Assessment of Genetic Diversity among Egyptian Alfalfa Varieties Using Agro-Morphological and Molecular Markers

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Abstract: Genetic variation among five Egyptian alfalfa varieties have been studied based on forage yield and yield components performance, isozymes and seed storage protein. A field trial was carried out at the experimental farm of Giza Research Station, ARC, Giza, Egypt. The trial included five Egyptian alfalfa varieties namely; Giza-1, Ismailia-1, Ismailia-94, New Valley and Siwa-1. Twenty cuts were obtained during 2013 and 2014. The combined analysis across the two years revealed that significant differences were found among the studied alfalfa varieties for plant height, tillers m⁻², leafiness and dry forage yield ton fed⁻¹. New Valley recorded the highest dry forage yield (20.04 ton fed⁻¹) followed by Siwa-1 (19.40 ton fed⁻¹), New Valley ranked also first regarding plant height (67.46 cm), Ismailia-94 (53.29 m⁻²) ranked first followed by New Valley (53.35 m⁻²) for tillers m⁻². Meanwhile, Giza-1 (48.67 %) ranked first followed by Siwa-1 (47.20 %) for leafiness % across the two years. The results for forage quality traits of alfalfa showed highly significant variation among the studied varieties for crude protein yield, crude fiber yield and ash yield (ton fed⁻¹) in the first season. Data revealed that Ismailia-1 ranked first (399.44 and 206.24 ton fed⁻¹) followed by New Valley (392.21 and 201.77 ton fed⁻¹) for crude protein and ash yield, respectively. New Valley recorded the highest value (452.47 ton fed⁻¹), while Giza-1 recorded the lowest value (337.38 ton fed⁻¹) of crude fiber yield. Great variation was recorded for the obtained values of phenotypic (PCV %) and genotypic (GCV %) coefficient of variability and heritability (H %) estimates for studied traits except leafiness which interpreted the significant differences possessed with studied genotypes. Leafiness had negative and significant phenotypic and genotypic correlations with all other traits. Highly significant positive correlation values were found between dry forage yield and each of plant height and tillers m⁻², crude protein yield, crude fiber yield and ash yield (ton fed⁻¹) for both the genotypic and phenotypic levels. The genetic makeup, which is the combination of alleles located on homologous chromosomes that determines a specific characteristic; some unique bands appeared in all markers systems in this study. The overall similarity matrix based on four isozymes and seed storage protein combined analysis revealed that the highest similarity was observed between Siwa-1 and Ismailia-1, while the lowest similarity was observed between Ismailia-1 and Ismailia-94. The dendrogram resulting from the combination of the two systems separated the five Egyptian alfalfa varieties into two main clusters. Where Ismailia-1 and Ismailia-94 were located in two main clusters reflecting the genetic dissimilarity while, Ismailia-1 and Siwa-1 occupied the same sub cluster indicating that the two varieties had the highest similarity among the studied varieties. The results could be used to direct parent selection for further breeding work in alfalfa improvement.

Key words: Alfalfa • Medicago sativa L. • Forage yield • Quality traits • Genetic parameters • Isozymes • Seed storage protein

INTRODUCTION

Alfalfa (Medicago sativa L.) is one of the most important forage crops all over the world. Alfalfa produces high quality forage for all classes of livestock and alone can provide energy, protein, minerals and vitamin requirements for dairy cattle. It is widely adaptable to diverse environmental conditions partly because of its deeply penetrable root system and its ability to fix atmospheric nitrogen in symbiosis with Rhizobium meliloti. These obvious soil-improving properties make it an important member of the crop rotation. Cultivated alfalfa is autotetraploid (2n=4x=32). All those characteristics confirm, that alfalfa is a really “queen” of
forages [1]. Mousa et al. [2] evaluated six alfalfa varieties and found significant differences for plant height, number of tillers/plant, leaf/stem ratio, total fresh and dry forage yields with individual cuttings in the first, second years and combined analyses. Abdel-Galil and Hamed [3] studied the values of genotypic coefficient of variation for fresh and dry forage yields and revealed relative variation among the tested cultivars which were less influenced by environment.

