



Introduction of dry yield-related traits to screen low-irrigation tolerant ecotypes in alfalfa (*Medicago sativa* L.)

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ABSTRACT

Alfalfa is one of the most important forage crops in the world and Iran. Due to the adverse effect of drought on alfalfa yield, screening drought-tolerant genotypes is essential in breeding efforts. In the present study, 11 alfalfa ecotypes were evaluated during two years under the low-irrigation stress condition. The statistical analyzes were done on the average of two-years data. The first and second factors, respectively as "forage-quantity factor" and "forage-quality factor", explained 70.40% of the data total variance. Factor analysis showed that, the traits of fresh-forage yield, plant height, stem fresh weight and regrowth rate, had the most positive effect on dry-forage yield, respectively. Qharaghezlou ecotype with the highest dry-forage yield, and Sedghiyan ecotype, with the highest forage quality, were the most drought-tolerant and high-quality ecotypes, respectively. Ecotypes of Mohajeran and Famenein showed the lowest dry-forage yield. Ecotypes were grouped in three separate clusters. The first and third clusters were identified as "dry-forage quality" and "dry-forage quantity" cluster, respectively. These two clusters had the highest genetic distance. Correctness of cluster grouping was confirmed by the discriminant function analysis. Fresh-forage yield, dry to fresh-forage yield ratio, plant height and regrowth rate were entered into the regression model respectively, as the most important traits affecting on dry-forage yield. The traits of fresh-forage yield and dry to fresh-forage yield ratio showed the most positive direct effect on dry-forage yield. Also, plant height, through increasing fresh-forage yield, and regrowth rate, through decreasing the dry to fresh forage yield ratio, had the largest positive and negative indirect effects on dry-forage yield, respectively. According to the results, the ecotypes showed a high diversity, which suggests the use of desirable traits and superior genotypes identified for use in future alfalfa breeding programs.

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1. Introduction

Unfortunately, the significant warm weather in recent years may increase the severity of drought, which is due to the decrease in rainfall and the increase in evaporation and transpiration in some regions of the world (Solomon *et al.*, 2007). Drought stress is the most important abiotic stress that reduces crop yield in arid and semi-arid regions, such as Iran (Nazari *et al.*, 2018). Based on the expansion of the range of this stress, conducting basic research to identify low-irrigation-tolerant ecotypes is a basic, economic and necessary solution (Ranjbar *et al.*, 2015; Neupane and Guo, 2019). Although various researches have been carried out in the field of breeding to improve tolerance to drought stress, but due to the significant interaction between genotype and environment, the process of these researches has been relatively complicated and slow.

Medicago sativa L. is a perennial plant, cross-fertilization with about 10% self-fertilization, from the legume family. Its primary origin is northwest Iran, northern Turkey and the Caucasus region. This plant, as the queen of forage crops, can produce about two tons of protein per hectare of cultivation annually (Nekoyanfar *et al.*, 2017). Considering the significant role of this plant in feeding livestock on the one hand and the spread of drought stress on the other hand, conducting basic research on alfalfa breeding to improve tolerance to drought stress is necessary and essential (Pour-Farhad *et al.*, 2008). The presence of high genetic diversity and selection is a requirement for success in breeding a species (Moosavi *et al.*, 2013). In this regard, multivariate statistical methods are useful methods to obtain efficient information in evaluating the diversity between different genotypes to identify superior genotypes and desirable traits. Among the various multivariate methods, principal component analysis, factor analysis, regression analysis, cluster analysis and causality analysis are widely used to separate the genotypes (Moosavi *et al.*, 2022). Multivariate statistical methods, especially the regression method and path analysis can be used to investigate the real relationships between the traits (Le-Singer *et al.*, 2005). Previously, these methods have been effectively used to understand the relationship between yield and agro-morphological traits affecting yield in crops. Le-Singer *et al.* (2005) used cluster analysis to group alfalfa genotypes using Ward's method. Bahar *et al.* (2005) classified alfalfa genotypes into 6 groups using cluster analysis to investigate Iranian alfalfa genetic diversity. Considering that some diverse agro-morphological traits have high heritability and can be easily measured, therefore, selecting based on them will be appropriate for improving yield (Julier *et al.*, 2000; Sharma and Hore, 1993). Due to the complexity of yield, it is possible that sometimes indirect selection to improve yield, through yield-related traits with high heritability, will lead to better selection response (Jomova *et al.*, 2005, Moosavi *et al.*, 2020). Researchers believe that because the average of several samples has a better estimate of the parameter, therefore it is better to carry out repeated sampling and use the average of the samples to analyze the data (Farshadfar *et al.*, 2007).

