Physiological and biochemical changes of Alfalfa (*Medicago sativa* L.) cultivars at different growth stages

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Abstract

Physio-biochemical changes in plants are a principal practice to manage harvesting time. The present study was aimed to investigate the effect of flowering stages (10%, 50%, and 100% flowering) on physiological and biochemical properties in four cultivars of three- and four-year-old Alfalfa (*Medicago sativa* L.) plants (Fasta, Hamadani, Meldor, and Sovrana) based on a randomized completely block design (RCBD). Results showed that four-year-old plants of Hamadani cultivar had higher total phenolic content (TPC) and total flavonoid content (TFC) at middle flowering stages. Catalase (CAT) and superoxide dismutase (SOD) activities increased by progressing the flowering stage and time of harvesting. Proline content increased by advancing the flowering stage as its highest level was observed at late flowering stage in Fasta cultivar. Middle flowering stage (50% flowering) represented the greater chlorophyll (Chl.) content compared with early and late maturity. Relative water content (RWC) decreased by progressing the maturity as its highest level was observed at early flowering stage. RWC differed from 65% in four-year-old plants of Sovrana at late flowering stage to 80% in three-year-old plants of Meldor cultivar at early flowering stage. Malondialdehyde (MDA) content and total soluble sugar (TSS) increased by advancing the maturity. Four-year-old Sovrana plants at late flowering stage contained higher MDA and TSS. Heat map analysis showed the significant role of all physiological and biochemical traits except total Chl. for clustering the cultivars. The study recommends Hamadani cultivar because of high phenolic compounds and the minimum variations in biochemical properties at year four.

Keywords: antioxidant capacity, heat map, malondialdehyde, phenolic content, proline

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Introduction

Alfalfa (*Medicago sativa* L.), as a popular forage for animals, has been cultivated for a long time. It is well-known for treating a wide range of degasses diseases in human being such as anima, diabetes, breast cancer, etc. Alfalfa contains various minerals, proteins, and amino acids (Noori et al., 2019; Guo et al., 2019). Hay making is the best method for conservation and utilization of alfalfa (Karayilanli and Ayhan, 2016). Alfalfa has a wide

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range of nutritional value in its leaves, which makes it as the best forage crop. In addition, it is widely consumed by animals in both green and hay forms (Zhang et al., 2019).

Physiochemical attributes of most plants particularly alfalfa are influenced by many factors such as the harvest time (Guo et al., 2019; Biazzi et al., 2017). By advancing maturity, biochemical changes like decreased crude protein (CP) and raised fiber contents in alfalfa forage have been reported in different studies (Guo et al., 2019; Noland et al., 2018). Finding the most proper harvesting time is critical to achieve the optimal yield of alfalfa (Karayilanli and Ayhan, 2016). Recently, it has been confirmed that the best practice to reach high quality alfalfa is harvesting time, and the early flowering stage has been suggested in this regard. However, this may be different in various climatic conditions. Most parts of Iran have arid and semi-arid climate. In these areas, the main stage of plants experience heat stress, which decreases nutritional value, and in turn mitigates relative feed value (RFV) by noticeable reduction of forage quality (Kiraz, 2011; Engin and Mut, 2018), having a significant effect on the production of high-quality alfalfa. The improvement in forage quality is economically important and is considered as a cost-effective factor in crops and legumes (Gou et al., 2019). Physiological and biochemical traits are the main factors to determine the plant strategies under different treatments. The changes in photosynthesis pigments, antioxidant capacity, and water content in different plant tissues are the initial step of plants to adapt to, and cope with, new conditions (Szymańska et al., 2017). For instance, the reduction of chlorophyll content and enhancement of antioxidant enzyme activities are the strategies of most plants to control the stress conditions (Szymańska et al., 2017).

Many research has emerged to find the effective management strategies on alfalfa quality particularly in areas with harsh environmental conditions. It has been generally approved that with advancing crop maturity, the main results are increases in phenolic contents and antioxidant capacity and decreases in water and chlorophyll contents (Lakhani et al., 2018) and forage digestibility (Palmonari et al., 2014; Yari et al., 2014). Previous studies focused on the nutritional values of alfalfa to show the response of plants during growth period. Increased DM and NDF have been reported by Yu et al. (2003) in alfalfa plants at late budding stages compared with those harvested at the early flowering stage. Gou et al. (2019) claimed that while CP of alfalfa silage decreased with approaching maturity, Digestibility dry matter (DDM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) increased. However, there is no information on physiological and biochemical properties of alfalfa cultivars regarding the influence of harvesting time in terms of flowering stages. Therefore, the present work was designed to investigate the effect of different flowering stages on growth, physiological, and biochemical attributes of four main alfalfa cultivars under semiarid conditions in Iran.

