Improving water deficit tolerance of *Salvia officinalis* L. using putrescine

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Research Article

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Abstract

To study the effects of foliar application of putrescine (distilled water (0), 0.75, 1.5, and 2.25 mM) and water deficit stress (20%, 40%, 60%, and 80% available soil water depletion (ASWD)) on the physiological, biochemical, and molecular attributes of sage, a factorial experiment was performed in a completely randomized design with three replications in the growth chamber. The results of qRT-PCR analysis showed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced cineole synthase, sabinene synthase, and bornyl diphosphate synthase relative expression. The highest concentration of 1,8-cineole, camphor, α-thujone, β-thujone, cineole synthase, sabinene synthase, and bornyl diphosphate synthase were obtained in the irrigation regime of 80% ASWD with the application of 0.75 mM putrescine. There was high correlation between expression levels of the main monoterpenes synthase and the concentration of main monoterpenes. The observed correlation between the two enzyme activities of APX and CAT strongly suggests they have coordinated action. On the other hand, the highest PO and SOD concentrations were obtained with the application of 0.75 mM putrescine under the irrigation regime of 40% ASWD. Putrescine showed a significant increase in LAI and RWC under water deficit stress. There was an increasing trend in endogenous putrescine when putrescine concentration was increased in all irrigation regimes. Overall, the results suggest that putrescine may act directly as a stress-protecting compound and reduced H_2O_2 to moderate the capacity of the antioxidative system, maintain the membrane stability, and increase secondary metabolites under water deficit stress.

Introduction

Polyamines are considered plant growth regulators with multiple functions and are implicated in many processes of plant growth and development, such as cell division and DNA replication[1]. The most abundant PAs in plants are putrescine, spermidine, and spermine. Several studies have reported on the protective effects of high endogenous levels due to either exogenous PAs treatment or transgenic modification in plant species under various stress conditions[2, 3]. Polyamines participate in plant stress responses under various adverse environmental conditions, including water deficit stress. These responses might be due to their ability to adjust osmosis[4] and scavenge free radicals[5]. According to some reports, it can be said that PAs are key compounds in signaling. Furthermore, they contribute to interactions with endogenous plant hormones and influence several defense mechanisms[6-8]. Exogenous PAs help to improve the function and activation of antioxidant enzymes under water deficit stress[5, 9-10]. Water deficit stress is considered the most limiting environmental factor for the growth and yield of many plant species in arid and semi-arid regions. One way to deal with water shortages is to apply herbs that have more tolerance. Generally, 49% of medicinal and aromatic plants whose native habitat is the Mediterranean region are notably tolerant and adaptable to dry conditions[11]. In addition to their economic value, these plants are proper crops for cultivation in dry areas[12]. There is a remarkable demand for sage and various related products all over the world. Accommodating this demand requires an increase in its cultivation area under water deficit conditions, particularly in arid and semi-arid areas. *Salvia officinalis* L.

was introduced in old Latin as a medicinal plant. It is one of the best known genera due to monoterpenes as major compounds used in food and pharmaceutical industries[13].Three of the most important monoterpene synthases in *Salvia officinalis* included cineole synthase, which directly generates 1,8-cineole, and produces the first step of α-thujone and ß-thujone by Sabinene synthase and bornyl diphosphate synthase, which generates the pioneer of camphor[14-16] (Fig. 1). These compounds have potent antioxidative and anti-inflammatory effects[17]. Water deficit stress affects plants at the morphological, physiological, biochemical, and molecular level[18]. It reduces plant growth by reducing cell turgor and RWC, which in turn decreases cell elongation and development, consequently reducing leaf area[19-21], And an imbalance between antioxidant defenses and the amount of ROS leads to oxidative stress under water deficit conditions[22, 23]. Excessive accumulation of ROS can destabilize the cell membrane and cause direct damage to DNA, pigments, proteins, lipids, and other essential cellular molecules, leading to cell death and loss of biomass[24]. Enhanced antioxidant enzyme activity helps in ROS scavenging and protecting against stress[24]. SOD, PO, and CAT activity is responsible for quenching ROS. Indeed, they are usually activated when excessive ROS is generated to protect plants against oxidative damage under various types of stress[25-27]. The expression of PO genes was increased in *Tamarix hispida* and the leaves of rice cultivars under water deficit stress conditions[28-29]. As the first line of protection against ROS, SOD catalyzed the superoxide radical (O_2) to O_2 and H_2O_2; then CAT catalyzed the H_2O_2 to water and molecular oxygen[30-31]. Peroxidase expression was increased by the application of putrescine and PEG[32]. A prevalent physiological response in various plants under abiotic stress is the accumulation of proline. This response is suggested to act as a resistance to stress[33]. Proline helps to stabilize antioxidant enzymes through ROS scavenging[34]. Proline, as a component of stress signal transduction pathways, could be the nitrogen and carbon source needed for stress recovery and finally to help enhance stress tolerance[35]. Zwenger and Basu[36] reported that many factors, i.e. water deficit, nutrient, ozone, and mechanical stress, contribute to the up-adjustment of terpene synthesis genes in Arabidopsis. They also showed a positive correlation in terpene transcription under salt stress in *Arabidopsis thaliana*. The essential oil yield and compounds in plants may change in water deficit stress[15, 37-38]. Plants generally produce higher levels of secondary metabolites under water deficit stress[16]. Increases in percentages of essential oils and main compounds have been reported in both *O. basilicum* and *O. americanum* under water deficit stress[39]. Bettaieb et al.[40] reported a significant increase in essential oil concentrations in *Salvia officinalis* under water deficit stress. A study by Nowak et al.[15] determined that the total content of monoterpenes in sage is significantly increased by moderate water deficit stress. Monoterpene biosynthesis is located in plastids, and monoterpene synthases are located in chloroplasts, which represent the key enzymes in monoterpene biosynthesis[14]. Some signal transduction pathways are generally involved in the regulation of monoterpene biosynthesis in response to stress[41]. The production of secondary metabolites and gene expression levels involved in their biosynthesis are strongly correlated to growth conditions[42]. It has been suggested that signal molecules may be employed directly or indirectly in the production of plant secondary metabolites[43]. The signal components involved in monoterpene biosynthesis might therefore serve as potential allelochemicals for the induction of

monoterpenes. Researchers have found that the expression of monoterpene synthase increased with increasing concentrations of gibberellin but decreased when gibberellin biosynthesis was blocked with daminozide. Increasing concentrations of gibberellin also increased 1,8-cineole and camphor contents[44]. Methyl jasmonate and its derivatives increase the induction of volatile terpenoids in *lima bean*[45] and *Catharanthus roseus*[46]. Induction of monoterpene cyclase levels, principally those of limonene cyclase, was reported in grand fir callus tissue after the addition of chitosan to a MS solid medium[47]. The environment under which basil is grown has been shown to significantly alter the content and composition of basil oils[48-50]. A survey of the literature indicated that salicylic acid (SA) can affect antioxidant enzyme activities and cause a moderate increase in the content of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2)[51-54], which acts as a second messenger in regulating plant defense responses[55-56]. Secondary metabolite production can be directly enhanced by using deliberate water deficit stress. This enhancement can be reached by applying special irrigation regimes that are both simple and inexpensive, but this approach requires intense examination to optimize secondary metabolites[16]. In the current study, putrescine in different concentrations was employed as an elicitor to study the physiological, biochemical, and molecular changes in sage under water deficit stress. The main goal of this study was to determine the effects of water deficit stress and putrescine on the contents of three main monoterpenes in sage. It was hypothesized that the expression pattern of the genes involved in monoterpene biosynthesis pathways and their connection with increases in essential oil compounds under water deficit stress and putrescine may facilitate the production of economically valuable medicinal plants through genetic engineering techniques.

Materials And Methods

Plant material, growth conditions, and sampling

A total of 48 *Salvia officinalis* plants were sown in 48 plastic pots containing a mixture of peat moss, soil, and sand (1:1:1) under 16/8-hour light/dark conditions with a light intensity of 300 $\mu E/m^2/S^{-1}$ in a greenhouse for 30 days. The plants used are not wild and 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). A factorial experiment was performed in a completely randomized design (CRD) with three replications. Treatments included irrigation regimes and putrescine, as follows:

1. Irrigation after depletion of 20% available soil water (control)
2. Irrigation after depletion of 40% available soil water
3. Irrigation after depletion of 60% available soil water
4. Irrigation after depletion of 80% available soil water

Four concentrations of putrescine (distilled water (0), 0.75, 1.5, and 2.25 mM) were also applied. All aboveground parts of each plant were exogenously sprayed 50 cm above the head of plant. Foliar

application of putrescine was performed one week before applying irrigation regimes. To investigate proper treatments, plants must have similar masses of foliage before treatments are applied. Therefore, no water stress or putrescine were applied in the first 14 days of the growth cycle. During this period, all pots were irrigated when 20% of the available soil water was depleted (ASWD). The depth of the irrigation water was assigned based on the available soil water and calculated using the following equations:

1) $ASWD = (\theta FC - \theta PWP) \times D \times 100$

2) $Id = ASW \times \rho$

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

where FC and PWP (%) are the volumetric soil water amounts, D (cm) is the soil layer depth, Id is the irrigation depth (cm), ρ is 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 is the irrigation efficiency (%) assumed at an average of 65%[57]. The applied irrigation water was measured (based on Eq. (3)) at each irrigation[58]. Irrigation treatments were implemented based on the maximum allowable depletion (MAD) from the percentage of ASWD. Each treatment was irrigated when the available soil water reached its threshold value[57]. The applied treatments were 20%, 40%, 60%, and 80% MAD of ASWD. A TDR probe (Time-Domain Reflectometry, Model TRIME-FM, Germany) was applied to measure soil water amount at a depth of 50 cm (root zone of *Salvia officinalis*). TDR probes were taken from the first experiment to the last experiment. Sampling was conducted from the sage leaves one week after the irrigation regimes were applied. The expression rates were considerably higher in young sage leaves (first and second node) than in fully mature leaves (nodes five and up)[59]. Accordingly, only first and second node leaves were used. These samples were cleaned with ethanol and paper tissues to remove any surface contamination, immediately frozen in liquid nitrogen, and stored at −80 °C for use in measuring physiological attributes and RNA extraction.

Essential oil extraction

The shade-dried foliage of collected *Salvia officinalis* was extracted by hydro-distillation in a Clevenger device with double-distilled water. The obtained essential oil was separated from the aqueous layer using a 100 ml capacity separatory hopper (a piece of laboratory glassware used in liquid-liquid extractions). The collected surplus aqueous essential oil was dried over anhydrous sodium sulfate. After extraction, the essential oil was conserved in vials sealed at 4 °C until GC/MS evaluation.

Identification of essential oil composition (GC–MS analysis)

Gas chromatography/mass spectrometry (GC/MS) analysis was accomplished using a Thermoquest-Finnigan TRACE GC–MS instrument (Manchester, UK) provided with the same gas chromatography situation as mentioned for the GC analyses. Helium was utilized as the vector gas at a flow rate of 1.1 ml min^{-1} with a split ratio of 1:50, and ionization voltage was 70 eV. The constituents of the essential oil

were analyzed based on the retention index (RI) of the series of n-terpenes (C5h8)n and the oil on a Ph-5 column under the same chromatographic conditions. Single constituents were identified based on comparisons of their mass spectra fragmentation designs with standard ones found in the literature in Wiley libraries or with those of valid compounds[60].

RNA preparation and cDNA synthesis

Total RNAs were extracted from the sage leaves using a RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA quality and concentrations were determined using 1.0% agarose gel electrophoresis and analysis by a NanoDrop 2000/2000c Spectrophotometer (Wilmington, DE 19810 U.S.A.). Moreover, cDNA was synthesized with a SuperScript III Reverse Transcriptase reagent kit (Thermo Fisher Scientific, USA).

qRT-PCR

qRT-PCR Primers for the assayed genes were designed according to the GenBank accession (Table 1). Real-time quantitative PCR was conducted with a Corbett Model (Rotor gene 6000). The qRT-PCR reaction for target gene transcript amplification was carried out in a final volume of 25 µL containing PCR buffer, 2.5 µL MgCl2, 0.5 µL dNTP, 2.5 µL of SYBR green I (Sigma-Aldrich), 0.1 µL of Taq, 1.25 µL of each forward and reverse primer, and 2 µL diluted cDNA. The PCR reaction conditions were denaturation at 95 °C for 5 min followed by 45 cycles at 95 °C for 20 s, primer annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min. Melting curve analysis included 95 °C for 60 s, 55 °C for 90 s, and 60 °C for 1 s. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GapC2), was used as the control to account for variations in cDNA template levels[61]. All reactions were done in triplicate.

Putrescine analysis

Leaf tissue was ground under liquid nitrogen using a mortar and pestle. Putrescine extraction followed by HPLC quantification were carried out in accordance with Lütz et al.[62].

Antioxidant enzymes (SOD, PO, CAT and APX)

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[63].

To estimate superoxide dismutase activity (SOD, EC 1.15.1.1), the Bayer and Fridovich[64] 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.

Peroxidase activity (EC 1.11.1.7) was determined utilizing the method introduced by Herzog and Fahimi[65]. 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.

Catalase activity (EC.1.11.1.6) was determined using the method of Aebi[66]. 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.

To estimate ascorbate peroxidase activity (APX, EC 1.11.1.11), the Nakano and Asada[67] 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.

Measurement of DPPH and Hydrogen peroxide (H_2O_2)

The free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined spectrophotometrically as described by Hung et al.[68]. In brief, 2 mL of different extract solutions (16–500 g/ml) were mixed with 2 mL of DPPH solution. The mixture was allowed to stand for 30 min to achieve complete reaction at room temperature. Finally, the absorbance of samples was determined at 515 nm.

To calculate leaf H_2O_2[69], 100 mg fresh tissue was extracted with 5 mL trichloro acetic acid (0.1%) and then centrifuged at 10,000 rpm for 10 min. Finally, supernatant was mixed with potassium phosphate buffer (pH 7.0) and potassium iodide (KI), and absorbance was read at 390 nm.

Measurement of lipid peroxidation (MDA) and glycine betaine (GB), proline and total reducing sugars (TRS)

Fresh plant was ground into a fine powder in liquid nitrogen and homogenized with 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was then centrifuged at 12,000 rpm. Aliquots of the supernatant were mixed with 0.5% TBA (prepared in 20% TCA). First, the mixture was heated to 95 °C and then cooled on ice. The mixture was further centrifuged at 12,000 rpm. The absorbance of the supernatant was read at 532 and 600 nm. The MDA content was calculated using its absorption coefficient at 155 mM^{-1} cm^{-1}, after the non-specific absorbance was reduced to 600 nm. Finally, MDA content was expressed as nmol g^{-1} of fresh weight[70].

