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The Study of Forage Yield and its Components in Relation with Resistance to Alfalfa Weevil (*Hypera postica* Gyll.) in Some *Medicago sativa* Genotypes in Hamedan

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ARTICLE INFO

Article history:
Received 17 January 2017
Revised 4 March 2017
Accepted 25 March 2017
Available online
29 May 2017

Keyword:
Alfalfa collection
Factor analysis
Forage yield
Principal component analysis

ABSTRACT

Alfalfa (*Medicago sativa* L.) is one of the most important perennial forage crops in Iran. The aims of this study were to evaluate phenotypical and genetical variabilities in alfalfa germplasm collections using multivariate analysis and to select the most promising ecotype for further breeding programs. Principal component analysis (PCA) and factor analysis were used for understanding data structure and traits correlation. Forty-six alfalfa ecotypes collected from different regions in Iran were sown and studied in the pattern of Completely Randomized Design (CRD) in a research field at Bu-Ali Sina University, Hamedan, Iran. Some important agronomic traits and the traits related to weevil pest attack were recorded during the growing season in 2012. The data were analyzed by multivariate statistics for determining groups based on their similarities. The studied ecotypes were classified into 4 clusters. Factor analysis was performed for all agronomic traits and reduced them to 4 common factors which accounted for 82% of the total variations among the ecotypes studied. Collected data were centered and scaled to unit variance and analyzed by principal component analysis. The results showed that the first four PCs contributed to 0.7% of the total variability among the ecotypes. In other words, data reduction would let the plant breeders to reduce field costs required to obtain the genetic parameter estimates necessary to construct selection indices.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a valuable forage crop grown throughout the world. Cultivated alfalfa (*M. sativa* L., 2n= 4x= 32) is an autotetraploid, open-pollinated species characterized by tetrasomic inheritance with multiple allelism and by pronounced inbreeding depression (Barcaccia et al., 1999). The most Serious pest is the alfalfa weevil, *Hypera postica* (Mazahery-Laghab, 1997). In Iran, among forage crops, alfalfa is a major forage crop which is cultivated to an extent of about 0.65 million hectares (Zare et al., 2009). Having high mineral contents, vitamins and proteins, alfalfa is one of the most nutritious crops that can be utilized as forage (Monteiro, 2003).

Knowledge about germplasm diversity and genetic relationships among breeding materials could be an invaluable aid in crop improvement strategies (Mohammadi and Prasanna, 2003). The genetic diversity between the genotypes is important as the genetically diverged parents are able to produce high heterotic effects (Falconer, 1960). Knowledge of germplasm diversity among elite breeding materials has a significant impact

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on the improvement of crop plants (Hallauer et al., 1988). The use of established multivariate statistical algorithms is an important strategy for classifying germplasm, ordering variability for a large number of accessions, or analyzing genetic relationships among breeding materials (Mohammadi and Prasanna, 2003). On the other hand, multivariate analysis is a very useful method because it reveals the relationships and correlation among variables studies (Tucak et al., 2009). The genetic diversity can be determined by several multivariate techniques (clustering methods, analysis by principal components and/or canonical variables), the procedures that are widely used in different crops (Tucak et al., 2011). Principal components analysis (PCA) is a technique for producing high-dimensional data, and using the dependencies between the variables to represent it in a more tractable, lower-dimensional form, without losing too much information, PCA is one of the simplest and most robust ways of doing such dimensionality reduction. Agronomic evaluation is an important step in the description and classification of crop germplasm as improvement depends upon the magnitude of genetic variability and it enables researchers to plan use of appropriate gene pools in crop improvement for specific plant attributes (Peeters and Martinelli, 1989). Mahalanobis et al. (1949), proposed a generalized distance formula known as Mahalanobis D2 statistic, which was shown to remain reasonably constant for varying sizes. Multivariate analysis for measuring the degree of divergence between biological populations and for assessing relative contribution of different characters to the total divergence was employed (Rao, 1960).

