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Effect of Drought Stress on some of the Biochemical Characteristics of Three Achillea Populations (*Achillea vermicularis*)*

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Abstract

The experiment was conducted in the form of a randomized completely block design with three replications. The main pots were kept under irrigation (at 20%, 40% and 70% of field capacity along with a well watered control). The sub-pots contained three *Achillea vermicularis* populations (Kahak, Khalkhal and Semnan). The results showed that drought stress had significant effects on photosynthetic pigments, proline, soluble sugar contents and peroxidase activity, but drought induction. Moreover, population variations had a significant effect on chlorophyll *a*, chlorophyll *b* and peroxidase activity. Among all the measured samples, the Khalkhal population had the highest content of photosynthetic pigments, soluble sugars and peroxidase activity. However, the Kahak population had the lowest value of the mentioned traits instead of the latter trait. Meanwhile, the severe drought stress produced the highest peroxidase activity, carotenoid, proline, protein, soluble sugar contents. By contrast, the lowest values of the mentioned traits were achieved in well-water treatment. The highest chlorophyll *a*, chlorophyll *b* and total chlorophyll contents were observed in 70% FC, while the lowest were recorded in 20% FC. The Khalkhal population × severe drought stress produced the highest carotenoid, soluble sugar content and peroxidase activity, and also, Khalkhal population × moderate drought stress recorded the highest contents of phosynthetic pigments. Therefore, the Khalkhal population could be introduced as suitable *Achillea vermicularis* population in Iran.

Keywords: *Achillea vermicularis*, drought stress, field capacity, medicinal plants.

Introduction

Iran is located in arid and semi-arid region. Having an average annual precipitation of 250 mm, Iran receives less than one third of global average precipitation (750 mm). In addition,

* **Abbreviations:** ROS – Reactive Oxygen Species; POX – Peroxidase; SOD – superoxide dismutase; GR – glutathione reductase; MDA – malondialdehyde.

the rainfall distribution pattern over the country is not the same everywhere. Bearing in mind such a climatic condition, many severe or mild droughts are inevitable to come up. Any drought can inflict a severe damage on the agricultural, domestic and industrial sectors of the country. Due to the growth of population and expansion of the agricultural, energy, and industrial sectors, the demand for water has increased extensively, and water scarcity has been occurring almost every year in many parts of the world (Mishra and Singh, 2010). Drought is known as a major abiotic factor that limits plant's growth and production. Although, the general effects of drought on plant growth are fairly well known, the primary effects of water deficit at the biochemical and molecular levels are not well understood (Bhatnagar-Mathur et al., 2009). Furthermore, the physiologic and metabolic responses of crops to dry environments have been well studied, but similar studies are lacking in medicinal and aromatic plant. Stress is a factor outside plant's body which damages plant growth (Kafi et al., 2000). Among the abiotic stresses, drought is the most important one which affects plants periodically in some growth stages, or permanently in all life cycle (Reddy et al., 2008). Drought stress usually occurs when available water in soil reduces and atmospheric conditions increase water loss through evapotranspiration (Jaleel et al., 2009). A primary symptom of low available water to plants is the loss of turgor pressure and reduction of cell development especially in stems and leaves. Reduction of cell development makes the plant smaller in size, which is the characteristic of drought stressed plants. Moreover, drought stress disturbs nutrient absorption and reduces leaves growth. Lower leaf area means lower light absorption and photosynthesis. All these events finally decrease plant growth and yield (Hsaio, 1973). Drought stress in induced when moisture at the rhizosphere falls below the permanent wilting point (PWP). So the plant is not able to take up sufficient water, resulting in cell dehydration. Dehydration is reversible until a certain point (elastic point); however, is irreversible if the water loss is too server (plastic point) (Kuchaki and Mahallati, 1992). However, the time, duration and frequency of drought stress incident, soil properties and so many other factors affect plant tolerance to drought, and different genotypes may also respond differently (Sarmadnia, 1993). Drought stress induces some morphophysiological responses in plant such as the reduction of leaf area, shoot growth, enhancement of root growth, stomata closure, the reduction of growth rate, sudden antioxidants and soluble compounds accumulation, and activation of some enzymes (Hughes et al., 1989). Safikhani (2006) studied the effect of 100%, 60% and 40% FC drought stresses on *Dracocephalum moldavica* and concluded that irrigation at 40% FC (severe drought stress) decreased plant height, leaf area, internodes length, shoot yield and essential oil yield compared with the two other treatments.