Glycine betaine was determined using the method of Grieve and Grattan[71], in which 5 ml of toluene-water mixture (0.5% toluene) was added to 0.1 g dried ground material. Afterwards, tubes were shaken for 24 h at 25 °C, the extract was filtered, and the volume was made to equal 100 ml. Then, 1 ml of 2N HCl

solution was added to 1 ml of filtrate, and an aliquot of 0.5 ml from this solution was mixed with 0.1 ml of potassium tri-iodide solution. After placing the mixture in an ice bath for 90 min, 4 ml of 1,2 dichloroethane was added to it. The optical density was determined spectrophotometrically at 365 nm. Proline was estimated using the method described by Bates et al.[72]. To measure total reducing sugars, the method introduced by Dubois et al.[73] was employed.

Relative water content (RWC) and leaf area index (LAI)

To calculate relative water content (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 one hour to record saturation weight (SW), and after that discs were oven dried at 75 °Ċ for 24 hours to record the dry weight (DW). Calculations were performed using the formula: RWC = (FW – DW)/ (SW – DW)[74].

LAI = Sum of leaf area (cm^2)/Ground area where the leaves were collected (cm^2)

Statistical analysis

Amplification data was analyzed using Rotor-Gene 6000 Series software (version 1.7). The threshold cycle (Ct) values of the triplicate PCRs were averaged and the relative quantification of the transcript levels was determined using the comparative Ct method [75]. The Ct value of the calibrator (the sample with the highest Ct value) was subtracted from every other sample to produce the $\Delta\Delta Ct$ value, and $2^{-\Delta\Delta Ct}$ was taken as the relative expression level for each sample. The data was subjected to analysis of variance (ANOVA) using SAS 9.3 software (SAS Institute, Cary, NC, USA). Mean comparisons were evaluated using the LSD Test at 5% probability levels. Pearson's correlation coefficients were determined using the CORR procedure.

Results

Concentrations of 1, 8-cineole, camphor, α-thujone, and β-thujone

The results of GC/MS analyses revealed that the main compounds of the essential oils were α- thujone, β-thujone, camphor, and 1,8-cineole, respectively. These compounds accounted for more than 95% of the total monoterpenes amount. Therefore, the regulation of the biosynthesis of 1,8-cineole, camphor, α-thujone, and β-thujone was investigated in this study. The ANOVA results showed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced the concentrations of 1,8-cineole, camphor, α-thujone, and β-thujone (Table 2).

The highest concentrations of 1,8-cineole, α-thujone, and β-thujone were obtained with the application of 1.5 mM putrescine under irrigation regimes of 20% and 60% ASWD (Figs. 2, 3, and 4). The highest concentration of camphor was observed with the application of 2.25 mM putrescine and distilled water under irrigation regimes of 20% and 60% ASWD, respectively (Fig. 5). The highest concentrations of 1,8-

cineole and β-thujone were observed with the application of 0.75 mM putrescine under the irrigation regime of 40% ASWD (Figs. 2 and 4). Moreover, the highest concentrations of camphor and α-thujone were achieved with the application of 0.75 and 1.5 mM putrescine under the irrigation regime of 40% ASWD (Figs. 3 and 5). Generally, the highest concentration of 1,8-cineole (17.95%), camphor (18.83%), α-thujone (45.50%), and β-thujone (10.10%) were observed in the irrigation regime of 80% ASWD with the application of 0.75 mM putrescine (Figs. 2-5).

Gene Expression Assay

Expression analysis revealed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced the main monoterpene biosynthesis, including cineole synthase, sabinene synthase and bornyl diphosphate synthase (Table 2).

The highest cineole synthase and sabinene synthase relative expression levels were obtained with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Figs. 6 and 7), and the highest bornyl diphosphate synthase relative expression was observed with the application of 2.25 mM putrescine under the irrigation regime of 20% ASWD (Fig. 8). The highest sabinene synthase and bornyl diphosphate synthase relative expression levels resulted from the application of 0.75 and 1.5 mM putrescine under the irrigation regime of 40% ASWD (Figs. 7 and 8). The highest cineole synthase relative expression level was achieved with the application of 0.75 mM putrescine under the irrigation regime of 40% ASWD (Fig. 6). The highest cineole synthase, sabinene synthase, and bornyl diphosphate synthase relative expression levels were obtained with the application of (0.75 and 1.5), 1.5, and 0.75 mM putrescine under the irrigation regime of 60% ASWD, respectively (Figs. 6-8). Generally, the highest relative expression levels of cineole synthase (18.06), sabinene synthase (18.48), and bornyl nindiphosphate synthase (11.46) resulted from the application of 0.75 mM putrescine under the irrigation regime of 80% ASWD (Figs. 6-8).

Measurement of lipid peroxidation (MDA) and glycine betaine (GB), proline and total reducing sugars (TRS)

The analysis of variance results showed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced significantly MDA, GB, proline, and TRS (Table 2).

The lowest MDA, GB, proline, and TRS contents were obtained with the application of 0.75 mM putrescine under irrigation regimes of 40%, 60%, and 80% ASWD (Figs. 9-12). Moreover, the lowest GB and TRS contents were observed with the application of 1.5 mM putrescine under irrigation regimes of 80% and 60% ASWD, respectively (Figs. 10 and 12). Generally, the lowest MDA (0.16 μmol g−1 FW), GB (0.06 mmol g-1 DW), proline (0.26 mg g-1 FW), and TRS (0.18 mg g-1 FW) contents were obtained with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Figs. 9-12).

Antioxidant enzymes (SOD, PO, CAT, and APX)

Based on the results of the analysis of variance, putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced SOD, PO, CAT, and APX levels (Table 2).

The highest SOD, PO, CAT, and APX values were obtained with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Figs. 13-16). Moreover, the highest SOD, PO, CAT, and APX values were observed with the application of 0.75 mM putrescine under the irrigation regimes of 40%, 60%, and 80% ASWD (Figs. 13-16). Furthermore, the highest CAT resulted from the application of distilled water and 1.5 mM putrescine under the irrigation regime of 80% ASWD (Fig. 15). Generally, the highest SOD (0.33 µmol mg-1 protein) and PO (3.32 µmol mg-1 protein) values were obtained with the application of 0.75 mM putrescine under the irrigation regime of 40% ASWD (Figs. 13 and 14), and the highest CAT (1.20 µmol mg-1 protein) and APX (0.11 µmol mg-1 protein) levels were obtained with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Figs. 15 and 16).

DPPH and Hydrogen peroxide (H_2O_2)

The analysis of variance results showed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced DPPH and H_2O_2 (Table 2).

The highest DPPH was obtained with the application of 1.5 and 2.25 mM putrescine under the irrigation regime of 20% ASWD (Fig. 17). The highest DPPH was also obtained by applying 0.75 mM putrescine under the irrigation regimes of 40%, 60%, and 80% (Fig. 17). The lowest H_2O_2 was observed with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Fig. 18) and by applying 0.75 mM putrescine under the irrigation regimes of 40%, 60%, and 80% ASWD (Fig. 18). Moreover, the lowest H_2O_2 resulted from the application of 1.5 mM putrescine and distilled water under the irrigation regime of 40% ASWD (Fig. 18). Generally, the highest and lowest DPPH (91.89%) and H_2O_2 (0.45 µmol g -1 FW) were observed with the application of 0.75 and 1.5 mM putrescine under the irrigation regimes of 80% and 20% ASWD, respectively (Figs. 17 and 18).

Relative water content (RWC) and leaf area index (LAI)

Based on the results of analysis of variance, putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced RWC and LAI (Table 2). The highest RWC was obtained with the application of 0.75 mM putrescine under the irrigation regimes of 40%, 60%, and 80% ASWD (Fig. 19) and with the application of 1.5 mM putrescine under irrigation regimes of 20% and 60% ASWD (Fig. 19). Generally, the highest RWC (92.93%) was observed with the application of 1.5 mM putrescine under the irrigation regime of 20% ASWD (Fig. 19).

The highest LAI was observed with the application of 0.75 mM putrescine under the irrigation regimes of 20%, 40%, 60%, and 80% ASWD (Fig. 20). The highest LAI also resulted from the application of 1.5 and 2.25 mM putrescine under the irrigation regime of 20% ASWD (Fig. 20). Generally, the highest LAI (0.54)

was shown with the application of 0.75 mM putrescine under the irrigation regime of 40% ASWD (Fig. 20).

Endogenous putrescine

Finally, to investigate whether the endogenous putrescine increased with the foliar application of putrescine, endogenous putrescine was measured. The results of analysis of variance showed that putrescine concentration, irrigation regime, and the two-way interaction between irrigation regime and putrescine concentration significantly influenced the concentration of endogenous putrescine (Table 2).

The current results revealed an increasing trend in endogenous putrescine when putrescine concentration was increased under irrigation regimes of 20%, 40%, 60%, and 80% ASWD. Moreover, the highest concentration of endogenous putrescine was obtained with the application of 2.25 mM putrescine under irrigation regimes of 20%, 40%, 60%, and 80% ASWD (Fig. 21). Generally, the highest concentration of endogenous putrescine (480.00 nmol g-1 FW) resulted from the application of 2.25 mM putrescine under the irrigation regime of 80% ASWD (Fig. 21).

Discussion

The current results showed a significant increase in compatible osmolytes (proline, TS, MDA and GB) under water deficit stress. Compatible osmolytes aid in the maintenance of turgor and stabilize macromolecular structures in response to stress[76]. The increase in sage proline indicates the important role of this osmolyte under water deficit stress. Indeed, the accumulation of proline and glycine betaine in stressed sage plants maintain membrane integrity, reduce oxidation of lipid membranes, stabilize ROS scavenging enzymes, and scavenge free radicals[77]. Generally, increases in compatible osmolytes stabilize redox potential and NAD(P)+/NAD(P)H ratio to prevent oxidative damage under stress conditions[78]. In response to water deficit stress, RWC and LAI were decreased in sage plants. Decreases in RWC subject the cell membranes to changes such as penetrability and decreased sustainability under water deficit stress[79] that probably aims to create osmotic adjustment[80]. There were negative correlations between MDA, TS, and proline and RWC (-0.52, -0.64, and -0.56; $p < 0.01$, respectively). There were also high correlations between MDA, TS, and GB and H_2O_2 (0.74, 0.68 and 0.71; $p < 0.01$, respectively). Indeed, the decrease in RWC under water deficit stress could be attributed to over-production of H_2O_2 which causes oxidative damage to lipids and increases the content of compatible osmolytes in sage. Foliar application of putrescine alleviated the detrimental effects of water deficit stress and increased LAI and RWC considerably. These results are concordant with those of Farooq et al.[5], Behnamnia et al.[81], Anjum et al.[82], and Mahesh et al.[83]. The possible effect of foliar spray is related to the fact that while in direct contact with leaf surface, PAs improved the water status of epidermal cells and underlying cells[5]. It seems that the contribution of putrescine to osmotic adjustment can be considered as a mechanism to retain RWC for better growth and productivity. Two reasons for the responses of polyamines under different adverse environmental conditions might be the ability to

scavenge ROS and adjust osmosis[5-6, 84]. In the present study, water deficit stress increased the production of H_2O_2 in plants, thus enhancing SOD, CAT, PO, and APX activities under water deficit conditions. The effectiveness of the antioxidant defense system function depends on the intensity of the water deficit stress[85,86]. The highest CAT, SOD and APX concentrations for scavenging ROS and protecting biomolecules were obtained in the 40%, 60%, and 80% ASWD irrigation regimes, respectively. This higher enzyme activity did not provide enough protection against ROS (H_2O_2). Indeed, to improve the damage oxidative stress imposes on sage, foliar applications of putrescine are required. Similar results were reported by Mahesh et al.[83] ,Li et al.[87] and Huang et al.[88]. Tajti et al.[89] achieved similar results on cadmium stress in wheat by increasing putrescine levels. Negative correlations between SOD, PO, and APX and MDA were observed (-0.66, -0.63, and -0.63; $p < 0.01$, respectively), GB (-0.66, -0.67, and -0.55; $p < 0.05$ and 0.01, respectively), and TS (-0.69, -0.75, and -0.73; $p < 0.01$, respectively). There were also negative correlations between SOD and APX and proline (-0.56 and -0.52; $p < 0.05$, respectively). The lowest compatible osmolytes and H_2O_2 and highest antioxidant enzymes including CAT and APX were obtained with the application of 1.5 mM putrescine; this showed the role of putrescine in stabilizing the cell membranes and preventing degrading of cell membranes by free radicals like H_2O_2. This response indicates a good H_2O_2 scavenging ability in the application of putrescine. Indeed, putrescine inhibits NADPH oxidase enzymes in cell walls, which ultimately leads to less H_2O_2 production in putrescine-treated plants[90-91]. The observed correlation between the two enzyme activities strongly suggests coordinated action between APX and CAT. Yiu et al.[10] reported similar results. In the present study, with the application of 0.75 mM putrescine, SOD and PO played an important role in protecting H_2O_2 under the 40% ASWD irrigation regime. On the other hand, SOD (as the first line of defense against ROS) and APX have significant negative correlations with H_2O_2 (-0.66, -0.74; $p < 0.01$, respectively).