Davodi et al. (2011), reported that, based on ward cluster analysis, 200 entries (200 accession alfalfa) were divided into 7 groups and the results of factor analysis were accounted for 81% of the total variance for the first six factors, the factorial analysis was done based on the principal component extraction and the varimax rotation methods. Khodarahmpour and Soltani (2013), in the study of alfalfa ecotypes, reported that factor analysis based on principal component analysis showed that three independent factors, determined 54, 17 and 14 percent of all the available variation among the data. Measurement of genetic distance should be very important for breeding when it is based on a broad range of traits relevant to breeding objectives (Davodi et al., 2011). The objective of this study was to analyze the phenotypic diversity in the alfalfa germplasm using multivariate analysis to examine the extent of genetic diversity ecotypes that could contribute to the improvement of yield and quality of alfalfa. Furthermore, the other objectives of the study were to (1) classify alfalfa cultivars based on their agronomic traits, (2) identify the genotypes with the best combinations of agronomic traits for future use in alfalfa modification.

2. Materials and methods

2.1. Plant materials and experimental design

Forty-six ecotypes of alfalfa from different geographical origins were evaluated in this study (Table1) (Selected from experimental field with 150 populations). The experiment was conducted on the experimental field of the faculty of agriculture at Bu-Ali Sina University in 2012. The field trial was arranged in a completely randomized design (CRD) with two replications (Because of seed limited and 150 types of alfalfa Population). The experiment was carried out in the research farm of Bu-Ali Sina University in Dastjerd region with the altitude of 1810 meters from sea level and with the longitude of 48º 88' and latitude of 34º 54' in September 2007. After the spring rainfalls, irrigation was done every 8 days according to the common rainfalls of the region. Data for the studied characteristics were recorded in different growth stages. Weeds were controlled by hand. Each experimental unit consisted of 2.00 meter rows with 50 cm row spacing. No insecticides were used in the field as the routine spraying operations in the region were done. A natural pest infection in the field occurred due to the outburst of alfalfa leaf weevil in early spring (depending on the ecological conditions of the region under study).

2.2. Phenotypic data collection and statistical analysis

During the investigation, eight phenotypic traits were observed in this study. The following traits were collected from plants of each ecotype:

1-The number of larvae per plant (larvae): during three stages and each stage in seven days trial steps and each step of one-third of the experimental unit, the number of larvae was counted by placing a white cloth at the foot of the plant and hitting the shoots three times.

Table 1. List of alfalfa ecotypes evaluated in the present study.

Code	Name	Type/species	Code	Name	Type/species
1	Hamedan 29	cultivar/sativa	24	Yazdi	cultivar/sativa
2	Bahar-e- Hamedan 28	cultivar/sativa	25	Talent 2	cultivar/sativa
3	Hamedan Ala 31	cultivar/sativa	26	Lahontan policrossamerica	cultivar/sativa
4	Hamedani 87	cultivar/sativa	27	Ladak	cultivar/sativa
5	Hamedani 106	cultivar/sativa	28	Policrosshamedan	cultivar/sativa
6	Hamedani 54	cultivar/sativa	29	Policross bam 10	cultivar/sativa
7	Afghani 81	cultivar/sativa	30	Naragamet	cultivar/sativa
8	Sarab 16	cultivar/sativa	31	Etlantic 1	cultivar/sativa
9	Calyfornia 19	cultivar/sativa	32	Argantin	cultivar/sativa
10	Harati 26	cultivar/sativa	33	Mahalo khoe	cultivar/sativa
11	Tafresh 42	cultivar/sativa	34	Policross bam 11	cultivar/sativa
12	Feez 48 (Fc32666)	cultivar/sativa	35	Grem–e-america	cultivar/sativa
13	Feez (varragensee)	cultivar/sativa	36	Policrossshiraz 8	cultivar/sativa
14	Feex (Vermal)	cultivar/sativa	37	Ardabili	cultivar/sativa
15	Yazdi 40	cultivar/sativa	38	Rezaeeh	cultivar/sativa
16	Shiraz 9	cultivar/sativa	39	Vernal	cultivar/sativa
17	Trnambdasoued	cultivar/sativa	40	Telantic 34044	cultivar/sativa
18	Mahali Shapor	cultivar/sativa	41	Devipoe 34630	cultivar/sativa
19	Mahali marandi	cultivar/sativa	42	Dupuits	cultivar/sativa
20	Mahali bami	cultivar/sativa	43	Hamedani 32	cultivar/sativa
21	Sun loez America 288	cultivar/sativa	44	Takboteh 51	cultivar/sativa
22	Mahali hamedan	cultivar/sativa	45	Lahontan	cultivar/sativa
23	Hamedani 292	cultivar/sativa	46	Eur	cultivar/sativa

