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Evaluation of Biochemical Characteristics of Advanced Lentil Genotypes (Lens Culinaris) Under Rainfed and Low Irrigation Conditions

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Abstract

In order to investigate the effect of drought stress or dry crop on biochemical properties of some advanced lentil genotypes, the experiment was conducted as split plots based on randomized complete block design with three replications and in dry and low irrigation conditions as the main factor and genotype As a subsidiary in 2 areas, Ardabil Agricultural Research Station and the central part of Germi city were cultivated in the years 2015-2016. The biochemical traits included proline accumulation, cytoplasmic membrane stability index, percentage of relative water content of the cell, peroxidase enzyme, ascorbate peroxidase enzyme, soluble sugars, phenolic compounds and quantitative trait including grain yield.Combined analysis of variance and mean comparison showed the significant difference between rainfed and low irrigation cultivation in most evaluated characteristics; as though, phenolic compounds, solved sugars, and ascorbate peroxidase enzyme were the highest amounts in rained irrigation condition. Seed performance in rainfed condition was 22% less than low irrigation. A and b chlorophyll and proline amounts were more in Germi region, but peroxidase and ascorbate peroxidase enzymes had the maximum amounts in low irrigation condition in Ardabil. The maximum solved sugar, peroxidase enzyme, cytoplasmic membrane stability, and proline were related to genotype 1, 3, 4, and 12 showing varieties and selectivity of cultivars for resistance against low irrigation The correlation between proline and peroxidase enzyme was positive and significant. In addition, the correlation between seed performance with cytoplasmic membrane stability, cell relative water content, ascorbate peroxidase enzymes and phenolic compounds was significant and positive.

Keywords: Drought Stress, Enzyme, Proline, Seed Performance.

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Introduction

Drought is one of the most important environmental stresses that influence various growth steps of the plant including germination, plantlet establishment, and production all over the world (Bacelar et al., 2007; Ben Ahmad et al., 2009). Drought stress has significantly increased in recent years because of changes in climate and increasing CO2 levels. Therefore, identification of the resistant plant varieties against drought stress in a necessity. Identification of biochemical or physiological mechanisms that interfere with drought resistance can help to select drought tolerant varieties. Nowadays, the great production part of legume is cultivated in rainfed regions, and the potential low performance of the current cultivars, using the limited agricultural inputs, selecting the improper production methods, and biological and non-biological stresses during growth seasons are the important reducing factors and performance fluctuations of this plant (Bayazid, 1995). Lentil with the scientific name of Lens culinaris is from leguminous, one-year, long-day, self-pollinated, and diploid family (2n=14) with mean 28.5% protein. Lens culinaris is resistant to drought. Lens culinaris is cultivated in tropical regions of West Asia and North Africa In the form of irrigated and winter (late January (Sadeghipour, 2001). Lens culinaris main cultivation in Iran in rainfed. When plants are exposed to stress, their morphology, physiology, and biochemistry change differently. Although the exact mechanism of peroxidase has not yet been determined, it seems that they play an important role in protecting the plant against stresses (Kanzok, 2001). Ascorbate peroxidase enzyme is one of the most important antioxidant enzymes that is connected to thylakoid membrane in two isozyme forms in chloroplasts, and solution presents in the stroma (Edreva, 2005).Ascorbate peroxidase enzyme plays an important role in the

production of hydrogen peroxide in both Mahler and Glutylone-Ascorbate cycles (Mittler, 2002).

Osmosis adjustment is considered as one of the plant adaptable mechanisms against drought stress which protect turangansation of cells and processes dependent on it in the low potential of water through soluble materials accumulation. Proline is one of the osmosis adjustors under the environmental stresses which bring high correlation in many plant species by tolerating these stresses (Azarpanah, 2013). Allahmoradi et al. (2013) showed about Lens culinaris that drought stress significantly increases proline. ROS system can disrupt the natural mechanism of the cell by damaging oxidative to lipids, proteins, and nucleic acids, damage cell membrane, and finally kill cell (Ozikur et al., 2009). The soluble sugars act as osmosis adjustors, the stabilizer of the cell membrane, and protector of cell turangansation. Actually, osmosis is better adjusted in plants with the accumulated sugars in response to drought stress (Slama, 2007). Genotypes with high soluble sugar concentrations are proposed as the resistant genotypes against drought in drought stress (Farshadfar et al., 2008). Plants are able to protect their cellular structures against the produced active radicals in stress conditions by producing antioxidant such as phenol and carotenoid (Bettaaieb et al., 2010).