Indeed, putrescine substantially improved the impacts of water deficit stress on the membrane stability index in sage plants by binding to the negatively-charged phospholipid head group[92]. It is well documented that PAs (e.g., putrescine) are able to induce adaptive changes to maintain plasma membrane integrity under water deficit stress. Moreover, the enzymatic antioxidant activity enhanced by putrescine seems to be the result of de novo synthesis and/or the activation of the enzymes, mediated by the transcription and/or translation of specific genes[93], that potentially aids stressed plants to resist against oxidative stress induced by water deficit stress. The overflow of electron forms (as a Mehler reaction originating from the photosynthetic electron transport chains) is caused by the enormous generation of toxic ROS radicals under water deficit stress[94]. Classical energy dissipating mechanisms for eliminating the overflow of electrons comprise non-photochemical quenching, photorespiration, and the xanthophyll cycle[31, 95-97]. Furthermore, the synthesis of secondary metabolites and biosynthesis of highly reduced compounds like isoprene are significantly involved in the dissipation of excess photosynthetic energy[98-99]. The amount of energy decomposed by isoprene emission could account for up to 25% of the net photosynthesis energy store under stress conditions[100]. Indeed, synthesis and increased secondary metabolites could be considered as the machinery to minimize the reduction equivalents. The current results showed that monoterpene concentration, monoterpene synthesis, and

H_2O_2 were increased under water deficit stress conditions with the highest contents obtained with the 80% ASWD irrigation regime. Indeed, it is well known that ROS as signal components are involved in the activation of monoterpene biosynthetic enzymes, and to some extent, oxidative bursts could induce monoterpene biosynthesis[41,43,101]. The highest levels of expression of the main monoterpene synthase and concentrations of main monoterpenes were obtained with the application of 0.75 mM putrescine and the 80% ASWD irrigation regime. On the other hand, there were positive correlations between CS, BO, and SS and 1,8-cineol, camphor, α-thujone, and β-thujone (0.98, 0.75 and 0.99/0.99; $p < 0.01$, respectively). The increase in the secondary metabolite contents in sage are due to a “passive” shift of biosynthesis as a result of an over-reduced status and an “active” up-regulation of the enzymes involved in the corresponding biosynthesis[59, 102]. The biosynthesis of terpenes is composed of two distinct paths: methylerythritol 4-phosphate (MEP) and mevalonate (MVA), occurring in the plastids and cytoplasm of plants. The MEP pathway is for the synthesis of carotenoids, isoprene, mono- and diterpenes, plant hormones [abscisic acid (ABA), gibberellins (GA)], phytol, the side chain of chlorophyll, tocopherols, phylloquinone, plastoquinones, etc[103]. The polyamines in plants are not only found in cytoplasm, but also in specified organelles like mitochondria, chloroplasts, and vacuoles[104-105]. The ratio of (1,8-cineol and cineole synthase), (camphor and bornyl diphosphate synthase) and (α- thujone, β-thujone and sabinene synthase) were obtained (3.16 and 3.65 times), (2.50 and 2.59 times), and (3.43, 4.41, and 4.04 times) with the application of 0.75 mM putrescine compared to the application of distilled water under the irrigation regime of 80% ASWD. In explaining this result, it should be said that putrescine enters the leaves by penetrating the cuticle or through the stomata before entering the plant cell, where they can be practical in metabolism and are further transported to other parts through plasmodesmata. Therefore, polyamines and monoterpenes were probably produced in the MEP pathway and essential oil content affected by putrescine. Schmiderer et al.[44] achieved similar results; they reported that the expression of monoterpene synthase and contents of 1,8-cineole and camphor were increased with the application of gibberellins. Methyl jasmonate (MJ) and salicylic acid (SA) have also been applied as abiotic elicitors to induce secondary metabolite biosynthesis as terpene metabolism in *N. sativa* and other plant species[106]. The highest amount of DPPH was observed with the application of 0.75 mM putrescine under the irrigation regime of 80% ASWD. High correlations were observed between DPPH and CS, BO, SS, 1,8-cineol, camphor, α- thujone, and β-thujone (0.64, 0.71, 0.77, 0.54, 0.55, 0.75 and 0.74; $p < 0.05, 0.01$, respectively). There were also high correlations between SOD and CS, BO, and 1,8-cineol (0.58, 0.64, and 0.55; $p < 0.05, 0.01$, respectively), between PO and BO (0.74; $p < 0.01$), and between APX and BO, SS, α-thujone, and β-thujone (0.63, 0,56, 0.52, and 0.52; $p < 0.05, 0.01$, respectively). High correlations were also observed between DPPH and SOD, PO, CAT, and APX (0.74, 0.64, 0.50, and 0.76, respectively). According to the current results, the content of compatible osmolytes and concentration of antioxidant enzymes were increased and decreased, respectively, with the application of 2.25 mM putrescine. There is evidence that SA causes a rise in the quantity of ROS in the cell, suggesting the existence of a self-induced SA H_2O_2 cycle[107]. This is not surprising, as a close correlation was recently reported between the endogenous PA and SA contents[108], which may be responsible for the negative effects of greater concentrations of putrescine. Some reports on SA have been in agreement with the current results[109-111].

The treatment of 2.25 mM putrescine resulted in an inhibitory effect compared with the 0.75 mM putrescine treatment. This is why stress-induced H_2O_2 accumulation was lower in the 0.75 mM than the 2.25 mM putrescine-treated plants. Putrescine reduced the accumulation of total monoterpenes in concentrations of 2.25 mM which, to some extent, can be attributed to excessive oxidative burst-induced putrescine in high concentrations. Indeed, there was a decreasing trend in the concentration of monoterpenes and the expression of monoterpene synthases genes with increasing putrescine concentrations under the irrigation regime of 80% ASWD; however, further studies are needed to determine whether the application of putrescine in high concentrations at the cellular level decrease monoterpenes in sage under water deficit stress conditions.

Conclusion

The current results showed a significant increase in compatible osmolytes (proline, TS, MDA, and GB) under water deficit stress conditions. Water deficit stress increased the production of H_2O_2 in plants and thus enhanced the SOD, CAT, PO, and APX activities under water deficit conditions. The results further showed that concentrations of monoterpenes and monoterpene synthases were increased under water deficit stress, the highest contents of which were obtained on the irrigation regime of 80% ASWD. Foliar application of putrescine alleviated the detrimental effects of water deficit stress and considerably increased LAI and RWC. The lowest compatible osmolyte and H_2O_2 contents and the highest antioxidant enzymes including CAT and APX were obtained with the application of 1.5 mM putrescine, which showed the role of putrescine in stabilizing cell membranes and preventing the degradation of cell membranes by free radicals like H_2O_2. This response indicates a good H_2O_2 scavenging ability in the application of putrescine. In the present study, SOD and PO in the application of 0.75 mM putrescine played important roles in protecting H_2O_2 under the irrigation regime of 40% ASWD. The highest levels of expression of the main monoterpenes synthase, concentrations of main monoterpenes, and DPPH were obtained with the application of 0.75 mM putrescine and the irrigation regime of 80% ASWD. Polyamines and monoterpenes were probably produced in the (MEP) pathway, and the essential oil content affected by putrescine. Indeed, putrescine could be a useful strategy to increase the main monoterpenes in sage plants. Because of the decreasing trend seen in the concentration of monoterpenes and the expression of monoterpene synthases genes with increases in putrescine concentration under the irrigation regime of 80%, however, further studies are needed to confirm the increase in monoterpenes by applying low concentrations of putrescine under water deficit stress conditions in sage.

Declarations

Statements on plant material

The plant material in this manuscript complies with relevant institutional, national and international guidelines and laws and can be obtained from various respective centers worldwide.

Author contributions

Maryam Mohammadi-Cheraghabadi, performed all the experiments and data analysis and wrote the article; Seyed Ali Mohammad Modarres-Sanavy and Fatemeh Sefidkon, supervised the project and provided editorial input into the writing; Sajad Rashidi-Monfared, supported and verified the analysis of qRT-PCR; Ali Mokhtassi-Bidgoli, advised compatible osmolytes and antioxidant enzymes and data analyses. All authors discussed the results and contributed to the final manuscript.

Competing interests

The authors declare no competing interests.

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Tables

Table 1. Description of reference genes and the monoterpene synthase primer sequences for qRT-PCR.

Gene	GeneBank accession number	Primer name	Primer sequence
1 ,8 cineole synthase	AF051899	CS (FW)	5-TTCAAGCACAATTTCAACAAGAG-3
		CS (RV)	5-AGCGTACCATAAATATCAAAGAC-3
Bornyl diphosphate synthase	AF051900	B-PP-S (FW)	5-TATTTCACAGCTCTTGGATTCAG-3
		B-PP-S (RV)	5-TGTAACATTCCCTTCGTATCTTG-3
sabinene synthase	AF051901	SS (FW)	5-AGGTGGTGATGAAATTGATGAAG-3
		SS (RV)	5-ATATTGAAGTTGAGTTTGGCGAG-3
Glyceraldehyde-3-phosphate dehydrogenase	JN083806.1	Gap C2 (FW)	5-CAGTGTATTGATGGATGGTATTC-3
		Gap C2 (RV)	5-CCAAACTCACTTACTTCAAACAG-3

Table 2. Analysis of variance (mean square) of lipid peroxidation (MDA), total reducing sugars (TRS), glycine betaine (GB), proline, superoxide dismutase activity (SOD), peroxidase activity (PO), catalase activity (CAT), ascorbate peroxidase activity (APX), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Hydrogen peroxide (H_2O_2), cineole synthase (CS), sabinene synthase (SS), bornyl diphosphate synthase (BO), 1,8-cineole, camphor, α-thujone, β-thujone, leaf area index (LAI), relative water content (RWC) and endogenous putrescine of *Salvia officinalis* influenced by irrigation regimes (I) and putrescine concentrations (P).

Sources of variation	I	P	I×P	Error
DF	3	3	9	32
MDA	0.11**	0.24**	0.05**	0.0005
TRS	0.004**	0.006**	0.002**	0.0001
GB	0.21**	0.64**	0.21**	0.0007
Proline	15.50**	1066.01**	5.06**	0.88
SOD	63.12**	350.47**	18.57**	0.23
PO	88.49**	435.27**	19.70**	0.35
CAT	150.71**	1025.31**	42.33**	0.48
APX	102.90**	856.03**	32.37**	0.33
DPPH	21259.03**	67051.47**	23922.83**	83.19
H_2O_2	10521.54**	325915.86**	8066.24**	184.94
CS	0.11**	0.24**	0.05**	0.0005
BO	0.004**	0.006**	0.002**	0.0001
SS	0.21**	0.64**	0.21**	0.0007
1,8-cineole	15.50**	1066.01**	5.06**	0.88
camphor	63.12**	350.47**	18.57**	0.23
α-thujone	88.49**	435.27**	19.70**	0.36
β-thujone	150.71**	1025.30**	42.33**	0.48
LAI	102.90**	856.03**	32.37**	0.33
RWC	21259.03**	67051.47**	23922.83**	83.19
Endogenous Putrescine	10521.54**	325915.86**	8066.24**	184.94

**: $\alpha \leq 0.01$

Figures

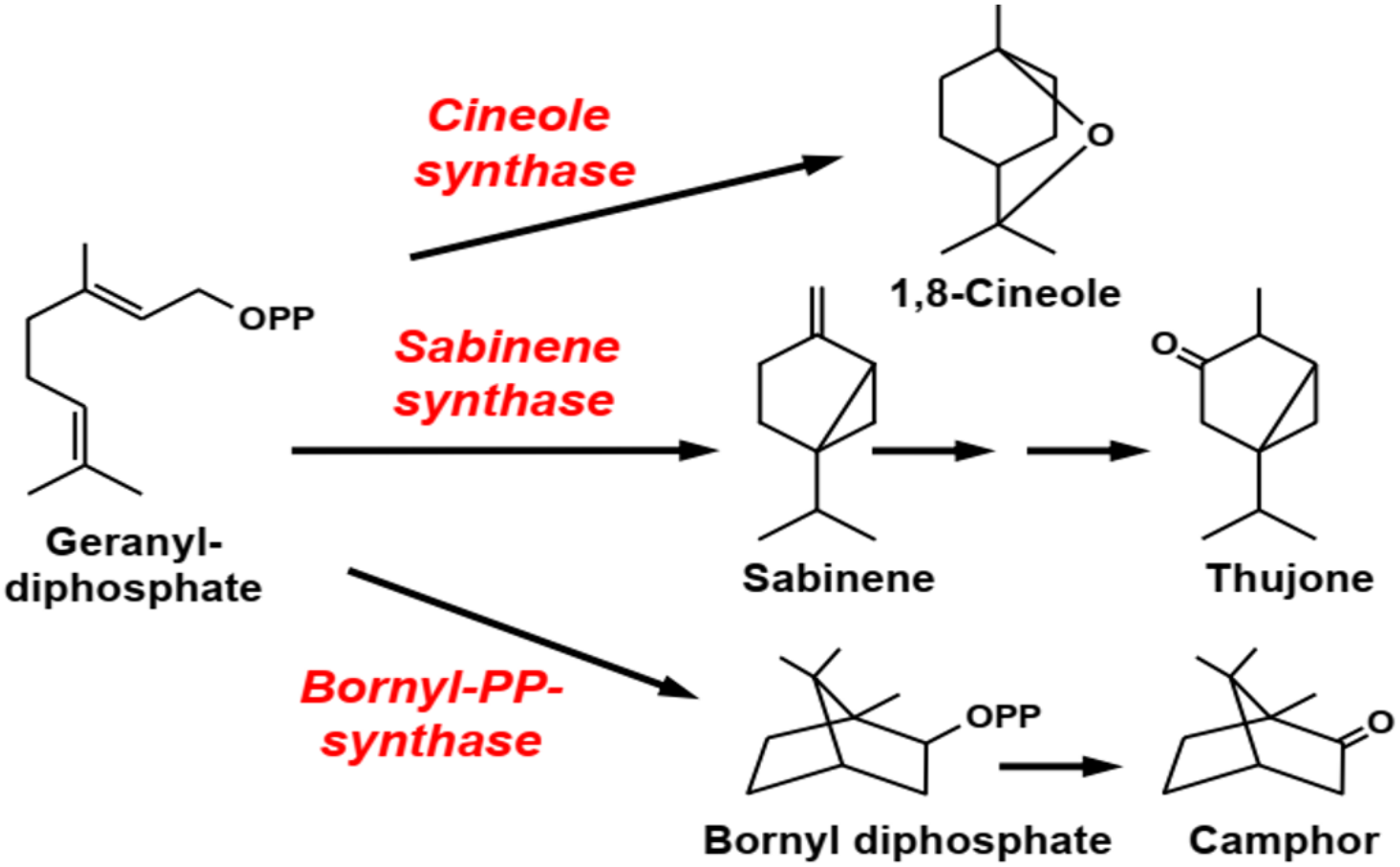

Figure 1

Three of the most important monoterpene synthases in Salvia officinalis included cineole synthase, which generates directly 1,8-cineole, produce first step of α- and ß-thujone by Sabinene synthase and the bornyl diphosphate synthase, which generates the pioneer of camphor (Wise et al., 1998; Nowak et al., 2010; Selmar and Kleinwachter, 2013).