2-Plant height in 10% flowering time (He1): Five plants were measured for this trait.

3-Plant height at the time of damage (He2): during the pest attack, this trait was measured from the top to five centimeters from of the collar in 3 plants in each experimental unit and recorded.

4-Chlorophyll amounts: Chlorophyll in 5 leaves was measured in 3 stages and 3 times in each stage during the pest attack using an American Spad as chlorophyll meter. The mean amount of chlorophyll was calculated and recorded as chlorophyll amount for each genotype.

5-Dry matter yield (DMY): 250 grams of fresh forage were weighed out and after being air-dried, were then transferred to an oven at 75°C for 24 hours. Then, the dry weights in grams were recorded and were generalized to the test plots.

6-Percentage of dry matter (PDM): in order to calculate this trait, the formula below was used: 100× (fresh forage yield/dry forage yield) = percentage of dry matter.

7-Amount of damage: During three seven-day steps at the time of pest attack (from April to May), considering the amount of damage caused to the shoots and leaves of the plants, the amount of damage was determined using scores 0 to 9 (zero for 1% to 10% and nine for 91% to 100% damage caused by larval feeding). In order to reduce experimental errors during scoring, the amount of damage to shoots was measured as facing the opposite direction of the sun shine. After transforming the score data to percentages, the amount of damage was analyzed as percentages.

8-Fresh forage yield (FFY): Alfalfa shoots of each ecotype was cut off from 5cm from the top of collar when flowering percent was at 10%. The forages were harvested starting from a meter to the middle of the experimental unit and weighed out as grams. Principal component analysis (PCA), factor analysis (FA) and hierarchical clustering were performed on all traits. SPSS (ver. 16) and Minitab (ver. 14) were used to calculate the traits for multivariate analysis.

3. Result and discussion

3.1. Cluster Analysis and Discriminant Function

Cluster analysis refers to a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster (Hair et al., 1995). Strong positive relationships have been found between genetic distance and heterosis in broad range of crop species (Humphreys, 1991). Cluster analysis was based on significant traits. Based on ward cluster analysis and Pearson methods, 46 entries were divided into 4 groups (Fig. 1). The results of this study indicated that selection of variables productivity factor (factor 2) could enable breeders to release the desirable increment in forage yield of alfalfa. The cluster analysis performed on the first two components grouped the majority of alfalfa ecotypes into four clusters of germplasm (Fig. 1). Cluster 1 contained twelve ecotypes (1, 41, 17, 42, 38, 46, 10, 34, 19, 11, 33 and 21), cluster 2 contained twelve ecotypes (2, 14, 12, 44, 40, 9, 36, 6, 39, 29, 35 and 30), cluster 3 contained seventeen ecotypes (3, 37, 7, 45, 13, 28, 26, 27, 37, 18, 31, 5, 8, 15, 12, 25, and 43) and cluster 4 contained five (4, 24, 16, 20 and 23). Azad et al., (2012), reported that, cluster analysis for *zea maize* hybrid genotypes could be improved and could have the chance to obtain higher heterosis with high performing crosses. According to table 2, the results of the discriminant function, group ecotype classification was fully confirmed. According to table 3, parameters within cluster sum of square, average and maximum distance from centroid of the cluster were shown. Table 4 showed the Standardized test performance tend to center (Average of clusters).