Quality of alfalfa forage is closely associated with the relative production of the leaf and stem components as measured by leafiness percentage, but more often as leaf/stem ratio (LSR) and the nutritive value of the stem. Johnson et al. [4] found positive correlations for crude protein (CP) with leaf / stem ratio and lodging and negative correlations for CP and regrowth height. Moreover, CP is considered an important indicator of forage quality [5, 6].

Biochemical genetic markers offer specific advantage in assessment of genetic diversity and trait-specific crop improvement. Such markers can facilitate appropriate choice of parents for crosses to mapping/tagging of gene blocks associated to economically important traits and in turn permits marker-assisted selection (MAS) in backcross, pedigree and population improvement programs [7]. Traditionally, genetic diversity is determined through the analyses of morphological parameters including serious limitations such as the influence of environmental conditions, giving results that represent just a part of total genetic diversity and differentiation of alfalfa ecotypes and populations [8]. The enzymatic markers were used to identify useful phytogenetic of alfalfa resources for their integration in programmes of safeguard and conservation as well as these markers are of practical interest in the background selection and improvement programmes [9]. Availability of genetic variation is important for genetic improvement of the crop. Local and exotic germplasm can be used as source of genetic variation. Protein markers can act effectively to study the genetic variation of germplasm for its utilization in crop breeding programs. Many workers used seed storage protein electrophoresis for characterization of cultivated and wild species [10]. Besides studying allozyme polymorphism, we can study quantification of SDS-PAGE differences of seed storage proteins. This method was used to detect genetic diversity in various plant species [11].

The goals of this study are (i) to estimate genetic parameters associated with forage yield, (ii) to evaluate phenotypic and genotypic correlations between yield and its components and (iii) to measure genetic diversity between varieties at the molecular level.

**MATERIALS AND METHODS**

**Field Experiment:** Field trial was carried out at the experimental farm of Giza Research Station, ARC, Giza, Egypt, during 2013-2014 on a clay loam soil. Five Egyptian alfalfa varieties (Giza-1, Ismailia-1, Ismailia-94, New Valley and Siwa-1) were used in this investigation. Egyptian varieties of alfalfa were sown into a plot size of 6 m² and the seeds were drilled in 6 rows 20 cm apart at the seeding rate of 20 kg fed⁻¹ in a randomized complete block design with three replications. The recommended practices for alfalfa production under Egyptian conditions were carried out. The trial was harvested 10 times each year by hand clipping to 7-cm stubble. Twenty cuts were obtained through the two growing years 2013 and 2014. The data were recorded at each harvest on five guarded plants plot⁻¹ for field performance and chemical composition. The field performance traits included: height; length of the main stem from soil surface to stem-tip, number of tillers m⁻², dry forage yield and leafiness [leaf weight x 100/(leaf + stem)] weight on dry basis.

**Statistical Analysis:** Data for all studied traits were subjected to regular statistical analysis of variance of the randomized complete block design (RCBD) according to Gomez and Gomez [12]. Bartlett test of variance homogeneity was carried out before the combined analysis. Mean comparison were performed using least significant difference (L.S.D.) at 5% level of probability. Phenotypic (PCV %) and genotypic (GCV %) coefficients of variability and heritability in broad sense (H²%) for the studied traits were calculated according to Johnson et al. [13] and Kumar et al. [14]. Relationships between the traits were studied through genotypic and phenotypic correlation and path coefficient. Genotypic and phenotypic correlation coefficients were calculated for each pair of traits according to Falconer [15]. All correlation coefficients were worked out between all possible combinations of traits. The statistical analysis and the relationships between germplasm were measured by calculating their Euclidean distance and complete linkage method as phenogram using SYSTAT version 7.0 [16].

**Chemical Analysis:** Chemical analysis followed the conventional method recommended by the Association of Official Agricultural Chemists (AOAC) [17] on the dried samples at 70°C for each cut of the first season to
Data Analysis: The polymorphic bands were scored as present (1) or absent (0). Genetic similarity among genotypes was estimated based on Euclidian coefficients. Cluster analysis was performed using the un-weighted pair group method based on arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

Field Experiment: Analysis of variance (Tables 1 and 2) indicated highly significant differences among the studied alfalfa varieties for plant height, number of tillers m⁻², leafiness and dry forage yield ton fed⁻¹ in both successive seasons and the combined across the two seasons. These results are in line with those obtained by Hefny [22], Abdel-Galil and Hamed [3] and Hamd Alla et al. [23] who found highly significant differences among alfalfa genotypes for dry forage yields, plant height and number of tillers m⁻² in both seasons.

