Saudi Journal of Biological Sciences 28 (2021) 7227–7240

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Phytochemical and physiological changes in *Salvia officinalis* L. under different irrigation regimes by exogenous applications of putrescine

Maryam Mohammadi-Cheraghabadi [a], Seyed Ali Mohammad Modarres-Sanavy [a,*], Fatemeh Sefidkon [b], Ali Mokhtassi-Bidgoli [a], Saeid Hazrati [c]

[a] Department of Agronomy, Faculty of Agriculture, Tarbiat Modares University, PO Box 14115-336, Tehran, Iran
[b] Research Institute of Forests and Rangelands, Tehran, Iran
[c] Department of Agronomy, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran

ARTICLE INFO

Article history:
Received 28 June 2021
Revised 20 July 2021
Accepted 8 August 2021
Available online 13 August 2021

Keyword:
Antioxidant enzymes
Phenolic
Sage
Regression
Water stress

ABSTRACT

Water stress is the major factor limiting plant productivity and quality in most regions of the world. In the present study, a two-year field experiment was conducted to determine the influence of putrescine (Put) on phytochemical, physiological, and growth parameters of *Salvia officinalis* L. under different irrigation regimes. The highest stem dry weight (56.05 and 65.21 g m^{-2}) plus leaf dry weight (124.51 g m^{-2}) were predicted in irrigation regimes of (20 and 40%) plus 20% available soil water was depleted (ASWD), respectively. Total phenolic content (TPC) was increased significantly under the irrigation regime of 80% with the application of distilled water in spring. TPC showed an increasing trend with increases in Put concentration under all irrigation regimes in both spring and summer. The highest total flavonoids content (TFC) in wavelengths of 415 and 367 nm were predicted in 2.25 mM Put. The highest ascorbate peroxidase (APX) activity (0.13 µmol mg^{-1} protein) was predicted in the irrigation regime of 20% with the application of distilled water in spring and summer. There was a significantly negative correlation coefficient between APX, TPC, and TFC. Indeed, there was a decreasing trend in APX and an increasing trend in TPC and TFC with increases in Put concentration under the irrigation regime of 20% ASWD. The highest hydroxyl radical scavenging activity (HRSA) values were obtained under irrigation regimes of 49.27% and 20% ASWD in spring and summer, respectively. There was an increasing trend in endogenous Put with increases in the Put concentration. The responses of compatible osmolytes to irrigation regime can be expressed by quadratic model, suggesting maximum proline (0.52 mg g^{-1}), total reducing sugars (TRS) (0.37 mg g^{-1}), xylose (0.68 mg g^{-1}), and mannose (0.37 mg g^{-1}) values would be obtained in irrigation regimes of 68.33%, 48.33%, 53.75%, and 56.25% ASWD, respectively.

1. Introduction

Salvia officinalis L. (sage) has the oldest history among medicinal plants (Altindal and Altindal, 2016). It is used as raw material in medicine, perfumery, and the food industry (Martins et al., 2015; Altindal and Altindal, 2016). Reports show the compositions of sage essential oil are different depending on environmental stress (Verma and Shukla, 2015; Ghorbanpour et al., 2016; Mohammadi et al., 2018). Overall, one of the most effective ways to protect plant cells against abiotic stress is to increase the activity of the antioxidant defense system (Ishikawa and Shigeoka, 2008; Chung et al., 2009). Plants have developed this system by activating enzymatic (e.g., ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (PO), and catalase (CAT) and non-enzymatic (e.g., flavonoids) mechanisms to resist oxidative stress (Ahmed et al., 2009). Flavonoids play a key role against oxidative stress, because they can inhibit the production of free radicals (Agati et al., 2007). The production of ROS (reactive oxygen species), such as O_2 and H_2O_2, disrupts the metabolic stability of cells in plants, leading to chlorosis and decreases in photosynthetic activity (Nxele et al., 2017; Sharma and Zheng, 2019). It is now well established that despite their potential for creating harmful oxidations, ROS are strong signaling molecules which stimulate enzymes and the accu-

* Corresponding author.
E-mail addresses: mohammadi.maryam@modares.ac.ir (M. Mohammadi-Cheraghabadi), modaresa@modares.ac.ir (S.A.M. Modarres-Sanavy), sefidkon@rifr-ac.ir (F. Sefidkon), mokhtassi@modares.ac.ir (A. Mokhtassi-Bidgoli), saeid.hazrati@azar-univ.ac.ir (S. Hazrati).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.08.024
1319-562X/

mulation of osmolytes, thus contributing to plant growth and development under stress (Foyer and Noctor, 2009). Considered one of the most important strategies for improving water stress tolerance in plants is the accumulation of compatible osmolytes (Ahanger et al., 2014). Soluble sugars are major organic osmolytes which act as antioxidants and osmoprotectants in plant cells (Nishizawa et al., 2008). Other metabolites such as polyamines (PAs) can also serve protective functions in chloroplasts, maintain membrane stability, and promote the scavenging of reactive oxygen species (Kubís et al., 2014). The metabolism of endogenous PAs is changed by the exogenous application of PAs and can affect plant tolerance to water deficit stress (Capell et al., 2004; Farooq et al., 2009). PAs in plants are found not only in the cytoplasm, but also in specified organelles like mitochondria, chloroplasts, and vacuoles (Foyer and Shigeoka, 2011). According to many researchers, PAs increase the activities of antioxidant enzymes and remarkably reduce the oxidative effects of ROS on membranes (Ahmad et al., 2010; Roychoudhury et al., 2011; Li et al., 2015; Ahanger et al., 2017). Hussain et al. (2019) reported significant increases in the activities of SOD, CAT, and peroxidase and a decrease in H_2O_2 when spermidine was foliar-applied to *Brassica juncea* plants. Environmental stresses and the application of PAs can change the synthesis of some essential oil compounds in plants. Exposure to abiotic stress increased the endogenous levels of phenolics in *Matricaria chamomilla*, *Mentha pulegium,* and *Nigella sativawere* (Kováčik et al., 2009; Oueslati et al., 2010; Verma and Shukla, 2015). In addition, the application of PAs increased secondary metabolites in *Ligustrum vulgare* (Agati et al., 2011) and *Triticum aestivum* (Ahanger and Agarwal, 2017). Polyphenolic compounds as secondary metabolites play an important role in physiological resistance mechanisms to cope with various types of stress (Edreva et al., 2008; Mazid et al., 2011; Huang et al., 2010; Lin et al., 2016; Razavi-Azarkhiavi et al., 2016; Alu'datt et al., 2017). Total phenolic content was reported to increase under salinity stress (Misra et al., 2014, Yucel and Heybet, 2016). Phenolic compounds have been used as natural antioxidants and preservatives in drug and food industries (Parke and Lewis, 1992; Das and Roychoudhury, 2014; Li and Vierstra, 2014).

To improve the potential of plants for phenolics and flavonoid production, strategies should be developed. Despite the widespread distribution of polyamines in the plant kingdom, however, information about the antioxidants and phenolic compounds of sage under water deficit stress or Put are few. In the same context, experiments were performed to investigate the effects of Put application on the physiological and biochemical parameters of sage under water deficit stress in two harvest times in two years; the results could establish a strong groundwork for the proposed investigations in this field.

2. Materials and methods

2.1. Site description

A two-year (2017–2018) field experiment was conducted at the Research Field of Agriculture Faculty of Tarbiat Modares University, located in Tehran, Iran (35°70′ N, 51°40′ E and 1200 m above

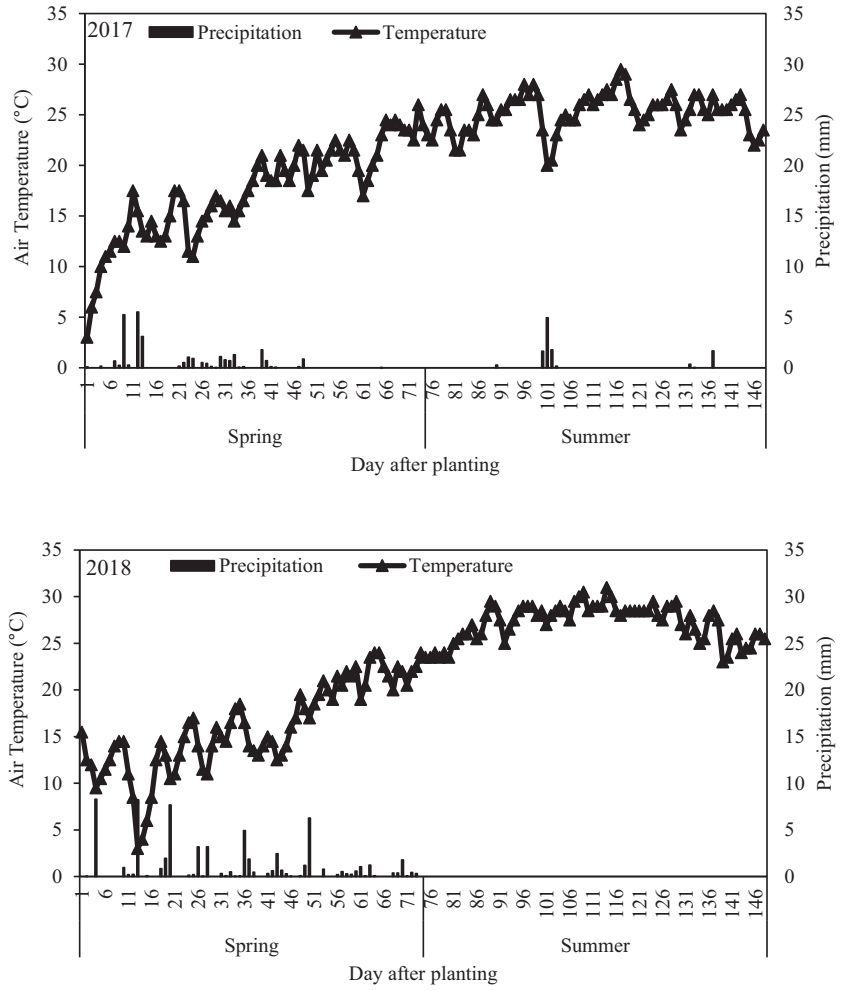


Fig. 1. Daily average air temperatures (°C), and precipitation (mm) recorded during the growing season in 2017 and 2018.

sea level). The research location is classified as a semi-arid climate according to the Koppen climate classification system. The long-term (30 years) mean annual rainfall and temperature are 232.6 mm and 22 °C, respectively (see meteorological data in Fig. 1). The physical and chemical properties of the field soil were assessed in 0–30 cm depth (Total N: 0.121%; organic carbon: 1.034%; E.C: 2.610 dSm^{-1}, texture: Silt loam; pH: 7.51; field capacity (FC): 22.51%; permanent wilting point (PWP): 8.01%. Initial soil test values (mg kg^{-1}) for P, K, Fe, Mn, Zn, Cu and B were 269, 68.8, 4.17, 8.91, 0.86, 1.09, and 1.77, respectively.