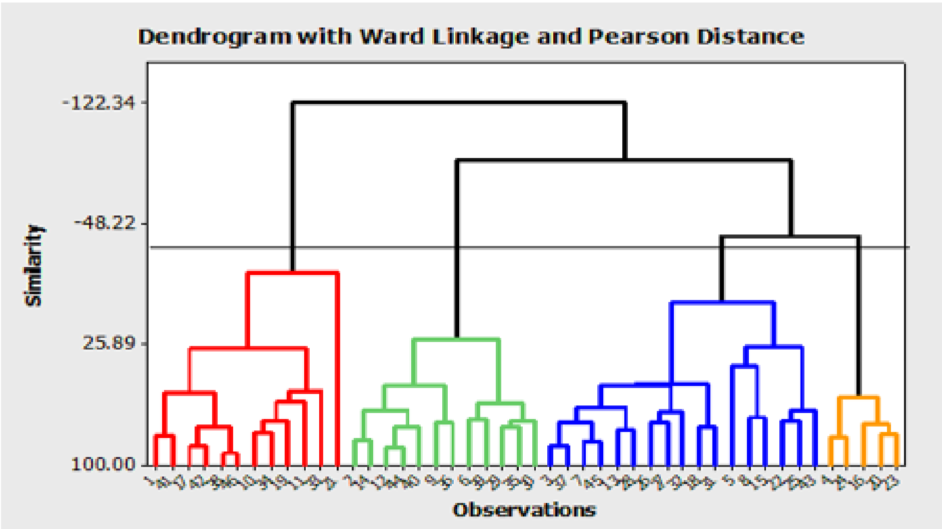

Fig 1. Dendrogram of 46 Alfalfa ecotypes by analyzing 8 traits using ward cluster analysis method. (See ecotype name in Table 1).

Table 2. Result of discriminant function for alfalfa ecotypes.

	Predicted Group Membership									
Ungrouped	1		2		3		4		Total	
Cases	Count	Percent	Count	Percent	Count	Percent	Count	Percent	Count	Percent
1	6	100	0	0	0	0	0	0	6	100
2	0	0	16	100	0	0	0	0	16	100
3	0	0	0	0	17	100	0	0	17	100
4	0	0	0	0	0	0	7	100	7	100

100% of original grouped cases correctly classified

Table 3. Scattering parameters of the cluster (Estimates of the standard variables).

	Number of Observation	Within Cluster Sum of Squares	Average Distance From Centroid	Maximum Distance From Centroid
Cluster 1	12	182398	107.544	253.147
Cluster 2	12	156970	103.101	213.244
Cluster 3	17	785815	178.037	530.634
Cluster 4	5	60277	99.483	147.742

Table 4. Standardized test performance tend to center (Average of clusters).

.	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Number of Larvae Per Plant	19.568	32.028	18.354	14.500
Amount of Damage	23.403	36.956	22.970	11.634
Plant Height in 10% Flowering Time	34.054	38.901	39.126	40.934
Chlorophyll Amounts	51.544	52.158	54.735	50.886
Fresh Forage Yield	532.875	794.458	867.529	820.800
Dry Matter Yield	216.590	303.693	302.745	229.558
Plant Height at the Time of Damage	51.442	53.636	55.833	67.398
Percentage of Dry Matter	43.250	38.465	35.808	28.750

Between the center of clusters 1 and 3, there is a distance of 345.729, i.e. ecotypes in clusters 1 and 3 on the base of K-means algorithm have the maximum distance or the lowest genetic similarity and they could be effective in plant breeding programs (Table 5). Table 6 showed Standardized Canonical Discriminant Function Coefficients.

Table 5. Centre distance matrix clusters (Estimates of the standardized variables).

