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Research Article

Changes of fatty acid profiles in clover genotypes induced by cold stress

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Abstract

Degradation of cell membranes is one of the consequences of cold stress. In this study, changes in profile of fatty acids (FA) were investigated in leaves of different clover genotypes exposed to cold stress and the most tolerant genotype was selected. Experimental treatments included factors, such as sampling time at two levels (autumn and spring), planting date at two levels, and 10 clover genotypes (7 cultivars of Persian clover and 3 cultivars of berseem, red ,and crimson clover). Lipid percentage was found to be influenced by season, planting date, and cultivar type. Persian clover (lately maturity) showed the highest lipid percentage by 3.5% and total unsaturated FA percentage by 79% and the lowest values were observed in crimson clover. Cold stress in autumn caused a 20% decrease in lipid percentage. Also, it caused a 21% increase in total saturated FA percentage, and 5 and 20% decrease in total unsaturated FA percentage and unsaturated /saturated FA ratio, respectively. Delay in planting date reduced percentage of lipid in clover cultivars. In all the cultivars, percentage of unsaturated FA was higher than that of saturated FA. Thus, the unsaturated FA percentage can be used as a criterion for selecting genotypes with respect to cold tolerance. In this regard, Persian clover (lately maturity) was the most tolerant genotype under cold stress conditions.

Keywords: Berseem clover, Crimson clover, Persian clover, Saturated fatty acids, Unsaturated fatty acids

Introduction

Due to the diversity of cultivated areas, clover species may be affected by cold stress. Cell membranes are the primary cellular structures damaged under chilling stress (Tang *et al*., 2012). The cell membrane is composed of lipids and proteins. Cell membrane lipids are of two types: Saturated and unsaturated fatty acids. Unsaturated fatty acids help maintain the fluidity of the membrane, which plays a pivotal role in plant survival under cold stress conditions. Sometimes, under cold stress, the cell membrane undergoes a stage of change from liquid (very fluid) to gel (harder) crystals. In the gel phase, fats are highly ordered, which interferes with routine physiological function and makes cell membranes more permeable (Cyril *et al*., 2002; Longo *et al*., 2017). Biochemical and biophysical changes occur to prevent phase changes in the membrane. Besides, the degree of unsaturation strongly affects the temperature range at which the cell undergoes a phase change. The increase in fatty acid unsaturation levels is mediated by fatty acid desaturases which is located in the chloroplast and in the endoplasmic reticulum (Barrero-Sicilia *et al*., 2017). Therefore, increasing the percentage of unsaturated fatty acids in membrane fatty acid composition is involved in maintaining plasma membrane stability, integrity as well as cell membrane function (Hu *et al*., 2017). Lipids containing more saturated fatty acids solidify faster, faster, and at higher temperatures compared with lipids containing less saturated fatty acids. Therefore, the relative contribution of these two fatty acids to plasma membrane lipids determines the membrane fluidity (Steponkus *et al*., 1993; Longo *et al*., 2017). At the transfer temperature, the cell membrane changes from semi-fluid to semi-crystalline. Cold-sensitive plants usually have a higher proportion of saturated fatty acids in their plasma membranes, so cold-sensitive plants have higher transfer temperatures. Conversely, cold-resistant plants have a higher proportion of unsaturated fatty acids and therefore lower transfer temperatures (Yadav, 2010). It is clear that the increase of unsaturated fatty acids content in cell membrane structure causes the stability of cellular membranes in transgenic plants, and as a result, acts as a factor of cold tolerance in plants. The conversion of stearic acid to oleic acid is fulfilled by the activity of D9-desaturase and continuously oleic acid to linoleic acid and linoleic acid to linolenic acid are conducted by D12- and D15-desaturases, respectively (Kazemi *et al*., 2013). Cold acclimation is a highly complex process that involves many physiological and biochemical changes such as changes in the composition of fatty acids and the accumulation of carbohydrates, amino acids and other osmolality (Catala *et al*., 2014). Most trienoic acids are present in thylakoid membranes, where the photosynthetic apparatus is