2.2. Experimental design

These experiments were implemented in two years, from 4 April 2017 to 29 August 2018. About 1200 cuttings were prepared from one-year-old mother plants of *S. officinalis* in the medicinal farm of the Institute of Medicinal Plants & Natural Products Research, Iranian Academic Center for Education, Culture & Research (ACECR) (Karaj, Iran). Cuttings were transferred to field after rooting (30 days). The plots included six rows, 0.5 m apart, and four seedlings in each row with a density of 40,000 plants ha^{-1}. No fertilizer was utilized according to the soil chemical properties. Weeds were controlled manually. The experiment was as split-split plot arrangement in a randomized complete block design with three replications. The main plot was the irrigation regime, the sub-plot was the Put concentration, and the sub-sub plot was the harvest time. Irrigation after depletion of 20, 40, 60 and 80% available soil water content. Four concentrations of Put (distilled water (0), 0.75, 1.5 and 2.25 mM) were applied. All aboveground parts in each plant were exogenously sprayed 50 cm above the head of plant using a hand atomizer sprayer equipped with an even nozzle (8001E) delivering 180.5 L ha^{-1} with a pressure of 250 kPa and a speed of 5 km h^{-1}. Foliar application of Put (Sigma-Aldrich) was performed twice each year, one week before applying irrigation regimes in each harvest time in two years. Further, the foliage was handpicked in spring and summer. To investigate proper treatments, plants should have been formed of similar masses of foliage, before applying treatments. Thus, no water stress and Put were applied in the first month of the growth cycle. In addition, during this period, all plots were irrigated, while 20% of available soil water was depleted (ASWD). The depth of irrigation water was assigned based on the available soil water and calculated using the following equations:

$$ASW = (\theta_{FC} - \theta_{PWP}) \times D \times 100 \quad (1)$$

$$Id = ASW \times \rho \quad (2)$$

$$Ig = (Id \times 100)/Ea \quad (3)$$

Where, FC and PWP (%) represent the volumetric soil water amounts. D (cm) is the soil layer depth; Id reflects the irrigation depth (cm); ρ shows the fraction of ASW that can be depleted from the root zone (20%, 40%, 60%, and 80%); Ig is the coarse depth of irrigation (cm), and Ea denotes the irrigation efficiency (%), averagely assumed at 65% (Govahi et al. 2015). The applied irrigation water (based on Eq. (3)) at each irrigation event was measured by flow meters installed in the pipe outlet delivering water to the plots (Bahreininejad et al. 2013). Irrigation treatments were implemented based on the maximum allowable depletion (MAD) from the percentage of ASW. Each treatment was irrigated when the available soil water reached its threshold value (Govahi et al. 2015). The applied treatments were 20%, 40%, 60%, and 80% MAD of ASW. Time-Domain Reflectometry, Model TRIME-FM, Germany (TDR) probe was applied to measure soil water level at a depth of 50 cm (root zone of *S. officinalis*). TDR probes were taken from the first experiment to the last experiment. The plants were harvested in spring and summer before flowering (70 days after planting) because of the highest content of sage essential oil. The foliage of plants was harvested 8–10 cm over the soil surface. Collections were performed on 11 June (spring harvest), and 29 August (summer harvest) in two years.

2.3. Growth parameters

Foliage of the plants were cut at the soil surface at the end of each harvest time. Then, to determine dry weights, leaf and stem were separated and dried at 70 °C for 48 h.

2.4. Total phenolic and flavonoids content

Folin-Ciocalteu reagent was used to estimate TPC. Briefly, dry leaf samples (100 mg) were extracted in 80% methanol and then centrifuged at 4000 rpm for 15 min at 4 °C. Crude extract was mixed with 10% Folin-Ciocalteu reagent and 7.5% Na_2CO_3. After samples had been incubated for 1.5 h at 25 °C, absorbance was read at 760 nm using a Cary 60 UV–Vis Spectrophotometer. Gallic acid was used as a standard, and the results were expressed as mg GAE/g Dw (Singleton et al., 1999).

Flavonoid content was measured using the methods of Zhishen et al. (1999), Chang et al. (2002), and Lamaison and Carnet (1990) with slight modification. Absorbance was read at three wavelengths of 367, 415, and 510 nm using a Spectrophotometer.

2.5. Putrescine analysis

Leaf tissue was ground under liquid nitrogen using a mortar and pestle. Put extraction followed by HPLC quantification were carried out in accordance with Lütz et al. (2005). For the assessment of endogenous Put, samples were injected in to 20 μl injector loop in RP-C18 Column, 15 cm × 4 mm i.d., 5 μm particle size, at 30 °C using Methanol: Water linear gradient from ratio of 50:50 to 80:20 (v/v) for 30 min. The last proportion was maintained at 1 ml/min. The detection of Put was carried out by measuring the fluorescence intensity at 254 nm of samples and making their comparisons with the peaks and retention times of standard Put. The standard Put was prepared at a concentration of 1 mM.

2.6. Antioxidant enzymes

Leaf samples (500 mg) were pulverized in Na–P buffer (pH 7.0) containing 1 mM EDTA and 1% soluble polyvinyl pyrrolidone (PVP) using a mortar and pestle. The leaf extract was then centrifuged at 12,000*g* for 20 min at 4 °C. The enzyme extracts obtained were used to determine the activity levels of superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase enzymes. Enzyme activity was expressed as enzyme unit (EU) mg^{-1} protein. Furthermore, the extraction buffer for ascorbate peroxidase enzyme contained 1 mM ascorbic acid.

The protein concentration was analyzed using the method reported by Bradford (1976) method. To estimate ascorbate peroxidase (APX) (APX, EC 1.11.1.11), the Nakano and Asada (1981) method was employed. The reaction mixture contained 50 mM Na–P buffer with pH 7.0, 0.5 mM ascorbic acid, 0.1 mM EDTA-Na2, 0.12 mM H_2O_2, and 20 μl of enzyme extract. Absorbance was read at 290 nm.

To estimate superoxide dismutase (SOD) (SOD, EC 1.15.1.1), the Bayer and Fridovich, (1987) method was used. Briefly, 3 ml reaction mixture (containing 100 mM phosphate buffer with pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin, and 100 μl enzyme extract was incubated under a light intensity of 3600 lux for 15 min, and the reaction was stopped by switching off the light. The absorbance was read at 560 nm using a Cary 60 UV–Vis Spectrophotometer.

Peroxidase (PO) activity (EC 1.11.1.7) was determined utilizing the method introduced by Herzog and Fahimi (1973). The reaction mixture contained 0.15 M sodium phosphate-citrate buffer (pH 4.4), 50% (w/v) gelatin, 0.6% H_2O_2, and 5 µl enzyme extract. Absorption levels were read at 465 nm (extinction coefficient 2.47 mM^{-1} cm^{-1}) for 3 min using a Cary 60 UV–Vis Spectrophotometer.

Catalase (CAT) activity (EC.1.11.1.6) was determined using the method of Aebi (1984). Enzyme extract (100 µl) was added to 900 µl reaction solution (containing 50 mM sodium phosphate buffer with pH 7.0 mM H_2O_2), and absorbance was read at 240 nm using a Cary 60 UV–Vis Spectrophotometer.

2.7. Hydroxyl radical scavenging activity and H_2O_2

To estimate HRSA, 50 µl of the sample (concentration 0.2–5 mg ml^{-1}) was mixed with 1 ml of 9 mM $FeSO_4$ and 1 ml of H_2O_2 (0.3%) in 0.5 ml of 9 mM salicylic acid–ethanol and then incubated under darkness for 30 min. Absorbance of the sample was read at 510 nm using a Cary 60 UV–Vis Spectrophotometer (Hifney et al., 2016). Ascorbic acid was used as positive control. HRSA (%) was calculated as follows: HRSA (%) = [{A0 (A1- A2)}/A 0] × 100, where A0 = the absorbance of control (without sample or ascorbic acid), A1 = the absorbance of sample or ascorbic acid, and A2 = the absorbance of blank without ascorbic acid.

To calculate leaf H_2O_2 (Velikova et al., 2000), 100 mg fresh tissue was extracted by 5 ml trichloro acetic acid (0.1%) and then centrifuged at 10,000 rpm for 10 min at 4 °C. Finally, supernatant was mixed with potassium phosphate buffer (pH 7.0) and potassium iodide (KI), and absorbance was read at 390 nm using a Cary 60 UV–Vis Spectrophotometer.