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	0.000			
Cluster 2	276.412	0.000		
Cluster 3	345.729	75.773	0.000	
Cluster 4	289.389	86.186	88.797	0.000

Table 6. Standardized canonical discriminant function coefficients.

Function	Variable							
	Number of Larvae Per Plant	Plant Height at the Time of Damage	Percentage of Dry Matter	Dry Matter Yield	Fresh Forage Yield	Chlorophyll Amounts	Amount of Damage	Plant Height in 10% Flowering Time
1	0.105	-0.232	0.508	-0.422	1.244	0.291	-0.160	-0.132
2	0.146	-0.409	1.933	-1.254	0.418	0.319	-0.039	0.674

3.2. Principal Component Analysis

Principal Component Analysis (PCA) is defined as “a method of data reduction to clarify the relationships between two or more characters and to divide the total variance of the original characters into a limited number of uncorrelated new variables” (Wiley, 1981). This will allow visualization of the differences among the individuals and identify possible groups; PCA can also be used to determine the optimum number of clusters in a study. In this case, the objective is to maximize the variation explained by the first PC of each cluster (Mohammadi and Prasanna, 2003). Biplot basis on the first and second components in alfalfa cultivars were divided into two groups (Fig. 2). The result of PCA showed that the first four PCs contributed to 70% of the total variability among the ecotypes (Table 7). The cluster analysis performed on the two first components grouped the majority of alfalfa cultivars and populations into three clusters of germplasm. Due to the high negative correlation coefficients of the first component with the trait He1 and FFY, the first component could be mentioned as yield component. The second component had a negative correlation with larvae number and damage percent traits. This component was associated with the pest weevil and it could be named as Resistance component the selection based on this trait causes the selection of genotypes with a few number of larvae and a low damage percentage and also a higher plant height. The third trait could be called as the component of yield reduction.

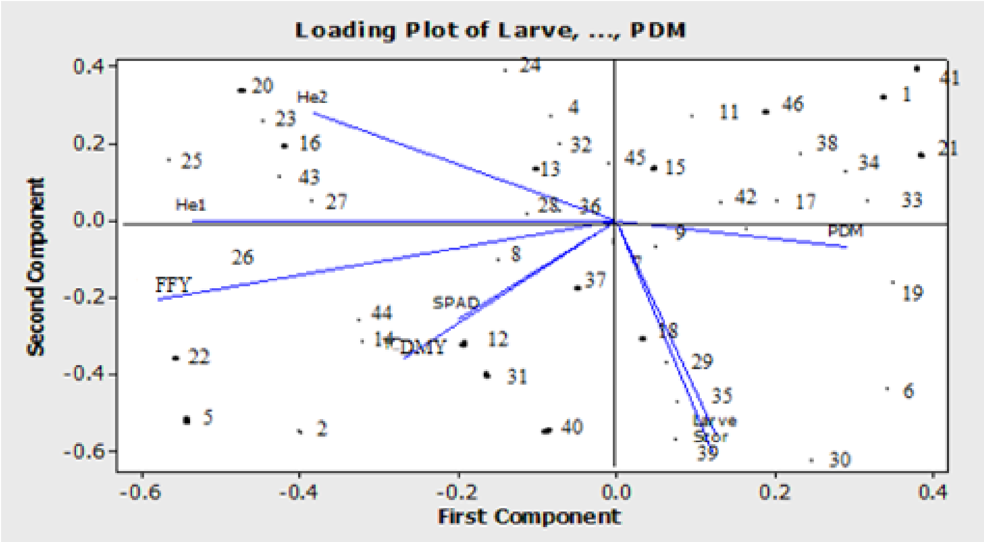

Fig 2. The biplot display of alfalfa cultivars on the first and second components. Larvae (number of larvae per plant), Score (Amount of damage), He1 (Plant height in 10% flowering time), SPAD (Chlorophyll amounts), TFW (Fresh forage yield), TDW (Dry matter yield), He2 (Plant height at the time of damage) and PDM (Percentage of dry matter).

Table 7. Principal component analysis on traits data: eigenvalues, proportion and cumulative accounted by the first three principal components.