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found, variations in their degree of unsaturation at low temperatures could play an important role in maintaining the photosynthetic capacity of the plants (Dominguez *et al*., 2010). Hu *et al*. (2017) by investigating two cold-sensitive and cold-tolerant bermudagrass cultivars and investigating changes in the concentrations of palmitic and stearic as saturated fatty acids and linoleic and linolenic as unsaturated fatty acids, showed that under cold stress, saturated fatty acids increased in the susceptible cultivar. Linoleic acid decreased in both cultivars, especially in the sensitive cultivars. The percentage of linolenic acid in the sensitive cultivar decreased slightly whereas increased slightly in the tolerant cultivar. The proportion of unsaturated to saturated fatty acids under normal conditions was higher in sensitive cultivars compared with tolerant cultivars but decreased by 30% due to cold stress, although did not change in tolerant cultivars. The results showed that the natural variability in cold stress tolerance in bermudagrass genotypes was mainly related to changes in the antioxidant defense system and fatty acid composition. In another bermudagrass study, Fontanier *et al*. (2020) demonstrated that greater unsaturation of fatty acids belonged to cultivar that had superior tolerance to chilling stress. Cruz *et al*. (2010) by studying alterations in fatty acid composition due to cold exposure at the vegetative stage in rice demonstrated that linolenic acid increased after cold exposure in cold-tolerant genotypes while palmitic acid decreased, and an opposite behavior was found in the cold-sensitive genotypes. They expressed that this evidence indicated that these fatty acids are potential molecular markers useful for breeding programs as well as for future basic studies on cold tolerance in rice. Cabiddu *et al*. (2017) by examining the profile of fatty acids in two cultivars of berseem clover under the influence of growth period and plant organs showed that the chemical composition of fresh herbage was affected by plant organs and relatively less affected by the growth stage. In both cultivars and at all phenological stages, the stem was the main storage site for soluble carbohydrates. The leaves were richer than the stem in terms of monounsaturated, polyunsaturated and total fatty acids in both vegetative and reproductive periods. However, different leaf-to-root ratios indicate different chemical compositions of herbage during the growing season. In a comparative study between grasses and legumes, it was shown that the total fatty acid concentrations in timothy (*Phleum pratense* L.) species, orchard grass (*Dactylis glomerata* L.), red clover and alfalfa in summer regrowth were 48%, 40%, 15% and 2.5% more than spring growth, respectively and in this respect, a significant difference was observed between grasses and legumes. Grasses and legumes had significant differences in individual fatty acid concentrations. Orchard grass and timothy had higher C18: 3 concentrations than legumes in summer regrowth. But the concentrations of C12: 0, C14: 0, C16: 0, C16: 1, C18: 0, C18: 1 and C18: 2 of legumes were higher than grasses. As a result, the total concentration of fatty acids didn't differ between grasses and legumes. Concentrations of C18: 1 and C18: 2 in timothy were higher than orchard grass. But the concentration of C12: 0 was lower than orchard grass. Red clover showed higher concentrations of C16: 1, C18: 1, C18: 2, C18: 3 and total fatty acids than alfalfa. Forage fats were mainly of leaf origin and leaf ratio is important in determining the concentration of fatty acids (Boufaied *et al*., 2003; Ranst *et al*., 2009). Dewhurst *et al*. (2001) analyzed the fatty acid composition of three ryegrass cultivars grown in the UK during a growing season, with three or five cuttings. All species had high concentrations of fatty acids and high ratios of C18: 3 during the growing season (late April), and fatty acid levels dropped dramatically after this date and improved by autumn. Concentrations of fatty acids were highest in the early and late seasons but lowest in the summer months and this pattern of change was associated to leaf ratio. Belanger and McQueen (1998) reported that in timothy the share of leaves in summer regrowth (0.5) was higher than spring growth (0.3). Tremblay *et al*. (2002) reported that the share of leaves in alfalfa in spring growth (0.39) was lower compered with summer regrowth (0.45). As a result, the greater difference in leaf contribution between spring and summer regrowth in timothy compared to alfalfa in the studies mentioned may explain somewhat greater differences in total fatty acid concentrations during growing periods (Boufaied *et al*. 2003).

Variation in lipid percentage and fatty acid profile in Persian clover cultivars compared to other clover species as well as the effect of cold stress and wintering periods and spring regrowth on the mentioned traits remains unknown. This study aimed to identify the possible variability in fatty acid profile and to determine the changes of these traits under cold stress in clover genotypes and use these traits as criteria for selecting the most cold-tolerate genotype.