Data in Table 3 revealed the highly significant differences among the investigated varieties for different traits in both seasons.

Data revealed that New Valley (65.89 and 69.02 cm) ranked first followed by Ismailia-94 (61.62 and 69.29 cm) for plant height in both seasons, respectively. However, New Valley (450.55 and 614.15) ranked first followed by Ismailia-94 (449.36 and 615.42) for tillers m⁻² in both seasons, respectively.

Table 1: Mean squares of forage yield and yield components of five Egyptian alfalfa varieties in two seasons.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td></td>
<td>9.31</td>
<td>7.38</td>
<td>11.07*</td>
<td>12.56**</td>
<td>21.81</td>
<td>16.09</td>
<td>6.14</td>
<td>7.14</td>
</tr>
<tr>
<td>Tillers m⁻²</td>
<td></td>
<td>8.35</td>
<td>4.60</td>
<td>18.19**</td>
<td>25.78**</td>
<td>0.56</td>
<td>1.90</td>
<td>1.99</td>
<td>1.63</td>
</tr>
<tr>
<td>Leafiness (%)</td>
<td></td>
<td>151.69*</td>
<td>198788.56**</td>
<td>13.25</td>
<td>14.55**</td>
<td>18.95</td>
<td>6.64</td>
<td>1.82</td>
<td>1.23</td>
</tr>
<tr>
<td>Dry forage yield (ton fed⁻¹)</td>
<td></td>
<td>5.44*</td>
<td>15.20**</td>
<td>13.73**</td>
<td>14.55**</td>
<td>1.82</td>
<td>1.23</td>
<td>1.81</td>
<td>0.39</td>
</tr>
</tbody>
</table>

** Significant at 1% level of probability, * Significant at 5% level of probability
While, Giza-1 (48.46 and 48.87%) ranked first followed by Siwa-1 (46.00 and 48.39%) for leafiness % in the both seasons, respectively. Regarding dry forage yield, New Valley ranked first in both seasons (19.87 and 20.22 ton fed\(^{-1}\)) followed by Siwa-1 (19.20 and 19.59 ton fed\(^{-1}\)). The combined data analysis across the two years revealed that New Valley had significant higher plant height (67.46 cm) than the other varieties. However, Ismailia-94 (532.39 m\(^{-2}\)) ranked first followed by New Valley (532.35 m\(^{-2}\)) for tillers m\(^{-2}\). Meanwhile, Giza-1 (48.67%) ranked first followed by Siwa-1 (47.20%) for leafiness %. On the other hand, New Valley ranked first in dry forage yield (20.04 ton fed\(^{-1}\)) followed by Siwa-1 (19.87 ton fed\(^{-1}\)). These results are in agreement with those reported by Bakheit [24], Abdel-Galil and Hamed [3] and Hamd Alla et al. [23].

**Genetic Parameters:** Highly significant differences were observed for all the traits. This considerable variability provides a good chance of improvement in studied alfalfa genotypes. In general, phenotypic coefficient of variability (PCV %) was higher than corresponding genotypic coefficient of variability (GCV %) for all the traits which demonstrated the effect of environment on dry forage yield (10.47 and 10.18%), leafiness % (4.99 and 4.67%), plant height (3.07 and 2.78%) and tillers m\(^{-2}\) (0.21 and 0.18%), respectively. This indicates the presence of exploitable genetic variability for these traits. Heritability (H\(^2\)) estimates were generally high for all studied traits and recorded values from 94.59% for dry forage yield to 82.11% for plant height, except tillers m\(^{-2}\) that recorded 78.38%. In general, all traits except tillers m\(^{-2}\) had high heritable variation. Hence, it can be assumed that genotypes of almost all the traits are mainly determined by their phenotypes. High estimates of heritability indicated that selection based on mean performance would be successful in improving these traits [25, 26, 3].