Lens culinaris is one of the importent plants in rainfed cultivation in West Asia and South Africa, and a high protein resource for the human. This plant grows in regions with low raining (300-400mm) annually in Mediterranean weather conditions (Dalbeer et al., 2013). Lack of water and drought in growth critical periods and absence of resistant cultivars against drought in this plant has reduced the performance and the economic benefit of its agriculture. Performance in stress conditions for the reciprocal effects of genotype*environment could be a proper and precise criterion to selected the drought tolerable genotypes, and the aim of preparing the drought-resistant cultivars has been their relative tolerance of stress in comparison to other genotypes and less performance drop in the similar environmental conditions (Zabet et al., 2003).

Allahmoradi et al. (2013) showed that drought stress in flowering step has the maximum significant effect on the seed performance, leave relative water content, stomatal resistance (RS), and proline. These researchers observed the maximum performance for seed in drought stress in native masses. The first result of drought stress in plasma damage (Levitt, 1980). Consequently, changes in cell membrane increase cell permeability which permeates electrolytes from cells (Blum and Ebercon, 1980).

The aim of this study was to investigate the effects of drought stress on biochemical indices and performance of advanced lentil genotypes, and correlations between these indices with each other and, finally, identification of drought resistant cultivars.

Methods and Materials

In order to study the biochemical characteristics and identification of drought tolerant cultivars, A split plot experiment based on randomized complete block design with 3 replications was conducted to evaluate 12 advanced genotypes of *Lens culinaris* (table 1) in rainfed and low irrigation conditions as the main factors and genotypes as the secondary factors in Ardabil Agriculture Research Station and Central of Germi city during 2015-2016 cropping seasons with different conditions and climates. Lentil genotypes were obtained from the Gene Bank of Ardabil Research Center.

Table 1- genotypes of *Lens culinaris*

Genotype no.	Genotype
1	ILL-2126
2	ILL-9893
3	Bilesavar (control)
4	FILIP-2007-11L
5	ILL-2580
6	ILL-10023
7	LOCAL CHECK
8	ILL-10315
9	ILL-10277
10	ILL-465
11	ILL-10721
12	ILL-10837

It was cultivated in two regions in Germi city on December 18-21, 2015 and on February 27, 2016 in Ardabil. Moghan (Germi) city is located in West of Iran in 110 km far from the center of Ardabil province with 3’48° longitude, and 1’30° latitude with 1023m altitude. Germi city has the semi-dry climate with relatively hot summer and cold winter.

The results of soil analysis of Germi cultivation place showed that pH=6.48 and its texture is clay loam. The annual raining of Germi was reported 326mm in 2015-2016. Cultivation soil pH in Ardabil was 7.91 with clay loam and its station raining as reported 300mm in 20015-2016. Ardabil agriculture research station is located in 12km far from Ardabil city with moderate and cool summer and very cold winter. Temperature is often under 0 and -25°C in winter. Its altitude is 1350m, and longitude and latitude of 20, 48 and 15, 38, respectively. Its mean annual raining is 310mm which doesn’t have a proper dispersion and makes problem by drought stress for rainfed agriculture.

All plots were simultaneously irrigated in two steps of flowering and pot filling in 10mm in low irrigation treatment in each region and weren’t irrigated in stress treatment or rainfed and just raining was used up to the end of harvest. Each test unit was made of 4 cultivation line with 4m length, 30cm row distance, and 133 bushes in each row. Hand weeding was done in several steps to fight with weeds. Each genotype is harvested in proportion to its biochemical consideration. In this step, two edge lines and 0.5m of two middle lines end were omitted to remove edge effect. Harvest was with hand. Seed performance was determined after threshing and separating the seeds from the straw based on seed weight (gr).

biochemical characteristics were measured after the plant sheathing in both rainfed and low irrigation types:

The relative water content of the cell

The relative water content of leaf was measured by Volaire et al. (1998). First, 0.5 gr fresh tissue (Wf) was weighted then was put in distilled water for 4 hr. finally, the sample was weighted again (was) and put in pockets inside the oven at 75 ° C for 24 hours to be dried carefully (wd) and the relative water content of leaf was calculated using the following formula:

$$\mathrm{RWC} = \frac{\mathrm{Wf} - \mathrm{Wd}}{\mathrm{Ws} - \mathrm{Wd}} \times 100$$