Variable	PC 1	PC 2	PC 3
Number of Larvae Per Plant	0.125	-0.562	0.321
Amount of Damage	0.120	-0.603	0.271
Plant Height in 10% Flowering Time	-0.538	-0.000	0.084
Chlorophyll Amounts	-0.201	-0.256	-0.190
Fresh Forage Yield	-0.580	-0.209	-0.019
Dry Matter Yield	-0.270	-0.359	-0.589
Plant Height at the Time of Damage	-0.383	0.280	0.128
Percentage of Dry Matter	0.289	-0.069	-0.645
Eigenvalue	2.1926	1.9826	1.4548
Proportion	0.274	0.248	0.182
Cumulative	0.274	0.522	0.704

3.3. Factor Analysis

Factor analysis (FA) was applied after PCA (Fig. 3) because it was generally recommended that PCA be performed as a first step before other multivariate analysis to check for abnormalities present in data set (Johnson, 1998). Ashofteh-Beiragi et al. (2012), in a study on the application of the multivariate analysis method for some traits in maize reported that the fourth factor was loaded by stem diameter and ear length, and it accounted for just 11.93% of the total variance. The factor analysis was done based on the principal components extraction and varimax rotation method. Table 8 shows the contribution of different significant characteristics of each factor. The axes represented 81% of the total variance for the first six factors. Factor 1, accounted for 27% of the variation, was associated with FFY, He1 and He2. This factor was regarded as quality factor. Factor 2, which was accounted for 24% of the variation, was named as productivity factor since it included Amount of damage yield, vegetation score and leaf/stem ratio. Third (CP and CF) and forth factors (plant height and node number) were important elements. Factor analysis based on principal component analysis and equimax rotation showed that four independent factors determined 27, 24, 18 and 12 percent of all variation data, respectively (Table 8). According to The results of the factors in table 8, TFW (factor 1), Larvae number and percent damage (factor 2), PDM (factor 3) and chlorophyll amount (factor 4) were determined.

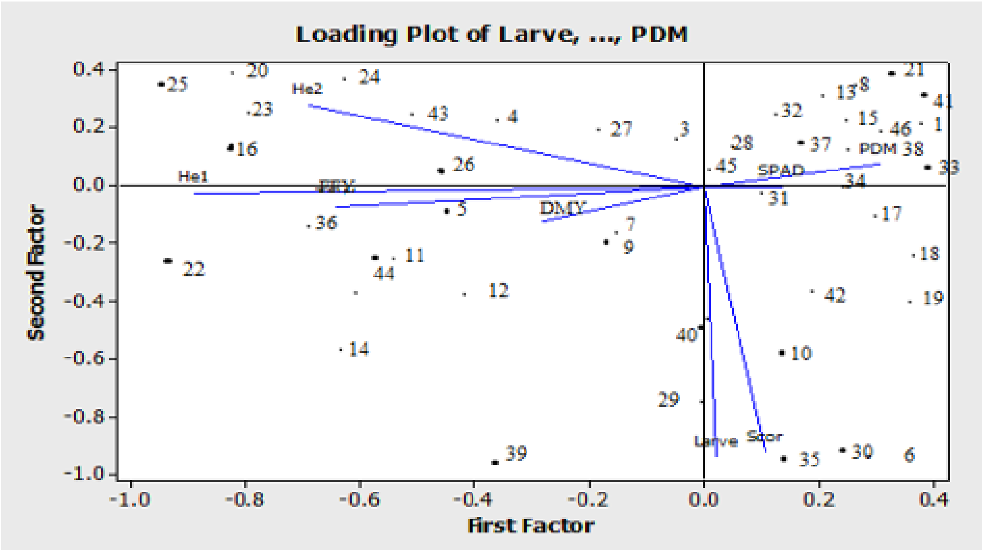

Fig 3. Scatter Plot of 46 alfalfa ecotypes for studied traits: (Number of larvae per plant, Amounts of damages, Plants height in 10% flowering time, Chlorophyll amounts, Fresh forage yield, Dry matter yield, Plant height at the time of damage and Percentage of dry matter.