Materials and Methods

Alfalfa plots management

Four cultivars (Fasta, Hamadani, Meldor, and Sovrana) of Alfalfa (*Medicago sativa* L.) were cultivated in 36 plots (5 m × 1 m) in Varamin (35°35′48″ N, 51°64′71″E, asl. 917 m), Iran, based on a randomized completely block design (RCBD). The ground was plowed to a depth of 30 cm using an iron plow. Alfalfa seeds of the cultivars under study were acquired from the Seedling and Seed Research Institute, Karaj and were sown in February after being disinfected in a sodium hypochlorite solution. Each plot contained four lines (5 m) with 50 cm distance. The study was conducted on 4- and 5- year-old plants. The alfalfa plants were harvested at three flowering stages including early stage (10% flowering), middle stage (50% flowering), and late stage (100 % flowering) for two subsequent years 2016 and 2017. No fertilizers, insecticides, and herbicides were applied during the experiment. Soil properties of the study area in two years were recorded at the beginning of each experiment (Table 1). The mean annual rainfall for 2016 and 2017 were 351 and 345.7 mm, respectively (Table 2). A fodder chopper was used with 2 cm in length of fresh matter samples.

Chlorophyll (Chl.) determination

Table 1
Soil properties used in the case study in two soil depth (0-20 cm and 20-40 cm)

Year	Depth	pH	EC	OC (%)	N%	P (mg/kg)	K (mg/kg)	Sand (%)	Silt (%)	Clay (%)
2016	0-20 cm	7.9	0.8	1.1	0.96	20	274	21	46	34
	20-40 cm	8.1	0.4	1.1	0.98	19	258	21	42	37
2017	0-20 cm	8.1	0.9	1.3	0.96	18	271	20	47	33
	20-40 cm	8.2	0.5	1.2	0.95	19	254	21	43	36

Table 2
Rainfall and temperature during the experiment

Month	Rainfall (mm)		Minimum temperature (℃)		Maximum temperature (^{o} C)	
	2016	2017	2016	2017	2016	2017
Jan	17.5	16.5	-2.34	-2.57	8.18	8.28
Feb	66.8	65.8	-3.96	-2.66	6.23	6.45
Mar	35.5	35.7	1.71	1.83	11.78	11.55
Apr	42.5	40.5	5.53	5.58	16.37	17.21
May	122	121	7.26	7.56	17.83	17.65
Jun	45.2	46.2	12.87	12.32	25.92	26.02
Jul	0	0	16.90	17.54	32.82	32.75
Aug	1.7	1.5	16.81	17.33	31.45	31.76
Sep	2.2	2.5	12.95	13.92	27.77	28.74
Oct	12.2	11.2	7.03	8.21	20.45	20.40
Nov	1.8	1.6	4.48	4.68	16.73	16.32
Dec	3.6	3.2	-4.35	-4.05	8.37	8.35

The contents of total Chl. were measured based on Arnon (1949) method. Initially, 0.1 g of each leaf sample was pulverized with a mortar and pestle with 3 ml of 80% acetone, increasing the volume of the extract to 15 ml. The extract was then filtered through a centrifuge at 3000 g for 15 min. A spectrophotometer (Shimadzu UV-160) was used to measure the absorbance of the samples. First, the device was blanked with 80% acetone, and then the absorption of the extract was read at 645 nm and 663 nm. Finally, the amounts were determined at 645 and 663 nm by the spectrophotometer.

Relative water content (RWC) measurement

To measure RWC, developed leaves were selected, and their fresh weights (FW) were recorded. After that, the leaf samples were placed on ice and were immediately sent to the cold room of the laboratory. They were immersed in distilled water and stored at a refrigerator condition (4 ℃). After 24 hours, the saturation weight (SW) of the leaves was measured, and subsequently, the leaves were placed in the oven set at 70 ℃ for 24 hours to record dry weight (DW). The RWC was obtained according to following equation (Dhopte and Manuel, 2002):

$$\mathrm{RWC} = \frac{(\mathrm{FW}-\mathrm{DW})}{(\mathrm{SW}-\mathrm{DW})} \times 100$$

Proline concentration

Proline accumulation was determined according to the method of Bates et al. (1973). Leaf samples (0.1) were extracted with 3% sulfosalic acid. Then, 2 ml of the extract was dissolved in 2 ml of ninhydrin and 2 ml of glacial acetic acid, and the mixture was placed in a Bain-marie container for one hour. Then, 2 ml of toluene was added to the solution and placed in ice and at a dark condition. The wavelength of 520 nm was determined to read the samples, and finally the content was expressed as µmol proline g^{-1} fresh weight.

Determination of total phenolic content (TPC)

Folin–Ciocalteu reagent was selected to measure TPC spectrophotometrically (Medina, 2011). The wavelength of 725 nm was used by a spectrophotometer after 90 min at 22 ℃. Standard curve was calibrated by Gallic acid. The

results were represented as mg Gallic acid (GA) g^{-1} dry weight.

Determination of total flavonoid content (TFC)

The amount of flavonoids was measured by aluminum chloride colorimetric method. The adsorption of the mixture was read at a wavelength of 415 nm. The quercetin standard was used to draw the curve (Zhishen et al., 1999

Antioxidant enzyme assays

The protein for assaying enzyme activity was extracted from 300 mg of leaf samples. The extraction was carried out using 2 mL of phosphate buffer (100 mM, pH 6.8), EDTA (1 mM), PMSF (100 Mm), and 2% PVP. The extract was centrifuged at 4 ℃ for 15 min at 15000 g, and the supernatant was kept at -80 ℃ for the enzymatic tests.