Material and methods

This study was conducted at Research Field in the Seed and Plant Breeding Research Institute of Karaj (longitude 51 degrees and 6 minutes east and latitude 35 degrees and 59 minutes north and altitude 1321 meters above sea level), Iran. Karaj region has a cold and semi-cold arid climate. The average annual temperature is 14.97 °C. The maximum temperature is related to July and August and the minimum temperature belongs to January. According to the two-year average, the highest number of frost days is related to December and January (average 19.5 days). The average rainfall of 30 years is 240 mm. Most of the rains took place in late autumn and early spring, with the least rainfall in July and August and the highest rainfall in April and November (Meteorological Organization of Iran).

Figure 1 shows the minimum, maximum and daily rainfall temperatures during the growing season. The number of frost days in two cropping years is shown in

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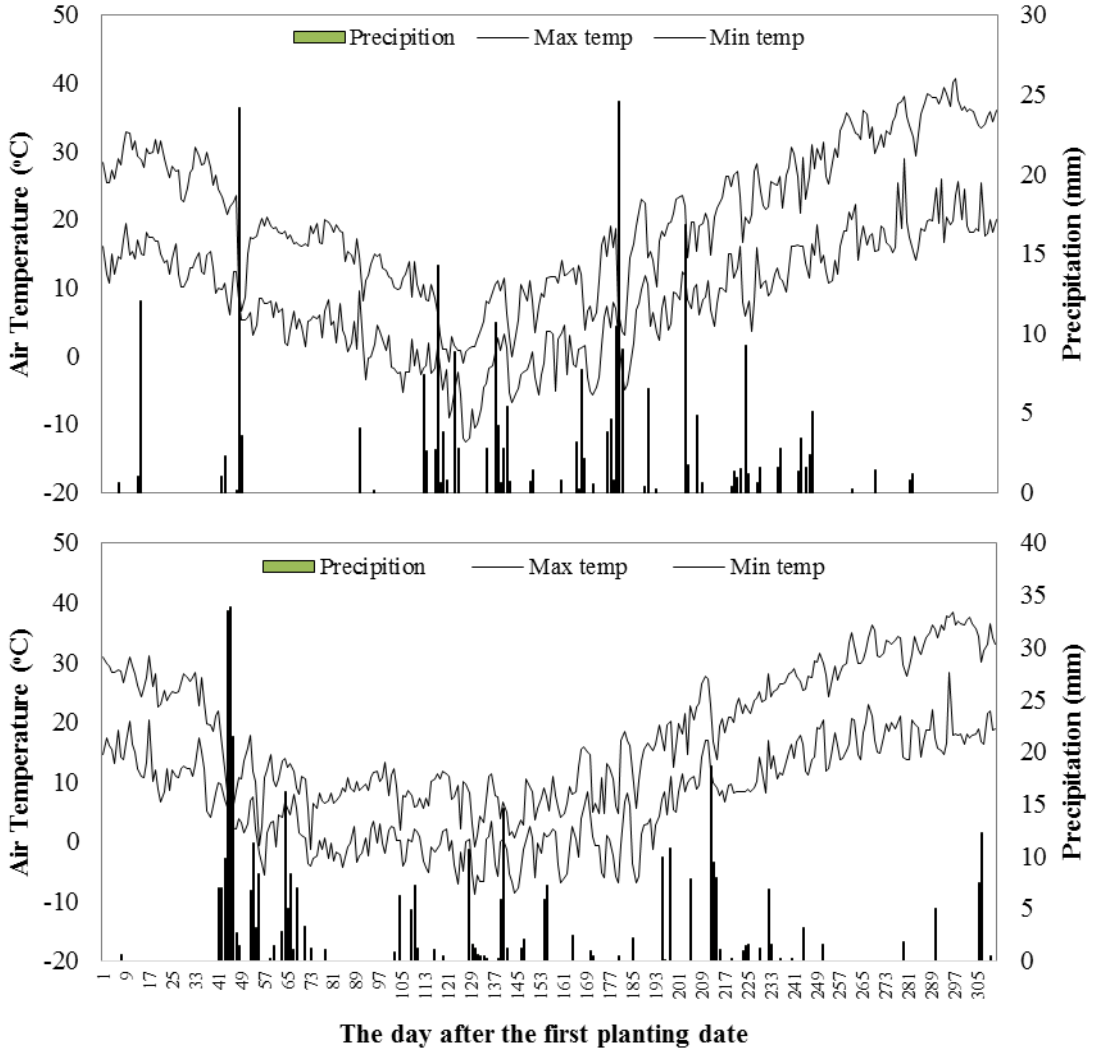