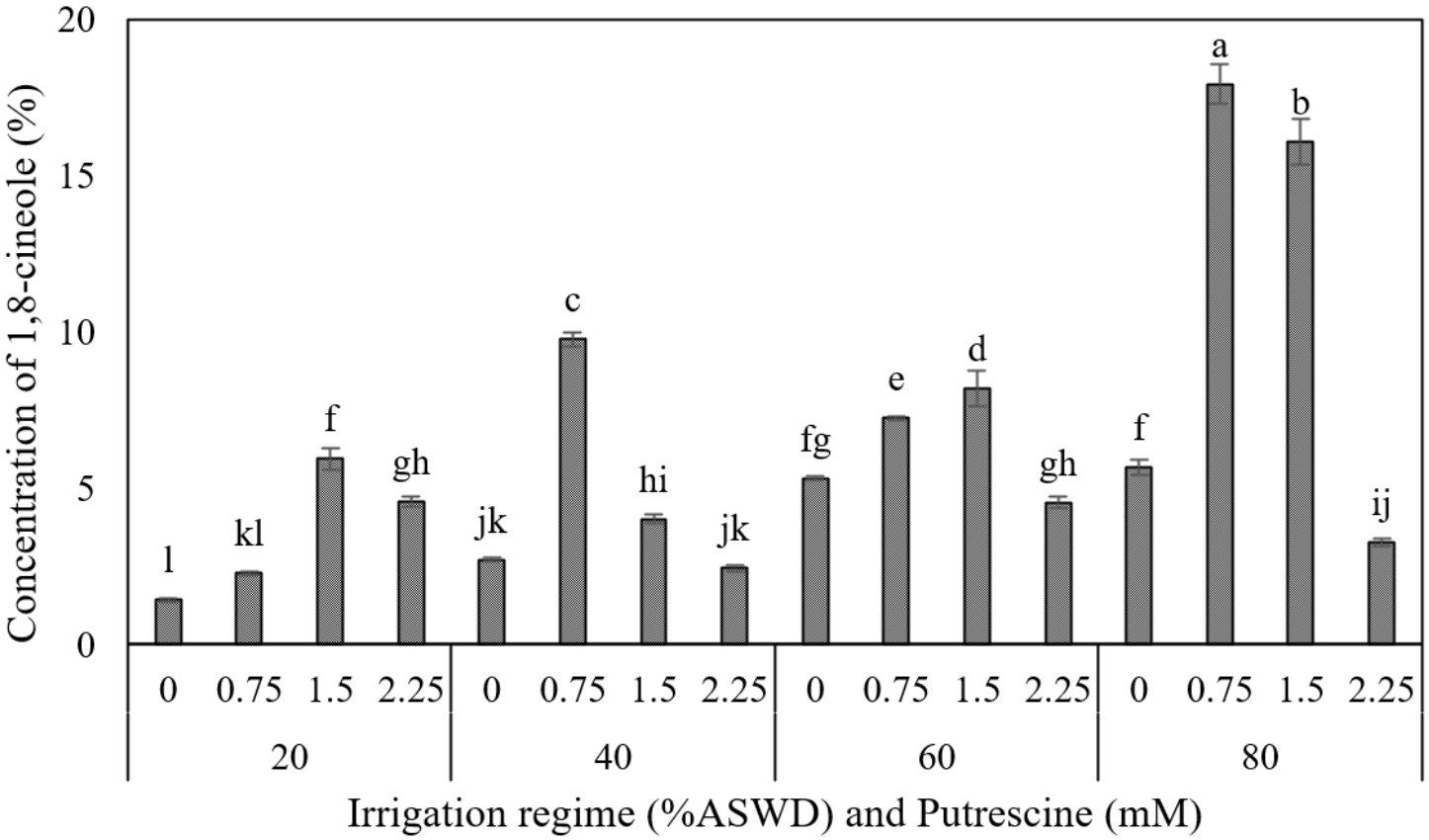

Figure 2

Interaction between irrigation regime and putrescine on concentration of 1,8-cineole. The different letters show significantly different at the level of 0.01. The error bars represent standard error. (Retention Index=1031)

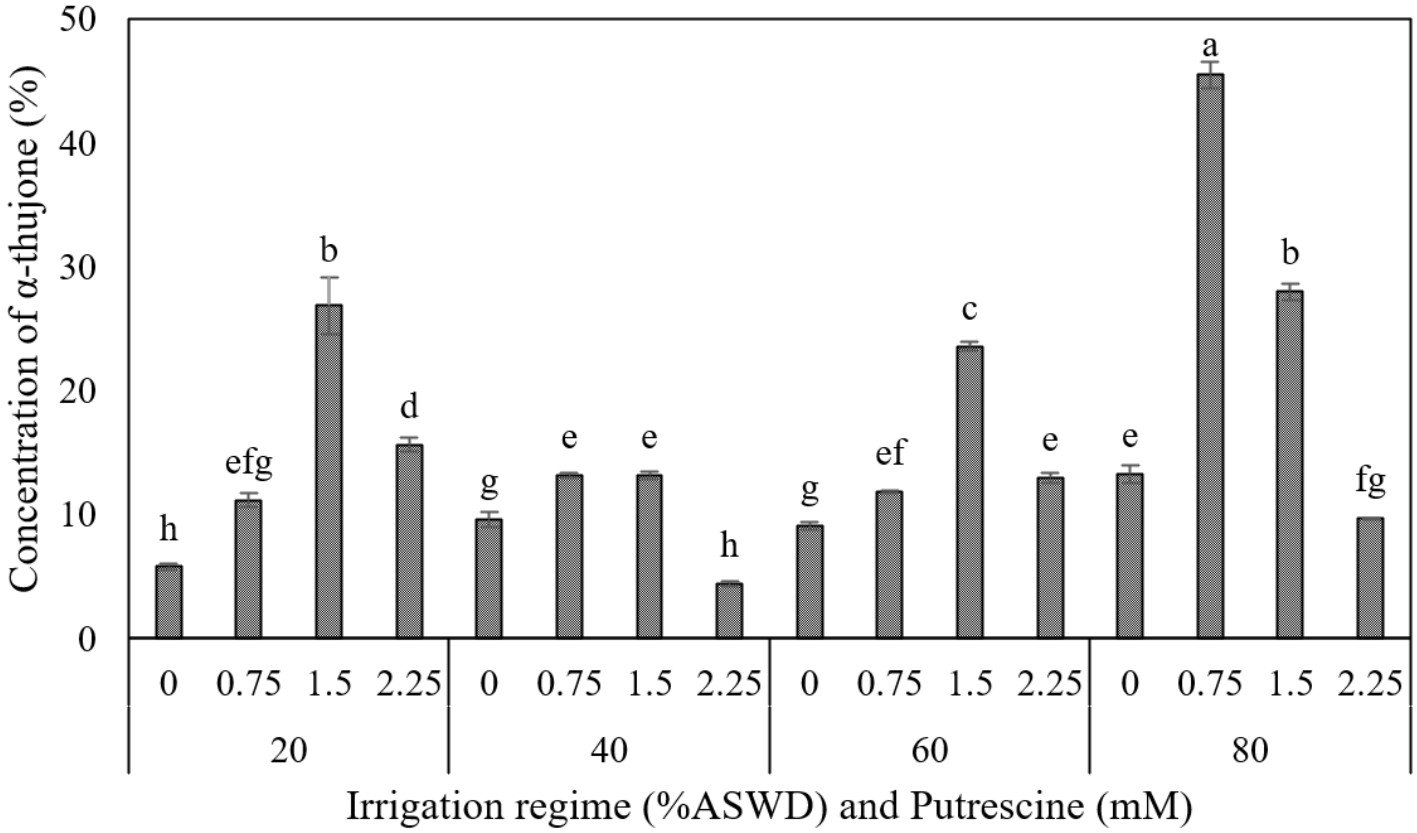

Figure 3

Interaction between irrigation regime and putrescine on concentration of α-thujone. The different letters show significantly different at the level of 0.01. The error bars represent standard error. (Retention Index = 1103)

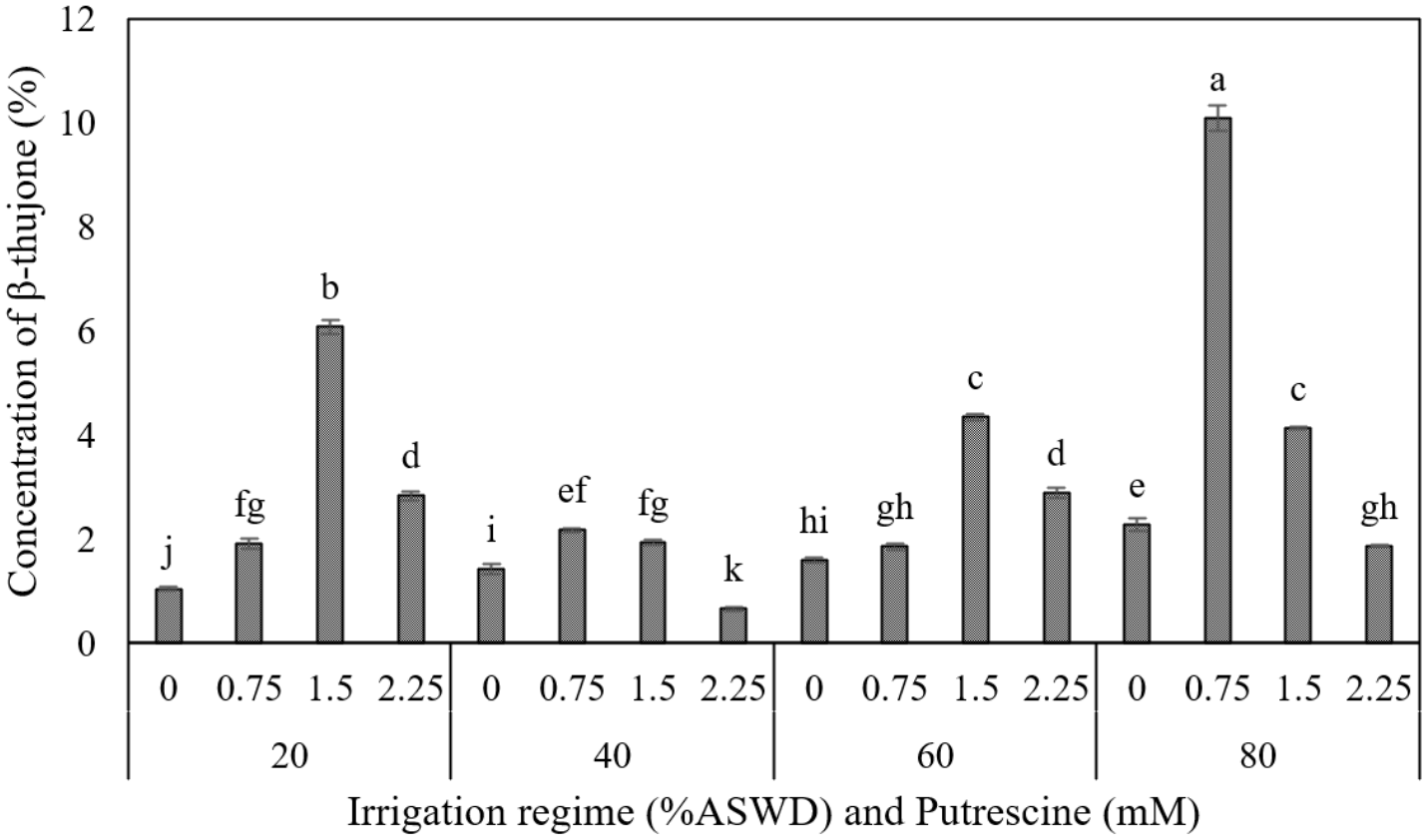

Figure 4

Interaction between irrigation regime and putrescine on concentration of β-thujone. The different letters show significantly different at the level of 0.01. The error bars represent standard error. (Retention Index = 1115)

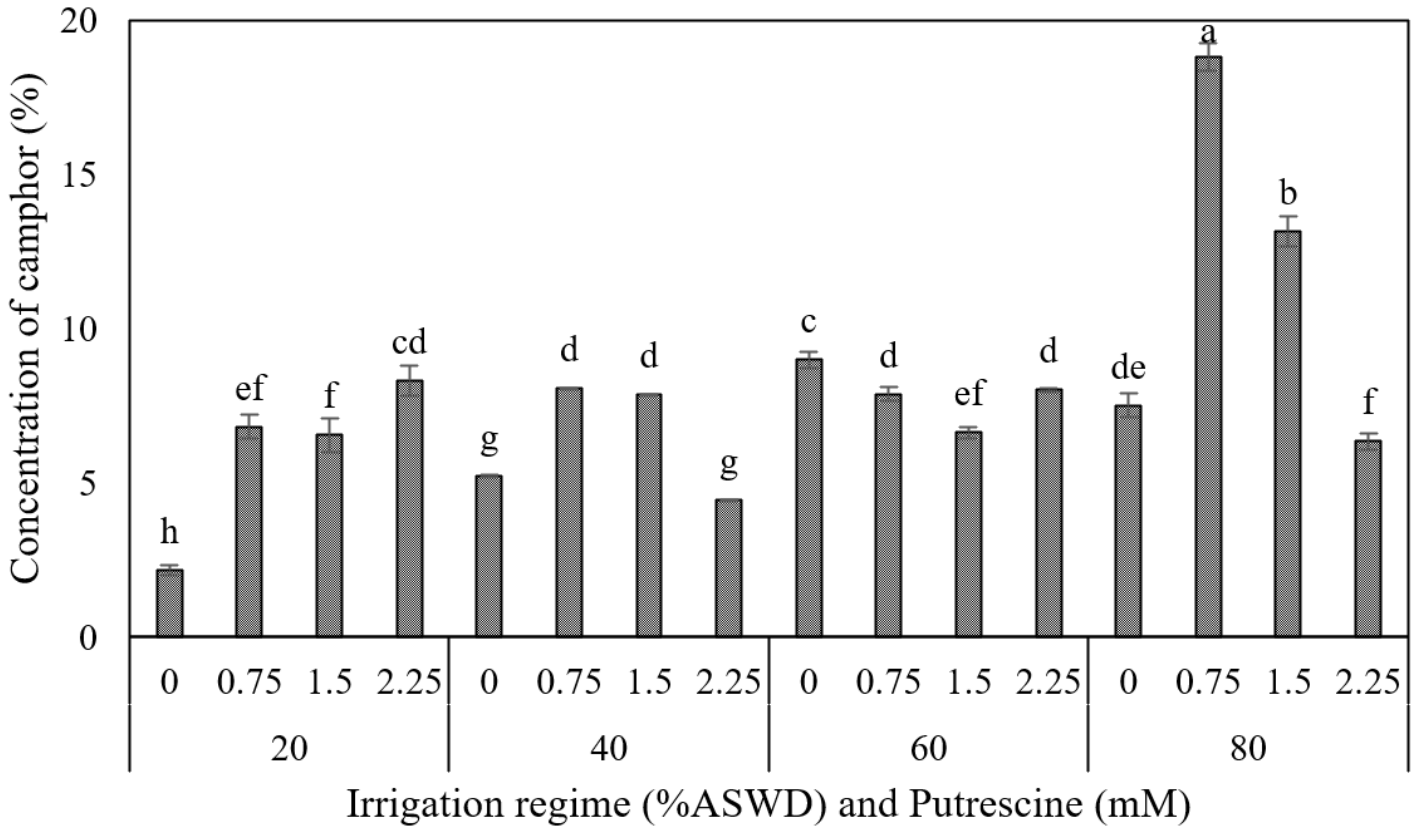

Figure 5

Interaction between irrigation regime and putrescine on concentration of camphor. The different letters show significantly different at the level of 0.01. The error bars represent standard error. (Retention Index = 1144)