Stephanie et al. (2005) reported that drought stress reduced stem length and root length of *Salvia splendens*. Lebaschy and Sharifi Ashoorabadi (2004) concluded that higher drought stress levels reduced plant height and shoot weight in some medicinal plants such as *Salvia officinalis* and *Achillea millefolium*. Sangwan et al. (1994) reported that mild drought stress decreased lemon grass height, leaf area and leaf weight. Finally, Ardakani et al. (2007) reported that drought stress affected shoot yield, essential oil percentage and yield, leaf yield, stem yield, height, the number of tillers, leaf area, stem diameter and the length of internodes in balm (*Melissa officinalis*). Water deficit (commonly known as drought) can be defined as the absence of adequate moisture necessary for normal plant grow and to complete the life cycle (Zhu, 2002). The lack of adequate moisture leading to water stress is common occurrence in rain fed areas, brought about by infrequent rains and poor irrigation (Wang et al., 2005). When plants are subjected to various abiotic stresses, some reactive oxygen species (ROS) such as superoxide (O_2·-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and singlet oxygen (O_2^* ($a^1\Delta_g$)) are produced. These ROSs may initiate destructive oxidative processes such as lipid peroxidation, chlorophyll bleaching, protein oxidation, and damage to nucleic acids. However, antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), catalase and peroxidase, and low-molecular antioxidants such as ascorbic acid, glutathione, α-tocopherol, flavonoids and carotenoids play a key role in scavenging those activated species. Modulation of the activity of these enzymes may be an important factor in the tolerance of various plants to environmental stress.

Researchers have also linked various physiological responses of plants to drought with their tolerance mechanisms, such as pigment content and stability, and high relative water content. When water availability is limited, Drought can also lead to pigment degradation, thus causing irreversible water-deficit damage to the photosynthetic apparatus (Terzi and Kadioglu, 2006).

In higher plants the oxygen toxicity is more serious under condition of water-deficit conditions. Water stress causes stomatal closure, which reduces the CO_2/O_2 ratio in leaves and inhibits photosynthesis (Jason et al., 2004; Moussa, 2006). These conditions increase the rate of reactive oxygen species (ROS) like superoxide radical ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) particularly in chloroplast and mitochondria (Mittler, 2002; Neill et al., 2002) *via* enhanced leakage of electrons to oxygen. The superoxide radicals and their dismutation product, hydrogen peroxide, can directly attack membrane lipid and inactivate SH-containing enzymes (Sairam et al., 2000). The hydroxyl radical, one of the most reactive oxygen species, is responsible for oxygen toxicity in vivo, causing damage to DNA, protein, lipids, chlorophyll and almost every other organic constituent of the living cell (Bacana et al., 1998). Plants protect the cellular and sub-cellular system from the cytotoxic effects of active oxygen radicals with anti-oxidative enzymes such as SOD, POX and CAT as well as metabolites like glutathione, ascorbic acid, tocopherol and carotenoids (Alscher et al., 2002). It has been reported which membranes are subject to damage rapidly with increasing water stress. This leakiness of membranes is caused by an uncontrolled increase in free radicals, which cause lipid peroxidation (Smirnoff, 1993). The stress induced burst in free radicals could also be partially related to the activity of lipoxygenase, which convert C18:2 and C18:3 to the corresponding hydroxyl peroxides (Bell and Mullet, 1991). Further damage to fatty acid could then produce small hydrocarbon fragments including malondialdehyde (MDA) (Alscher et al., 2002). It is hypothesized that modulation of the activities of these enzymes at early growth stage may be important in imparting resistance to a plant against environmental stresses. Therefore, in the present investigation the relative significance of antioxidative enzymes, MDA, H_2O_2 content, PRO, GB accumulation, photosynthetic activity and membrane permeability has been examined at seedling stage in drought-tolerant and susceptible maize cultivars (Helal and Samir, 2008). Plants subjected to environmental stress evolved a complex and efficient antioxidant system, which includes enzymatic antioxidants and nonenzymatic antioxidants to counteract the detrimental effects of active oxygen species (Zhu et al., 2009). These are toxic intermediates that result from a reduction in molecular O_2, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Dat et al., 2000). The role of antioxidative defence systems in plant responses to drought stress was comprehensively documented in *Gypsophila aucheri*, which is a xerophytic plant (Sekmen Esen et al., 2012).