Cell membrane stability index

Shaoyun et al. (2009) method was used to measure the cell membrane stability index. In this method, first 1 gr of each leaf sample was selected and added to 10ml ionized distilled water in test tube. Tubes closed with plastic cap and kept at 25°C. Their electric conductivity was measured after 12 hr by EC system. Then samples were boiled in 100°C for 2hr. electric conductivity was measured after cooling and reaching to 25°C. Cell membrane stability was calculated by the following relation.
The second measured EC: C2
The first measured EC: C1
MSI= (1- C1/ C2)

Measuring proline

The fresh plant leaf sample was milled in liquid nitrogen presence inside the porcelain mortar and then homogenized by sulfosalicylic acid. After centrifugation, the solution was replaced with nine hydronic acid and acetic acid solution in warm water bath and then was put in ice to stop the reaction after increasing toluene, the absorbance of proline-containing colored fluids was measured by spectrophotometer at 520 nm wavelength and finally, proline concentration was calculated using standard proline curves (Bates, 1973) .

Enzymes

First, Lens culinaris was extracted to measure the enzymes. Enzymes were determined after preparation of buffer and were extracted Beauchamp and Fridovich (1971) by the method.

A- Peroxidase enzyme

Cesar (2010) method was used to measure the kinetic activity of POD enzyme.

The reaction mixture contained phosphate buffer, ethylenediamine tetraacetic acid (EDTA) and hydrogen peroxide and enzyme extracts, and enzyme activity was measured at 470 nm wavelength.

B- Ascorbate peroxidase enzyme

Nakano and Asada (1981) method were used to measure the kinetic activity of this enzyme.

1- solution with 25 mM phosphorus and pH 7
2- Buffer containing potassium phosphate buffer, EDTA 0.1 mg and 0.5 mM ascorbic acid and 10 mM H2O2 The enzyme activity was measured by measuring the oxidation of ascorbate by spectrophotometer at 290 nm for 1 minute.

Soluble sugars:

Irigoyen et al. (1992) method was used to measure the soluble sugar.

Measurement of phenolic compounds:

McDonald et al (2001) method was used to measure the total phenol compounds.

Before performing the statistical calculations, the data was first tested first. If needed, the data in percentages were ArcSin and the data obtained from the counts were normalized by rooting. Simple variance analysis of traits was performed under dry and wet conditions. Before comparing the mean of composite analysis of the traits, the experimental error was analyzed by simple variance analysis in two varieties uniformity testing environments. By analyzing variance assumptions and testing the error error uniformity test in two locations, the analysis of the combined variance was carried out. For this purpose, the max-FF test, the ratio of the largest variance to the smallest variance, was calculated and traits with a Fmax of less than or equal to 5 (uniform variance) And integration) with compound variance analysis, and traits with a Fmax greater than 5 were analyzed by simple variance analysis. The mean comparisons with Duncan's multiple range test at 5% probability level with MSTATC softwares were performed.SPSS software was used to determine the linear correlation in some effective characteristics on resistance indexes to stress and also cluster analysis. Ward cluster analysis and Euclidean distance criterion were used to determine the studied genotype relativity and their classification based on the important agricultural characteristics. The most rupture methods based on the sudden change in the difference between the two intervals of sequential integration and the second root number of individuals were used to determine the number of clusters, and their accuracy was evaluated based on the detection function, and finally, the proper number of clusters was determined .

Results and Discussion

Table of the combined variance analysis (Table 2) showed that the significant difference was observed among proline, and peroxidase enzyme values of two cultivation methods. There was a significant difference in proline (µm/gr of wet weight) in Germi and Ardabil, but the amount of enzyme peroxidase (µgr of enzyme/gr of leaf) was higher in Ardabil. Proline accumulation is a popular biochemical response of plants to the extensive range of bio and

non-bio stresses (Geravandi et al., 2011). The maximum proline was in Germi; as though, stress increased 23% of proline than Ardabil condition. In Gunes et al. (2008) research, drought stress after pollination significantly increased proline .