The activity of CAT was determined based on Aebi (1974) method. For this, 300 ml of enzyme extract and 200 ml of H_2O_2 (1%) were added to 50 mM phosphate buffer (pH 7). Enzymatic activity was determined by measuring the total oxygen produced during the enzymatic dissociation of H_2O_2 in complete darkness for one minute. Reduction in H_2O_2 absorbance at 240 nm (ε = 40 mM^{-1} cm^{-1}) was used to determine the evolution of oxygen. The activity was given as absorbance of enzyme unit mg^{-1} of protein.

Superoxide dismutase (SOD) was assessed by preventing the enzyme from photochemically degrading nitroblue tetrazolium (NBT) at 560 nm. The reaction solution was made up of 50 mL of enzyme extract, 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 0.075 mM NBT, 0.1 mM EDTA, 150 mL deionized water, and 0.002 mM riboflavin. The tubes received radiation for 10 min.

Determination of total soluble sugar (TSS)

Leaf samples (0.5 g) were ground with 5 ml of 95% ethanol to release sugar, which was then crushed with liquid nitrogen to determine the TSS. Following that, 5 ml of 70% ethanol was added twice before the mixture was centrifuged for 10 min at 3500 rpm, and then it was refrigerated for a week. The stored stock was then mixed with 0.1 ml of the 3 ml antron solution, which contained 150 mg antron and 100 ml sulfuric acid 72%. The solution was heated to 90 ℃ in a Bain-mari container for 15 min. A UV-Vis spectrophotometer was used to detect the absorbance at 625 nm (Jayaraman and Jayaraman, 1981).

Determination of malondialdehyde (MDA)

To determine MDA, leaf samples (0.1 g) were homogenized in one ml TCA (5 %) and centrifuged at 8,000 rpm for 30 min. After that, 1 ml of the supernatant was incubated with 4 ml TCA 20% containing 0.5% thiobarbituric acid for 30 min at 95 ℃. The reaction was stopped by cooling on ice for 10 min, then were centrifuged at 6000 rpm for 20 min. Extinction coefficient was 155 mM^{-1} cm^{-1} and MDA content was expressed as mmol g^{-1} fresh weight (Heath and Packer, 1969).

Statistical Analysis

All data used in the study were analyzed by SAS software (version 9.3). The statistical significance was determined with Duncan test evaluated at P≤0.05. Clustered image map (CIM) mining program created the heat map.

Results

Total phenolic and flavonoid contents

According to ANOVA, the interaction of year, cultivar, and harvesting time was significant (P≤0.05) on TPC and TFC (Table 3). Different cultivars of alfalfa showed significant variations in leaf TPC and TFC. Four-year-old plants had higher TPC (Fig. I. a) and TFC (Fig. I. b) compared to the three-year-old plants. In Hamadani cultivar at early flowering stage, four-year-old plants increased TPC and TFC by 13% and 70%, respectively compared to the three-year-old plants. Hamadani cultivar showed higher TPC and TFC compared to other experimental cultivars. For flowering stages, the plants harvested at middle flowering stage showed greater TPC and TFC as compared to early and late flowering stages. Therefore, Hamadani cultivar at middle flowering stages in fourth year showed higher TPC and TFC.

Table 3
Analysis of variance for traits of alfalfa plants under study

S.O.V	df	MS						
		TPC	TFC	CAT	SOD	Proline	Chl	RWC
Year (Y)	1	30.16**	20.59**	0.229**	28.75**	0.006*	0.0001^{ns}	13.3*
Y (rep)	4	0.18^{ns}	0.15^{ns}	0.001^{ns}	0.04^{ns}	0.006^{na}	0.0011^{ns}	4.4^{ns}
Cultivar (Cv)	3	2.97**	1.02**	0.064**	1.09**	0.107**	0.0134**	36.8**
flowering stage (FS)	2	4.91**	1.90**	0.162**	2.83**	0.069**	0.0070**	364.0**
Y*Cv	3	0.35*	0.97**	0.010**	0.64*	0.075**	0.0001^{ns}	58.5**
Y* FS	2	0.40*	0.08^{ns}	0.004*	0.41*	0.017**	0.0001^{ns}	3.4^{ns}
Cv* FS	6	0.29*	0.36**	0.012**	0.11^{ns}	0.011**	0.0009^{ns}	16.3**
Y*Cv*FS	6	0.24*	0.20*	0.003*	0.43**	0.024**	0.0001*	9.6**
Total error	44	0.11	0.10	0.001	0.15	0.001	0.001	1.9
C.V.	-	3.8	9.1	4.9	8.8	6.6	1.9	1.8