By identifying genotypic groups with the greatest genetic distance through cluster analysis, it is possible to identify different parents for future crosses. So that different parents produce more heterosis and increase the probability of obtaining superior differentiated progeny through the phenomenon of transgressive segregation. On the other hand, determining the characteristics and grouping of germplasm allows breeders to avoid re-sampling of populations (Sharma and Hore, 1993). Despite the fact that the main efficiency of cluster analysis is to identify real groups and reduce the volume of data, sometimes it is possible that this statistical method creates unexpected groups. In this case, they should be analyzed through the decomposition of the discriminant function (Moghadam *et al.*, 1995). Analysis of the discriminant function is used when certain groups already exist and the goal is to test the difference between these groups (Rabiei *et al.*, 2008).

In general, multivariate statistical methods are a common method for identifying the most important traits affecting yield and determining the relative contribution of each of them on total variance. The use of multivariate statistical methods in different plants has been reported by researchers. For example, some researches, such as Rabiei *et al.* (2008) in potato, Abdullahi-Mandolkani (2011) in alfalfa, Hazeq-Jafari *et al.* (2014) in alfalfa, Kakai and Mazahery- Laqab (2014) in alfalfa, Khodarahmpour and Motamedi (2016) in alfalfa, Tucak *et al.*, (2009) in alfalfa and Moosavi *et al.* (2020 and 2022) in wheat, can be mentioned.

The purpose of this research was to evaluate the genetic diversity and introduce the most desirable traits effective in increasing dry-forage yield to screen alfalfa ecotypes under low-irrigation conditions.

2. Materials and methods

2.1. Plant materials and experimental design

In this research, 11 alfalfa ecotypes (Table 1) were evaluated in the form of randomized complete block design with 3 replications during 3 years. This plant germplasm is the best ecotypes of cold regions of Iran, which were compared and evaluated in this research. The first year was considered as the year of seedling establishment. Therefore, the traits were measured during the second and third year (two consecutive year). The land preparation consisted of turning plow, two perpendicular discs and a trowel, which was done before sowing the seeds. Then the required fertilizers were used based on the soil-test results. Then, rows with a distance of 50 cm were created using a furrow machine. Each experimental plot consisted of 6 rows with a length of 8 meters, and the distance between the rows was equal to 50 cm. Accordingly, the area of each plot was 24 square meters. The distance between plots was 50 cm and the distance between replicates was 250 cm. Planting was done manually in the beginning of September and immediately after planting, irrigation was done. Irrigation was carried out in a leaky manner. Low-irrigation stress was done by increasing the length of the irrigation period from 7 days to 12 days. This stress was applied after the establishment of seedlings and the development of their roots. During the experiment, the fight against pests and diseases was carried out. Then, different traits were measured in four cuts in the second and third years of planting.

Table 1. Information of 11 alfalfa ecotypes used in the experiment

Ecotype code	Ecotype name	Ecotype code	Ecotype name	Ecotype code	Ecotype name	Ecotype code	Ecotype name
1	Ghahavand	4	Gharaghezlou	7	Malek-kandi	10	Galebani
2	Ordoubad	5	Gharghelough	8	Hamedan	11	Mohajeran
3	Chaleshtar	6	Sedghiyan	9	Famenein	---	---