In the second year, the highest phenotypic and genotypic coefficient of variability were recorded for dry forage yield (5.14 and 4.79%), leafiness% (4.33 and 4.04), plant height (3.05 and 2.84%) and tillers m\(^{-2}\) (0.58 and 0.57 %), respectively. Heritability estimates were generally high for all studied traits and recorded values from 93.62% for dry forage yield to 86.86% plant height.

**Chemical Analysis:** Analysis of variance (Table 4) indicated highly significant differences among the studied alfalfa varieties for crude protein yield (ton fed\(^{-1}\)), crude fiber yield (ton fed\(^{-1}\)) and ash yield (ton fed\(^{-1}\)) in 2013 growing seasons.

Data in Table 5 revealed the highly significant differences among the investigated varieties for different quality traits in both seasons.
Table 5: Means performance of quality traits of five Egyptian alfalfa varieties in 2013 growing season.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>Crude protein yield (ton fed⁻¹)</th>
<th>Crude fiber yield (ton fed⁻¹)</th>
<th>Ash yield (ton fed⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giza-1</td>
<td>323.89</td>
<td>337.38</td>
<td>174.60</td>
</tr>
<tr>
<td>Ismailia-1</td>
<td>399.44</td>
<td>442.77</td>
<td>206.24</td>
</tr>
<tr>
<td>Ismailia-94</td>
<td>348.14</td>
<td>394.34</td>
<td>181.55</td>
</tr>
<tr>
<td>New Valley</td>
<td>392.21</td>
<td>452.47</td>
<td>201.77</td>
</tr>
<tr>
<td>Siwa-1</td>
<td>388.62</td>
<td>413.30</td>
<td>192.32</td>
</tr>
<tr>
<td>Mean</td>
<td>370.46</td>
<td>408.05</td>
<td>191.30</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>29.37</td>
<td>36.05</td>
<td>20.32</td>
</tr>
<tr>
<td>PCV%</td>
<td>8.86</td>
<td>11.22</td>
<td>6.95</td>
</tr>
<tr>
<td>GCV%</td>
<td>8.52</td>
<td>10.89</td>
<td>6.14</td>
</tr>
<tr>
<td>H²</td>
<td>92.47</td>
<td>94.17</td>
<td>78.06</td>
</tr>
</tbody>
</table>

PCV% = Phenotypic coefficient of variability, GCV% = Genotypic coefficient of variability and H²= Heritability (Broad sense)

Table 6: Estimates of phenotypic (p) and genotypic (g) correlation coefficient of yield, some yield components and quality traits of five Egyptian alfalfa varieties in 2013 (above diagonal) and 2014 (below diagonal) growing seasons.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Plant height (cm)</th>
<th>Tillers m⁻²</th>
<th>Leafiness (%)</th>
<th>Dry forage yield (ton fed⁻¹)</th>
<th>Crude protein yield (ton fed⁻¹)</th>
<th>Crude fiber yield (ton fed⁻¹)</th>
<th>Ash yield (ton fed⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height, cm</td>
<td>p 0.82**</td>
<td>-0.90*</td>
<td>0.68**</td>
<td>0.62**</td>
<td>0.75**</td>
<td>0.65**</td>
<td></td>
</tr>
<tr>
<td>g 0.91**</td>
<td>-1.00**</td>
<td>0.73**</td>
<td>0.67**</td>
<td>0.82**</td>
<td>0.71**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tillers m⁻²</td>
<td>p 0.89*</td>
<td>-0.71*</td>
<td>0.47**</td>
<td>0.36**</td>
<td>0.63**</td>
<td>0.49**</td>
<td></td>
</tr>
<tr>
<td>g 1.00**</td>
<td>-0.76**</td>
<td>0.56*</td>
<td>0.44*</td>
<td>0.74**</td>
<td>0.66*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leafiness, %</td>
<td>p -0.90*</td>
<td>-0.78*</td>
<td>-0.89*</td>
<td>-0.87**</td>
<td>-0.92*</td>
<td>-0.91*</td>
<td></td>
</tr>
<tr>
<td>g -0.96**</td>
<td>-0.89**</td>
<td>-0.95**</td>
<td>-0.93**</td>
<td>-1.00**</td>
<td>-1.00**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry forage yield, ton fed⁻¹</td>
<td>p 0.96**</td>
<td>0.94*</td>
<td>-0.85**</td>
<td>0.98**</td>
<td>0.98**</td>
<td>0.95*</td>
<td></td>
</tr>
<tr>
<td>g 1.00**</td>
<td>1.00**</td>
<td>-0.97**</td>
<td>0.99**</td>
<td>0.98**</td>
<td>0.98**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein yield, ton fed⁻¹</td>
<td>p -</td>
<td>-</td>
<td>-</td>
<td>0.94*</td>
<td>0.96**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.94**</td>
<td>0.99**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude fiber yield, ton fed⁻¹</td>
<td>p -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.95*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g -</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.99**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** = Highly significant at P=0.01, * = Significant at P=0.05, ns = Not significant at P=0.05 # =Data for the first season.