** and * were significant at 1 and 5% level, respectively, while ns is not significant difference.

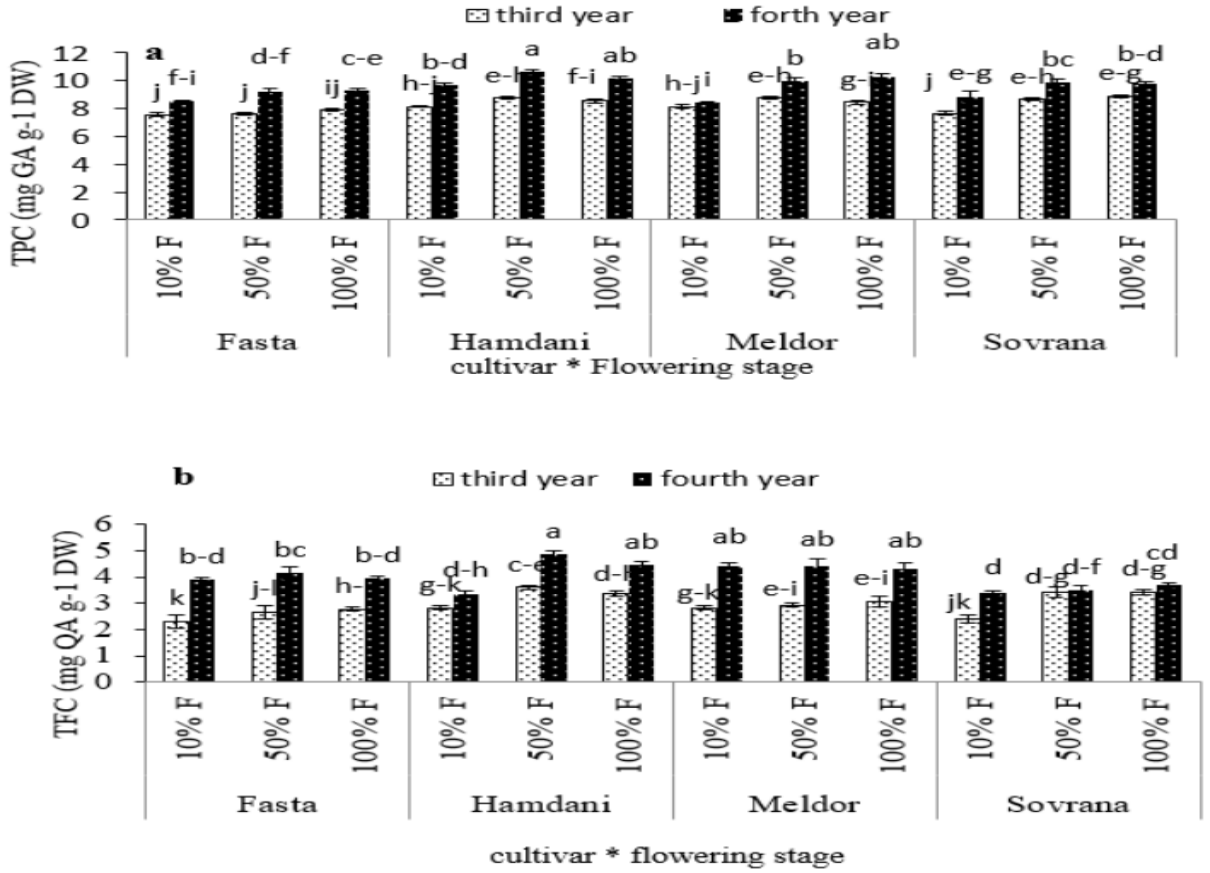

Fig. I. Total phenolic content (TPC) and total flavonoid content (TFC) of alfalfa cultivars at different flowering stages; values are the means ± standard errors of three replicates (n=3). Statistically significant differences between treatments (P≤ 0.05) are shown with different letters.

Enzymes activity

The ANOVA results showed that the interaction of year, cultivar, and flowering stage was significant (P≤0.05) on TPC and TFC (Table 3). Fig. II. shows CAT and SOD activities in different alfalfa cultivars at flowering stages in two consecutive years. The cultivars showed variation in CAT activity during two experimental years. Fasta cultivar had higher CAT activity as compared to other cultivars. In addition, the CAT activity increased by progressing the flowering stage. In the third year Fasta cultivar