2.2. Trait measurement and data collection

Recording of the traits (Table 2) was done during the second and third year of planting in four cuts in each year. The time interval between greening in spring and when 50% of the plants in each plot have at least one flower was considered as the trait of days to flowering. Before forage harvest, 10 plants were randomly selected from each experimental plot and plant height was recorded in centimeters. After removing 50 cm from the beginning and end of each row and after removing the marginal rows at the time of harvesting, the bushes of the remaining rows were harvested and immediately the fresh forage yield per area unit was recorded. Then, the above samples were kept for 48 hours in an oven with a temperature of 75 degrees Celsius and the dry

forage yield per area unit was recorded. 250 grams of fresh forage samples were randomly selected in each plot at the time of harvesting and after separating the stems and leaves, the stem and leaf fresh weight was recorded. At the time of harvesting, from each experimental plot with a length and width of 50 cm, it was harvested separately and the stems number per unit of 0.25 square meters was counted and then converted to the number of stems per square meter. Stems less than 20 cm were not counted. To record the regrowth rate, 17 days after harvesting each cut, that is, half the time of each cut, the height of 10 plants was randomly measured.

Table 2. Information of the measured traits in the present experiment

Trait code	Trait name (unit)	Trait code	Trait name (unit)
1	Days to flowering (day)	6	Stem fresh weight (g)
2	Plant height (cm)	7	Leaf fresh weight (g)
3	Dry forage yield (area unit)	8	Leaf to stem fresh weight ratio
4	Fresh forage yield (area unit)	9	Stem number per square meter
5	Dry to fresh forage yield ratio	10	Regrowth rate

2.3. Data analysis

Statistical analysis was done based on two-year average data. These analyzes included factor analysis, cluster analysis, discriminant function analysis, stepwise regression analysis, and causality analysis, which were performed using SPSS and Minitab software. Factor coefficients were calculated based on the principal components. Cluster analysis was done using Minitab software based on Pearson's squared distance matrix and Ward's method for grouping ecotypes. The discriminant function was analyzed to determine the difference between the groups using SPSS software. Stepwise regression analysis was performed using the forward method to identify traits with the greatest effect on dry forage yield and determine the relative contribution of each trait using Minitab software. Causality analysis in order to determine the cause-and-effect relationship of traits affecting dry forage yield, and to evaluate their direct and indirect effects using Path software.

3. Results

3.1. Factor analysis results

The first and second factors explained 45.3% and 25.1%, respectively, and together 70.40% of the total data variance (Table 3). The first factor showed a positive and strong relationship with the traits of stem fresh weight, fresh forage yield, plant height, regrowth rate and dry- forage yield (Table 3). Accordingly, the first factor was named "dry-forage quantity factor". Meanwhile, the second factor showed a strong and positive relationship with the traits of leaf fresh weight, stem number per square meter, and leaf-to-stem fresh weight ratio. Based on this, the second factor was named "dry-forage quality factor".

Table 3. The results of the coefficients of the first and second factors for different traits under low-irrigation conditions

Factor	Variance (%)	Days to flowering	Fresh forage yield	Dry forage yield	Plant height	Dry to fresh forage yield ratio	Stem fresh weight	Leaf fresh weight	Leaf -to- stem fresh weight ratio	Stem number per square meter	Regrowth rate
1	45.30	-0.38	0.78	0.49	0.71	-0.43	0.84	-0.84	-0.88	0.06	0.64
2	25.10	-0.72	-0.42	-0.77	-0.21	-0.78	-0.06	0.60	0.45	0.49	0.26
Sum	70.4										