Table 7: Similarity matrix among five Egyptian alfalfa varieties based on isozymes and seed storage protein.

<table>
<thead>
<tr>
<th>Proximity Matrix</th>
<th>Matrix File Input</th>
<th>Case</th>
<th>Ismailia-1</th>
<th>Ismailia-94</th>
<th>New Valley</th>
<th>Siwa-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Giza-1</td>
<td>0.759</td>
<td>0.880</td>
<td>0.864</td>
<td>0.761</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ismailia-1</td>
<td>0.735</td>
<td>0.735</td>
<td>0.763</td>
<td>0.893</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ismailia-94</td>
<td></td>
<td>-----</td>
<td>0.873</td>
<td>0.784</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Valley</td>
<td></td>
<td>-----</td>
<td>0.852</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siwa-1</td>
<td></td>
<td>-----</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data revealed that Ismailia-1 (399.44 ton fed⁻¹) ranked first followed by New Valley (392.21 ton fed⁻¹) for crude protein yield. New Valley (452.47 ton fed⁻¹) recorded the highest value, while Giza-1 (337.38 ton fed⁻¹) recorded the lowest value of crude fiber yield. Regarding ash yield, Ismailia-1 ranked first (206.24 ton fed⁻¹) followed by New Valley (201.77 ton fed⁻¹).

The highest phenotypic and genotypic coefficient of variability were recorded for crude fiber yield (11.22 and 10.89 %), crude protein yield (8.86 and 8.52 %) and ash yield (6.95 and 6.14 %), respectively. This indicates the presence of exploitable genetic variability for these traits. Heritability (H%) estimates were generally high for all quality traits and recorded values from 94.17% for crude fiber yield to 92.47% for crude protein yield, except ash yield that recorded 78.06%. In general, all quality traits except ash yield had high heritable variation. Hence, it can be assumed that genotypes of almost all the traits are mainly determined by their phenotypes.

**Phenotypic and Genotypic Correlations:** Phenotypic and genotypic correlation coefficients (r) for all possible combinations for traits under study in two seasons are presented in Table 6. A positive value of (r) shows that the changes of two variables are in the same direction, that is, high values of one variable are associated with
high values of other and *vice versa*. In general the magnitude of genotypic correlations ($r_g$) was higher than those of phenotypic correlations ($r_p$). This revealed that association among these characters was under genetic control and indicating the preponderance of genetic variance in expression of characters. It might be due to depressing effect of environment on character association as reported earlier for alfalfa crop [26, 3, 22, 27, 23, 28].

When value of $r_p$ was greater than $r_g$, it showed that apparent association of two traits was not only due to genes but also due to favorable influence of environment. By contrast, if value of $r$ was zero or insignificant (Table 7), this showed that these two traits are independent.

In the first year, the dry forage yield fed$^{-1}$ showed significant positive correlations with each of plant height ($r_p$ = 0.73**) and tillers m$^{-2}$ ($r_p$ = 0.56*) at genotypic level. Meanwhile, it exhibited negative and significant association with leafiness at both phenotypic and genotypic levels ($r_p$ = -0.89*, $r_g$ = -0.95**). Such results could help the breeder to select high dry forage yield through selection for one or more of these traits.