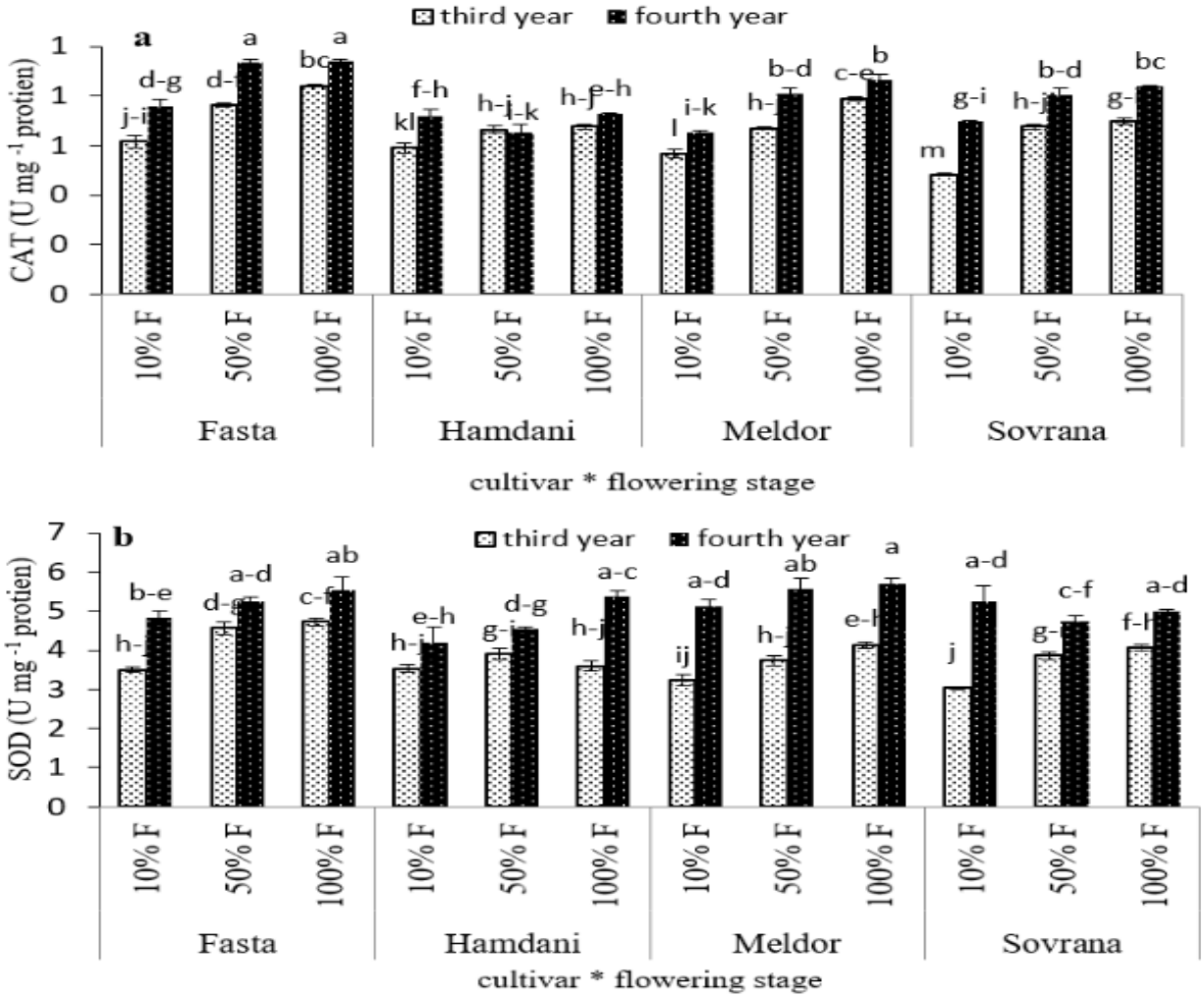

Fig. II. Catalase (CAT) and superoxide dismutase (SOD) activity in alfalfa cultivars at different flowering stages; values are the means ± standard errors of three replicates (n=3). Statistically significant differences between treatments (P≤ 0.05) are shown by different letters.

plants late flowering stage increased CAT activity by 36% in comparison to early flowering stage. Compared to the third-year plants, a 36% enhancement of CAT activity was observed in plants harvested in year 4. This behavior was approximately found for SOD. It differed from 3.03 U mg^{-1} protein in three-year-old Sovrana plants at early flowering stage to 5.7 U mg^{-1} protein in four-year-old Meldor plants at late flowering sage. Totally, CAT and SOD activities increased by progressing the flowering stage and time of harvesting (year).

Proline concentration

Proline content was significantly (P≤0.05) affected by the interaction of year, cultivar, and flowering stage (Table 3). Proline concentration significantly changed under cultivars and flowering stage. Alfalfa cultivars showed different behavior in proline concentration. Fasta represented the higher variation of proline as it reached the maximum value in the fourth year (0.60 µ mol g^{-1} FW). Proline content increased by advancing the flowering stage as its highest amount was observed at late flowering stage. We obtained 52% augmentation of proline concentration at late flowering stage compared to early flowering stage (Table 4).

Chlorophyll and relative water content

The interaction of year, cultivar, and flowering stage was significant (P≤0.05) on Chl. and RWC (Table 3). Although total Chl. content significantly changed under cultivars and year, the flowering

Table IV
Analysis of variance for the studied traits at different flowering stages

Year	Cultivar	Flowering stage	Proline	Chl. content	RWC
Third year	Fasta	10%	0.35±0.026j	1.69±0.009cd	76.3±055bc
		50%	0.49±0.008c-f	1.70±0.007b-d	73.3±0.73de
		100%	0.53±0.016b-d	1.66±0.009d	67.0±1.27fg
	Hamadani	10%	0.39±0.016h-j	1.76±0.022ab	76.7±0.55bc
		50%	0.44±0.012f-h	1.78±0.022a	74.7±0.48b-e
		100%	0.48±0.009c-f	1.72±0.019a-d	69.0±0.55f
	Meldor	10%	0.42±0.012g-i	1.71±0.012b-d	80.0±0.73a
		50%	0.47±0.012d-f	1.72±0.017a-d	76.0±0.27b-d
		100%	0.45±0.009e-g	1.73±0.007a-c	77.3±0.48b
	Sovrana	10%	0.38±0.019ij	1.72±0.012a-d	76.7±1.27bc
		50%	0.46±0.002e-g	1.74±0.016a-c	75.0±0.48b-e
		100%	0.50±0.002b-e	1.69±0.004cd	66.7±0.99fg
Fourth year	Fasta	10%	0.53±0.009bc	1.70±0.005b-d	77.3±0.48b
		50%	0.60±0.002a	1.70±0.002b-d	75.0±0.73b-e
		100%	0.55±0.021ab	1.66±0.007d	67.7±0.73f
	Hamadani	10%	0.36±0.012j	1.75±0.023a-c	77.0±0.99bc
		50%	0.37±0.016ij	1.78±0.022a	76.7±0.55bc
		100%	0.38±0.009ij	1.73±0.027a-c	74.3±0.48c-e
	Meldor	10%	0.39±0.007h-j	1.72±0.018a-d	76.0±0.73b-d
		50%	0.44±0.021f-h	1.73±0.017a-c	73.0±0.48e
		100%	0.49±0.012c-f	1.72±0.007a-d	67.3±0.73fg
	Sovrana	10%	0.50±0.018b-e	1.73±0.015a-d	74.7±0.48b-e
		50%	0.49±0.004c-f	1.74±0.018a-c	74.3±0.48c-e
		100%	0.50±0.019c-f	1.70±0.0.12b-d	65.0±0.55g