Fig. 1. Daily maximum and minimum air temperatures (°C), and precipitation (mm) recorded during the growing seasons

Table 1 (Meteorological Organization of Iran). Physico-chemical properties of the soil of the experimental field are given in Table 2. The split-split-plot in time experiment was performed in a randomized complete block design (RCBD) with four replications. The experimental treatments included sampling time (autumn and winter) as the main plot two planting dates (October 7 and 22 October) (Zamanian, 2004) as the sub-plot and 10 clover genotypes (Persian clover (seven cultivars), berseem clover (one cultivar), red clover (one cultivar) and crimson clover (one cultivar)) (Table 3) as the sub-sub-plot. The number of experimental units in each block was 10, the distance between replications was one meter, each treatment consisted of 8 lines of 5 meters with a line spacing of 50 cm.

To conduct this research, a uniform plot of the field with an area of about 1500 square meters was selected for planting, which was in the previous year of fallow. In the first stage, about 50 kg of triple superphosphate fertilizer and 40 kg of nitrogen fertilizer (starter fertilizer) were spread on the ground and then the farrows were constructed. The experiment was carried out on two dates by the labor force in the form of dry-bed seeding. The date of the first irrigation was considered as the planting date.

The leaves were sampled after the occurrence of cold stress. Samples were oven-dried at 75 °C for 24 hours and powdered by the mill. lipid percentage of samples was measured by Soxhlet (methylation) method. Profile of fatty acids and separation of type of saturated and unsaturated fatty acids of treatments performed by GC device (Kaushik and Agnihotri, 1997) (International Standard ISO 5509 and International Standard ISO). Simple statistical analysis was performed on the data SAS software package (version 9.0, SAS Institute Inc., Cary, NC, USA) using the general linear model procedure. The means were compared by Duncan's method.

Results and discussion

Analysis of variance of autumn and spring showed that the effect of season, planting date and cultivar on lipid percentage was significant at the level of 1%. However, there was no significant effect of two-way interactions between season and planting date, season and cultivar, planting date and cultivar and three-way interactions

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Table 1. Meteorological statistics in the months of clover species growth in Karaj region (2010-2011 and 2011-2012)

number of frost days	number of sunny days	year	months	number of frost days	number of sunny days	year	months
12	25	2010	19 Feb. - 19 March	0	31	2010	22 Aug. - 21 Sep.
17	27	2011		0	31	2011	
2	29	2010	20 March - 19 Apr.	0	30	2010	22 Sep. - 21 Oct.
0	30	2011		0	30	2011	
0	29	2010	20 Apr. - 20 May	0	28	2010	22 Oct. - 20 Nov.
0	30	2011		4	23	2011	
0	31	2010	21 May - 20 Jun.	3	30	2010	21 Nov. - 20 Dec.
0	31	2011		20	29	2011	
0	31	2010	21 Jun. - 21 July	24	26	2010	21 Dec. - 19 Jan.
0	31	2011		15	26	2011	
0	31	2010	22 July - 21 Aug.	21	28	2010	20 Jan. - 18 Feb.
0	31	2011		17	28	2011	

Table 2. Physico-chemical properties of the soil of the experimental field (depth of 0–30 cm) before the beginning of the experiment

Year	depth	Soil texture	EC dS m^{-1}	pH	O.M[1]	N[2]	P[3]	K[4]
					%		mg kg^{-1}	
	(0-20)	loam	3.52	8.1	0.92	0.1	18.7	163.1
2010-2011	(20-40)	loam	2.45	8.16	0.96	0.09	24	155.2
	(0-20)	loam	4.10	8.1	0.90	0.07	19.3	165.2
2011-2012	(20-40)	loam	2.51	8.2	0.95	0.05	23.00	150.8

[1] organic matter, [2] total nitrogen, [3] available phosphorus, [4]exchangeable potassium