Significant positive correlations were also detected between dry forage yield fed$^{-1}$ and protein yield ($r_p$ = 0.98**, $r_g$ = 0.99**), fiber yield ($r_p$ = 0.98**, $r_g$ = 0.98**) as well as ash yield ($r_p$ = 0.95*, $r_g$ = 0.98**) at both phenotypic and genotypic levels. Avci *et al.* [29] reported that significant differences were found among alfalfa lines and cultivars in dry matter yield, plant height and quality traits in three respective years.

Regarding leafiness, significant negative correlations were found with plant height ($r_p$ = -0.90*, $r_g$ = -1.00**), fiber yield ($r_p$ = -0.92*, $r_g$ = -1.00**) and ash yield ($r_p$ = -0.91*, $r_g$ = -1.00**) at both phenotypic and genotypic levels as well as with tillers m$^{-2}$($r_p$ = -0.76**) at genotypic level.

Tillers m$^{-2}$ was significantly and positively correlated with each of plant height ($r_p$ = 0.91**), protein yield ($r_p$ = 0.44*), fiber yield ($r_p$ = 0.74**) and ash yield ($r_p$ = 0.66*) at genotypic level.

Plant height showed positive associations with protein yield ($r_p$ = 0.67**), fiber yield ($r_p$ = 0.82**) and ash yield ($r_p$ = 0.71**) at genotypic level. Julier *et al.* [30] found positive correlation between plant height and yield and negative correlation between plant height and quality.

In second season, the dry forage yield fed$^{-1}$ showed strong significant positive correlations with each of plant height ($r_p$ = 0.96**, $r_g$ = 1.00**) and tillers m$^{-2}$ ($r_p$ = 0.94*, $r_g$ = 1.00**) at both phenotypic and genotypic. Otherwise, it was significantly and negatively associated with leafiness ($r_g$ = -0.97**) at genotypic level. Hoda *et al.* [28] detected highly significant positive correlation values between dry forage yield and each of plant height and number of shoots m$^{-2}$ at both the genotypic and phenotypic levels in both seasons. Phenotypic correlations in alfalfa, specifically sativa, generally indicate higher yielding plants will be taller, more mature and have reduced nutritive value [31].

Strong significant positive correlations between plant height and tillers m$^{-2}$ were observed ($r_p$ = 0.89*, $r_g$ = 1.00**). In contrast, plant height showed significant negative correlation with leafiness ($r_p$ = -0.90*, $r_g$ = -0.96**) at both phenotypic and genotypic levels. Davodi *et al.* [32] reported that dry matter yield was positively correlated with each of plant height and stem number. In contrast, it was negatively correlated with leaf/stem ratio.

This study revealed a wide phenotypic variability for many of the assessed traits in the Egyptian alfalfa varieties.

**Molecular Studies**

**Isozymes Analysis:** Five varieties of alfalfa were studied against four isozymes systems are shown in Figure (1). The banding patterns of four isozymes i.e., alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), esterase (EST) and peroxidase (PRX) revealed wide variation of different bands ranging from 6 to 8 polymorphic bands. Esterase was the highest polymorphic isozyme giving rise to 8 bands.

**Alcohol Dehydrogenase (ADH):** The ADH isozyme activity showed band polymorphism in Figure 1A. Six bands were detected which were divided into 4 monomorphic and 2 polymorphic with 33.33% polymorphism.

**Malate Dehydrogenase (MDH):** The results of total malate dehydrogenase activity gave eight bands as total bands that gave 37.5% polymorphism (Figure 1B). Band No. 3 is present in Siwa while was absent in the other varieties. This band could be used to fingerprint Siwa1 using malate dehydrogenase.

**Esterase (EST):** The electrophoretic patterns of esterase isozyme are illustrated in Figure 1C, the five varieties of alfalfa revealed six different bands. Bands No. 2 and 5 were present in New Valley while they were absent in other varieties, Band No. 3 was present in Siwa-1 only. Bands no. 2 and 5 could be considered specific markers for New Valley while band no.3 could be considered specific marker for Siwa-1.
Fig. 1: Activity analysis of isozymes in Egyptian alfalfa varieties based on PAGE electrophoreses. Varieties: 1: Ismailia-94, 2: Giza-1, 3: Ismailia-1, 4: New valley and 5: Siwa-1. Isozymes: A: Alcohol dehydrogenase (ADH), B: Malate dehydrogenase (MDH), C: Esterase (EST) and D: Peroxidase (PRX).