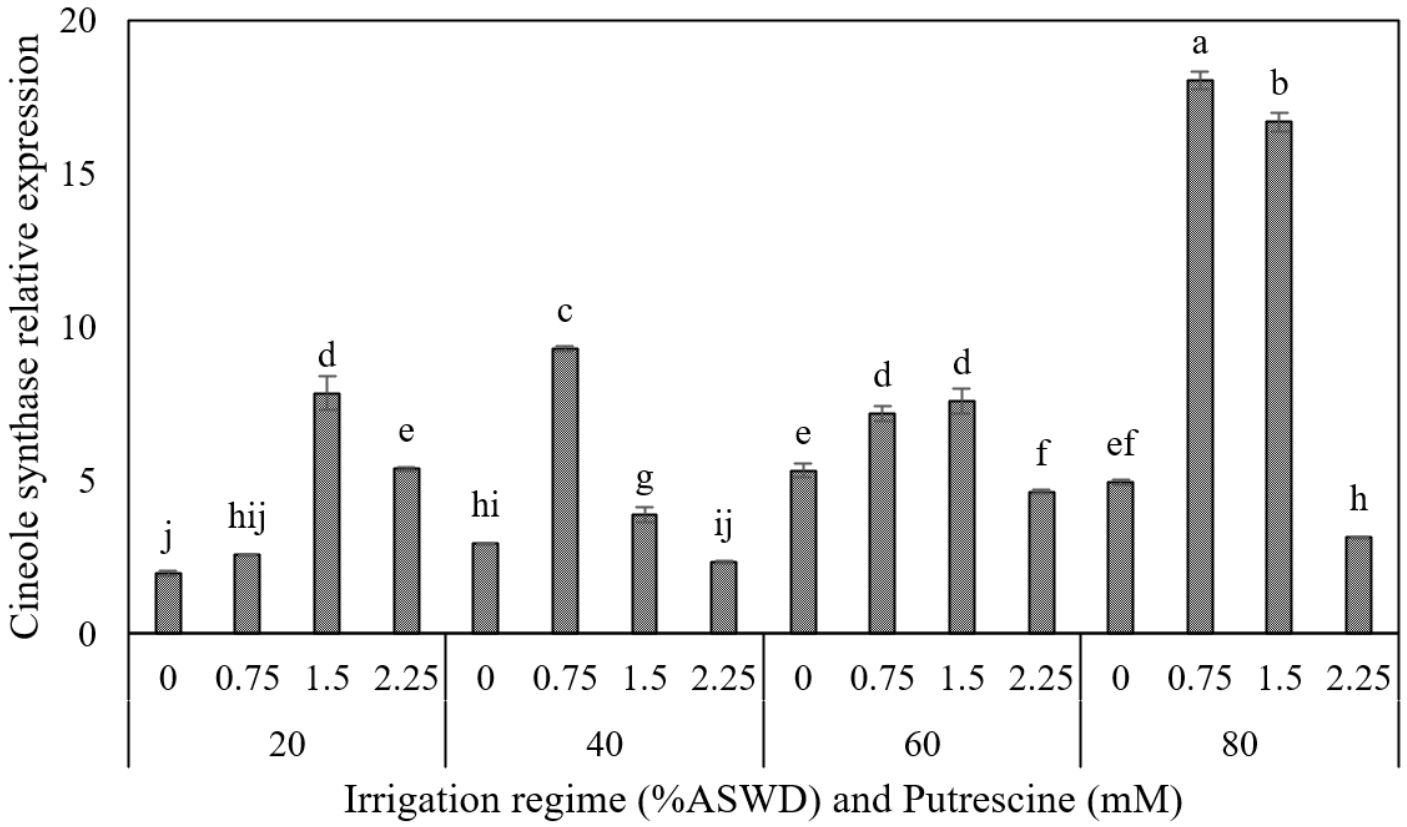

Figure 6

Interaction between irrigation regime and putrescine on cineole synthase relative expression. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

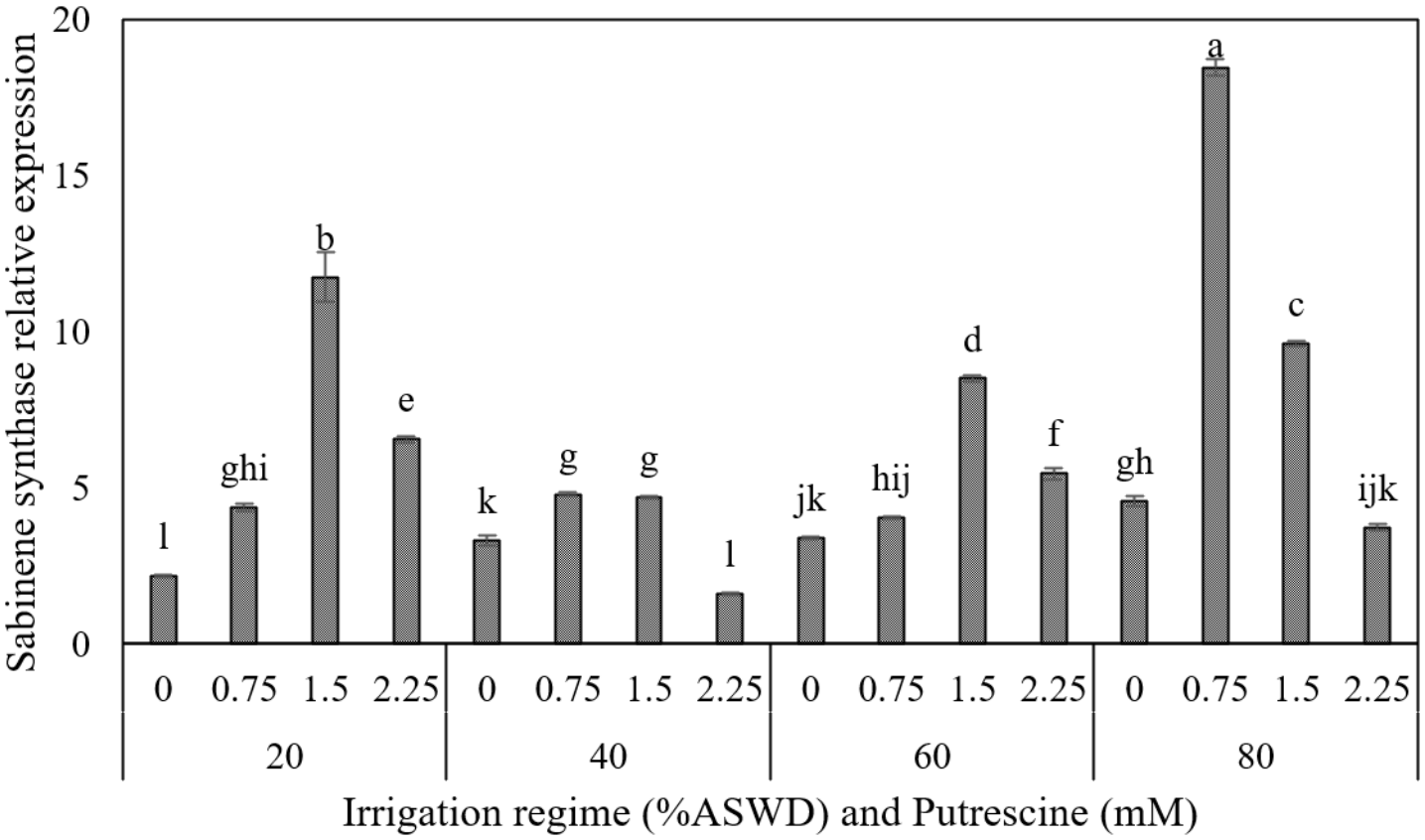

Figure 7

Interaction between irrigation regime and putrescine on sabinene synthase relative expression. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

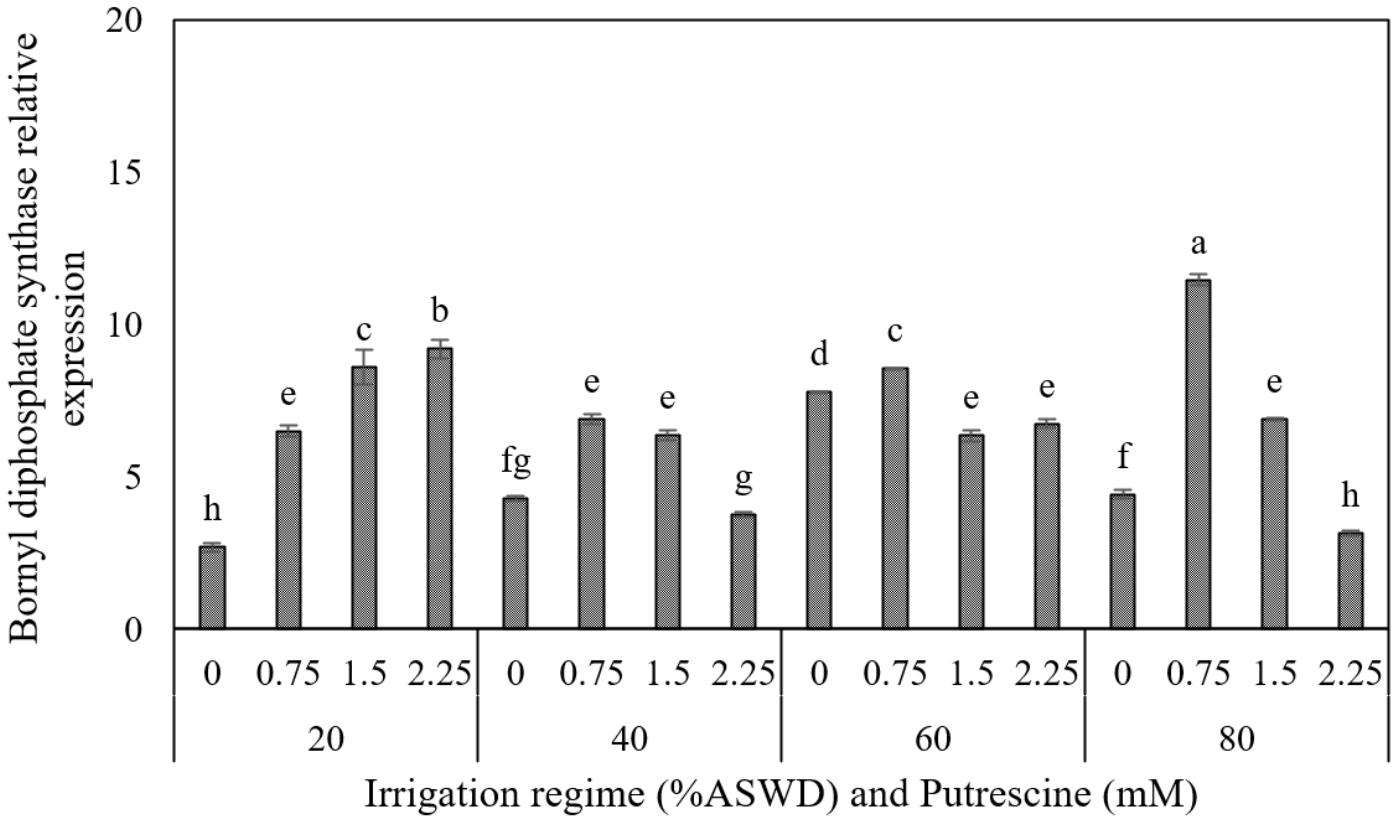

Figure 8

Interaction between irrigation regime and putrescine on bornyl diphosphate synthase relative expression. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

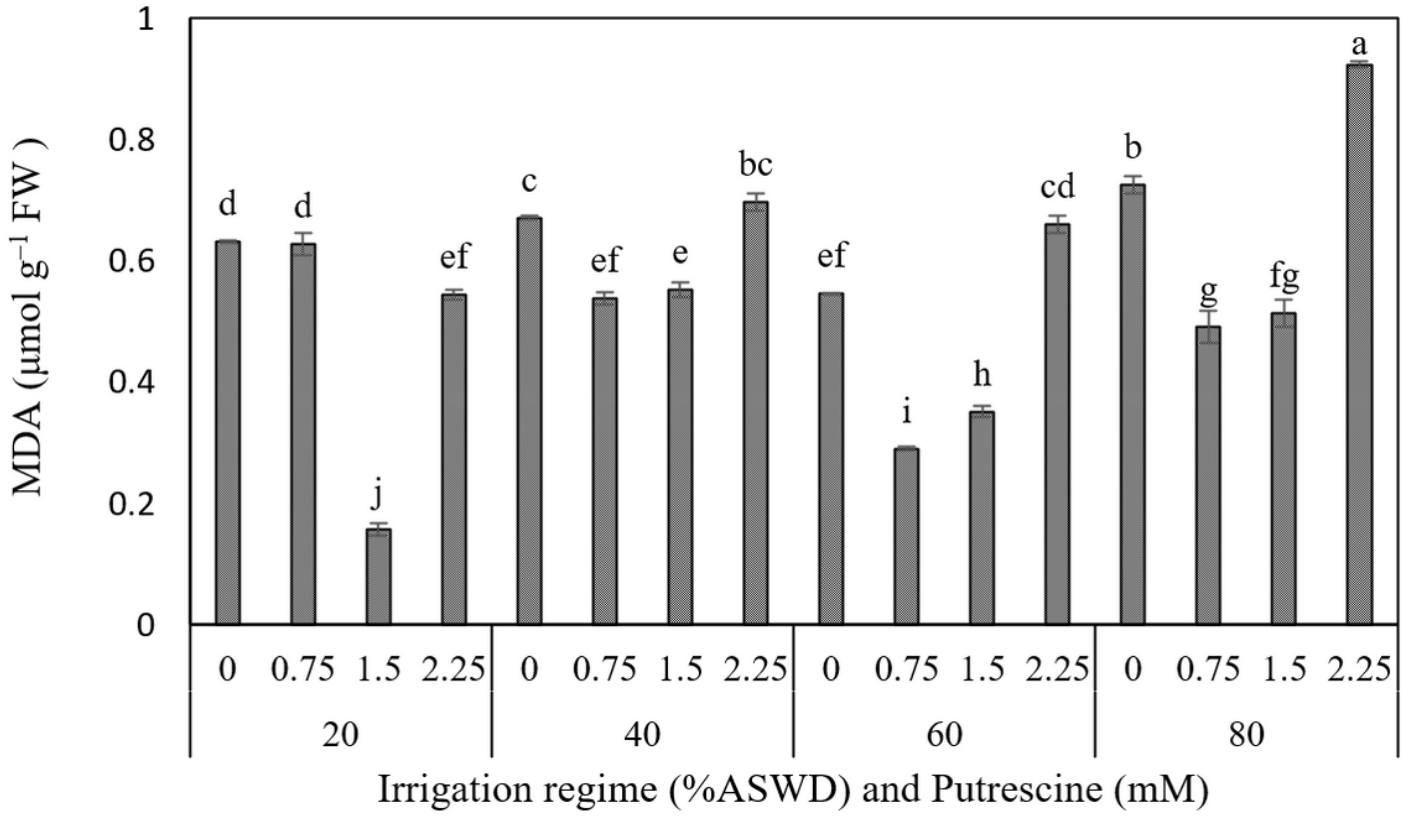

Figure 9

Interaction between irrigation regime and putrescine on MDA. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