Table 3. Name and origin of clover genotypes

Genotyps No.	Genotype name	Scientific name	Cultivar name	Origin	Type growth
1	Persian clover	*Trifolium resupinatum* L.	KPC-PL	Iran	Lately maturity
2	Persian clover	*Trifolium resupinatum* L.	KPC-PM	Iran	Moderate maturity
3	Persian clover	*Trifolium resupinatum* L.	KPC-PE	Iran	Very early maturity
4	Persian clover	*Trifolium resupinatum* L.	KPC-OC	Iran	One cutting
5	Persian clover (line 13)	*Trifolium resupinatum* L.	KPC-L/13	Iran	Line 13
6	Persian clover (line 7)	*Trifolium resupinatum* L.	KPC-L/7	Iran	Line 7
7	Persian clover	*Trifolium resupinatum* L.	KPC-PCh/Egh	Iran	population
8	berseem clover	*Trifolium alexsandrinum* L.	KBC-Toli.K	Italia	Lately maturity
9	red clover	*Trifolium pratense* L.	Nassim	FAO	Lately maturity
10	crimson clover	*Trifolium incarnatum* L.	Alborz1	FAO	Very early maturity

between season, planting date and cultivar. The results showed that the main effects of season, planting date and cultivar, and two and three-way interactions had different effects on the percentage of individual fatty acids. Clover cultivars were different in total unsaturated fatty acids, linoleic acid and eicosenoic acid (Table 4). The comparison of means showed that the highest lipid percentage was related to Persian clover (lately maturity) with 3.49% and the lowest amount was related to red clover with 2.40% (Table 5) (Fig. 2). The highest and the lowest percentage of total unsaturated fatty acids were observed in Persian clover (lately maturity) with 78.90% and crimson clover with 73.18%, respectively. Crimson clover with 3.46%, red clover with 20.01% and Persian clover (line 13) with 0.38% showed the highest percentages of linoleic acid (C18:2) and eicosenoic acid (C20:1), respectively. Persian clover (one cutting) with 1.73%, Persian clover (moderate maturity) with 17.07% and Persian clover (lately maturity) with 0.20% had the lowest percentage of linoleic acid and eicosenoic acid, respectively (Table 5). The high degree of unsaturation was an important role in maintaining the photosynthetic capacity of plants because most trienoic acids were present in the thylakoid membrane, where the photosynthesis machinery was found (Dominguez *et al*., 2010). Increased production of C18:3 accompanies cold acclimation in many plants (Graham and Patterson, 1982), and a positive relationship exists between a higher degree of fatty acid desaturation and both cold and freezing tolerance (Steponkus *et al*., 1993; Zhang *et al*., 2010). A higher proportion of saturated fatty acids was observed in plasma membranes of cold-sensitive plants than tolerant plants, so cold-sensitive plants have

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Table 4. Analysis of variance (mean squares) for lipid percentage and fatty acids percentage of clover cultivars and planting date over seasons

Source of variation	df	lipid	Palmitic acid C 16:0	Linoleic acid C 18:2	Linolenic acid C 18:3	Arachidonic acid	Eicosenoic acid	Other fatty acids	Total saturated fatty acids	Total unsaturated fatty acids	ratio of unsaturated to saturated fatty acid
Season (A)	1	16.79**	338.74**	9.84*	824.78**	16.04**	3.02**	0.58^{ns}	464.78**	475.59**	0.144**
Error 1	4	2.00	12.53	16.30	71.30	0.19	0.042	4.59	21.49	35.42	0.007
Planting date (B)	1	2.63**	38.64**	0.35^{ns}	150.14**	0.84*	0.001^{ns}	19.82**	47.91*	130.52**	0.021*
(B) × (A)	1	1.09^{ns}	15.66^{ns}	38.69**	43.37^{ns}	0.19^{ns}	0.054^{ns}	12.85**	39.76*	5.50^{ns}	0.012^{ns}
Error 2	4	1.32	15.94	9.17	37.25	0.08	0.014	6.52	17.79	38.67	0.007
Cultivar (C)	9	1.15**	3.23^{ns}	9.70**	33.93^{ns}	0.18^{ns}	0.068*	6.41**	10.99^{ns}	28.80*	0.005^{ns}
(A) × (C)	9	0.34^{ns}	3.11^{ns}	5.21*	8.53^{ns}	0.22^{ns}	0.030^{ns}	2.26^{ns}	5.09^{ns}	5.06^{ns}	0.002^{ns}
(B) × (C)	9	0.14^{ns}	3.30^{ns}	2.57^{ns}	21.11^{ns}	0.18^{ns}	0.043^{ns}	3.55*	5.38^{ns}	11.79^{ns}	0.002^{ns}
(A) × (B) × (C)	9	0.13^{ns}	3.05^{ns}	5.74*	4.52^{ns}	0.08^{ns}	0.028^{ns}	3.78*	3.93^{ns}	7.24^{ns}	0.002^{ns}
Error 3	72	0.24	5.08	2.29	20.17	0.15	0.03	1.42	9.64	12.84	0.004
CV (%)		16.47	13.05	8.48	8.38	24.19	21.3	19.27	15.4	4.67	23.38