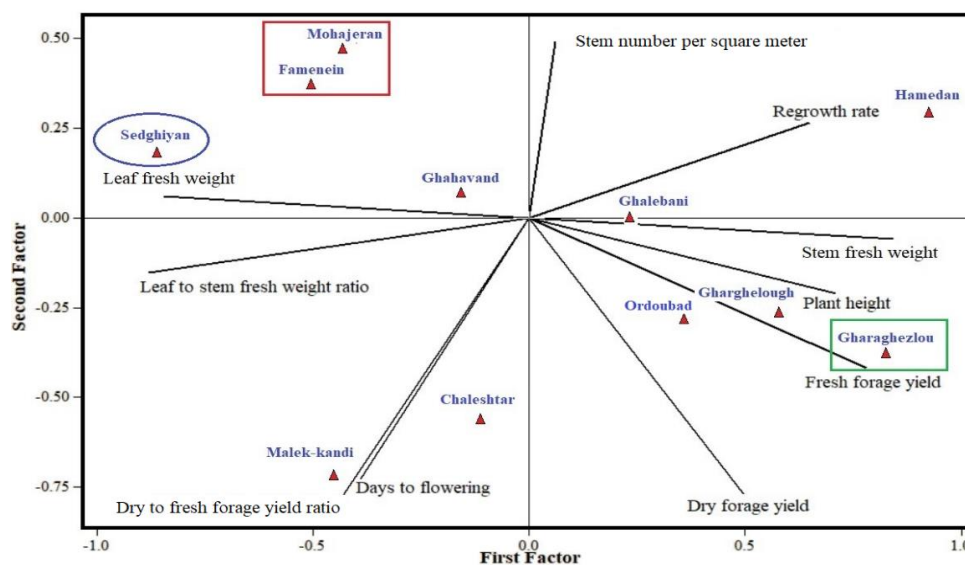


Fig. 1. The biplot of the first and second factors for different ecotypes and traits under low-irrigation conditions.

3.2. Cluster analysis results

According to the results (Figure 2), the studied ecotypes were placed in three separate clusters. According to the grouping of ecotypes in the first and third clusters, these two clusters were identified as "forage quality cluster" and "forage quantity cluster", respectively. Clusters No. 1 and 3 showed the greatest genetic distance from each other (Table 4).

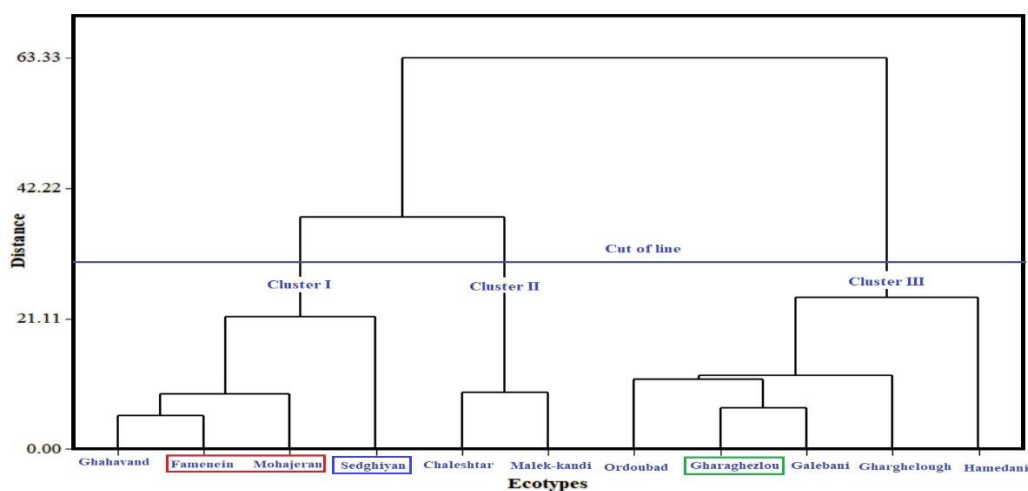


Figure 2. Cluster analysis for different ecotypes and traits under low-irrigation conditions.

Table 4. Distances between cluster centroids

	Cluster 1	Cluster 2	Cluster 3
Cluster 1	0.00	3.75	3.93
Cluster 2	3.75	0.00	3.85
Cluster 3	3.93	3.85	0.00

3.2. Discriminant function results

The purpose of analyzing the discriminant function is to investigate the differentiation between groups in terms of several variables. Also, one of its goals is to verify cluster results (Farshadfar, 1998). The results of the analysis of the discriminant function confirmed the correctness of the grouping obtained from the cluster analysis one hundred percent (Table 5).