In another study, antioxidative and physiological responses of 2 sunflower (*Helianthus annuus*) cultivars under drought stress were evaluated, and the efficiency of antioxidative systems in coping with drought effects was clear (Baloğlu et al., 2012). It was also shown that a plant's ability to cope with abiotic stress is mainly related to an altered biochemical profile and produces a varied range of secondary metabolites. Secondary metabolite production is a critical part of the defence response to stress conditions. The role of lipid peroxidation in initiation of secondary metabolites has been documented by some researchers. Consequently, the accumulation of secondary metabolites is mainly related to membrane lipid protection from oxidative stress, and reactive oxygen species (ROS) are the mediators in the biosynthesis of particular secondary metabolites (Zhu et al., 2009).

Water stress decreases growth of some medicinal plants, including *Hypericum brasiliense* Choisy (Nacif de Abreu and Mazzafera, 2005) and *Bupleurum chinense DC* (Zhu et al., 2009). On the other hand, many studies have shown that drought enhances the amount of secondary metabolites in a wide variety of plant species, such as *Rehmannia glutinosa (Gaertn.) DC* (Chung et al., 2006). Conversely, drought caused a significant reduction in all growth parameters and essential oil yield and percentage in some medicinal plants such as peppermint (*Mentha piperita L.*) (Khorasaninejad et al. 2011; Saeidnejad et al., 2013).

Proline is one of the protective molecules that can unite oxygen and free radicals caused by stress. Therefore, one of the roles of proline in tomato shrubs is probably reacting against drought stress. Proline's role as an osmotic factor is already established and low water stress increases the proline content in plants. Water shortage trigger to LEA proteins production that their work hasn't identified completely however, some evidence indicated that these proteins play role in increasing plant resistance against drought stress. Reduction of Chlorophyll *a* and Chlorophyll *b* in resistant tomato plant under low water condition indicated that drought stress changes the amount of chlorophyll in plant. Reduction of chlorophyll was due to chloroplast decomposition and disappearing thylakoid structures (Ghorbanli et al., 2012). To cope with drought stress, plants

respond with physiological and biochemical changes. These changes aim at the retention of water in spite of the high external osmoticum and the maintenance of photosynthetic activity, while stomatal opening is reduced to counter water loss. Accumulation of low molecular compounds, such as glycine betaine, sugars, sugar alcohols and proline, is a mechanism aimed at balancing water potential following drought. In addition to synthesis of these osmolytic compounds, specific proteins and translatable *m*RNA are induced and increased by drought stress (Parida et al. 2007).