^{ns} non-significant * Significant at the 0.05 probability levels, ** Significant at the 0.01 probability levels

Table 5. Main effect of genotypes on lipid and individual fatty acids

Clover species	Lipid	Linoleic acid C18:2	Eicosenoic acid C20:1	Total unsaturated fatty acids
	(%)			
Persian clover (Lately maturity)	3.49^{a}	18.28^{b}	0.20^{c}	78.90^{a}
Persian clover (Moderate maturity)	2.97^{bc}	17.07^{b}	0.45^{a}	76.38^{ab}
Persian clover (Very early maturity)	3.08^{abc}	17.41^{b}	0.35^{abc}	77.15^{ab}
Persian clover (One cutting)	3.34^{ab}	18.03^{b}	0.33^{abc}	77.39^{ab}
Persian clover (line 13)	3.13^{abc}	17.65^{b}	0.38^{ab}	76.01^{abc}
Persian clover (line 7)	3.05^{abc}	17.34^{b}	0.29^{bc}	77.47^{ab}
Persian clover (population)	2.89^{bc}	17.08^{b}	0.36^{ab}	77.13^{ab}
berseem clover	2.79^{cd}	18.32^{b}	0.36^{ab}	75.17^{bc}
red clover	2.40^{d}	20.01^{a}	0.22^{bc}	77.02^{ab}
crimson clover	2.73^{cd}	17.17^{b}	0.35^{abc}	73.18^{c}
Mean	2.98	17.83	0.32	76.58

Means within a column followed by the same letter are not significantly different at the level of 5%.

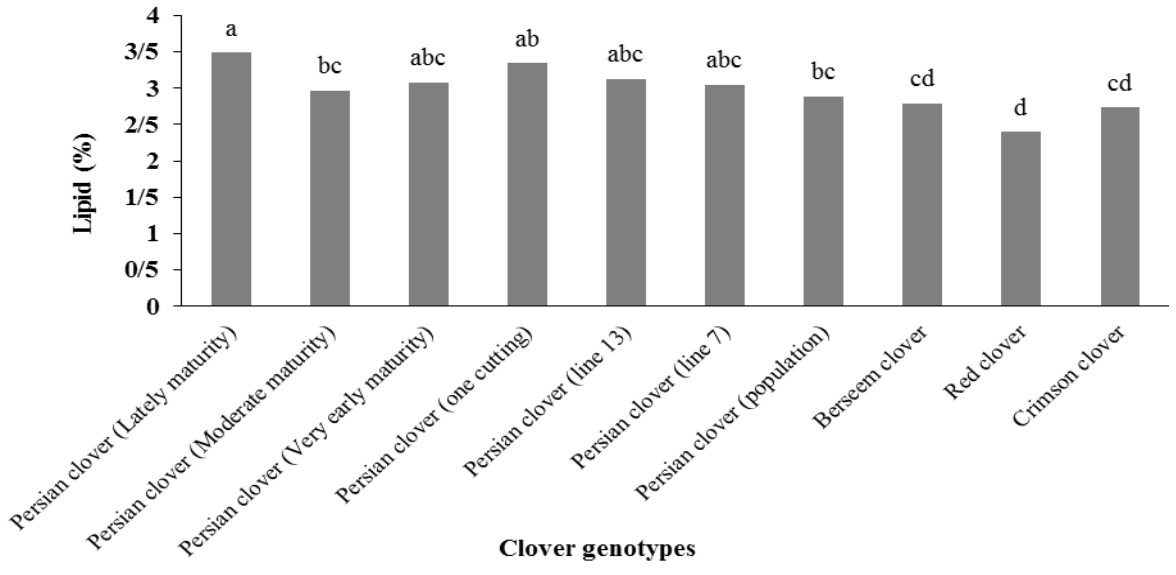

Fig. 2. Lipid percentage of clover cultivars. Significant differences at P ≤ 0.05 have been indicated with different letters.