Values are the means ± standard errors of three replicates (n=3). Statistically significant differences between treatments (P≤ 0.05) are shown by different letters.

stage did not remarkably modify this attribute. Alfalfa cultivars showed different behavior of proline concentration. Hamadani cultivar showed the higher Chl. content (1.78 mg g^{-1} FW) in both third and fourth year plants at middle flowering stage compared to other cultivars. RWC decreased by progressing the flowering stage as its highest level was observed at early flowering stage. RWC differed from 65% in fourth-year plants of Sovrana cultivar at late flowering stage to 80% in third-year plants of Meldor cultivar at early flowering stage (Table 4).

Malondialdehyde content and total soluble sugar

The MDA and TSS were significantly (P≤0.05) affected by the interaction of year, cultivar, and flowering stage (Table 3). MDA showed different amounts depending on cultivars and flowering stages. The main change in MDA was corresponding to the flowering stage, where MDA significantly increased by progressing flowering stage. In fourth year plants, the increases by 25, 9, 35, and 30% were observed for MDA in Fasta, Hamadani, Meldor, and Sovrana, respectively, compared with early flowering stage (Fig. III. a). Like MDA, TSS increased by maturity in alfalfa plants. In addition, the cultivars showed different TSS. Hamadani represented the minimum increase in TSS during growth stages. The highest TSS was observed in four-year-old Sovrana plants at late flowering stage (7.9 mg g^{-1} FW) (Fig. III. b).

Heat map analysis

Distribution of physiological traits in different cultivars are shown in Fig. IV. According to heat map analysis, Fasta was placed in one cluster and Hamadani, Meldor, and Sovrana were in a distinguished cluster. The distribution of attributes was from the least amount (blue) to the most amount (red). The majority of characteristics

Table IV
Analysis of variance for the studied traits at different flowering stages

Year	Cultivar	Flowering stage	Proline	Chl. content	RWC
Third year	Fasta	10%	0.35±0.026j	1.69±0.009cd	76.3±055bc
		50%	0.49±0.008c-f	1.70±0.007b-d	73.3±0.73de
		100%	0.53±0.016b-d	1.66±0.009d	67.0±1.27fg
	Hamadani	10%	0.39±0.016h-j	1.76±0.022ab	76.7±0.55bc
		50%	0.44±0.012f-h	1.78±0.022a	74.7±0.48b-e
		100%	0.48±0.009c-f	1.72±0.019a-d	69.0±0.55f
	Meldor	10%	0.42±0.012g-i	1.71±0.012b-d	80.0±0.73a
		50%	0.47±0.012d-f	1.72±0.017a-d	76.0±0.27b-d
		100%	0.45±0.009e-g	1.73±0.007a-c	77.3±0.48b
	Sovrana	10%	0.38±0.019ij	1.72±0.012a-d	76.7±1.27bc
		50%	0.46±0.002e-g	1.74±0.016a-c	75.0±0.48b-e
		100%	0.50±0.002b-e	1.69±0.004cd	66.7±0.99fg
Fourth year	Fasta	10%	0.53±0.009bc	1.70±0.005b-d	77.3±0.48b
		50%	0.60±0.002a	1.70±0.002b-d	75.0±0.73b-e
		100%	0.55±0.021ab	1.66±0.007d	67.7±0.73f
	Hamadani	10%	0.36±0.012j	1.75±0.023a-c	77.0±0.99bc
		50%	0.37±0.016ij	1.78±0.022a	76.7±0.55bc
		100%	0.38±0.009ij	1.73±0.027a-c	74.3±0.48c-e
	Meldor	10%	0.39±0.007h-j	1.72±0.018a-d	76.0±0.73b-d
		50%	0.44±0.021f-h	1.73±0.017a-c	73.0±0.48e
		100%	0.49±0.012c-f	1.72±0.007a-d	67.3±0.73fg
	Sovrana	10%	0.50±0.018b-e	1.73±0.015a-d	74.7±0.48b-e
		50%	0.49±0.004c-f	1.74±0.018a-c	74.3±0.48c-e
		100%	0.50±0.019c-f	1.70±0.0.12b-d	65.0±0.55g

Values are the means ± standard errors of three replicates (n=3). Statistically significant differences between treatments (P≤ 0.05) are shown by different letters.