2.8. Relative water content, electrolyte leakage, proline, total reducing sugars, xylose, and mannose

To calculate RWC, twenty leaf discs were punched, and their fresh weight (FW) was recorded. The same leaf discs were kept in Petri dishes containing distilled water for six hours to record saturation weight (SW), and after that, discs were oven dried at 75 °C for 24 h to record the dry weight (DW). Calculations were performed using the formula: RWC = (FW – DW)/ (SW – DW) (Gonzalez and Gonzalez-Vilar, 2001).

To estimate EL in the leaves, 10 fresh leaf discs 5 mm in diameter were immersed in test tubes filled with deionized water and the electrical conductivity (EC1) was recorded. Then they were heated at 50 °C for 25 min in a water bath and the electrical conductivity (EC2) was measured again. Finally, they were heated at 100 °C for 10 min, and again the electrical conductivity (EC3) was recorded (Dionisio-Sese and Tobita, 2000). The percentage of EL was calculated the following formula: EL% = [(EC2 –EC1) / (EC3)] × 100.

Proline was estimated using the method of Bates et al. (1973).

TRS, xylose, and mannose were measured using the method of Dubois et al. (1956).

2.9. Statistical analysis

The method as type3 in MIXED procedure of SAS v. 9.3 (SAS Institute, Cary, NC, USA) was used to analyze data. The main effects of irrigation regimes, Put concentrations, harvest times and their two- and three-way interactions were considered fixed effects, and years, replicates × years and years × irrigation regimes × Put concentrations × harvest times were considered random effects. The PDIFF option of least square means was used for mean comparisons. The interactions between experimental factors were separated by slicing method. Polynomial orthogonal contrasts were used to test the significance of linear and quadratic regression models and significant models ($P \leq 0.05$) were presented. Pearson's correlation coefficients were determined using the CORR procedure.

3. Results

3.1. Growth parameters

Analysis of variance showed that the main effects of harvest time were significant for leaf and stem dry weights. Irrigation regime also affected stem dry weight (Table 1). Leaf and stem

Table 1
Analysis of variance (mean square) and analysis of regression (mean square of linear and quadratic models) of leaf dry weight (g m^{-2}), stem dry weight (g m^{-2}), total phenolic content (TPC) (mg g^{-1}), total flavonoids content (mg g^{-1}) in wavelengths 510, 415 and 367, superoxide dismutase (SOD)(µmol mg^{-1} protein), peroxidase(PO) (µmol mg^{-1} protein), catalase (CAT) (µmol mg^{-1} protein), ascorbate peroxidase (APX) (µmol mg^{-1} protein), H_2O_2 (µmol g^{-1} FW), hydroxyl radical scavenging activity (HRSA) (%), putrescine endogenous (nmol g^{-1} FW), relative water content (RWC) (%), electrolyte leakage (EL) (%), proline (mg g^{-1} FW), total reducing sugars (TRS) (mg g^{-1} FW), xylose (mg g^{-1} FW) and mannose (mg g^{-1} FW) of *Salvia officinalis* influenced by irrigation regimes (I), putrescine concentrations (Put) and harvest times (H).

Sources of variation	I	Put	H	I × Put	I × H	Put × H	I × Put × H	Put-Linear	Put-Quadratic	I-Linear	I-Quadratic
Num DF	3	3	9	1	3	3	9	1	2	1	2
Den DF	31	31	31	31	31	31	31	190	189	190	189
Leaf dry weight	2.20^{ns}	0.74^{ns}	212.93^{**}	1.01^{ns}	1.73^{ns}	0.06^{ns}	1.30^{ns}	0.00^{ns}	0.71^{ns}	4.22^{*}	2.21^{ns}
Stem dry weight	11.25^{**}	1.38^{ns}	93.19^{**}	0.63^{ns}	2.67^{ns}	0.84^{ns}	1.91^{ns}	1.27^{ns}	0.93^{ns}	22.03^{**}	12.76^{**}
TPC	0.99^{ns}	1454.52^{**}	205.83^{**}	17.93^{**}	13.43^{**}	9.84^{**}	5.00^{**}	241.32^{**}	120.12^{**}	0.05^{ns}	0.03^{ns}
TFC (510 nm)	0.17^{ns}	42.51^{**}	0.54^{ns}	2.31^{*}	1.44^{ns}	1.26^{ns}	0.55^{ns}	179.98^{**}	89.55^{**}	0.14^{ns}	0.11^{ns}
TFC (415 nm)	0.04^{ns}	10.77^{**}	16.96^{**}	0.04^{ns}	0.04^{ns}	0.02^{ns}	0.05^{ns}	9.23^{**}	4.59^{*}	0.00^{ns}	0.00^{ns}
TFC (367 nm)	0.35^{ns}	208.68^{**}	3.61^{ns}	0.54^{ns}	0.22^{ns}	0.17^{ns}	0.73^{ns}	125.69^{**}	62.56^{**}	0.04^{ns}	0.03^{ns}
SOD	0.19^{ns}	0.56^{ns}	24.70^{**}	0.54^{ns}	0.13^{ns}	0.25^{ns}	0.68^{ns}	0.99^{ns}	1.49^{ns}	0.00^{ns}	0.26^{ns}
PO	0.07^{ns}	0.19^{ns}	24.00^{**}	0.17^{ns}	0.19^{ns}	0.12^{ns}	0.24^{ns}	0.02^{ns}	0.28^{ns}	0.13^{ns}	0.13^{ns}
CAT	0.38^{ns}	0.19^{ns}	28.29^{**}	0.10^{ns}	0.20^{ns}	0.05^{ns}	0.09^{ns}	0.00^{ns}	0.29^{ns}	0.67^{ns}	0.47^{ns}
APX	63.10^{**}	139.56^{**}	168.35^{**}	120.13^{**}	127.64^{**}	17.56^{**}	24.21^{**}	23.55^{**}	12.48^{**}	10.93^{**}	6.06^{**}
H_2O_2	0.14^{ns}	0.22^{ns}	29.68^{**}	0.12^{ns}	0.14^{ns}	0.09^{ns}	0.19^{ns}	0.03^{ns}	0.32^{ns}	0.22^{ns}	0.20^{ns}
HRSA	1.38^{ns}	1.58^{ns}	13.78^{**}	0.74^{ns}	3.17^{*}	0.02^{ns}	0.49^{ns}	0.03^{ns}	1.80^{ns}	4.00^{*}	2.12^{ns}
Putrescine endogenous	0.25^{ns}	1.48^{ns}	23.53^{**}	0.18^{ns}	0.12^{ns}	0.74^{ns}	0.13^{ns}	9.27^{**}	4.74^{**}	0.91^{ns}	0.61^{ns}
RWC	1.91^{ns}	0.99^{ns}	29.76^{**}	0.75^{ns}	0.71^{ns}	0.38^{ns}	0.17^{ns}	4.17^{*}	2.20^{ns}	10.50^{**}	5.59^{**}
EL	5.14^{**}	0.54^{ns}	6.68^{*}	0.31^{ns}	1.41^{ns}	0.28^{ns}	0.47^{ns}	1.38^{ns}	0.89^{ns}	38.01^{**}	21.07^{**}
Proline	1.99^{ns}	0.11^{ns}	17.56^{**}	0.29^{ns}	0.56^{ns}	0.14^{ns}	0.48^{ns}	0.05^{ns}	0.08^{ns}	6.24^{*}	3.48^{*}
TRS	0.89^{ns}	0.08^{ns}	0.99^{ns}	0.78^{ns}	0.19^{ns}	0.49^{ns}	0.59^{ns}	0.69^{ns}	0.36^{ns}	0.00^{ns}	5.15^{**}
Xylose	1.32^{ns}	0.11^{ns}	0.41^{ns}	0.91^{ns}	0.09^{ns}	0.60^{ns}	0.54^{ns}	0.74^{ns}	0.37^{ns}	0.11^{ns}	5.82^{**}
Mannose	0.85^{ns}	0.09^{ns}	1.09^{ns}	0.78^{ns}	0.26^{ns}	0.76^{ns}	0.70^{ns}	0.79^{ns}	0.44^{ns}	0.00^{ns}	4.58^{*}

ns: non-significant
*: $\alpha \leq 0.05$
**: $\alpha \leq 0.01$

dry weight were higher in summer than in spring (Table 2). The highest stem dry weight was obtained in irrigation regimes of 20% (56.05 g m^{-2}) and 40% ASWD (65.21 g m^{-2}). The responses of leaf dry weight and stem dry weight to irrigation regime can be expressed by linear model, suggesting that leaf and stem dry weights would reach their minimum when the intensity of water deficit stress is increased (Fig. 2A and 2B). The highest leaf dry weight (124.51 g m^{-2}) and stem dry weight (62.53%) were predicted under the irrigation regime of 20% ASWD (Fig. 2A and 2B).

Table 2
Main effect of harvest time on leaf dry weight, stem dry weight, total flavonoids content (TFC) (415 nm) (mg g^{-1}), relative water content (RWC) (%), electrolyte leakage (EL) (%), superoxide dismutase (SOD) (μmol mg^{-1} protein), peroxidase (PO) (μmol mg^{-1} protein), catalase (CAT) (μmol mg^{-1} protein), H_2O_2 content (μmol g^{-1} FW) and proline (mg g^{-1} FW) of *Salvia officinalis*.