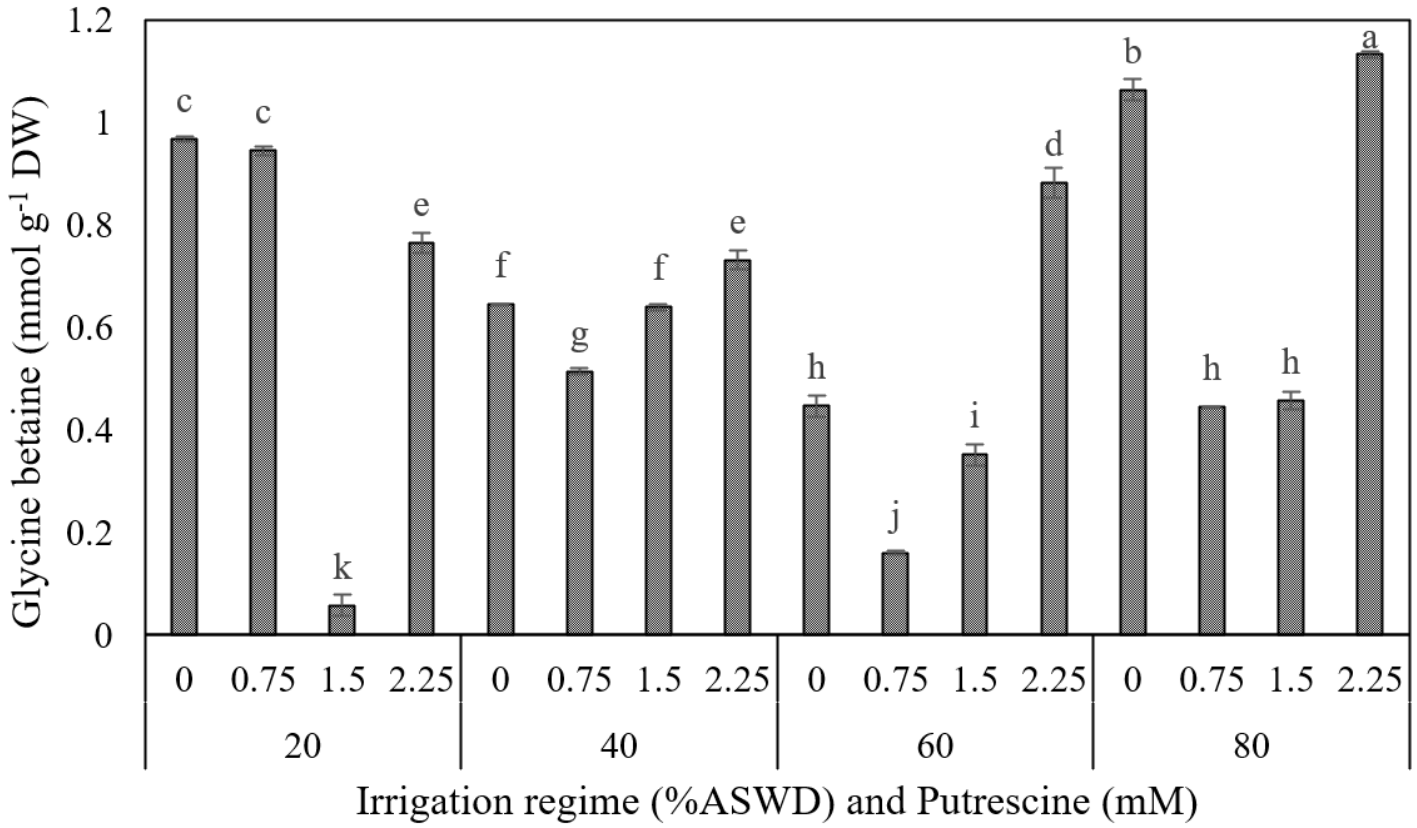

Figure 10

Interaction between irrigation regime and putrescine on glycine betaine. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

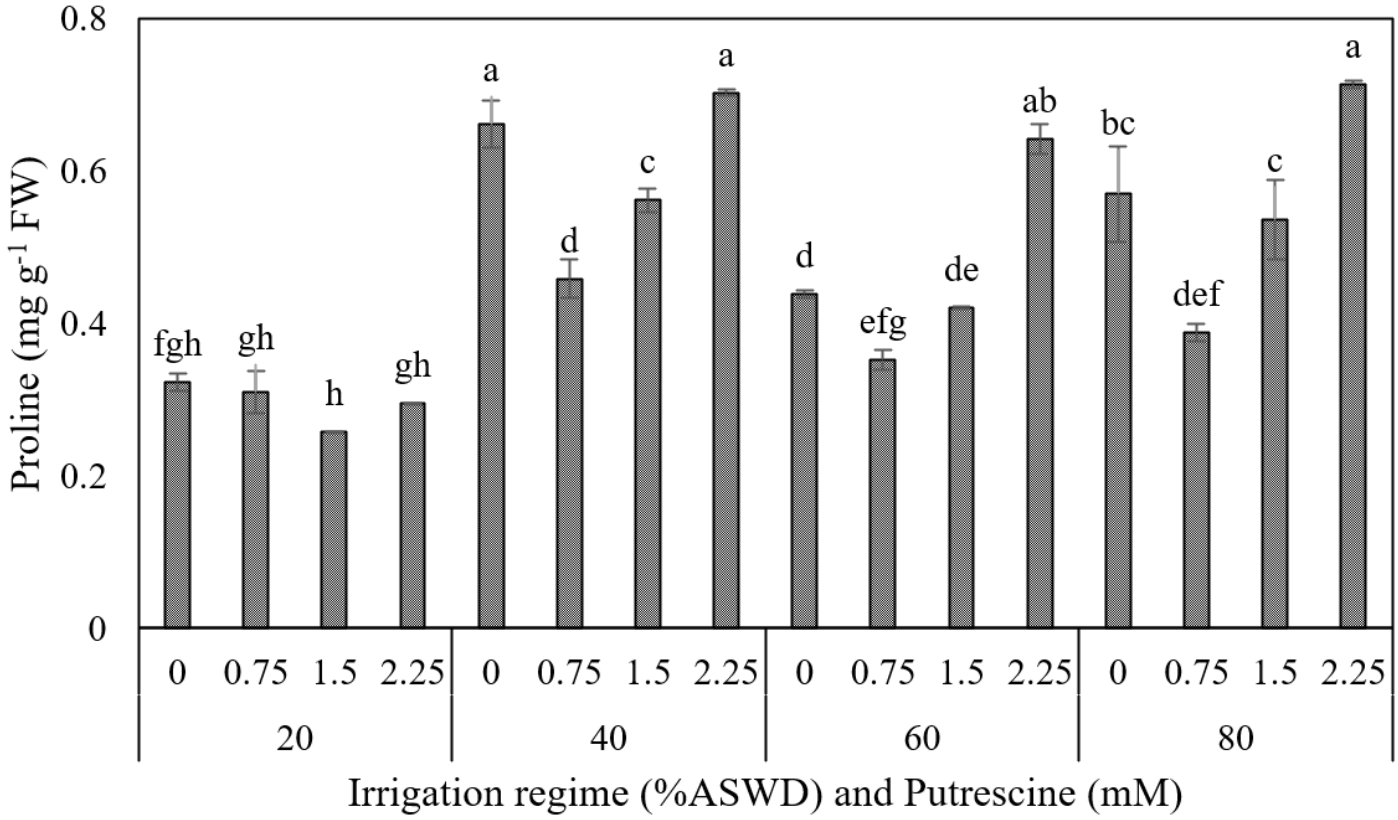

Figure 11

Interaction between irrigation regime and putrescine on proline. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

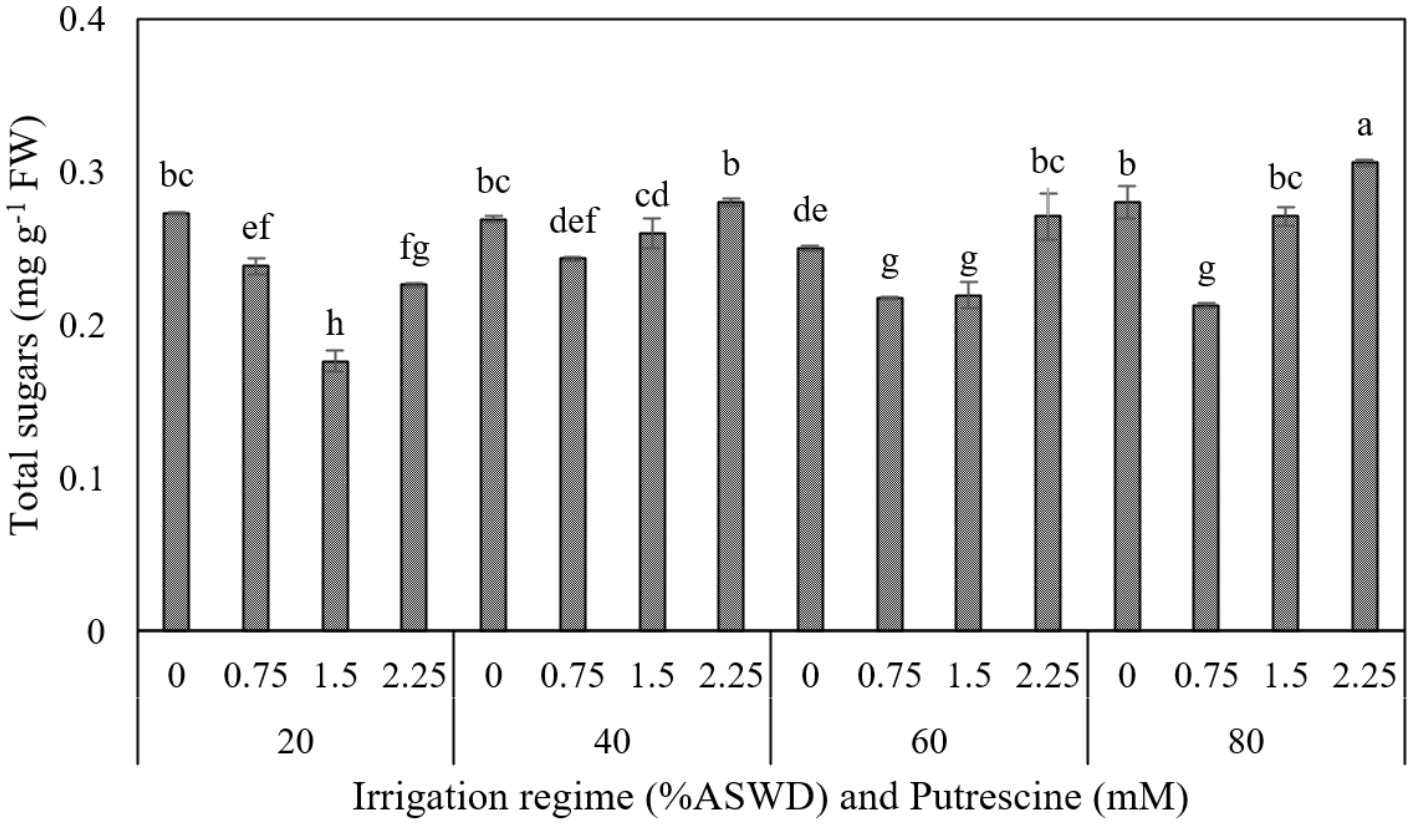

Figure 12

Interaction between irrigation regime and putrescine on total sugars. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

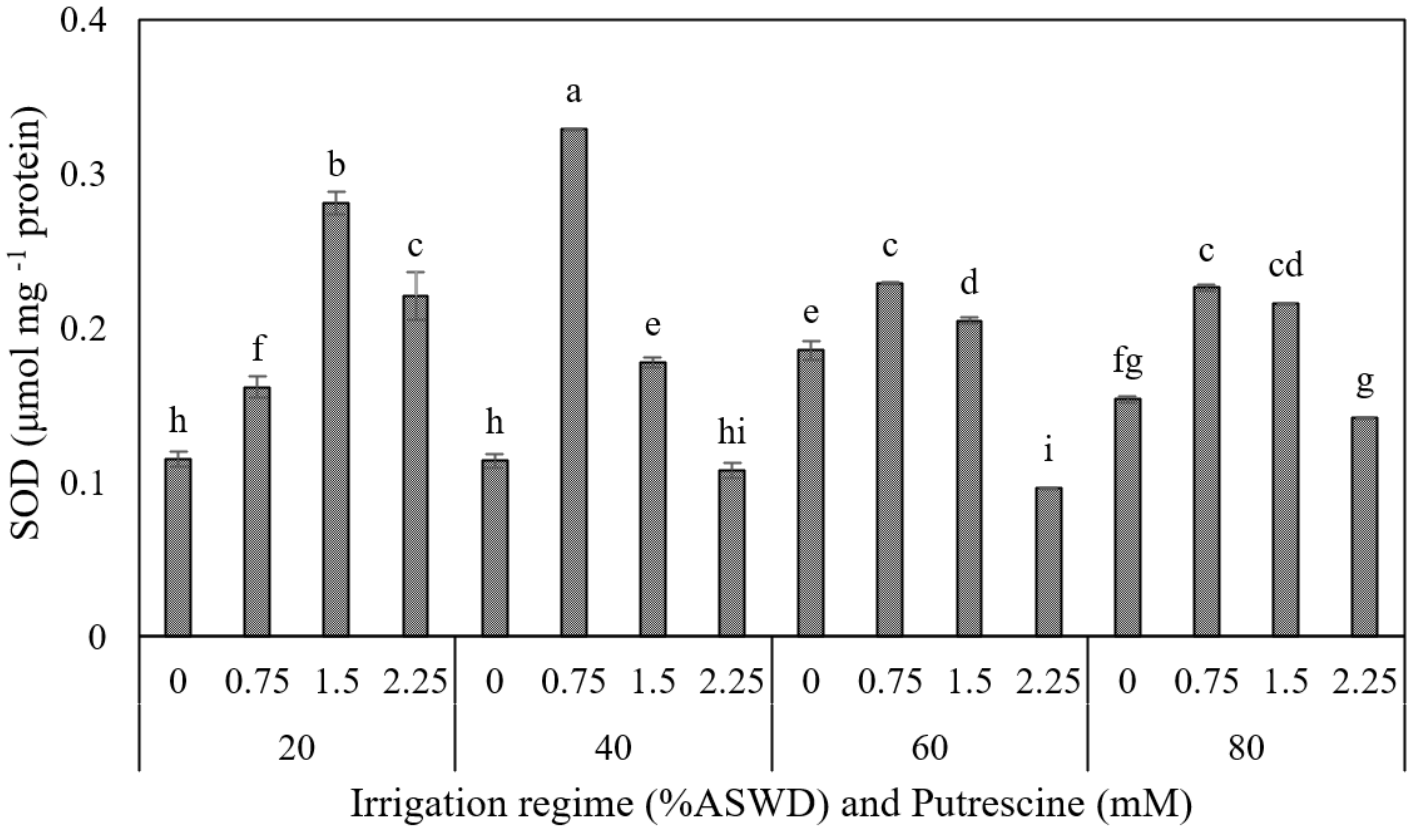

Figure 13

Interaction between irrigation regime and putrescine on SOD. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