The genus Achillea (*Asteraceae*), named after the mythological Greek warrior Achilles, comprises of approximately 85 species, most of which are endemic to Europe and the Middle East. The Turkish flora possesses 40 Achillea species and 20 of them are endemic (1). On the other hand, some Achillea species have been known to be ethnopharmacologically used in folk remedies for various purposes such as haemorrhoid and wound healing (2). Especially, *A. millefolium* is frequently used against diarrhea, abdominal pain and stomachache in Turkish traditional medicine (3–5). Several biological activity studies have been performed on various Achillea species, including antibacterial, antioxidant, anti-inflammatory and antispasmodic activities (6–10). Moreover, Achillea species are well-known to contain essential oil and their chemical compositions as well as antimicrobial activities have been well studied (Esra et al., 2007).

Regarding the expansion of arid regions and decreasing amount of cultivatable lands, exploitation of drought tolerant plant seems to be great importance to alleviate low performance of the crops cultivated in these areas. Determination of the growth aspects of medicinal plants under water stress can provide valuable information about the possibility of the cultivation of these plants in dry lands (Alaei et al., 2013).

The aim of this study was to investigate the effects of mild, moderate and severe drought stress on photosynthetic pigments, proline, total protein, soluble sugars and peroxidase activity in three populations of *Achillea vermicularis* under greenhouse condition.

Material and methods

The experimental conditions

This experiment was conducted under greenhouse conditions in August 2009 at Alborz Research Station, dependent of Research Institute of Forests and Rangelands (RIFR), Karaj, Iran. Alborz Research station is located in 5 km south east of Karaj (35° 48'N, 51° E, 1320 m above the sea level). Average annual precipitation at the site is 235 mm, minimum air temperature is -20°C and maximum air temperature is 38°C. The dominant winds at the area blew from east and south east.

A pot experiment was conducted in the form of randomized completely block design with three replications. Each replication consisted of 4 treatments. Seeds of three *Achillea vermicularis* populations, namely Kahak, Khalkhal and Semnan, were collected from Research Institute of Forests and Rangelands, Karaj, Iran, and planted in plastic pots with 15 cm diameter and 25 cm length containing soil, peat and sand in the ratio of 1:1:1, and grown under greenhouse conditions in August. Irrigation was regularly conducted according to the prepared map. Due to induce the drought stress, 100% of FC was considered as the well water and 70%, 40% and 20% of FC were considered as mile, moderate and severe drought stress treatments, respectively. Irrigation was done daily during the first 3 months. After 3 months from sowing, a cycle of drought was induced by stopping irrigating the potted plants for 45 days. The amount of irrigation was determined based on soil field capacity. A control set was maintained by irrigating the potted plants regularly. The control plants received 400 ml water every 2–3 days and the mild, moderate and severe drought stress treatments involved 300 ml, 200 ml and 100 ml water every 2–3 days, respectively. The plants were placed in 4 rows with 3 replications for each treatment. The leaf samples were collected from control and treated plants after 45 days of drought for estimations of various biochemical parameters.

Extraction and estimation of photosynthetic pigments

Fresh leaves (0.5 g) were thoroughly homogenized in chilled 80% acetone in a mortar and pestle in the dark at 4°C and the homogenates were centrifuged at 10000 g for 10 min. The supernatants were collected, and the absorbance of acetone extracts was measured at λ=663, 646 and 470 nm using a UV–visible spectrophotometer (Spectra Max Plus; Molecular Devices, USA). Chlorophyll content was estimated based on mg g^{-1} FW.

The Chl *a*, Chl *b*, total chlorophylls and total carotenoid (μg g^{-1}) contents were calculated using the following equations of Lichtenthaler (1987):

Chlorophyll *a* = 12.21×A 663 – 2.81×A 646;

Chlorophyll *b* = 20.13×A 646 – 5.03×A 663;

Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*;

Carotenoids = (1000×A 470 – 3.27 Chlorophyll *a* – 104; Chlorophyll *b* – 229.