Some dependent traits	Harvest time	
	Spring	Summer
Leaf dry weight	53.4797 b	169.09 a
Stem dry weight	28.4809 b	69.4218 a
TFC (415 nm)	0.3395 a	0.2883 b
RWC	87.8307 a	73.4957 b
EL	41.3833 a	37.1067 b
SOD	0.08858 b	0.1600 a
PO	0.4542 b	1.2816 a
CAT	0.1676 b	0.4672 a
H_2O_2	0.2368 b	0.6363 a
Proline	0.1627 b	0.2917 a

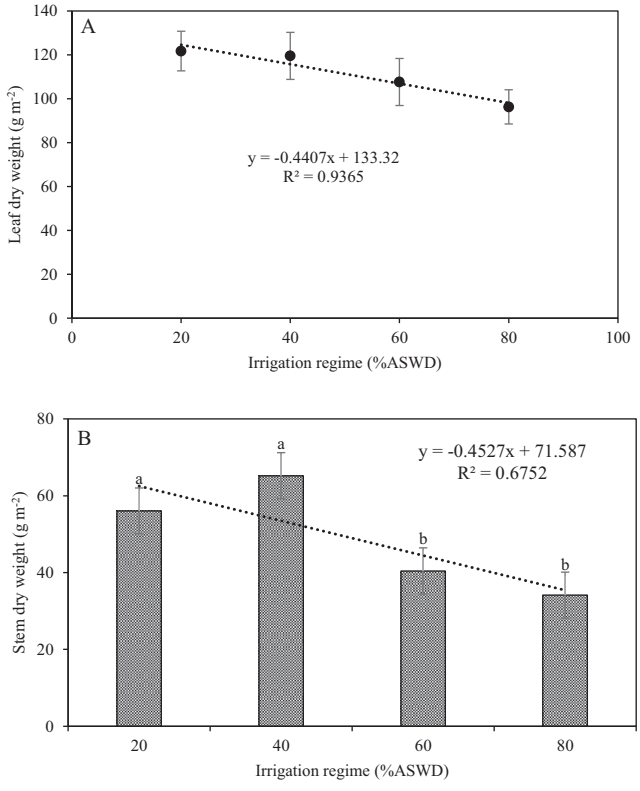


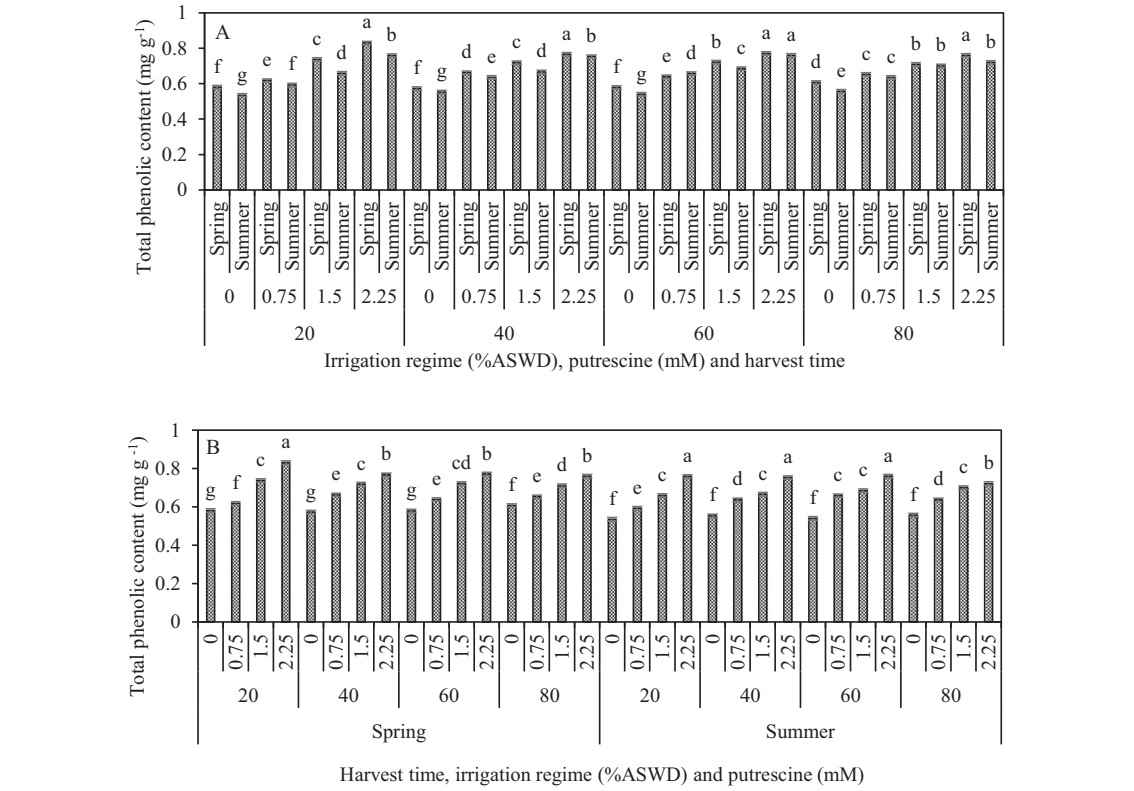
Fig. 2. Significant linear relationship between irrigation regime (Available soil water was depleted (ASWD)) and leaf dry weight (A). Main effect of irrigation regime on stem dry weight (B). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

3.2. Total phenolic content

Analysis of variance showed the main effects of Put concentration and harvest time and the three-way interaction between irrigation regime, Put concentration, and harvest time significantly influenced TPC. Also, analysis of variance indicated the two-way interactions between irrigation regimes and Put concentration, irrigation regime and harvest time, and Put concentration and harvest time significantly affected TPC (Table 2). The highest TPC was obtained in spring with the application of 2.25 mM Put under the irrigation regimes of 20, 40, and 80% ASWD. Moreover, the highest TPC was obtained in spring and summer with the application of 2.25 mM Put under the irrigation regime of 60% ASWD (Fig. 3A). A significant increase in TPC was observed under the irrigation regime of 80% compared with the irrigation regimes of 20, 40, and 60% in spring (Fig. 3B). The highest TPC was obtained with the application of 2.25 mM Put under the irrigation regime of 20% ASWD (0.83 mg g^{-1}) in spring (Fig. 3B). In summer, however, the highest TPC was obtained with the application of 2.25 mM Put under irrigation regimes of 20% (0.76 mg g^{-1}), 40% (0.75 mg g^{-1}), and 60% (0.76 mg g^{-1}) ASWD (Fig. 3B). Generally, the highest TPC (0.83 mg g^{-1}) was obtained with the application of 2.25 mM Put under the irrigation regime of 20% ASWD in spring. The response of TPC to Put concentration in all irrigation regimes in spring and summer can be expressed by a linear model, suggesting that TPC would reach maximum with increased Put concentrations in all irrigation regimes in both spring and summer. As, the highest TPC was predicted with the application of 2.25 mM Put under irrigation regimes of 20% (0.82 mg g^{-1}), 40% (0.78 mg g^{-1}), 60% (0.78 mg g^{-1}), and 80% (0.76 mg g^{-1}) ASWD in spring (Fig. 3C). In summer, however, the highest TPC was predicted with the application of 2.25 mM Put under irrigation regimes of 20% (0.75 mg g^{-1}), 40% (0.75 mg g^{-1}), 60% (0.76 mg g^{-1}), and 80% (0.74 mg g^{-1}) ASWD (Fig. 3D).

3.3. Total flavonoid content

Analysis of variance showed the main effects of Put concentration and two-way interaction between irrigation regime and Put concentration significantly influenced the concentration of TFC (510 nm). In addition, Put concentration significantly affected TFC (367 and 415 nm). The main effect of harvest time was to affect TFC (415 nm) (Table 1), which was higher in spring than in summer (Table 2). The highest TFC (510 nm) was obtained with the application of 2.25 mM Put under irrigation regimes of 20%, 40%, 60%, and 80% ASWD (Fig. 4A). There were significant differences between TFC (510 nm) under irrigation regimes of 40, 60, and 80% ASWD and the irrigation regime of 20% ASWD with the application of 0.75 mM Put. Differences were also observed between TFC (510 nm) under irrigation regimes of 80% and 20% ASWD with the application of 2.25 mM Put (Fig. 4B). Generally, the highest TFC (510 nm) (0.66 mg g^{-1}) was obtained with the application of 2.25 mM Put under the irrigation regime of 20%. The response of TFC (510 nm) to Put concentration in all irrigation regimes can be expressed by a linear model, suggesting TFC (510 nm) would reach maximum with increases in Put concentration. The highest TFC (510 nm) was predicted with the application of 2.25 mM Put under irrigation regimes of 20% (0.65 mg g^{-1}), 40% (0.62 mg g^{-1}), 60% (0.62 mg g^{-1}), and 80% (0.60 mg g^{-1}) ASWD (Fig. 4C). The highest TFCs (415 and 367 nm) were obtained with the application of 2.25 mM Put (0.36 mg g^{-1} and 0.16 mg g^{-1}, respectively) (Fig. 4D and 4E). The response of TFC in wavelengths of 415 and 367 nm to



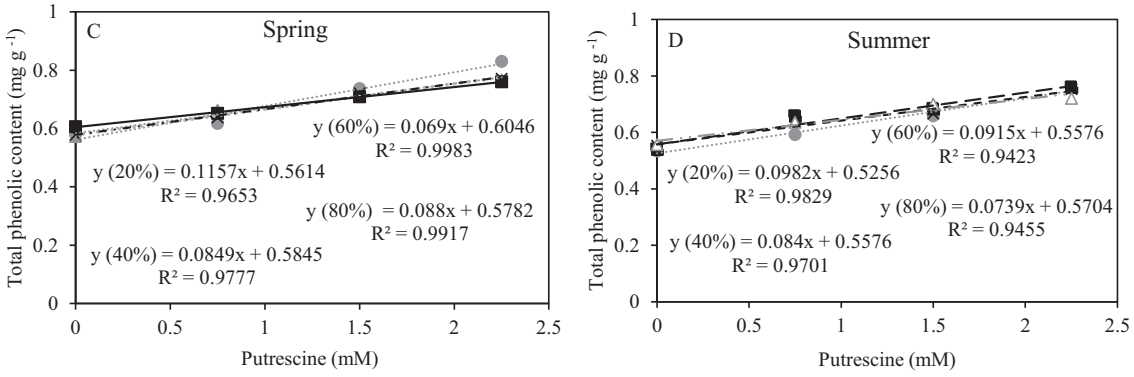


Fig. 3. Interaction between irrigation regime (Available soil water was depleted (ASWD)), putrescine and harvest time on total phenolic content (A: sliced by irrigation regime; B: sliced by harvest time). Significant linear relationship between putrescine concentrations and total phenolic content for irrigation regimes in spring (C) and summer (D). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

Put concentration can be expressed by a linear model, suggesting TFC would reach maximum with increases in the concentration of Put (Fig. 4D and 4E).