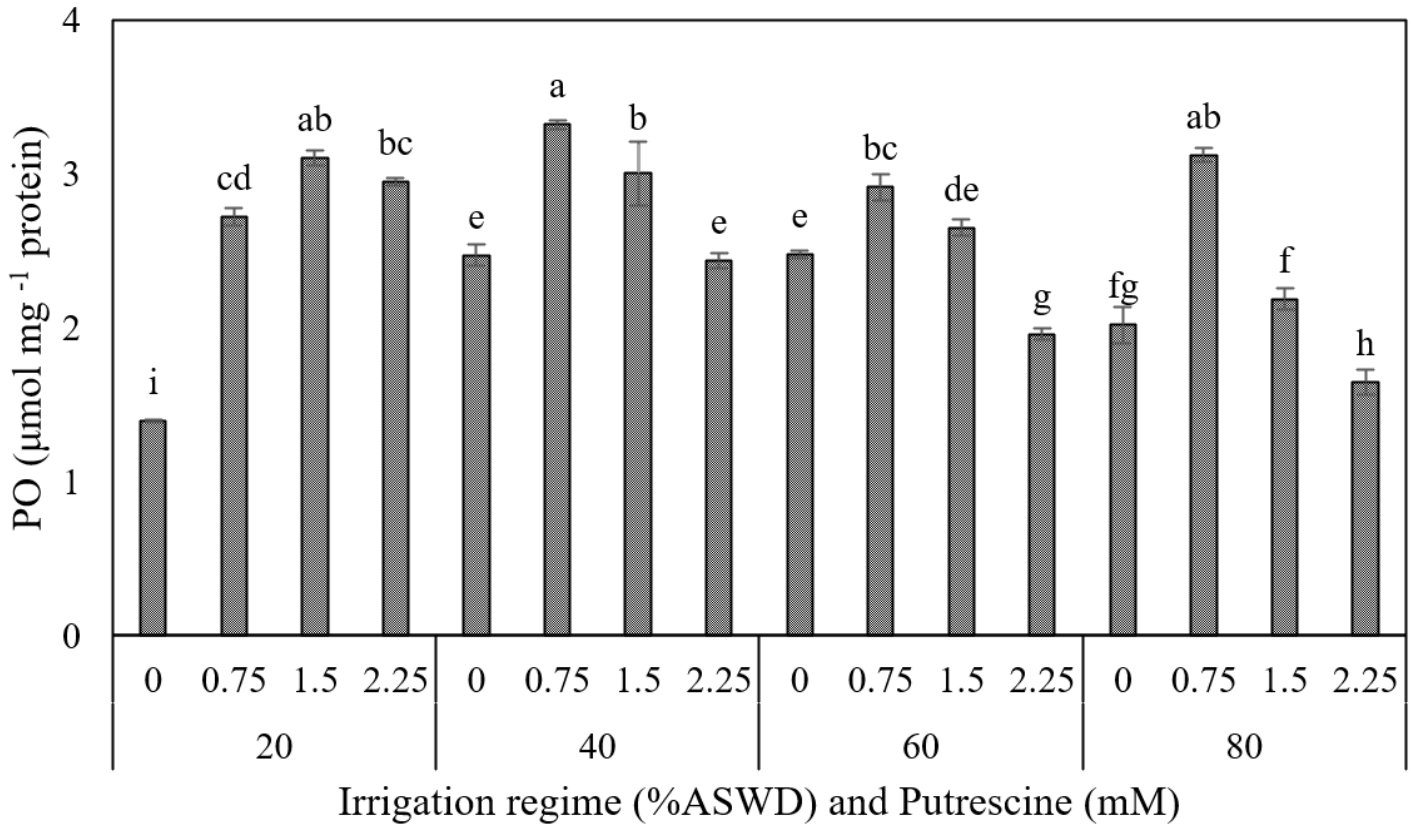

Figure 14

Interaction between irrigation regime and putrescine on PO. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

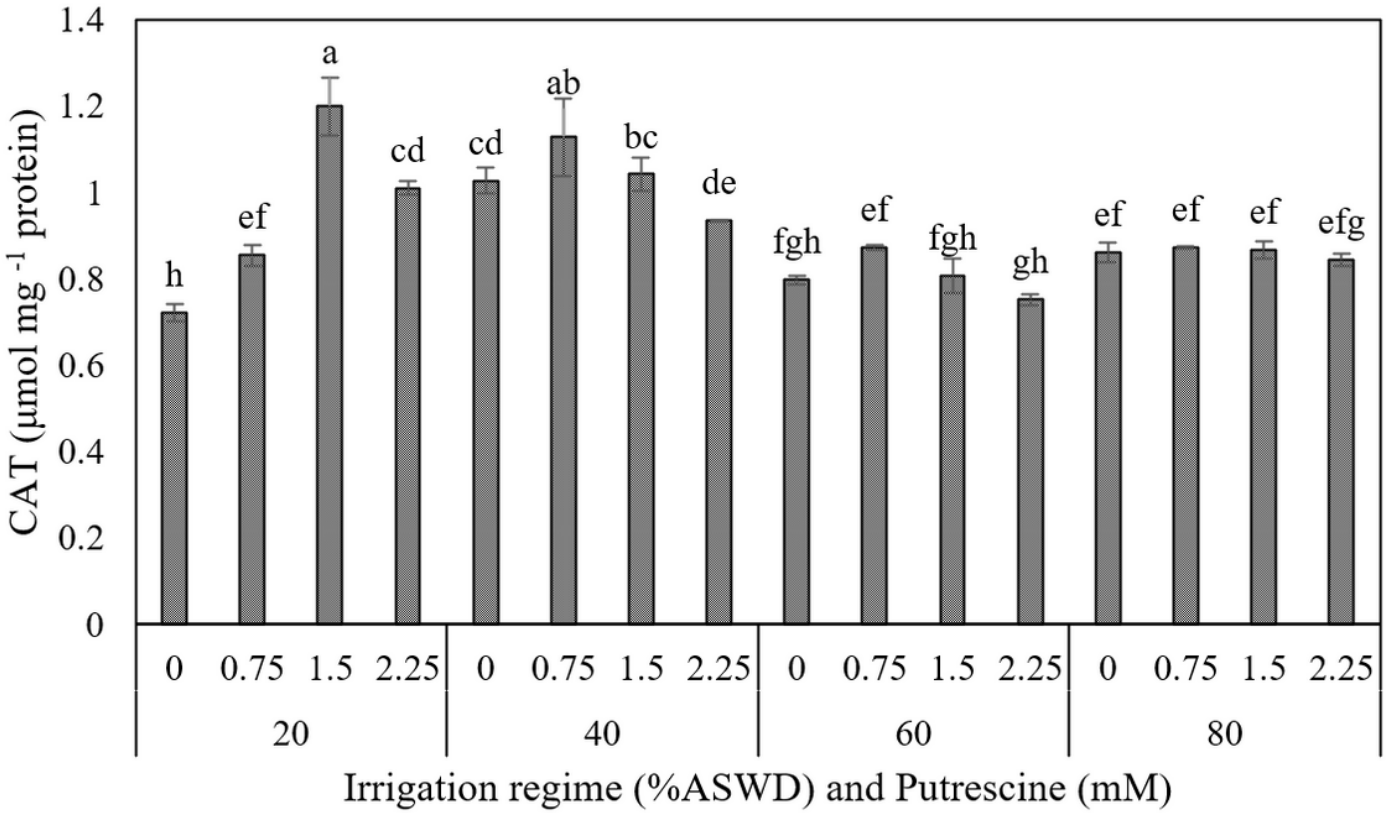

Figure 15

Interaction between irrigation regime and putrescine on CAT. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

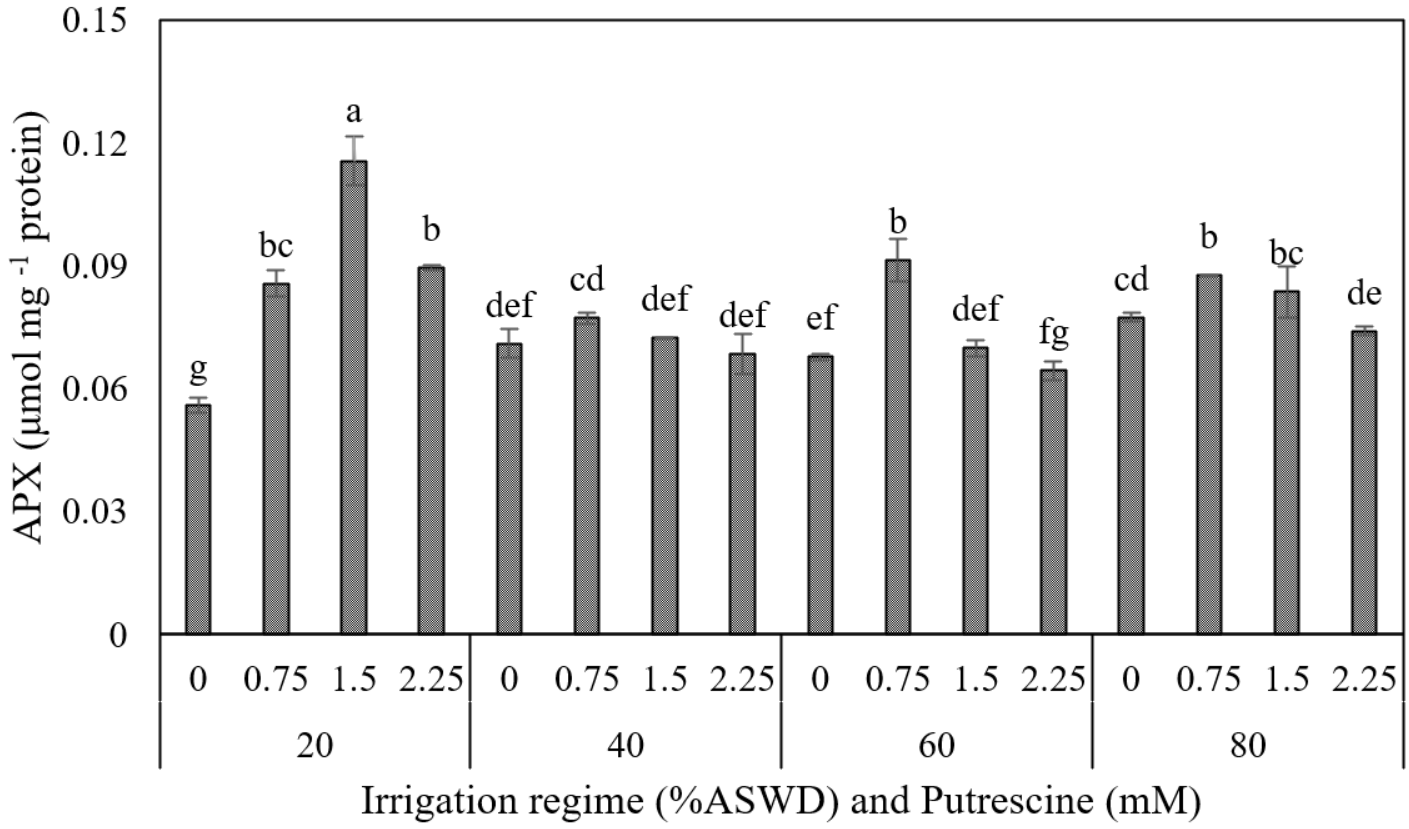

Figure 16

Interaction between irrigation regime and putrescine on APX. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

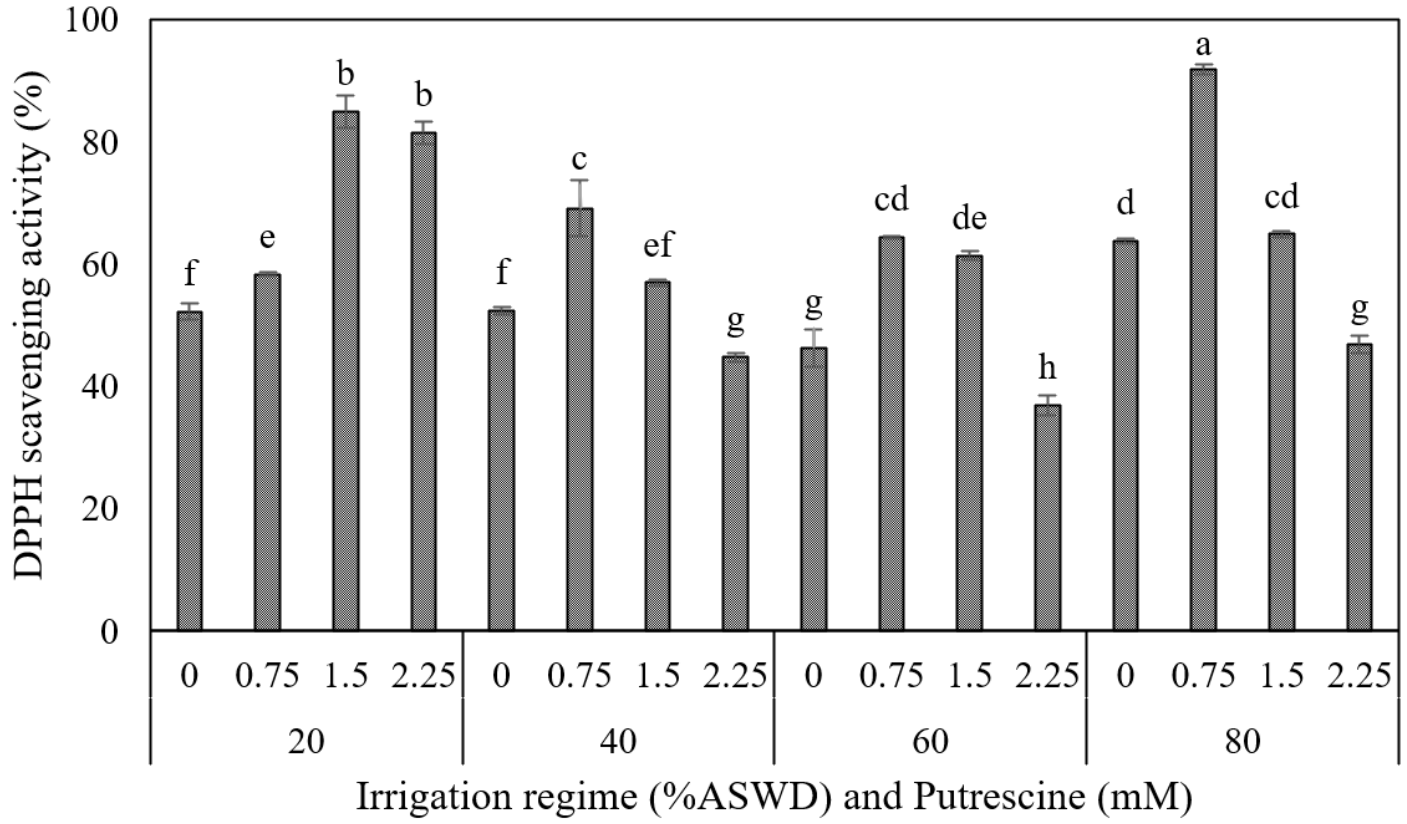

Figure 17

Interaction between irrigation regime and putrescine on DPPH. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