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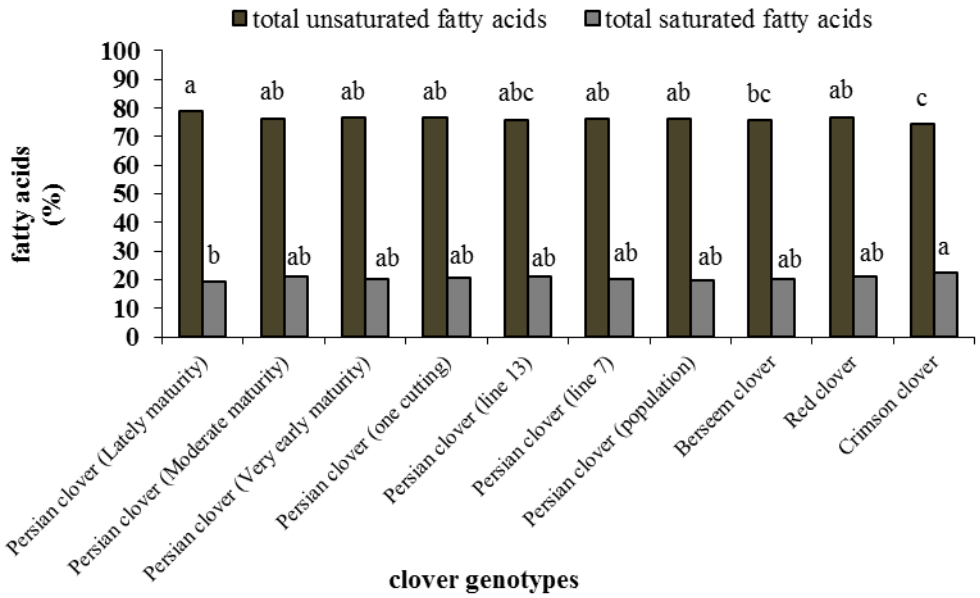

Fig. 3. Total unsaturated and saturated fatty acids in clover genotypes. Significant differences at P ≤ 0.05 have been indicated with different letters.

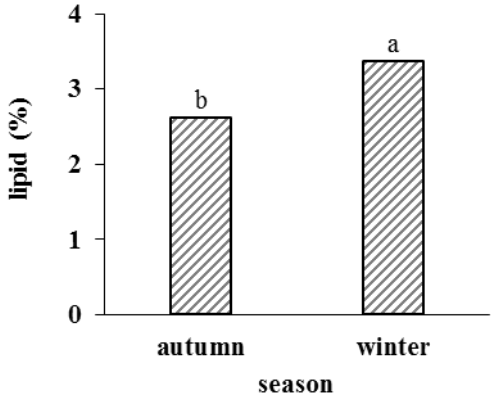

Fig. 4. Effect of season on lipid percentage. Significant differences at P ≤ 0.05 have been indicated with different letters.

higher transfer temperatures than tolerant plant (Yadav, 2010). Modifying the level of unsaturated fatty acids present in membrane lipids contributes to improved cold tolerance in cold-sensitive crops (Dominguez *et al.*, 2010). Based on the results, we can use these traits as criteria for selecting genotypes regarding cold tolerance. Persian clover (lately maturity) showed the highest percentage of lipid and unsaturated fatty acid and can be considered as the most tolerant genotype in our study.

In all cultivars, the percentage of unsaturated fatty acids was higher than saturated fatty acids (Fig. 3). In this regard, Hebeisen *et al*. (1993) reported that fodder plants have higher levels of unsaturated fatty acids compared with saturated fatty acids. Linoleic acid and oleic acid were consistent to the results of this study.

Significant effect of season on lipid percentage, palmitic acid, linoleic acid, linolenic acid, arachidonic acid, eicosenoic acid, total unsaturated fatty acids, as well as the ratio of unsaturated to saturated fatty acid were observed (Table 4). Cold stress in autumn made a decrease of 20% in lipid percentage (Fig. 4). It caused an increase of 21% in total saturated fatty acids, and a decrease of 5% and 20% in total unsaturated fatty acids (Fig. 5) and the ratio of unsaturated to saturated fatty acid (Fig. 6), respectively. These results were consistent to the results of Szyszkowska and Sowinski (2001) and Kendall and Stringer (1985) who reported the effect of cold on the reduction of unsaturated fatty acids and lipid percentage and reduced plant tolerance to cold.