Estimation of peroxidase activity

For the peroxidase activity, the leaf, petiole and roots were homogenized on ice in 10 ml cold sodium phosphate buffer (pH=7.0). Activity was determined spectrophotometrically according to Rodriguez and Sanchez (1982). POD activity was analyzed in 50 mM phosphate buffer (pH=6.5) containing 40 mM guaiacol (2-methoxyphenol) and 26 mM H_2O_2. The increase of absorbance at λ=420 nm was recorded within 180 sec after adding of 26 mM H_2O_2. Protein content was determined according to Bradford (1976). Bovine serum albumin was used as the standard.

Extraction and estimation of free proline

Free proline content was estimated following the method of Bates et al. (1973). Fresh leaves (0.5 g) were extracted in 3 % sulphosalicylic acid and the homogenates were centrifuged at 10000 g for 10 min. 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene and mixed vigorously with a vortex mixture for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance measured at λ=520 nm using toluene as blank. Proline concentration was calculated from a standard curve using 0–100 μg of *L*-proline (Sigma-Aldrich, UK). The free proline content was expressed as μg g^{-1} FW.

Estimation of total protein

Protein was estimated by the Lowry method (Lowry et al., 1951). Reagent A: 1% Na_2CO_3 in 0.5 *N* NaOH; Reagent B: 1% $CuSO4{\cdot}5H_2O$; Reagent C: 2% sodium tartrate ($Na_2C_4H_4O_6$); Reagent D: Mix 0.5 ml of reagent C with 0.5 ml of reagent B and 10 ml of Reagent A and Reagent E: Folin 0.2 *N* soluble proteins were extracted from 2 g dry weight of each sample into 5 ml of Tris-HCl buffer (pH=8.0) containing 26.8 ml of 0.2 *N* HCl, 17.2% sucrose, 1% ascorbic acid and then was centrifuged. 1 ml of Reagent D was added into 0.05 ml of the resulted solution and kept in temperature room. Then, 3 ml of Reagent E was added and the sample was kept in Bain-marie water bath at 50°C. The absorbance was measured spectrophotometrically at λ=625 nm. Protein was calculated based on μM g^{-1} FW.

Extraction and estimation of total soluble sugars

Total soluble sugars were estimated in 20 ml of 80% (v/v) ethanol extract at 95°C for 1 h from 100 mg of leaf powder frozen in liquid nitrogen. After centrifugation at 10000 g for 10 min, starch was measured in the pellet according to Jarvis and Walker (1993). Total soluble sugars were analyzed by reacting 0.25 ml of the supernatant with 3 ml of freshly prepared anthrone reagent [0.06% (w/v) anthorone in 95% H_2SO_4] and placing in boiling water bath for 10 min. After cooling to room temperature, the absorbance at λ=625 nm was measured and total sugar was quantified according to Irigoyen et al. (1992). Reducing sugars were estimated following alkaline copper method as described by Parida et al. (2002) using arsenomolybdate reagent. Absorbance was recorded at λ=520 nm and reducing sugar content was determined from a standard curve prepared against pure glucose (0–50 μg).

Statistical Analysis

Statistical analyses were performed with the SPSS (Statistical Package for the Social Science, version 13.0) software (SPSS Inc., Chicago, IL, USA). Data were subjected to variance analysis and means were compared by using Duncan's New Multiple Range Test (DNMRT) at $P \leq 0.05$.

Chemicals

Tris-Phosphate Buffer, photodynamic phosphate and EDTA were purchased from LabScan (Dubline, Ireland), $NaNO_3$ and NaDPH were purchased from Flula (Buchs, Switzerland), acetone and acetic acid were purchased from Sigma-Aldrich (Buchs, Switzerland).