Table 8. Results of factor analysis on traits data, percent variation accounted by the first four factors.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Number of Larvae Per Plant	0.185	-0.791	0.388	-0.269
Amount of Damage	0.178	-0.850	0.327	-0.106
Plant Height in 10% Flowering Time	-0.797	-0.001	0.102	-0.390
Chlorophyll Amounts	-0.298	-0.360	-0.230	0.675
Fresh Forage Yield	-0.859	-0.294	-0.023	0.146
Dry Matter Yield	-0.400	-0.505	-0.710	-0.145
Plant Height at the Time of Damage	-0.567	0.394	0.154	-0.320
Percentage of Dry Matter	0.428	-0.097	-0.778	-0.364
Variance	2.1926	1.9826	1.4548	0.9686
% Var	0.274	0.248	0.182	0.121

4. Conclusion

The alfalfa weevil (*H. postica* Gyll) is a serious pest that adversely affects alfalfa forage yield and completely destroys the harvest in the early season every year (Kakaei et al., 2015). Statistical analysis of agronomic traits could be informative in alfalfa breeding program since agronomic traits are a reflection of gene effects. The present investigation revealed a wide phenotypic variability for many of the assessed traits in the alfalfa collections (Tucak et al., 2009). Different statistical procedures have been used in modeling crop yield, including principal component analysis and factor analysis. Factor analysis identified some similar characters as the most important for classifying the variation among alfalfa population. PCA analysis separated the majority ecotypes into four clusters of germplasm. Positive traits for breeding were found in all clusters. Valuable plants from the most promising materials could be used for future activities in our alfalfa breeding program. The groups created by the cluster analysis are believed to have unique ecotype group properties that might be considered for the prediction of the results should the best ecotypes grown on these areas. However, stability analysis of different traits on the already established groups of the current study requires further investigations based on sufficient data that were collected during different years and at different experimental locations. Ecotypes distribution in the biplot space from the analysis to main components with the distribution of ecotypes in biplot space from factorial analysis was consistent. These results were consistent with the results of the cluster analysis. For example, a consistent number of clusters (4), ecotypes 4, 16, 20, 23 and 24, were spaced in the second bi-plot from principal component analysis and factors analysis. So, it could be confidently useful. Shoushi-Dezfuli et al. 2016 has also used principal component analysis and cluster analysis in study some of alfalfa Genotype and observed diversity.

5. Acknowledgements

The authors wish to thank Dr. Hamzeh Mazahery-Laghab and Dr. Leila Zarei for reading the manuscript and their valuable comments and are grateful to the MSc. Plant breeding Students of Bu-Ali Sina University who assisted with the collection of the data ecotypes and Mr. Qadrdan for ecotypes seed received from gene bank of Tehran University.

Reference

Ashofteh-Beiragi, M., Siah-Sar, B. A., Sadeghi-Geive, H., Alhossini, N., Rahmani, A., Bakhtiari-Gharibdoosti, A., 2012. Application of the multivariate analysis method for some traits in maize. African Journal of Agricultural Research, 7 (10): 1524-1533.

Azad, M. A. K., Biswas, B. K., Alam, N., Alam, Sk. S., 2012. Genetic diversity in maize (*Zea mays* L.) inbred lines. The agriculturists, A scientific journal of krishi foundation, 10 (1): 64-70.

Barcaccia, G., Albertini, E., Tavoletti, S., Falcinelli, M., Veronesi, F., 1999. AFLP fingerprinting in *Medicago spp.:* Its development and application in linkage mapping. Plant Breeding, 118 (4): 335-340.

Davodi, M., Jafari, A. A., Assadian, G., Ariapour, A., 2011. Assessment of relationships among yield and quality traits in alfalfa (*Medicago sativa* L.) under dry land farming system, Hamadan, Iran. Journal of Rangeland Science, 1 (3): 247-254.