There was a significant difference between rainfed and low irrigation conditions based on variance analysis table (table 4). In a way that the comparison among means, seed performance, the relative water content of a cell in low irrigation condition had the maximum amounts, and the mean of phenol compounds characteristics and the soluble sugars in the rainfed condition were maximum (table 4). Free proline accumulation in response to the osmosis stress is considered as an adaptable reaction that is seen not only in plants but also in all live animals (Kocheva and Gorgiev, 2003). When plants are exposed to the stress, the different morphological, physiological, and biochemical changes happened in them based on these conditions. Measuring the plant water is proposed as on if the important index in identification plant response to drought stress; as though, the high relative water content of leaf and low speed of dehydration showes adaptation with drought in genotypes and is used as a selectivity criterion to tolerate drought (Winter et al., 1988; Sivakumar and Singh, 1987).

Table 2- combined variance analysis *of* biochemical characteristics in Lens culinaris genotypes average of squares

Sources Change	Degrees of freedom	seed performance	Proline content	Stability of cytoplasmic membrane	Percentage of relative water content of the cell
location (L)	1	1686558.8*	523.1*	3093.2*	1357.7**
Block inside location (L) R	4	250157.9 ns	71.7 ns	460.4 ns	317.9*
Crop conditions (A)	1	4329128.3**	216.9 ns	10042.1**	1534.6**
Cultivation conditions × location (LA)	1	100225.5 ns	0.05 ns	8320.1**	0.3 ns
Test error (Ea)	4	101877.2	40.4	161.5	20.7
Genotype (B)	11	383121.1**	133.9**	265.5**	59.4*
Genotype × location (LB)	11	111467.3*	33.6 ns	157.1 ns	57.2*
Culture conditions × genotype (AB)	11	39789.1 ns	12.7 ns	133.7 ns	22.1 ns
location × cultivation conditions × genotype(LAB)	11	67445. 9 ns	29.4 ns	102.7 ns	11.8 ns
Test error (Eb)	88	46626.1	19.7	87.1	29.8
Changes coefficient (%)		16/01	29/7	23/62	9/55

** and *, and ns are significant in 1%, 5%, and non-significant

Continue Table 2- combined variance analysis *of* biochemical characteristics in Lens culinaris genotypes average of squares

Sources Change	Degrees of freedom	Enzyme peroxidase	Ascorbate enzyme peroxidase	Soluble sugars	Phenolic compounds
location (L)	1	0.15*	0.006 ns	12115.2 ns	17858.1 ns
Block inside location (L) R	4	3.8 ns	0.028 ns	18975.1 ns	18349.1 ns
Crop conditions (A)	1	22.2 ns	0.486*	4837986.2*	122905.4*
Cultivation conditions × location (LA)	1	15.9 ns	0.027 ns	8161.5 ns	1808.2 ns
Test error (Ea)	4	6.85	0.048	45726.1	6290.4
Genotype (B)	11	11.2**	0.021 ns	17610.4*	5.5909 ns
Genotype × location (LB)	11	3.2 ns	0.012 ns	8633.1 ns	1997.3 ns
Culture conditions × genotype (AB)	11	3.1 ns	0.016 ns	10558.2 ns	1138.3 ns
location × cultivation conditions × genotype(LAB)	11	3.1 ns	0.014 ns	7500.2 ns	4122.6 ns
Test error (Eb)	88	2.4	0.015	7432.2	5534.11
Changes coefficient (%)		25/72	13/74	24/99	27/60

** and *, and ns are significant in 1%, 5%, and non-significant

Table 3- comparison results of the simple effects means of location for some biochemical characteristics of Lens culinaris

Location	Proline (µmol/gr wet weight)	Peroxidase enzyme (µgr enzyme/gr leaf)
Ardabil	13/04 b	6/13 a
Germi	16/85 a	6/065 b

Table 4. Results of comparisons of mean simple effects of culture conditions on some traits

condition	seed performance (kg/ha)	Percentage of relative water content of the cell (%)	Ascorbate peroxidase Enzyme µgr enzyme/gr leaf)	Soluble sugars mg/ml	Phenolic compounds (mg of gallic acid per ml)
Low irrigation	1515 a	60/45 a	0/21 b	287 b	46/71 b
rainfed	1168 b	53/92 b	0/45 a	403 a	50/5 a

Based on Duncan's multi-domain test, the meanings of the same letters in the list at the 5% probability level do not have a significant difference with each other.

Table 5- the mean comparison results of cultivation condition* location for some biochemical characteristics in Lens culinaris

Location	Cultivation condition	Cytoplasmic membrane stability (%)
Ardabil	low irrigation	50/82 a
	Rainfed	18/92 b
Germi	low irrigation	44/89 a
	Rainfed	43/39 a

According to Duncan's multiple range test, means have similar letters in 5% sig. level in each column.