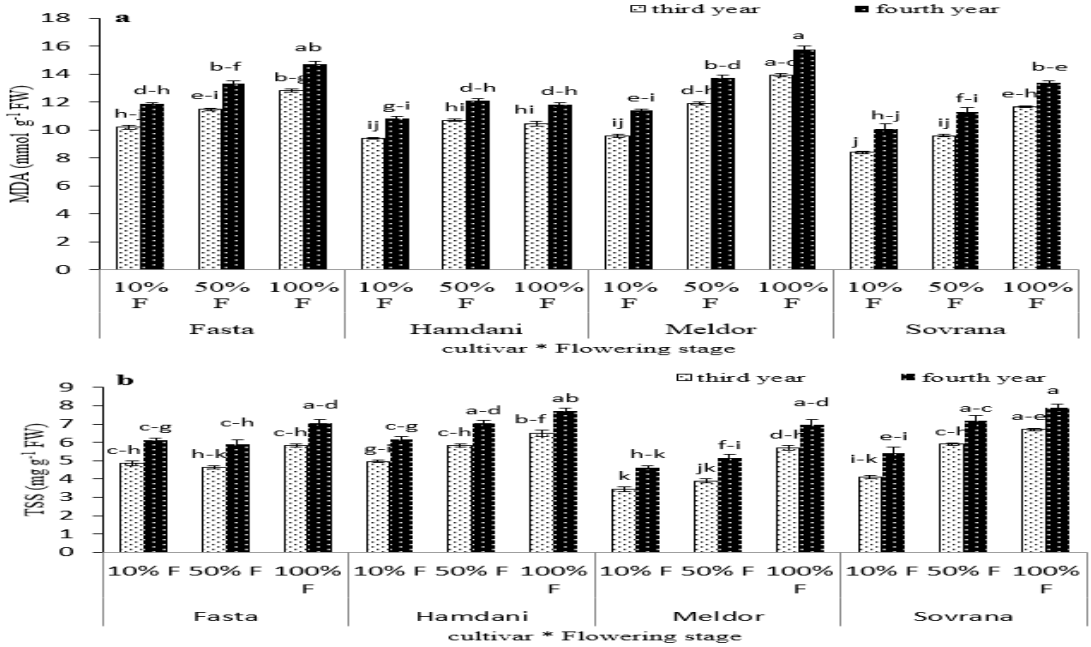

Fig. III. Malondialdehyde (MDA) and total soluble sugar (TSS) in alfalfa cultivars at different flowering stages; values are the means ± standard errors of three replicates (n=3). Statistically significant differences between treatments (P≤ 0.05) are shown by different letters.

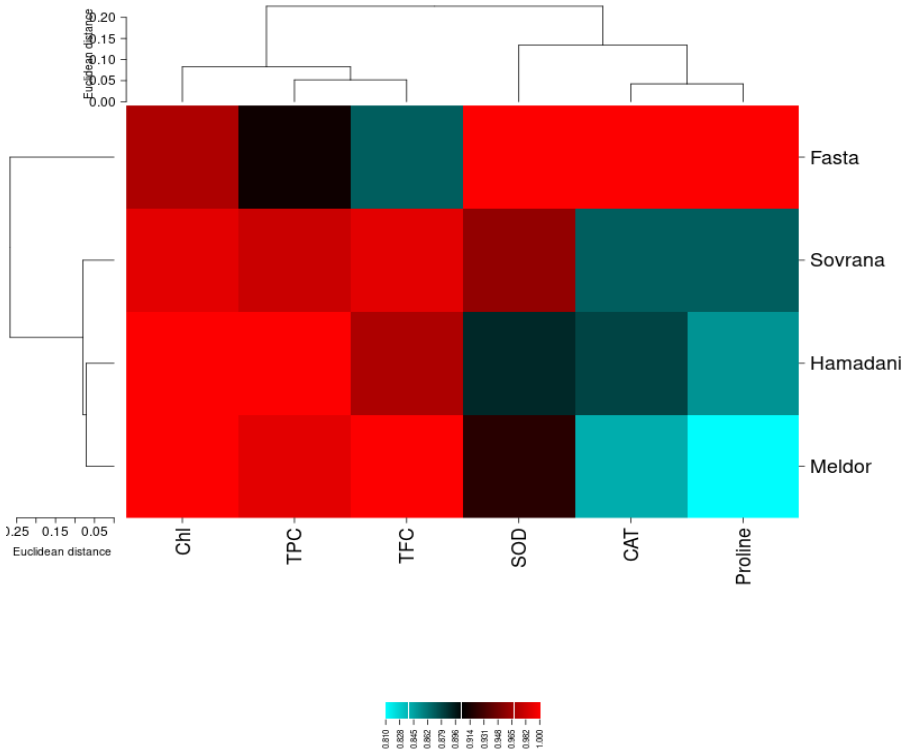

Fig. IV. Heat map analysis for physiochemical traits in clustering the cultivars

clusters, although Chl. failed to reveal any notable variations between cultivars.