This study showed that the percentage of linoleic (C 18:2) and palmitic acid (C 16:0) in clover cultivars was higher in autumn than in the spring (Table 6). Meanwhile, Mirmohammadi Meybodi and Tarkesh Esfahani (2004) reported that the percentage of linoleic acid and palmitic acid in cold-sensitive plants decreased rapidly when exposed to temperatures of +5 °C. Increased levels of different saturated fatty acids such as stearic acid, palmitic acid, lauric acid, pentadecanoic acid and myristic acid were observed in capsella bursa-pastoris exposed to 10 C (Wani *et al*., 2018). In cold-sensitive plants, some changes were made in the lipid of membrane during cold acclimation. Kazemi *et al.*

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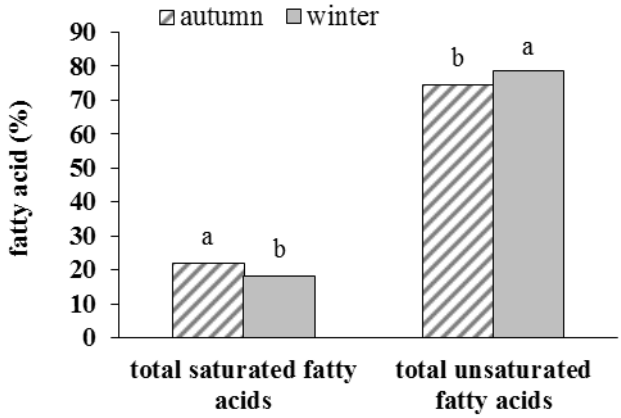

Fig. 5. Effect of season on total saturated fatty acids and total unsaturated fatty acids

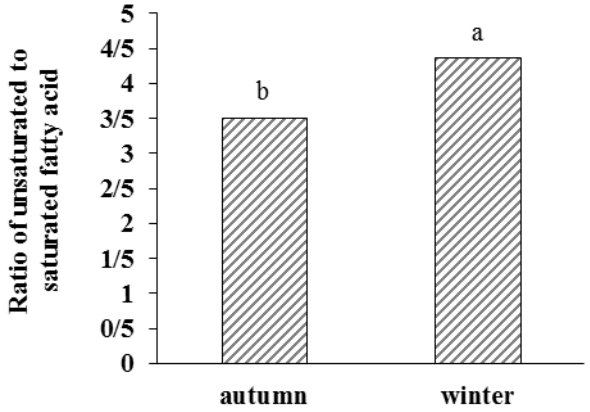

Fig. 6. Effect of season on ratio of unsaturated to saturated fatty acid

Table 6. Main effect of season on individual fatty acids

season	Palmitic acid C 16:0	Linoleic acid C 18:2	Linolenic acid C 18:3	Arachidonic acid C 20:0	Eicosenoic acid C 20:1
			%		
Autumn	18.94 [a]	18.12 [a]	50.96 [b]	1.07 [a]	0.49 [a]
Spring	15.58 [b]	17.55 [b]	56.20 [a]	0.34 [b]	0.17 [b]

Means within a column followed by the same letter are not significantly different at the level of 5%.

(2013) studied the changes in membrane fatty acid compositions and cold-induced responses in chickpea. They showed an increase in the ratio of unsaturated to a saturated fatty acid which is a sign of cold tolerance, especially after the cold acclimation phase. The increasing of membrane unsaturated fatty acid ratio compare to the saturated ones conducted by desaturases activity plays a critical role during cold stress and guarantees ion permeability, as well as appropriate fluidity and stability of membrane (Kazemi *et al*., 2013). Significant effects of planting date were observed on lipid percentage, linolenic acid, total unsaturated fatty acids, and the ratio of unsaturated to the saturated fatty acid.

Conclusion

Cold stress in autumn made a decrease of 20% in lipid percentage. It caused an increase of 21% in total saturated fatty acids, and a decrease of 5% and 20% in total unsaturated fatty acids and the ratio of unsaturated to saturated fatty acid, respectively. Clover cultivars were different in total unsaturated fatty acids, linoleic acid and eicosenoic acid. The highest lipid percentage was related to Persian clover (lately maturity) with 3.5% and the lowest amount belonged to red clover with 2.40%. The highest and the lowest percentage of total unsaturated fatty acids were observed in Persian clover (lately maturity) with 79% and crimson clover with 73%, respectively. We can use these traits as criteria for selecting genotypes regarding cold tolerance. Persian clover (lately maturity) showed the highest percentage of lipid and unsaturated fatty acid and can be considered as the most tolerant genotype in our study.

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