Comparison the mean reciprocal effects of cultivation conditions in the place showed (table 5) that Ardabil in rainfed condition has the highest percentage (18.92) of the cell cytoplasmic membrane stability so it has more resistance against stress. However, low irrigation condition in Ardabil and Germi and rainfed condition in Germi didn't show the significant difference. The first result of drought stress is damaging plasma (Levitt, 1980). Consequently, change in cell membrane increases cell permeability which permeates electrolytes from the cell (Blum and Ebercon, 1980).

Table 6. Comparison results of the simple effects means of location for some biochemical characteristics of *Lens culinaris*

Genotype no.	Genotype	Proline(µmol/gr wet weight)	Cytoplasmic membrane stability (%)	Peroxidase enzyme (µgr enzyme/gr leaf))	Soluble sugars mg/ml)(
1	ILL-2126	13.68 bc	42.42 ab	7.32 ab	401.1 a
2	ILL-9893	8.20 d	43.28 ab	6.23 bc	381.3 ab
3	Bilesavar(control)	16.10 ab	35.77 abc	8.17 a	311.1 bcd
4	FILIP-2007-11L	15.59 ab	46.04 a	6.36 bc	391.9 ab
5	ILL-2580	12.94 bcd	39.95 abc	5.39 c	356 abcd
6	ILL-10023	10.22 cd	40.82 abc	6.03 bc	342.6 abcd
7	LOCAL CHECK	17.47 ab	40.98 abc	6.13 bc	284.2 d
8	ILL-10315	14.48 bc	33.94 bc	4.89 c	325.1 abcd
9	ILL-10277	16.98 ab	34.71 abc	4.78 c	367.9 abc
10	ILL-465	18.15 ab	42.93 ab	6.14 bc	294.8 cd
11	ILL-10721	15.44 ab	42.85 ab	6.39 bc	365.1 abcd
12	ILL-10837	20.09 a	30.40 c	5.27 c	319.2 abcd

According to Duncan's multiple range test, means have similar letters in 5 % sig. level in each column

Comparison results of genotypes mean simple effects showed that (table 6) proline in genotype ILL-10837 (20.09 µmol/gr wet weight) has the significant difference in 1% level with other genotypes. As a result, the highest drought resistance in proline was genotype number 12 with 60% increase in proline relative to the lowest amount of proline in genotype number 2. Ataishaikh (2005) used test of leaf cytoplasmic membrane sensitivity. Since the obtained results have the reverse relationship with cytoplasmic membrane stability, According to Table 6, it can be concluded that genotype FILIP-2007-11L has the highest tolerance to drought, having the highest stability of the cytoplasmic membrane (46.04). The genotype ILL-10837 has the lowest cytoplasmic membrane stability (30.43%). Protecting the cell membrane totality after tolerating drought stress is an important strategy to improve drought tolerance in plants (Vasquez –Tello et al., 1990). The criterion of cell membrane stability is used to diagnose plants tolerance against drought (Gavuzzi, 1997; Kocheva and Gorgiev, 2003). Bilesavar cultivar was considered as a control sample with the maximum peroxidase enzyme and ILL-2126 showed the maximum soluble sugars (401.1mg/ml) than other genotypes

The comparison results of the genotype reciprocal effects in the place (table 7) showed that genotype 1 had the maximum seed performance (1902kg/ha) and genotype 6 had the maximum relative water content of the cell (66.64) in Germi .The simple correlation coefficients among the most biochemical and quantitative characteristics (cytoplasmic membrane stability, the relative water content of the cell, ascorbate peroxidase enzyme, and phenolic compounds had the positive and significant relationship with seed performance (table 8).

Table 7. The mean comparison results of genotype* location for some biochemical characteristics in *Lens culinaris*

Genotyp no.	Location	Genotype	Seed yield (kg /ha)	Percentage of relative water content of the cell (%)
1	Ardabil	ILL-2126	1435 bcdefg	53/02 e