Results and discussion

The results showed that drought stress had significant effects on photosynthetic pigments, proline, soluble sugar contents and peroxidase activity, but drought induction. Moreover, population had a significant effect on chlorophyll *a*, chlorophyll *b* and peroxidase activity. Among all the measured samples, the Khalkhal population had the highest photosynthetic pigments, soluble sugar content and peroxidase activity. However, the Kahak population had the lowest value of the mentioned traits instead of the latter trait. Meanwhile, the severe drought stress produced the highest peroxidase activity, carotenoid, proline, total protein, and soluble sugar contents. By contrast, the lowest values of the mentioned traits were achieved in well-water treatment. The highest contents of chlorophyll *a*, chlorophyll *b* and total chlorophyll were observed in 70% FC, but the lowest were recorded in 20% FC. The Khalkhal population × severe drought stress produced the highest carotenoid, soluble sugar content and peroxidase activity, and also, the Khalkhal population × moderate drought stress recorded the highest levels of phosynthetic pigments.

Changes in photosynthetic pigments

The data of this investigation showed that drought stress had a significant effect on chlorophyll content (Table 1). The Khalkhal population had the highest content of chlorophyll *a* (0.78 mg g^{-1} fw) compared with the Kahak (0.67 mg g^{-1} fw) and the Semnan population (0.64 mg g^{-1} fw). In fact, the mild drought stress (70% FC) increased the content of Chlorophyll *a* (0.78 mg g^{-1} fw) compared with well-watered treatment (0.73 mg g^{-1} fw). The Chlorophyll *a* content was decreased significantly ($P \leq 0.05$) from 0.73% in control plants to 0.58% in severe drought stress level (20% FC), but there was not a significant ($P \leq 0.05$) difference between the moderate and severe drought stress treated plants, 0.68% and 0.58%, respectively. The interactions between the drought stress × population had a significant ($P \leq 0.05$) effect on this trait (Table 3). The Khalkhal population under the moderate (0.84 mg g^{-1} fw) and mild (0.83 mg g^{-1} fw) drought stress showed the highest Chlorophyll *a* content. However, Semnan and Kahak populations under severe drought stress had the least Chlorophyll *a* content (0.55 mg g^{-1} fw).

As regards to the Chlorophyll *b* content, the Khalkhal population had the maximum Chlorophyll *b* content (0.31 mg g^{-1} fw) in comparison to the Kahak population (0.24 mg g^{-1} fw) and the Semnan population (0.23 mg g^{-1} fw) (Table 1). The Chlorophyll *b* content increased slightly from 0.29 mg g^{-1} fw in well-watered treatment to 0.32 mg g^{-1} fw in mild drought stress treated plants. However, under severe drought stress, Chlorophyll *b* content sank (0.20 mg g^{-1} fw) significantly ($P \leq 0.05$) compared with control plants (0.29 mg g^{-1} fw). The interactions between the drought stress × population were significant ($P \leq 0.05$) (Table 3). The Khalkhal population under mild and moderate drought stress showed the highest Chlorophyll *b* content (0.35 mg g^{-1} fw), while Semnan and Kahak populations under severe drought stress sank significantly to 0.18 mg g^{-1} fw. In other words, the Semnan population under severe drought stress decreased 0.6-fold compared with well-water treated plants.

According to the results, the mild drought stress had a significant ($P \leq 0.05$) effect on the total chlorophyll content (1.1 mg g^{-1} fw) (Table 1). In other words, the lowest content of total chlorophyll was obtained at severe drought stress (0.78 mg g^{-1} fw). The data of this investigation showed that mild drought stress (70% FC) enhanced the total chlorophyll content in 3 *Achillea vermicularis* populations. The interactions between drought stress × population were not significant ($P \leq 0.05$) (Table 3).

The carotenoid content was enhanced under drought stress conduction. In fact, well-water treated plants had the least amount of carotenoids (0.32 mg g^{-1} fw) (Table 1). There was a slight difference between severe drought-stressed plants (0.45 mg g^{-1} fw) compared with well-water (0.32 mg g^{-1} fw), mild (0.37 mg g^{-1} fw) and moderate (0.35 mg g^{-1} fw) drought-stressed plants, but it was not significant (P≤0.05).