3.4. Antioxidant enzymes and H_2O_2

Analysis of variance indicated that the main effects of irrigation regime; Put concentration; and harvest time and the three-way interaction between irrigation regime, Put concentration, and harvest time significantly affected ascorbate peroxidase (APX) activity. Also, analysis of variance showed the two-way interactions between irrigation regime and Put concentration; irrigation regime and harvest time; and Put concentration and harvest time significantly influenced APX activity (Table 1). In addition, the main effect of harvest time significantly affected H_2O_2, superoxide dismutase (SOD), peroxidase (PO), and catalase (CAT) activity, which were higher in summer than in spring (Table 2). The highest APX activity levels (0.13 μmol mg^{-1} protein) were observed in spring (0.12 μmol mg^{-1} protein) and in summer (0.10 μmol mg^{-1} protein) with the application of distilled water under irrigation regimes of 20%, 40%, and 80% ASWD, respectively. Moreover, the highest APX activity (0.11 μmol mg^{-1} protein) was observed in spring with the application of 1.5 mM Put under the irrigation regime of 60% ASWD (Fig. 5A). The highest APX activity was observed with the application of distilled water under the irrigation regime of 20% ASWD in spring (0.13 μmol mg^{-1} protein) and summer (0.13 μmol mg^{-1} protein) (Fig. 5B). The response of APX activity to Put concentration under the irrigation regime of 20% ASWD in spring and summer can be expressed by linear model, suggesting that APX activity would reach minimum with increasing the concentration of Put. The highest APX activity was predicted by the application of distilled water under the irrigation regime of 20%

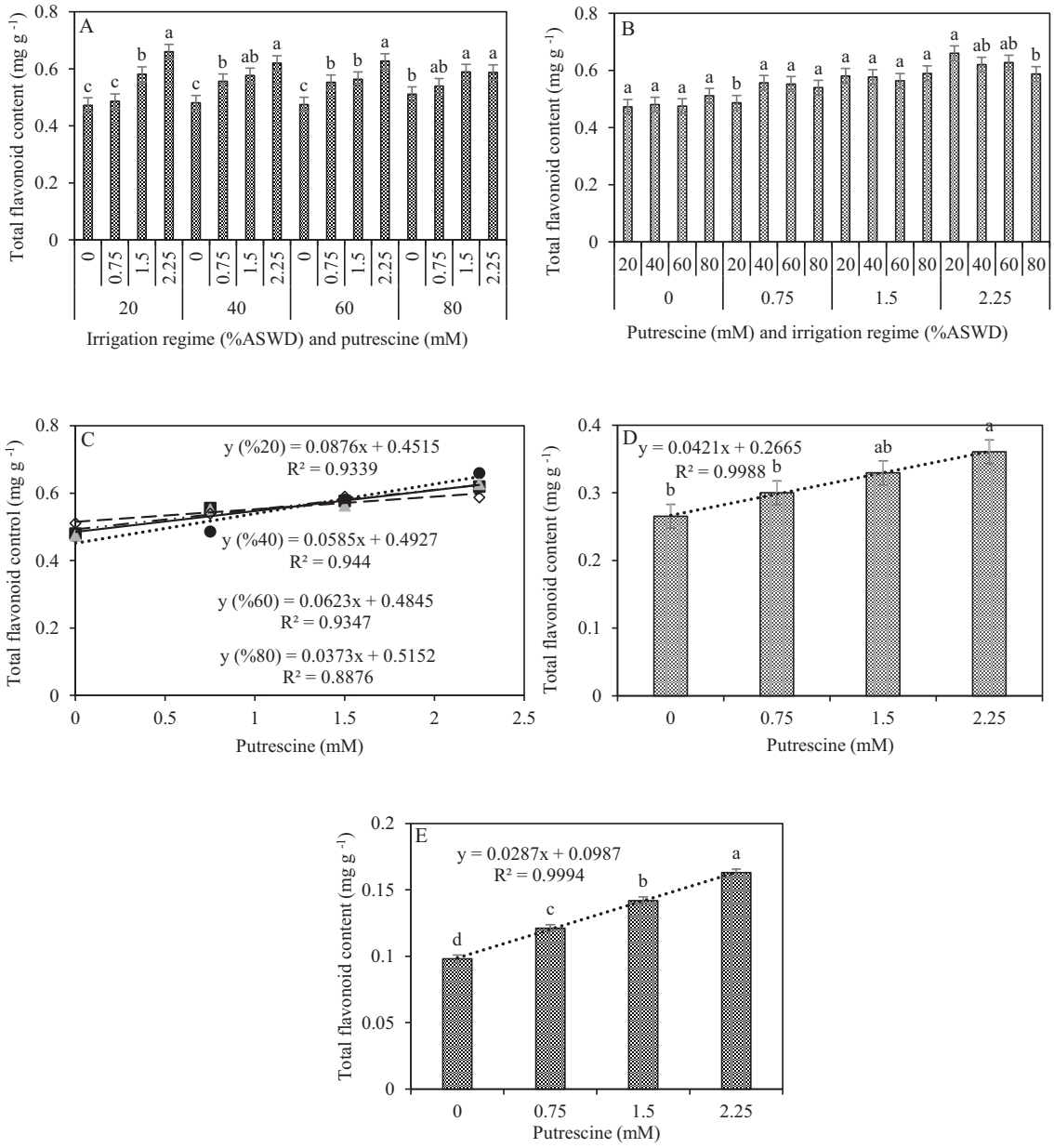


Fig. 4. Interaction between irrigation regime (Available soil water was depleted (ASWD)) and putrescine on total flavonoid content (510 nm). (A: sliced by irrigation regime; B: sliced by putrescine), Significant linear relationship between putrescine concentration and total flavonoid content (510 nm) for irrigation regimes (C), Main effect of putrescine on total flavonoid content (415 nm) (D) and Main effect of putrescine on total flavonoid content (367 nm) (E). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

ASWD in spring (0.13 μmol mg^{-1} protein) and summer (0.13 μmol mg^{-1} protein) (Fig. 6A and 6B). The response of APX activity to Put concentration under the irrigation regime of 40% ASWD in spring and summer can be expressed by quadratic and linear models, respectively, suggesting that APX activity would reach minimum with increasing concentrations of Put under the irrigation regime of 40% ASWD in summer. Based on the differentiation of a quadratic model under the irrigation regime of 40% ASWD in spring, the highest APX activity was predicted by the application of distilled water under the irrigation regime of 40% ASWD in spring (0.12 μmol mg^{-1} protein) and summer (0.09 μmol mg^{-1} protein) (Fig. 6A and 6B). The response of APX activity to Put concentration under the irrigation regime of 60% ASWD in spring can be expressed by quadratic model, and based on the differentiation of the quadratic model, the highest APX activity (0.10 μmol mg^{-1} protein) was predicted with the application of 1.29 mM Put under the irrigation regime of 60% ASWD in spring (Fig. 6A). The response of APX activity to Put concentration in the irrigation regime of 80% ASWD in summer can be expressed by quadratic model, and based on the differentiation of the quadratic model, the highest APX activity (0.10 μmol mg^{-1} protein) was predicted with the application of distilled water under the irrigation regime of 80% ASWD in summer (Fig. 6B). The response of APX activity to irrigation regime with the application of distilled water and 0.75 mM Put in spring and summer can be expressed by quadratic model. The highest APX activity was predicted under the irrigation regime of 20% ASWD with the application of distilled water (0.19 and 0.20 μmol mg^{-1} protein) and 0.75 mM Put (0.09