2		ILL-9893	1160 ghijk	58/77 bcde
3		Bilesavar (control)	1351 cdefghi	53/50 e
4		FILIP-2007-11L	1390 bcdefgh	52/60 e
5		ILL-2580	902 k	54/51 de
6		ILL-10023	1099 hijk	53/55 e
7		LOCAL CHECK	1327 defghi	54/59 de
8		ILL-10315	1316 defghi	56/83 bcde
9		ILL-10277	1326 defghi	55/29 cde
10		ILL-465	1124 hijk	51/84 e
11		ILL-10721	1305 efghi	52/46 e
12		ILL-10837	1063 ijk	52/38 e
1	Germi	ILL-2126	1902 a	61/60 abcd
2		ILL-9893	1217 fghij	62/02 abc
3		Bilesavar (control)	1386 bcdefgh	56/26 bcde
4		FILIP-2007-11L	1648 b	62/29 abc
5		ILL-2580	1008 jk	58/31 bcde
6		ILL-10023	1375 bcdefgh	66/64 a
7		LOCAL CHECK	1338 defghi	53/20 e
8		ILL-10315	1536 bcde	61/22 abcd
9		ILL-10277	1608 bcd	61/24 abcd
10		ILL-465	1493 bcdef	62/58 abc
11		ILL-10721	1251 efghij	63/15 ab
12		ILL-10837	1636 bc	54/50 de

According to Duncan's multiple range test, means have similar letters in 5% sig. level in each column.

Table 8. Linear correlation of some studied characteristics of Lens culinaris

characteristics	seed performance	Proline accumulation	Stability of cytoplasmic membrane	Relative water content of the cell	Peroxidase enzyme	Enzyme Ascorbate Peroxidase	Soluble sugars	Phenolic compounds
Seed performance	1							
Proline accumulation	0.07	1						
Stability of cytoplasmic membrane	0.32**	-0.07	1					
Relative water content of the cell	0.33**	-0.08	0.34**	1				
Peroxidase enzyme	-0.07	0.21*	-0.14	-0.20*	1			
Enzyme Ascorbate Peroxidase	-0.19*	0.14	-0.14	-0.09	0.22**	1		
Soluble sugars	-0.16	0.02	-0.14	-0.04	0.03	0.17*	1	
Phenolic compounds	-0.24**	-0.06	-0.14	-0.12	0.04	0.15	0.18*	1

** and * are sig. level in 1% and 5%,, respectively

Relative water content (RWC) of cell showed the positive correlation of 0.33% with seed performance, it can be concluded that plant without drought stress and using the stored water in its cells can grow and develop its organs and finally will increase the performance. The correlation of proxidase enzyme with proline accumulation was (0.21) positive and significant. Proline protects plats against the environmental stresses through various mechanisms of the osmotic adjustment, detoxification of reactive oxygen species and the stability of the enzymes or proteins. It has been proved in some plants that changes in proline amounts are related with their ability to tolerate or adapt with stress conditions and can be used as a parameter to select the resistant plant against stress (Niknam et al., 2006). In contrary, some reports say that proline can't be used as a valid index to select the resistant plant species against stress (Yazici, 2007).

Cluster analysis

Cluster analysis using Ward Minimum Variance was used to group the genotypes. Cluster analysis is used to classify genotypes based on the obtained results, the dendrogram cutting was classified into 4 classes based on cluster analysis of genotypes at Euclidean distance 4. The first class includes genotypes of 7, 12, 10, and 3; the second class includes genotypes of 6, 11, and 2; the third class includes genotypes of 8, 9, and 4; the fourth class includes genotype 1, and the fifth class includes genotype of 5 (fig. 1).

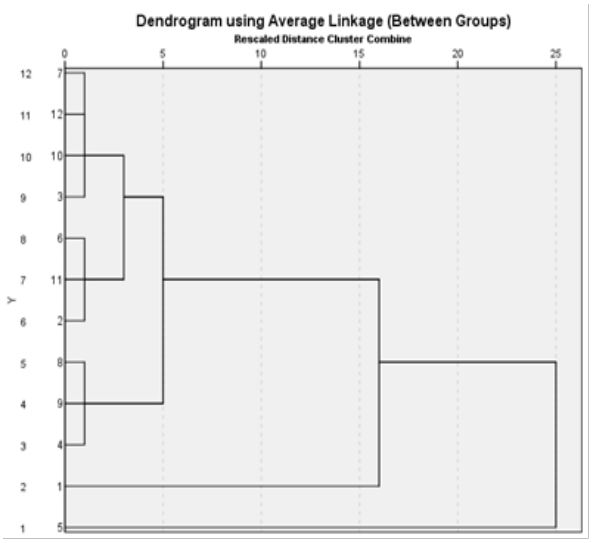

Figure 1- Dendrogram *Lens culinaris* based on biochemical characteristics.

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