Discussion

Phenolic derivatives are described as a main part of antioxidant systems in plants (Afshari and Rahimmalek, 2018). The content of total phenol compounds of alfalfa cultivars showed variability during the flowering stages. According to the results obtained by Farhat et al. (2020), changes in phenolic compounds can characterize vegetative and reproductive status of plants during growth stages. TPC and TFC increased up to middle flowering stage and then decreased in late flowering stage. The reduction of TPC and TFC at late growth stages was also reported in *Achillea aucheri* (Afshari and Rahimmalek, 2018) and *Achillea millefolium* (Farhadi et al., 2020). In line with our results, the variation of secondary metabolites, particularly phenolic compounds during phenological stages were addressed in *Trifolium pretense* (Vlaisavljević et al., 2017) and *Salvia aegyptiaca* (Farhat et al., 2015). The variation of TPC and TFC during the plant growth is related to changes in metabolic pathways of these compounds by progressing plant growth (Vlaisavljević et al., 2017). The flowering stages was directly related to variations in temperature in our study. Temperature, as a critical environmental agent, can influence plant growth and in turn, production of secondary metabolites. Temperature was reported to negatively correlate with the phenolic accumulation in plants (Albert et al., 2009; Sampaio et al., 2011; Farhadi et al., 2020). The plant age is another main factor influencing TPC and TFC (Farhadi et al., 2020). Increased TPC and TFC in older plants was recorded in our study, which can be attributed to the fact that plants accumulate more secondary metabolites during their life.

Increased CAT and SOD were observed in late flowering stage and in the four-year-old plants. By advancing the flowering stage, the antioxidant capacity increased. This can be due to the fact that the plants were exposed heat stress at late flowering stage, which leads to production of reactive oxygen species (ROS). The main mechanism to scavenge ROS is increasing

antioxidant enzyme activities (Gomes et al., 2018). Like our study, the difference among cultivars under oxidative stress condition was reported by Nasirzadeh et al., (2020). Hamadani cultivar showed lower variability in its physiological attributes owing to its resistance during flowering stages and the corresponding stresses. According to numerous reports, antioxidant enzymes can increase resistance to a variety of abiotic stressors (Nasirzadeh et al., 2020). According to heat map results, SOD was significantly stronger in clustering the cultivars; therefore, it can be used as the main factor in cultivars' response to maturity.

Total Chl. content is the main parameter in photosynthesis status in plants. In all cultivars, total Chl. synthesis was impaired with progressing the flowering stage (Table 4). Plants typically modify their Chl. content in response to environmental variations. Inhibition of photosynthetic electron transport chain and the enzymes involved in Chl. biosynthesis are responsible for decreased Chl. content in alfalfa cultivars exposed to abnormal conditions (Siddiqui et al., 2015). Also, environmental changes alter the anatomical structure in leaves, namely stomatal size and density and stomata closure, which affect the inputs (water and CO_2) to photosynthesis process (Lipiec et al., 2013). Thus, the amount of Chl. content is greatly influenced by the physiological reactions and stress tolerance of the species (Siddiqui et al., 2015). Under different environmental stresses, the accumulation of osmolytes like proline plays a critical function in modulating oxidative damage (Siddiquiet al., 2013, 2014). In all plant genotypes, proline were more accumulated at late flowering stage (Table 4).

Reduction of RWC for coping abnormal conditions is a physiological strategy in plants to tolerate stress. We observed decreased RWC at late flowering stage due to increased temperature. Similarly, Siddiqui et al. (2015) showed reduced RWC under high temperature in different cultivars of fava bean. Loss of turgidity and RWC leads to reduced growth in plants (Kesici et al., 2013). The change in temperature and other environmental stresses might alter cell division and cell elongation, which result in reduced growth. Therefore, it can be noticed that RWC is a suitable parameter in determining physiological water status of various alfalfa cultivars.

The increased TSS was the response of all cultivars by progressing the maturity. TSS is considered as a biochemical indicator in plants under environmental variations (Ghodke et al., 2018). Similarly, increased TSS during maturity has been reported in four Andean blackberry cultivars by Samaniego et al. (2020). Accumulation of sugar in plant tissues is a defense mechanism of plants to increases their antioxidant systems by advancing maturity. In addition, MDA also increased by maturity in all alfalfa cultivars. MDA is a naturally occurring product of lipid peroxidation and the its level is calculated as a biomarker of lipid quality in plants (Guemmaz et al., 2018). Confirming our results, Pilarska et al. (2017) showed increased MDA in green leaves of tobacco by advancing maturity.

Conclusions

The objective of the current study was to determine the physiochemical responses of different alfalfa cultivars at flowering stages in three- and four-year-old plants. According to our results, plant yield in three-year-old plants were significantly higher than four-year-old plants. Total Chl. and RWC decreased while antioxidant activity increased with advancing the flowering maturity. Cultivars had different responses to flowering stages, and Hamadani cultivar had significantly higher TPC and TFC compared to other experimental other cultivars. Therefore, we suggest early up to middle flowering stage to harvest alfalfa cultivars, particularly Hamadani cultivar, to achieve optimum concentration of biochemical traits.

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