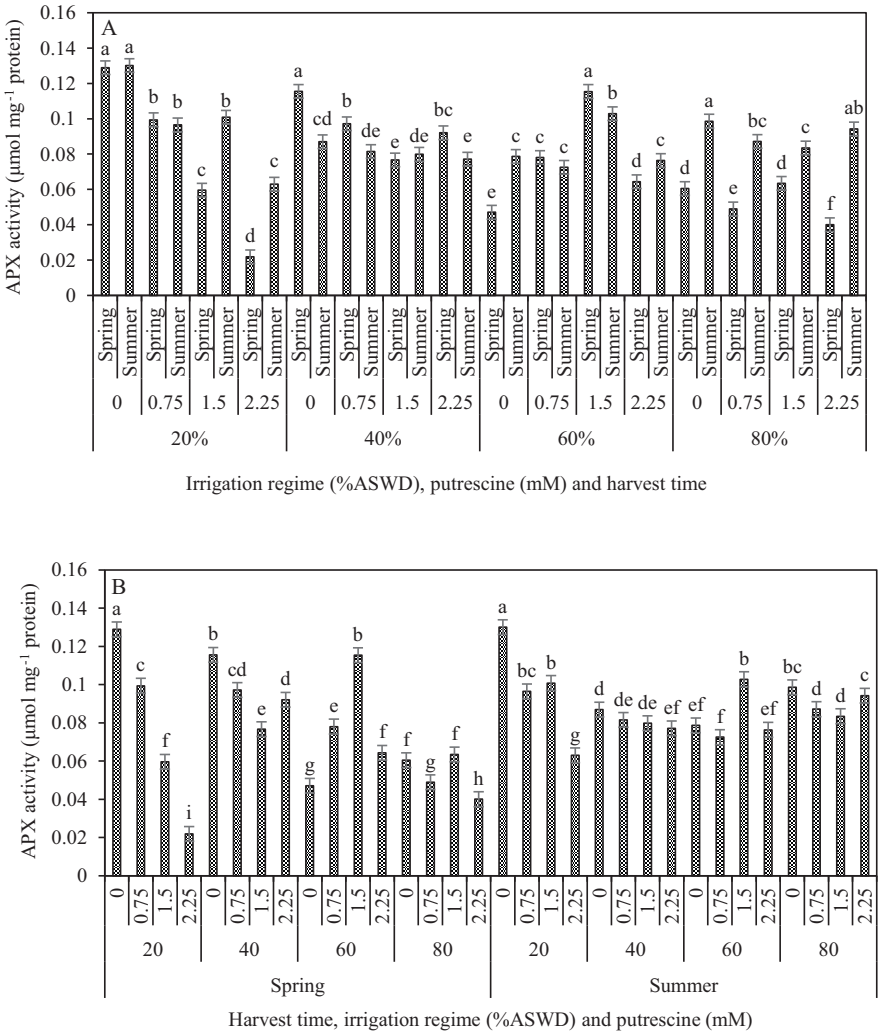


Fig. 5. Interaction between irrigation regime (Available soil water was depleted (ASWD)), putrescine and harvest time on ascorbate peroxidase activity (A: sliced by irrigation regime; B: sliced by harvest time). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

and 0.13 μmol mg^{-1} protein) in spring and summer, respectively (Fig. 6C and 6D). The response of APX activity to irrigation regime with the applications of 1.5 and 2.25 mM Put in spring can be expressed by quadratic model, suggesting that APX activity would reach maximum in irrigation regimes of 57.5% and 50% ASWD in 1.5 (0.11 μmol mg^{-1} protein) and 2.25 (0.08 μmol mg^{-1} protein) mM Put in spring, respectively (Fig. 6C). The response of APX activity to irrigation regime with the application of 2.25 mM Put in summer can be expressed by linear model, suggesting that APX activity would reach maximum with increases in the intensity of water deficit stress. The highest APX activity (0.09 μmol mg^{-1} protein) was predicted in the irrigation regime of 80% ASWD in summer (Fig. 6D).

3.5. Hydroxyl radical scavenging activity

Analysis of variance indicated that the main effects of irrigation regime and the two-way interaction between irrigation regime and harvest time significantly influenced HRSA (Table 1). HRSA was higher in summer than in spring under irrigation regimes of 20 and 80% ASWD (Fig. 7A). The highest HRSA was observed under the irrigation regimes of 40% ASWD in spring (55.76%) and 20% ASWD (63.97%) in summer (Fig. 7B). HRSA response to irrigation regime in spring and summer can be expressed by quadratic model, suggesting that it would reach maximum (49.27% and 64.15%) under irrigation regimes of 44.34% and 20% ASWD in spring and summer, respectively (Fig. 7C).

3.6. Relative water content, electrolyte leakage, and endogenous putrescine

Analysis of variance indicated that harvest time significantly affected the RWC, EL and endogenous Put. Also, the main effect of irrigation regime significantly influenced EL (Table 1). RWC, EL, and endogenous Put were higher in spring than in summer (Table 2). The response of RWC to the application of Put can be expressed by linear model, suggesting that RWC would reach minimum with increasing concentrations of Put. The highest RWC (83.06%) was predicted with the application of distilled water (Fig. 8A). The response of RWC to irrigation regime can be expressed by quadratic model, suggesting that RWC would reach maximum (83.72%) under the irrigation regime of 15.69% ASWD (Fig. 8B). There were significant differences between EL under irrigation regimes of 40%, 60%, and 80% ASWD and the irrigation regime of 20% ASWD. The response of EL to irrigation regime can be expressed by quadratic model, suggesting that EL would reach

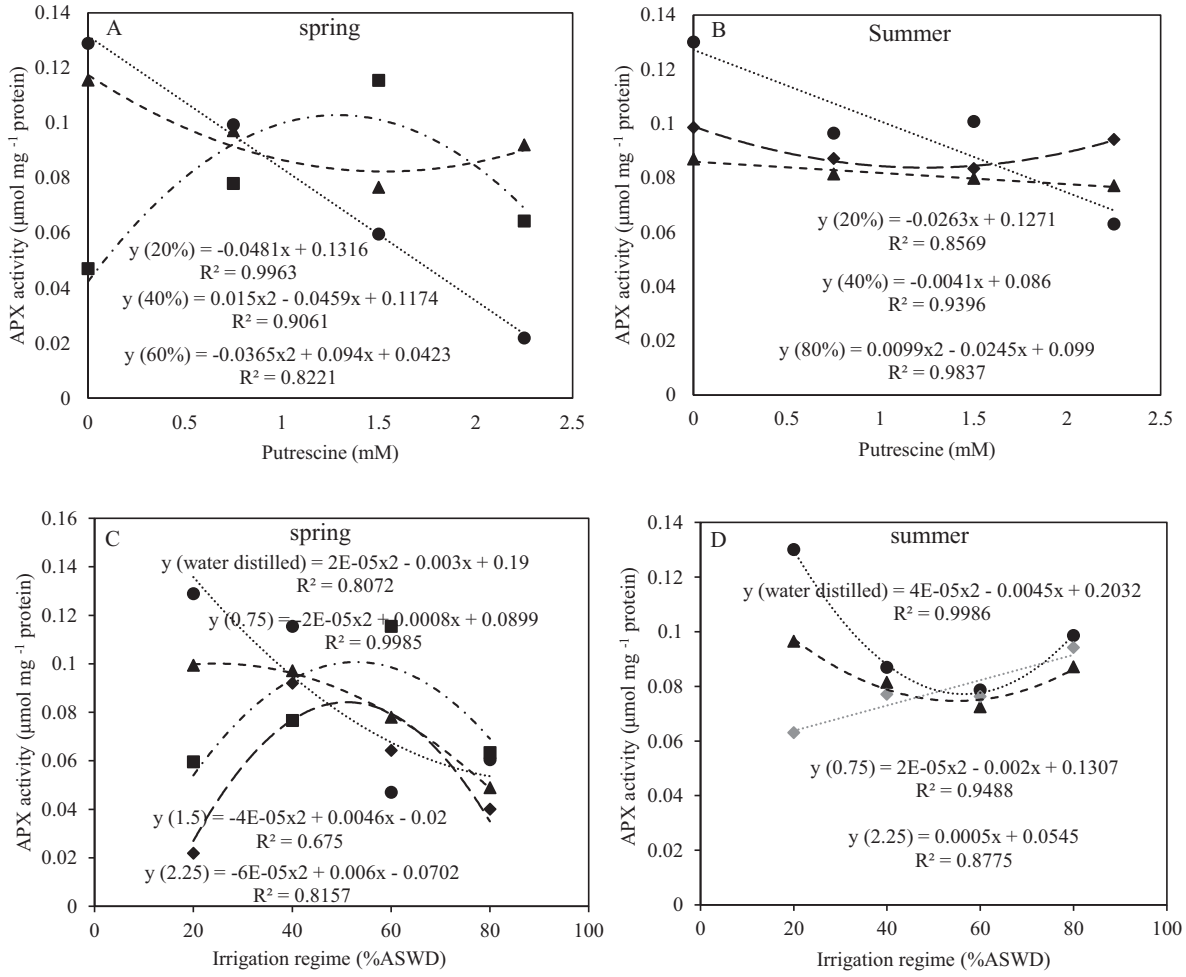


Fig. 6. Significant linear and quadratic relationship between putrescine concentrations and ascorbate peroxidase activity for irrigation regimes (Available soil water was depleted (ASWD)) in spring (A) and summer (B), Significant linear and quadratic relationship between irrigation regime and ascorbate peroxidase activity for putrescine concentration in spring (C) and summer (D). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

maximum with increasing intensities of water deficit stress. The highest EL (42.58%) was predicted by the irrigation regime of 80.88% ASWD (Fig. 8C). The response of endogenous Put to the application of Put can be expressed by quadratic model, suggesting that endogenous Put would reach maximum (190.45 nmol g^{-1} FW) with the application of 2.25 mM Put (Fig. 8D).

3.7. Proline, total reducing sugars, xylose, and mannose

Analysis of variance indicated that the main effect of harvest time significantly influenced proline (Table 1), which was higher in summer than in spring (Table 2). The response of proline, TRS, xylose, and mannose to irrigation regime can be expressed by quadratic model, suggesting that proline, TRS, xylose, and mannose would reach maximum (0.24 mg g^{-1}, 0.37 mg g^{-1}, 0.40 mg g^{-1} and 0.37 mg g^{-1}, respectively) under irrigation regimes of 68.33%, 56.25%, 48.33% and 53.75% ASWD, respectively (Fig. 9A, 9B, 9C and 9D).

4. Discussion

The highest leaf dry weight and stem dry weight of *S. officinalis* were produced in potential yield conditions without water stress with the irrigation regime of 20% ASWD. There was a decreasing trend in leaf and stem dry weights with increasing intensity of water deficit stress. Similar to the current results, Nowak et al. (2010) reported that biomass decreased under water deficit stress in sage. There was a positive correlation between leaf and stem dry weight ($r = +0.88$, $p < 0.01$).