Table 5. The results of the discriminant function to evaluate the correctness of the grouping obtained from the cluster analysis of 11 alfalfa ecotypes

The groups resulting from cluster analysis	Predicted groups based on the discriminant function							
	1		2		3		Total	
	Number of members in each group	Approval percentage of members of each group	Number of members in each group	Approval percentage of members of each group	Number of members in each group	Approval percentage of members of each group	Number of members in each group	Approval percentage of members of each group
1	4	100	-	-	-	-	4	100
2	-	-	2	100	-	-	2	100
3	-	-	-	-	5	100	5	100

3.3. Stepwise regression results

Stepwise regression was used to determine the traits with the greatest effect on the two-year average yield of dry-forage and then to determine the contribution of each trait in the variance of dry forage yield (Table 6). Fresh-forage yield, dry to fresh forage-yield ratio, plant height and regrowth rate, as the most important traits affecting dry-forage yield, were entered into the regression model in order and explained 99.52% of changes in dry forage yield.

Table 6. Stepwise regression analysis for dry yield, as dependent variable, and other traits, as independent variables, in 11 alfalfa ecotypes

Regression step	Constant	Regression coefficient for traits				Cumulative detection coefficient (R ²)
		1- Fresh forage yield	2- Dry to fresh forage yield ratio	3- Plant height	4- Regrowth rate	
1	309.4	0.215	-	-	-	73.50**
2	-906.1	0.221	45.10	-	-	93.51**
3	-1827.7	0.163	56.50	15.60	-	98.40**
4	-2014.4	0.155	65.40	12.40	11.20	99.52**

**; indicates significance at the one percent level.

3.4. Causality analysis (path analysis) results

According to the obtained results, two traits of fresh forage yield and dry to fresh forage yield had the most positive direct effect on dry forage yield. Also, plant height traits, through increasing fresh forage yield, and regrowth rate trait, through decreasing the dry to fresh forage yield ratio, had the largest positive and negative indirect effect on dry forage yield, respectively (Table 7).

Table 7. The results of causality analysis for dry yield with traits entered in the step-by-step regression model

Trait	Direct effect of traits on dry yield	Indirect effect of a trait on dry yield by other traits				Correlation with dry yield
		Fresh yield	Dry to fresh yield ratio	Plant height	Regrowth rate	
Fresh yield	0.654	---	-0.034	0.171	0.082	0.873
Dry to fresh yield ratio	0.636	-0.035	---	-0.091	-0.111	0.599
Plant height	0.247	0.452	-0.232	---	0.119	0.586
Regrowth rate	0.173	0.312	-0.404	0.171	---	0.252

Residual effect= 0.132

4. Discussion

4.1. Factor analysis discussion

The obtained results (Figure 1, Table 3) indicate that the selection of ecotypes based on high values of the first factor will lead to the selection of ecotypes with high dry forage yield. Meanwhile, the selection based on the high value of the second factor will lead to the selection of high-quality forage genotypes. Generally, there is a negative relationship between quantity and quality of forage. That is, the genotypes that have a high performance usually have a lower forage quality. Considering the positive and negative relationship between forage yield and the first factor and the second factor, respectively, a high amount of the first factor and a low

amount of the second factor are desirable. Based on this, the fourth region of the bi-plot was chosen as the most desirable region of the bi-plot (Figure 1).

The ecotypes located in the fourth region, including the Qharaghezlou ecotype, had the highest fresh and dry forage-yields under low-irrigation conditions (Figure 1). This ecotype was selected as the most favorable genotype tolerant to drought stress. Meanwhile, if the goal of choosing ecotypes is based on forage quality, the most desirable area of the bi-plot is the second area of the bi-plot. Sedghiyan ecotype, as an ecotype with the highest forage quality, is located in the second area of the biplot diagram (Figure 1).

According to the results of the factor analysis, the traits of fresh forage yield, plant height, stem fresh weight and regrowth rate, respectively, had the most positive correlation with the increase of dry forage yield (Figure 1). Qharaghezlou ecotype with the highest dry forage yield, and Sedghiyan ecotype, with the highest quality of forage, were screened and identified as the most drought-tolerant and high-quality ecotypes, respectively. Meanwhile, the two ecotypes of Mohajeran and Famenein showed the lowest dry forage yield.