Falconer, D. S., 1960. Introduction to quantitative genetics. Oliver & Boyd, London. 304 p.

Hair, J. R., Anderson, R. E., Tatham, R. L., Black, W. C., 1995. Multivariate data analysis with readings. 4th edition, Prentice-Hall, En strateglewood Cliffs, NJ.

Hallauer, A. R., Russell, W. A., Lamkey, K. R., 1988. Corn breeding. In: corn and corn improvement. 3rdedn. AgronMonogr 18, ASA-CSSA-SSSA, Madison, Wisconsin, USA, 469- 564.

Humphreys, M. O., 1991. A genetic approach to the multivariate differentiation of perennial ryegrass (*Lolium perenne* L.) populations. Heredity, 66: 437-443.

Johnson, D. E., 1998. Applied multivariate methods for data analysts. Pacific Grove, CA: Duxbury Press.

Kakaei, M., Mazahery-Laghab, H., Shararbar, H., 2015. Resistance mechanisms and protein expression of screening genotypes of alfalfa field germplasm to alfalfa weevil (*Hypera postica* Gyll) (Col., Curculionidae). Journal of Entomological Society of Iran, 7: 77-89.

Khodarahmpour, Z., Soltani, A., 2013. Multivariate analysis of alfalfa (*Medicago sativa* L.) cultivars for salinity tolerance at germination stage. Technical Journal of Engineering and Applied Sciences, 3 (2): 139- 144.

Mahalanobis, P. C., Majumdar, D. N., Rao, C. R., 1949. Anthropometric survey of the United Provinces: a statistical study. Sankhya: The Indian Journal of Statistics, 89-324.

Mazahery-Laghab, H., 1997. Endogenous resistance to insect pests in alfalfa; engineering for enhanced resistance. Durham Ph.D. Thesis.

Mohammadi, S. A., Prasanna, B. M., 2003. Analysis of genetic diversity in crop plants-salient statistical tools and considerations. Crop Science. 43 (4): 1235-1248.

Monteiro, M., Appezzato-da-Gloria, B., Jose Valarini, M., Alberto de, O., Lucia Carneiro Vieira, M., 2003. Plant regeneration from protoplasts of alfalfa (*Medicago sativa* L.) via somatic embryogenesis. Scientia Agricola, 60 (4): 683- 689.

Peeters, J. P., Martinelli, J. A., 1989. Hierarchical cluster analysis as a tool to manage variation in germplasm collections. Theoretical and Applied Genetics, 7 (1): 42-48.

Rao, C. R., 1960. Multivariate analysis: an indispensable statistical aid in applied research. Sankhya: The Indian Journal of Statistics, 22: 317- 338.

Shoushi-Dezfuli, A. A., Mohammadi-Dehcheshmeh, Sh., Rafiei-Boroujeni, F., Shiran, B., 2016. Evaluation of salinity tolerance of alfalfa genotypes during germination stage using multivariate analysis. Journal of Applied Environmental and Biological Sciences, 6 (3): 51-56.

Tucak, M., Popovic, S., Cupic, T., Simic, G., Gantner, R., Meglic, V., 2009. Evaluation of alfalfa germplasm collection by multivariate analysis based on phenotypic traits. Romanian Agricultural Research, 26: 47-52.

Tucak, M., Popovic, S., Cupic, T., Spanic, V., Jug, I., 2011. Phenotypic diversity of alfalfa (*Medicago sativa* L.) germplasm. Poljoprivreda, 17 (1): 36- 41.

Wiley, E. O., 1981. Phylogenetics: The theory and practice of phylogenetics and systematics. John Wiley, New York.

Zare, N., Valizadeh, M., Tohidfar, M., Mohammadi, S. A., Malboobi, M. A., Habashi, A. A., 2009. Selection of regenerative genotypes from Iranian alfalfa cultivars. Journal of Food, Agriculture and Environment,7: 567-572.