TPC and TFC (510 nm) were increased significantly under the irrigation regime of 80% compared to 20%, 40%, and 60% ASWD in spring. Similar to the current results, it has been reported that environmental stresses affect the levels of essential oil, because environmental factors are key determinants for variations in essential oil content (Gouvea et al., 2012; Maik et al., 2014; Verma and Shukla, 2015). Therefore, for an obvious explanation for this event, plants under water deficit stress and 20% ASWD synthesized and accumulated the same compounds of essential oil, while some of the reduced compounds (like terpenoids, phenols, or alkaloids) potentially depend on growing conditions, such as the temperature, irrigation regime, nutrient supply and etc. (Gershenzon, 1984; Falk et al., 2007). There was an increasing trend in TPC, TFC (510 nm), TFC (415 nm) and TFC (367 nm) with increases in Put concentration in the irrigation regimes of 20, 40, 60, and 80% ASWD, which Put affects the increases in TPC, TFC (510 nm), TFC (415 nm), and TFC (367 nm) in sage. On the other hand, TPC and TFC (510 nm) were significantly increased under the irrigation regime of 20% ASWD than the irrigation regimes of 40%, 60%, and 80% ASWD with the application of Put. Generally, the highest TPC was obtained with the application of 2.25 mM Put under the irrigation regime of 20%

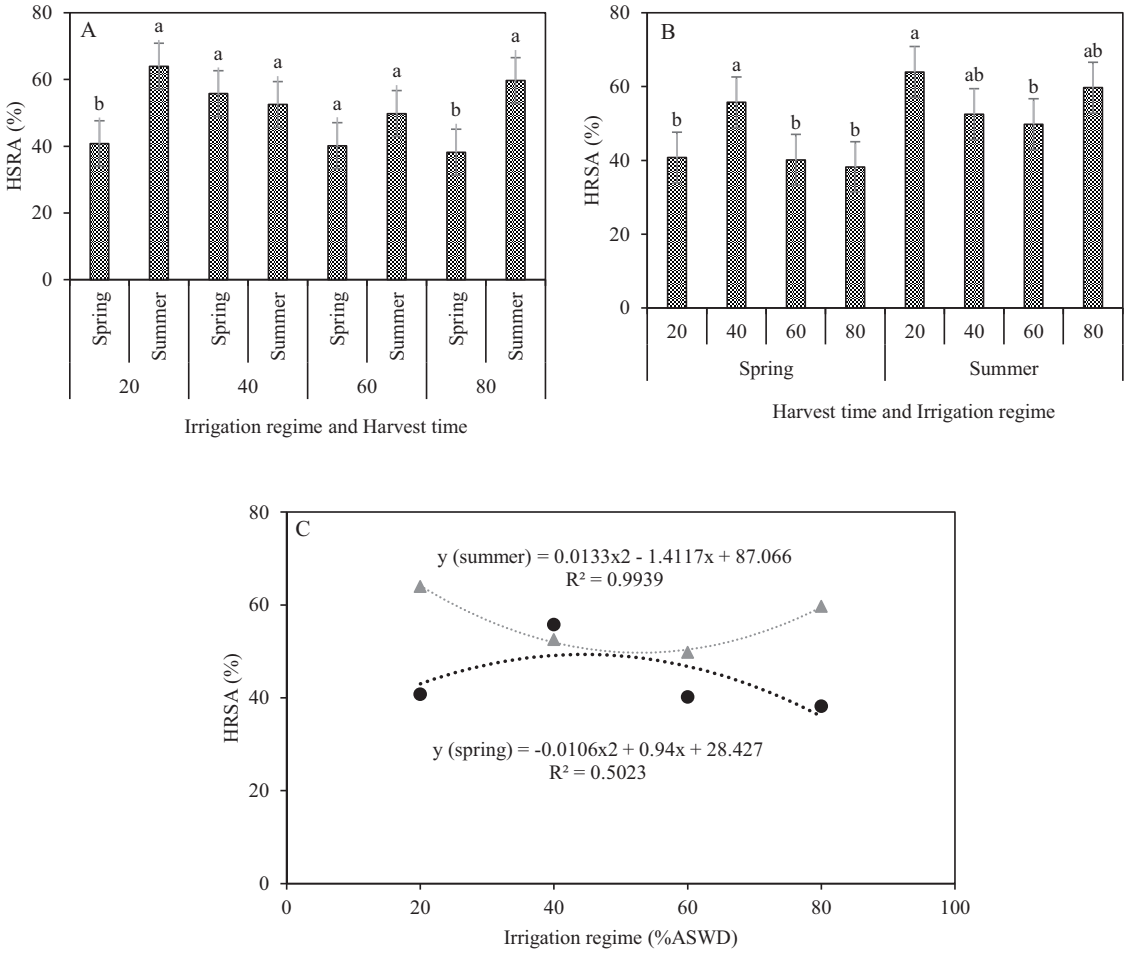


Fig. 7. Interaction between irrigation regime (Available soil water was depleted (ASWD)) and harvest time on hydroxyl radical scavenging activity (A: sliced by irrigation regime; B: sliced by harvest time) and significant quadratic relationship between irrigation regime and hydroxyl radical scavenging activity for harvest time (C). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

ASWD in spring. There was a negative correlation between TFC (415 nm) and SOD, CAT, and PO ($r = -0.36$, $p < 0.05$; $r = -0.54$, $p < 0.01$; and $r = -0.50$, $p < 0.01$, respectively). There was a positive correlation between H_2O_2 and SOD ($r = 0.78$, $p < 0.01$), PO ($r = 0.99$, $p < 0.01$), and CAT ($r = 0.96$, $p < 0.01$). Indeed, antioxidant enzymes (SOD, PO and CAT) were increased with increases in H_2O_2. One of the responses of plants to oxidative damage is the formation of a connection with antioxidant enzymes for balance between the production and elimination of ROS (Miller et al., 2010; de Carvalho, 2013). The highest APX was predicted under irrigation regimes of 20% and 40% ASWD with the application of distilled water in spring and summer. Also, the highest APX was predicted under irrigation regimes of 57.5% and 50% ASWD with the application of 1.5 and 2.25 mM Put in spring, respectively. The highest APX was predicted with the application of 1.29 mM Put under the irrigation regime of 60% ASWD in spring. On the other hand, the highest APX was predicted under the irrigation regime of 80% ASWD with the application of 2.25 mM Put in summer. It may be that the greater accumulation of phenolic and flavonoid in sage with the application of Put occurred to prevent massive generation of ROS. Generally, with increases in endogenous PAs, their catabolism also increases as well as H_2O_2 and ROS levels and antioxidant systems (Minocha et al., 2014). Indeed, PAs are regulators of redox homeostasis that play a double role in plants under oxidative stress (Saha et al., 2015). PAs biosynthesize, signaling nitric oxide (NO) and H_2O_2. It is likely that the nitrogen (N) of Put is the acceptor of singlet oxygen and produced NO in chloroplasts. NO reacts quickly with superoxide anion to generate peroxynitrite (Lindermayr et al., 2005; Ahmad et al., 2012). Moreover, flavonoids are secondary metabolites and, as reported, when toxic free radicals are transferred diffused from chloroplast and transported into vacuole, they are neutralized by flavonoids, thereby preventing damage to key cellular structures and their functions (Fini et al., 2011). The main effect of harvest time on TFC (415 nm), leaf dry weight, and stem dry weight were higher in spring and summer, respectively. Furthermore, a negative correlation between TFC (415 nm) and leaf dry weight ($r = -0.53$, $p < 0.01$) and stem dry weight ($r = -0.52$, $p < 0.01$) could be attributed to inter- and intra-seasonal weather fluctuations resulting from the tested years and harvest times over both years. precipitation was higher, approximately 0.64 mm more, in spring than in summer, while temperatures were lower (nearly 13.47 °C) (Fig. 1). It is probable that better growth and establishment of sage for the increasing of secondary compounds (like TFC) occur in spring rather than in summer. Based on the current results, Put could significantly increase antioxidant enzymes like APX for neutralizing oxidative injury through inhibition of the production of superoxide and hydrogen peroxide under water deficit stress in spring. Conversely, there was a significantly negative cor-