Kakai and Moosavi (2016), in a study of 19 chickpea genotypes, identified three factors of "grain yield", "harvest index", and "plant vigor". These three factors together explained 68.8% of the total variance. In research by Kakai and Mazahery-Laghab (2014), it was reported that the first three factors together explained 65.70% of the total data variation. Hazeq-Jafari et al. (2014), the first five factors explained 77.02 percent of the total variance.

Farshadfar *et al.* (2007) also stated that 70/69 of the data variance is explained by the first three factors. They reported that plant height traits, wet forage yield and dry forage yield showed the highest correlation with the first factor. Ba-Safa and Taheryan (2006) in the study of 21 ecotypes of alfalfa, separated the number of 22 variables into 2 factors, one of which was called the quantity factor and the other was named the forage quality factor.

Tucak *et al.*, (2009) showed that the first four principal components contributed to 89.02% of the total variability among the populations and cultivars. The yields of green mass and dry matter, vigour, growth habit, plant regeneration and length of central leaflet were the most important traits for the yield. Moosavi *et al.* (2020) reported that the first and second factors, as "yield and yield-components" and "vegetative growth" factors, respectively, explained 76.4% of the data on the total variance. Sayed *et al.*, (2022) showed that the principal analysis grouped the studied genotypes into groups and remained scattered in all four quadrants based on all studied traits.

4.2. Cluster analysis discussion

Cluster analysis is one of the most important common statistical methods for evaluating the diversity, grouping and screening of genotypes based on different traits. This method plays an important role in plant breeding selection programs. According to the grouping of ecotypes in the first and third clusters, these two clusters were identified as "forage quality cluster" and "forage quantity cluster", respectively. According to the genetic distance of cluster 1 and 3 ecotypes, they can be used for crossbreeding program and the possibility of obtaining the desired diversity for interbreeding activities in future programs.

Ba-Safa and Taheryan (2006), divided the number of 21 alfalfa ecotypes into 4 groups, and the second group had high plant height, high dry and fresh forage yield, and fast regrowth rate, and was identified as a desirable cluster.

Pour-Farhad *et al.* (2008) separated different alfalfa ecotypes into 5 groups with the help of Ward's cluster analysis. Hazeq-Jafari *et al.* (2014), 49 alfalfa genotypes were grouped into three clusters. The placement of some foreign and domestic cultivars in a cluster was an indicator of

the non-compliance of the existing genetic diversity with the geographical distribution. In research on 20 alfalfa genotypes, Khodarahmpour and Motamedi (2016) clustered the genotypes into three separate groups. In the first cluster, there were lines that were optimal in terms of forage yield. In the second cluster, there were lines that were low both in terms of quantity of forage and quality of forage. The third cluster included the cultivars, which were optimal in terms of forage quality. The results of cluster analysis (Abdollahi-Madoulakani, 2012), examined 110 genotypes of 11 different populations of alfalfa and placed them in two main groups, each group including two distinct subgroups. Kakai and Mazahery-Laghab (2014) classified 30 alfalfa populations into three groups. The greatest genetic distance in this study was observed between Karaj 2122 and Zabul 15 populations. It is expected that the crossing of the mentioned populations in the diverging generations will provide a favorable diversity for future breeding programs. Tucak *et al.*, (2009) showed that the cluster analysis performed on the two first components grouped the majority of alfalfa cultivars and populations into three clusters of germplasm. In most cases, clustering did not depend on the country of origin. In research on wheat (Moosavi *et al.*, 2020) showed that the bi-plot and cluster results revealed a remarkable genetic diversity among the genotypes; therefore, these results might be helpful to identify donor parents in wheat breeding crosses for yield increment. In another research (Sayed *et al.*, 2022) revealed that the genotypes F18, F49, K75, S35, P20, P5 and P13 showed the highest values for all studied traits compared with other clusters.

4.3. Discriminant function discussion

According to the results, four ecotypes with low forage yield, two ecotypes with medium forage yield and five ecotypes with high forage yield were placed in the first, second and third clusters, respectively. The analysis results of the recognition function confirmed this grouping with 100% accuracy. Ahmad Khan, in the study of chickpea genotypes (2009), used detection function analysis to verify cluster analysis grouping, and the accuracy of grouping was confirmed to be about 80. In research (Moosavi *et al.*, 2022), the results of the cluster analysis were confirmed 100% by the analysis of the discriminant function. According to the results of the discriminant function analysis by Khoda-Rahmpour and Motamedi (2016), the genotypes were 100% correctly assigned to the clusters resulting from the cluster analysis.