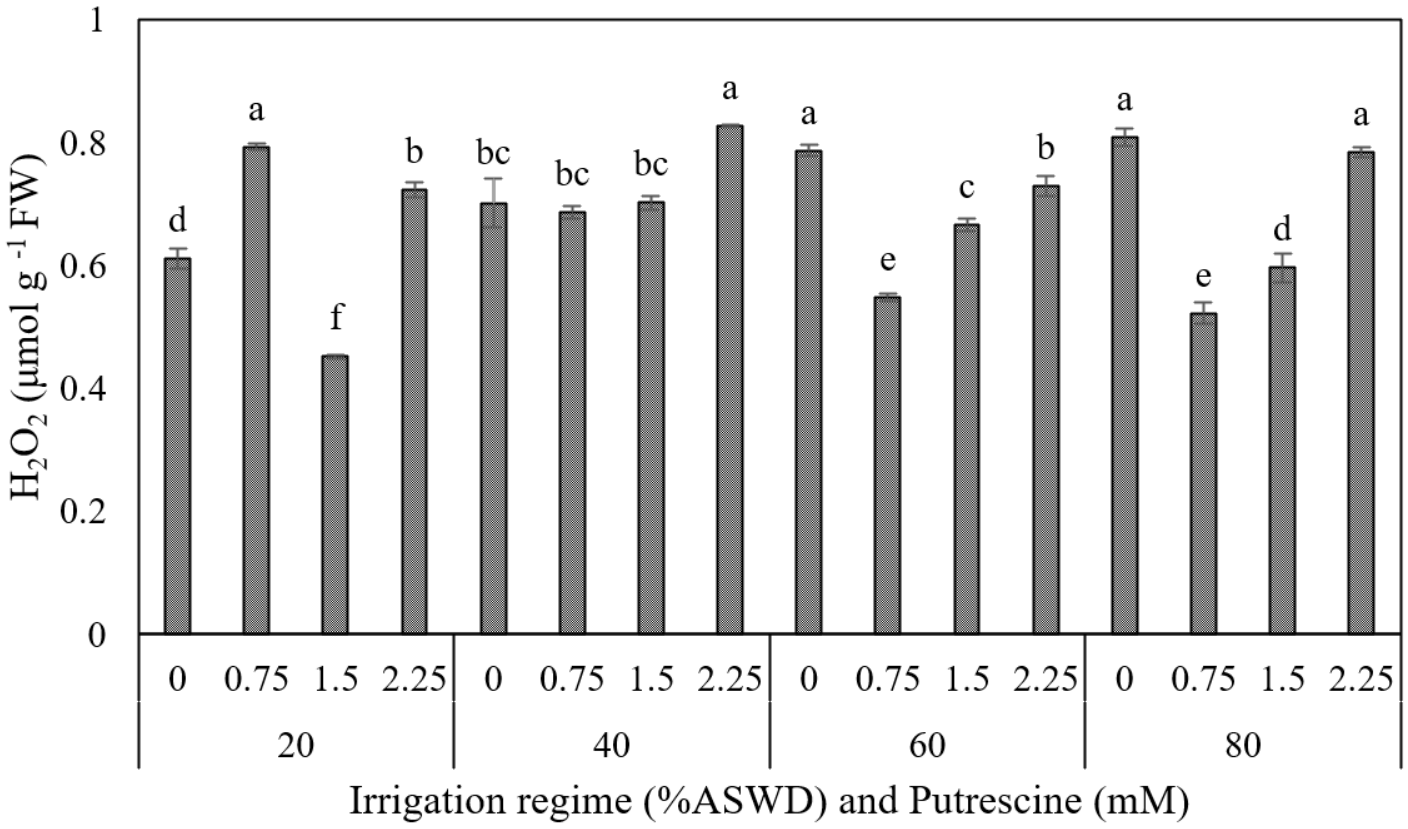

Figure 18

Interaction between irrigation regime and putrescine on H2O2. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

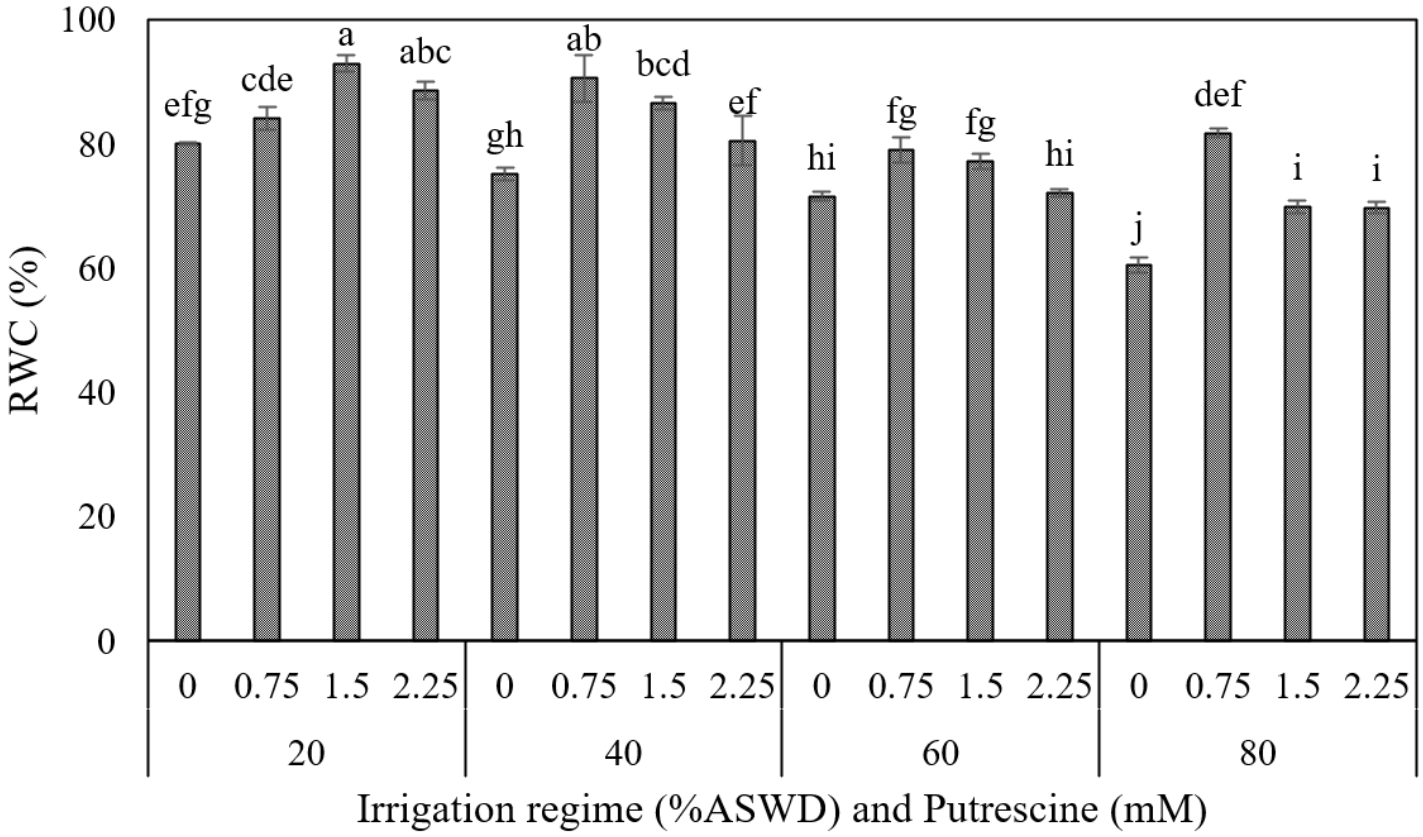

Figure 19

Interaction between irrigation regime and putrescine on RWC. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

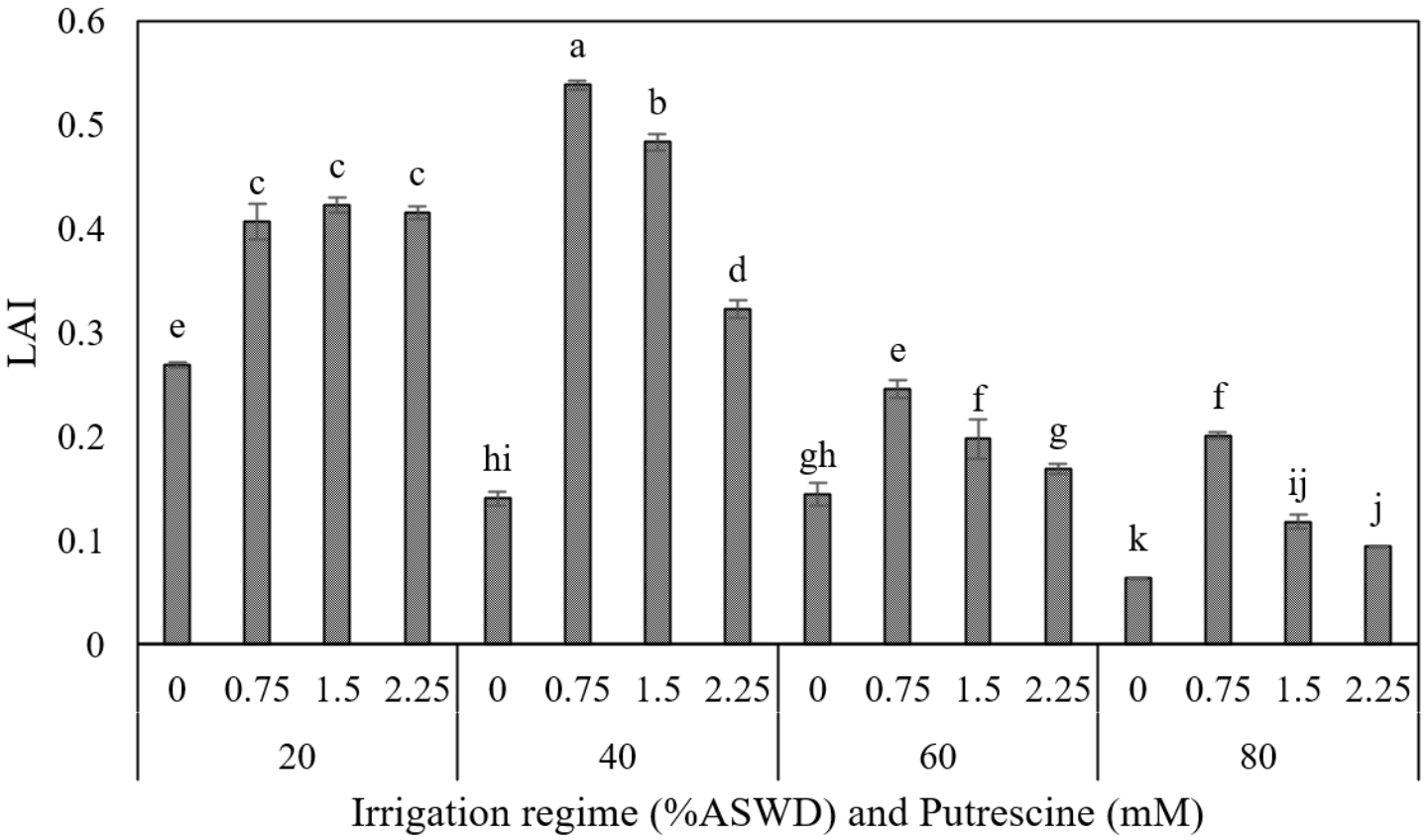

Figure 20

Interaction between irrigation regime and putrescine on LAI. The different letters show significantly different at the level of 0.01. The error bars represent standard error.

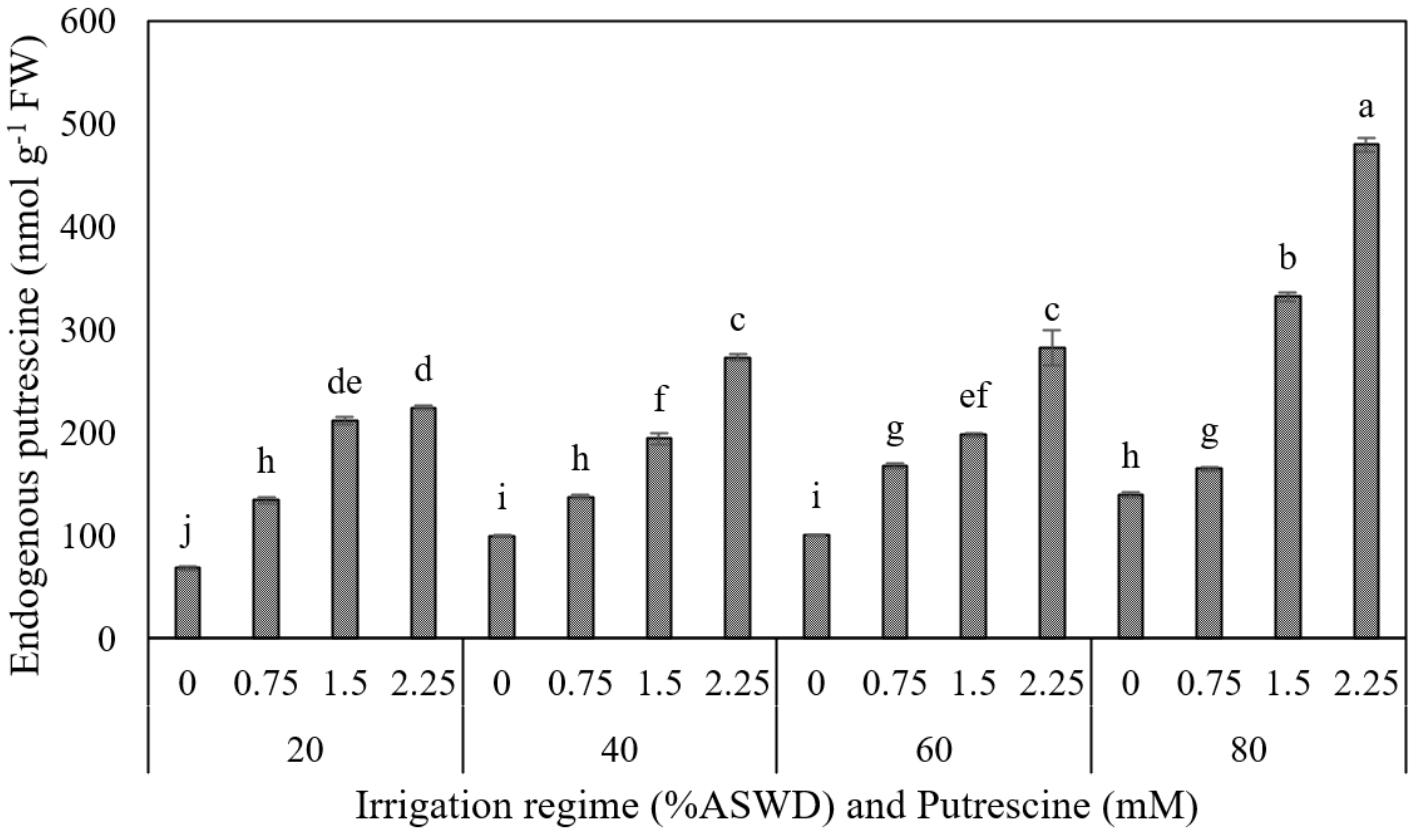

Figure 21

Interaction between irrigation regime and putrescine on endogenous putrescine. The different letters show significantly different at the level of 0.01. The error bars represent standard error.