Fig. 2: SDS protein banding patterns for water soluble protein of Egyptian alfalfa varieties. 1: Giza-1  2: Ismailia-1  3: Ismailia-94  4: New valley  5: Siwa-1
**Peroxidase (PRX):** The results revealed seven bands for peoxidase isozymes, which gave four monomorphic and three polymorphic bands with 42.85 % polymorphism as shown in Figure 1D. The chosen enzyme systems successfully identified 3 out of the five studied alfalfa varieties indicating that isozyme polymorphism could be successfully used to identify varietal differences, using more isozyme systems is expected to identify the five varieties. These results are in agreement with those obtained by Morales and Crespo [33] who reported that electrophoretic patterns for isozymes could aid in the characterization and identification and detection of genetic variation of alfalfa varieties.

**SDS- Protein Electrophoresis:** SDS- PAGE electrophoresis patterns for water- soluble proteins for all varieties showed band polymorphism (Figure 2). Twenty-nine bands were detected with the molecular masses ranging from 14.4 up to 116.0 kDa in five Egyptian alfalfa varieties, which were not necessarily present in all varieties. Polymorphic bands (22) giving polymorphism rate of 75.86 %. The highest number of bands belonged to New Valley (16) and the least number was related to Siwa-1. Similarity matrix among varieties is shown in Table 7 as calculated according to Nei [34]. The highest similarity value was estimated between Siwa-1 and Ismailia-1 (0.893) while the lowest one was recorded between Ismailia-94 and Ismailia-1 (0.735) other similarity estimates were between these two values. The dissimilarity between Ismailia-1 and Ismailia-94 could be justified by pedigree. These results are in agreement with Jaramillo et al. [35] who reported that SDS- PAGE was widely used to separate proteins, which are directly related to genetic makeup of wild, cultivars, or newly derived cereal plants. Also, Bertozo and Valls [36] reported significant variation among different species for seed storage proteins, therefore it is suggested to use different species to increase the genetic diversity of the germplasm.

Nei’s standard distance matrix among the five Egyptian alfalfa varieties utilizing isozymes markers and seed protein is shown in Table 7. It was carried out using UPGMA computer program. The similarity relationships ranged between 0.893 and 0.759. Siwa-1 generally showed the lowest distance with Ismailia-1.While the highest distance was observed between Giza and Ismailia-1 and New Valley was in between conformance.

A dendrogram for the genetic relationships among varieties is present in Figure (3). The varieties were separated in two main clusters, cluster 1 included Giza-1 and Ismailia-94 while the second cluster was separated into two subclusters. The first subcluster included New Valley only and the second subcluster included Siwa-1 and Ismailia-1 varieties. These data reflect that different cultivars are genetically divergent and that Ismailia-1 and Siwa-1 are the most closely related cultivars (89.3%). Li et al. [37] confirmed that both isozymes and protein results appear to play an important role in the differentiation among different cultivars.

Based on the forage dry yield, plant height, number of tillers, leafiness, crude protein, crude fiber and ash, data of the five Egyptian alfalfa varieties were combined; a dendrogram separated the five Egyptian alfalfa varieties into two main clusters as shown in Figure 4. The cluster constructed on the morphological and chemical traits was identical to that based on molecular analysis indicating the accuracy of both morphological and molecular traits in identifying alfalfa varieties. Cluster 1 included Giza-1 and Ismailia-94 while the second cluster was separated into two subclusters. The first subcluster included New Valley only and the second subcluster included Siwa-1 and Ismailia-1 varieties.
The present investigation revealed a significant correlation between morphological and observed molecular distances. The obtained data suggest that isozymes and seed protein could be useful and powerful tools for estimation of genetic diversity among diverse alfalfa germplasm. Also, the usefulness of detecting genetic variation among genotypes in selecting diverse parents to carry out a new successful crossing program.

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