4.4. Stepwise regression discussion

Stepwise regression analysis is a prerequisite method for causality analysis in order to identify the most important traits affecting dry-forage yield. In research (Khoda-Rahmpour and Motamedi, 2016), it was stated that the two traits of fresh-forage yield and days to flowering were entered into the regression equation as the most important traits affecting dry forage yield. Moosavi *et al.*, (2022) reported that water use efficiency, fertile spikes number per plant, seed number per the main spike, biological yield per plant, and water use (with a negative regression coefficient), as the most important traits, were entered into the regression model, respectively. Moosavi *et al.*, (2020), reported that in a stepwise regression, the traits of fertile spikes number/m², spike weight/m², grain number per spike and plant harvest index, as the most effective variables, explained 94.06% of grain yield variance in wheat.

4.5. Causality analysis discussion

The purpose of causality analysis is to provide a proper analysis of the correlation between variables by creating cause-effect models. To identify ecotypes with high yield, it is necessary to identify traits that have a significant relationship with dry forage yield. Then, by selecting

these traits, he proceeded to collect desirable genes in breed cultivars. Although correlation coefficients are widely used in identifying trait relationships, sometimes these relationships may be misleading. In fact, the high correlation between two traits may be the result of indirect effects of other traits, on the basis of which, the use of simple correlation analysis cannot explain the relationships between traits (Ali *et al.*, 2003). Therefore, it is necessary to separate the direct and indirect effects of effective traits on dry-forage yield. Causality analysis can separate the direct effects of each yield-component on the final yield from the indirect effects (Emam and Borjan, 2000). For this reason, plant breeders use the causality analysis method as a tool to determine the importance of traits affecting yield. Considering that the correlation coefficient between dry-forage yield and fresh-forage yield and dry to fresh-forage yield ratio was approximately equal to the causation coefficient (direct effect), in this case the correlation coefficient indicates the real relationship between the two variables. Therefore, direct selection through these two traits can be useful in increasing the dry-forage yield of alfalfa. Therefore, based on the results obtained in this research, selecting cultivars with higher forage yield and dry to fresh forage yield ratio will increase the dry forage yield of the plant. In research on wheat (Moosavi *et al.*, 2022) the most direct effect on increasing grain yield was water use efficiency. Seed number per plant and fertile spikes number per plant, due to increased water use efficiency, showed the most indirect effect on grain yield. Fertile spikes number/m² and spike weight/m² (Moosavi *et al.*, 2020) revealed the maximum direct and positive effect on grain yield enhancement. The first and second factors, as “yield and yield-components” and “vegetative growth” factors, respectively, explained 76.4% of the data on the total variance.

5. Conclusion

According to the results, the ecotype of Qharaghezlou was identified as the most favorable ecotype tolerant under the conditions of low irrigation stress. Also, the increase of traits such as fresh-forage yield, plant height, dry to fresh-forage yield ratio and regrowth rate are desirable traits for selection for moisture stress conditions. The most desirable ecotype in terms of quality was the Sedghiyan ecotype, which was located in the second area of the bi-plot and in the forage-quality cluster. Two traits of fresh forage yield and dry to fresh-forage yield ratio had the most positive direct effect on dry-forage yield. Also, the plant height, through increasing the fresh-forage yield, and the trait of regrowth rate, through the reduction of the dry to fresh-forage yield ratio, had the largest positive and negative indirect effects on dry-forage yield, respectively. In general, the diversity of the mentioned ecotypes was confirmed based on various multivariate analyses, which can be used for future alfalfa breeding programs. Considering the significant genetic distance between the ecotypes of the first and third clusters, the crossing of these ecotypes can be used to create diversity in future alfalfa breeding programs.

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Conflict of Interest

The authors declare no conflict of interest.

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