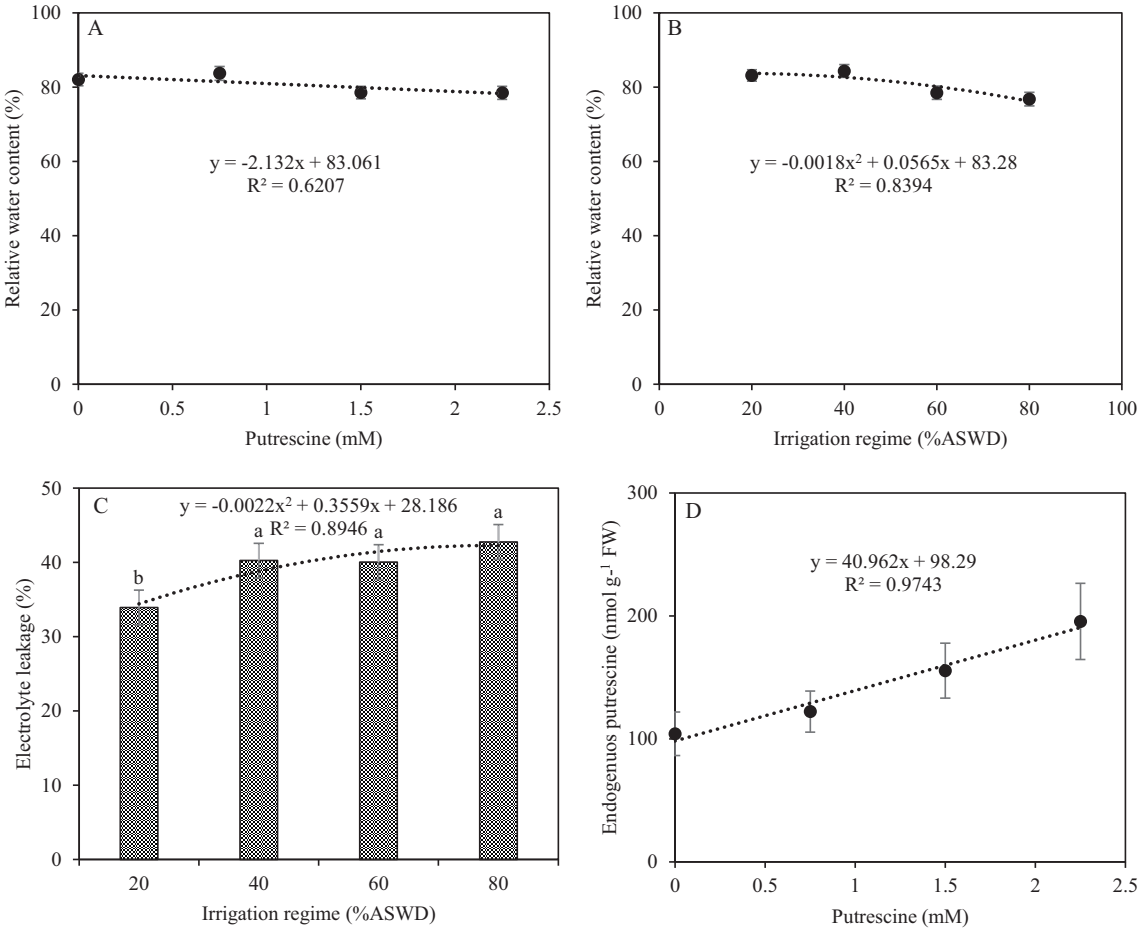


Fig. 8. Significant linear relationship between putrescine concentration and relative water content (A), Main effect of irrigation regime (Available soil water was depleted (ASWD)) on relative water content (B), Main effect of irrigation regime on electrolyte leakage (C) and Main effect of irrigation regime on endogenous putrescine (D). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

relation between APX and TPC, TFC (510 nm), TFC (415 nm), and TFC (367 nm) (r = −0.49, $p < 0.01$; r = −0.49, $p < 0.01$; r = −0.44, $p < 0.05$; and r = −0.39, $p < 0.05$, respectively). Indeed, TPC and TFC were increased, but APX was decreased with increasing the Put concentration under the irrigation regime of 20% SAW. The highest HRSA was obtained under irrigation regimes of 49.27% and 20% SAW in spring and summer, respectively. Probably, HRSA was increased to suppress ROS compounds under water deficit stress. There was an increasing trend in endogenous Put with increases in the foliar application of Put. The current results are in agreement with those of Tajti et al. (2018).

There was a decreasing trend in RWC with increasing intensity of water deficit stress and Put concentration. Our results corroborates findings of Ebeed et al. (2017). There was a negative correlation between H_2O_2 and RWC (r = −0.81, $p < 0.01$); indeed, increasing H_2O_2 led to a reduction in RWC. EL was increased more under water deficit stress than with 20% ASWD. This ionic interaction regulates the structure, function, and synthesis of lipids that are main components of cell membranes (Bouchereau et al., 1999). However, there was a significant positive relationship between H_2O_2 and EL (r = 0.44, $p < 0.05$). Indeed, H_2O_2 led to electrolyte leakage through increasing lipid peroxidation (Ahmed et al., 2009). There was also a significant negative correlation between El and leaf and stem dry weight (r = −0.50, $p < 0.01$; r = −0.49, $p < 0.01$, respectively).

Regression results showed that an increasing trend in TRS, xylose, mannose, and proline was obtained with increasing intensity of water deficit stress. These results are in agreement with those of Smeekens and Hellmann (2014). Proline and Put are connected through the activity of diamine oxidase and g-aminobutyric acid metabolism (Aziz et al., 1998). The presence of a higher concentration of proline could be indirect evidence of Put in the defense mechanism against water deficit stress. Moreover, proline protects the thylakoid membrane of chloroplast under abiotic stress (Alia and Mohanty, 1997). There was a positive correlation between proline and SOD, PO, CAT, HRSA, and H_2O_2 (r = 0.65, $p < 0.01$; r = 0.67, $p < 0.01$; r = 0.65, $p < 0.01$; r = 0.35, $p < 0.05$; and r = 0.68, $p < 0.01$, respectively). Increasing compatible osmolality is the first defense response. Proline probably protects cellular structures and function by maintaining water content and scavenging ROS. In addition to its direct effect on ROS scavenging, proline can fix and preserve the enzymes involved in ROS scavenging and can also turn on alternating ROS-detoxifying pathways (Hamilton and Heckathorn, 2001). However, there were negative correlations between APX and TFC (415 nm) and proline (r = −0.49, $p < 0.01$; and r = −0.44, $p < 0.05$, respectively). In fact, more plant energy is used to produce compatible osmolytes than to produce TFC and APX under water deficit stress.

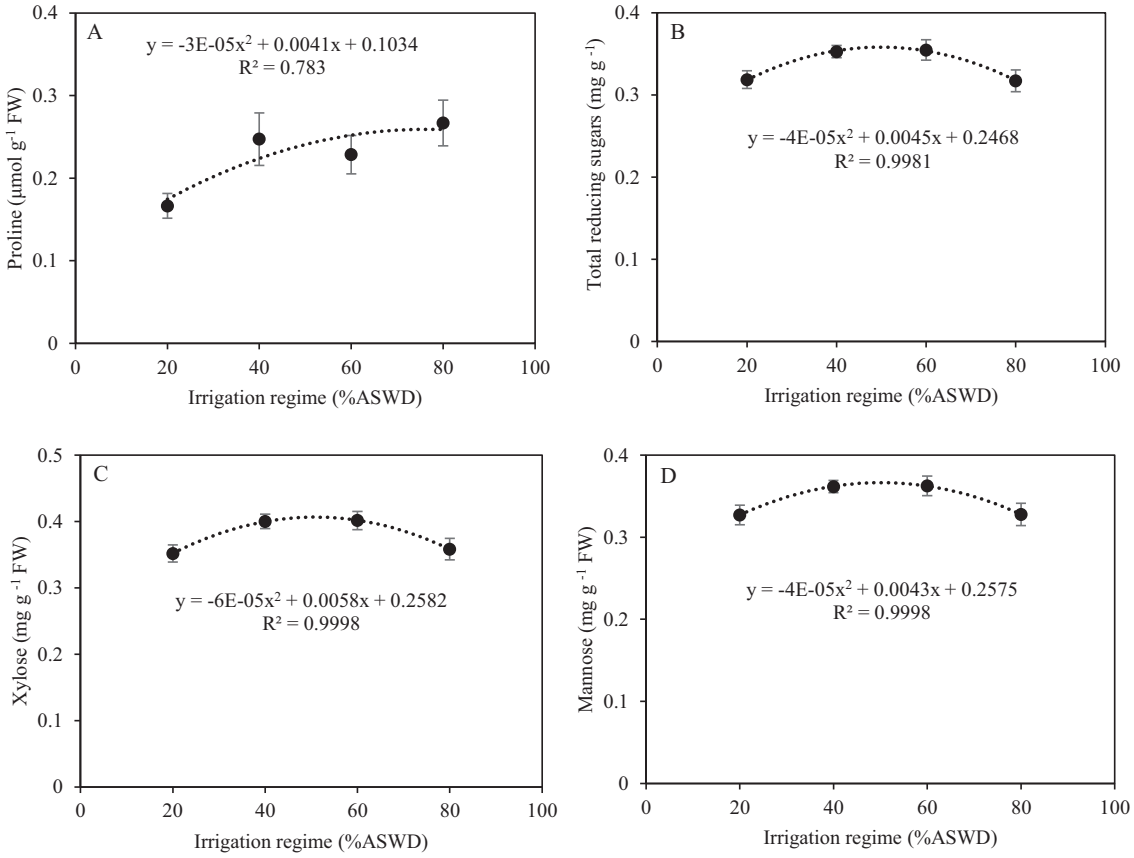


Fig. 9. Significant quadratic relationship between irrigation regime (Available soil water was depleted (ASWD)) and proline (A), Significant quadratic relationship between irrigation regime and total reducing sugars (B), Significant quadratic relationship between irrigation regime and xylose (C) and significant quadratic relationship between irrigation regime and mannose (D). The different letters show significantly different at the level of 0.05. The error bars represent standard error.

5. Conclusion

Increasing trends were observed in endogenous Put, TPC, and TFC with increasing concentrations of Put; the highest concentrations of these compounds were obtained with the application of 2.25 mM Put. APX activity was increased by the application of Put under water deficit stress during spring season. The highest HRSA was obtained under the irrigation regimes of 49.27% and 20% SAW in spring and summer. A decreasing trend was observed in RWC when Put concentration and the intensity of water deficit stress were increased. EL and proline, however, were increased under water deficit stress. Generally, soluble sugars and proline up-regulate the ROS scavenging mechanisms, leading to the protection of antioxidant enzymes activity as well as maintaining their cellular redox state and their beneficial role in preventing osmotic and ionic stress. In general, the important achievement of this study is the determination of the effect of Put on increasing TPC and TFC as well as APX with the application of Put under water deficit stress in sage. Therefore, applying Put at higher levels could be suggested as an important strategy for improving the phenolic compounds of sage.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors wish to extend their gratitude and appreciation to Tarbiat Modares University and Medicinal Plants Research Division of Research Institute of Forests and Rangelands (RIFR).

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