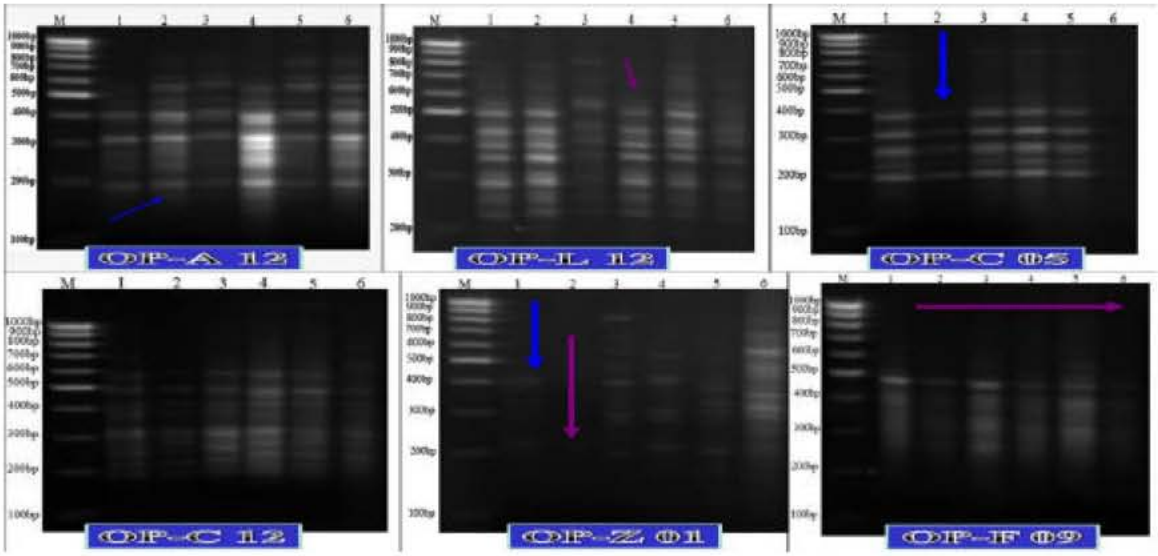

Fig. 6: RAPD analysis of six Egyptian clover cultivars with six primers OP-a12, OP-L12, OP-C05, OP-C12, OP-Z01 and OP-Lane M: DNA Marker; 1-6: 1=Fahl, 2=Sakha, 3=Gemmiza1, 4=Serw, 5=Giza 6 and 6=Helaly, the arrows indicated to the unique bands; the arrows were indicated to variation and unique bands.

tomato cultivars to adapt to a wide range of climates. Mirshamsi *et al.* [28] used RAPD markers to estimate genetic distances and determine the correlation between genetic distance and hybrid performance of 29 tomato lines that were the parents in a diallel mating design.

Genetic Similarity and Cluster Analysis: The RAPD data developed by all primers of this study were used to estimate the genetic similarities among six Egyptian clover cultivars. The genetic similarity matrix based on all possible pairs of cultivars ranged from 0.0 to 100% (Table 8). The lowest genetic similarity value was between cultivar Giza 6 and Sakha 4 followed by Sakha 4 and Gemmiza 1(25.0%). While, the highest genetic similarity values was Serw1 and Fahl (100 %) followed by (93.0%) between Helaly and Sakha 4. The dendrogram based on genetic similarity (Fig. 7) separated the six Egyptian clover cultivar into main cluster, two cultivars; Fahl and Helaly were grouped in the first cluster, while all the other cultivars were grouped in the second cluster, which was separated into subclusters where cultivars Sakha 4 and Giza 6 were grouped in the same group together in

Table 8: Similarity percentages of six Egyptian clover cultivars based on RAPD-PCR analysis.

Varieties	Sakha 4	Helaly	Gemmiza 1	Serw 1	Giza 6
Helaly	9.3	-	-	-	-
Gemmiza1	25	45.6	-	-	-
Serw1	29.9	35.2	33.8	-	-
Giza 6	0	64.1	46.6	22.8	-
Fahl	75.8	36.4	49.3	100	384

Table 9: Similarity percentages of six Egyptian clover cultivars based on RAPD-PCR and protein analysis.

Varieties	Sakha 4	Helaly	Gemmiza 1	Serw 1	Giza 6
Helaly	13.9	-	-	-	-
Gemmiza1	22.4	47.9	-	-	-
Serw1	25.5	37.2	30.2	-	-
Giza 6	100.0	66.2	44.7	21.0	-
Fahl	77.9	33.5	54.1	0.0	44.7

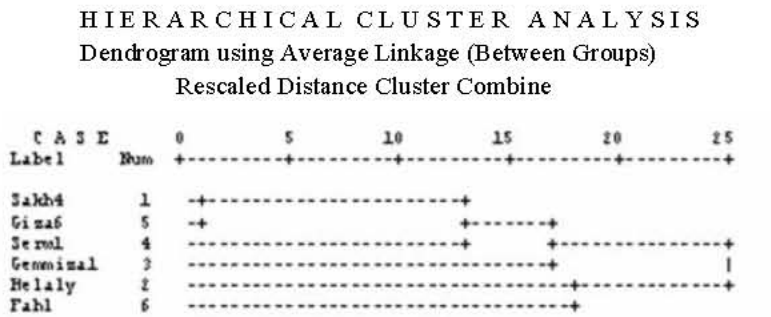

Fig. 7: Dendrogram of five Egyptian clover cultivars based on RAPD-PCR analysis.

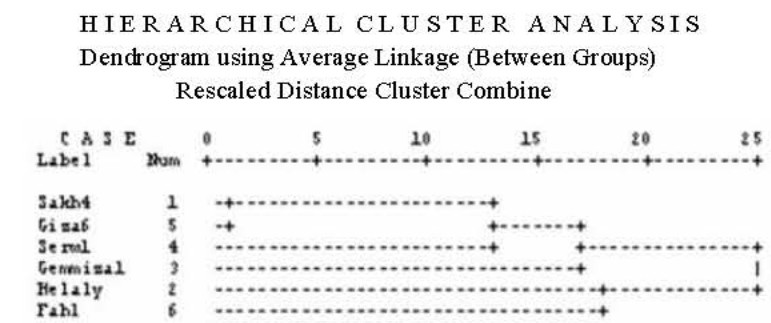

Fig. 8: Dendrogram of five Egyptian clover cultivars based on combine two system analysis (RAPD-PCR and protein SDS-PAGE)

the same subcluster group 1, on the other hand, the cultivar Serw 1 was only in the second group in the subcluster 1 and the sub cluster 2 had cultivar Gemmiza 1 alone.

RAPD and Protein Combined Analysis: Based on RAPDs and protein combined analysis, the overall similarity matrix (Table 9) revealed that the highest similarity matrix was 100% occurred between Giza 6 and Sakha 4, the lowest similarity matrix was 0% occurred between Serw 1 and Fahl. The dendrogram resulting from the combination two systems, RAPD and proteins (Fig. 8) separated the six Egyptian clover cultivars into main cluster. The highest similarity was observed between Sakha 4 and Gemmiza 1 (88.6 %), while, the lowest between Helaly and Giza 6 (42.3%). The rest four varieties also made independent clusters.

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