The results are in agreement with Nyachiro et al. (2001), who described a significant decrease of Chlorophyll *a* and *b*, caused by water deficit in six *Triticum aestivum cultivars* species. The decreased or unchanged chlorophyll level during drought stress has been reported in other species, depending on the duration and severity of drought. A decrease of total chlorophyll levels with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments (Mafakheri et al., 2010).

The decrease in Chlorophyll contents in drought-stressed plants might possibly be due to changes in the lipid protein ratio of pigment–protein complexes or increased chlorophyllase activity. Our results agree with several reports of decreased contents of chlorophyll, but in contrast to carotenoids by the drought or salt stress as reported in a number of plant species (Parida et al., 2007).

Table 1: Effect of drought stress, population and their interaction on the photosynthetic pigments

Treatments	Chlorophyll *a* (mg g^{-1} fw)	Chlorophyll *b* (mg g^{-1} fw)	Total Chlorophyll (mg g^{-1} fw)	Carotenoid (mg g^{-1} fw)
20% FC	0.58 b	0.20 b	0.78 b	0.45 a
40% FC	0.68 ab	0.25 ab	0.93 ab	0.35 b
70% FC	0.78 a	0.32 a	1.10 a	0.37 b
Control	0.73 a	0.29 a	1.02 a	0.32 b
Kahak	0.67 ab	0.24 b	0.91 b	0.36 a
Khalkhal	0.78 a	0.31 a	1.09 a	0.40 a
Semnan	0.64 b	0.23 b	0.88 b	0.36 a
S1P1	0.55 b	0.18 c	0.73 b	0.43 ab
S1P2	0.66 ab	0.23 abc	0.90 ab	0.48 a
S1P3	0.55 b	0.18 c	0.73 b	0.43 ab
S2P1	0.60 ab	0.20 bc	0.80 ab	0.35 b
S2P2	0.84 a	0.35 a	1.20 a	0.37 ab
S2P3	0.60 ab	0.20 bc	0.80 ab	0.35 b
S3P1	0.82 a	0.33 ab	1.15 a	0.37 ab
S3P2	0.83 a	0.35 a	1.18 a	0.39 ab
S3P3	0.69 ab	0.28 abc	0.98 ab	0.36 b
S4P1	0.70 ab	0.27 abc	0.97 ab	0.31 b
S4P2	0.79 ab	0.31 abc	1.10 ab	0.35 b
S4P3	0.72 ab	0.28 abc	1.00 ab	0.31 b

Notes: Means in a column followed by the same letter are not significantly different at P≤0.05; S1 – 20% FC; S2 – 40% FC; S3 – 70% FC; S4 – Control; P1 – Kahak; P2 –Khalkhal; P3 – Semnan.

Changes in peroxidase activity

Experimental findings on antioxidant system indicate that three Achillea populations followed the same trend. The peroxidase activity (mg/Unit protein) enhanced continuously with increasing drought induction in three populations (Table 2). Peroxidase, highly important H_2O_2 scavenging enzyme, increased significantly (P≤0.05) as drought stress levels increased. The Khalkhal population had the most peroxidase activity compared with two other populations. Peroxidase activity is enhanced 7.14-fold in severe drought stress treatment compared with well-water treatment. The interactions between drought stress × population had a significant (P≤0.05) effect on this trait (Table 3). Severe drought stress increased remarkably peroxidase activity in all

three populations, Khalkhal, Semnan and Kahak – 9.5-fold, 3.8-fold, and 17-fold, respectively compared with well-watered plants.

Enhancement in POX activity under various stress conditions has been linked with protection from oxidative damage, lignifications and cross-linking of the cell wall to prevent from such adverse conditions (Helal and Samir, 2008).

Table 2: Effect of drought stress, population and their interaction on the peroxidase activity, proline, protein and soluble sugar contents

Treatments	Peroxidase activity (unit mg^{-1} protein	Proline (µg g^{-1} fw)	Protein (µ mol g^{-1} fw)	Soluble Sugar (mg g^{-1} fw)
20% FC	16.44 a	24.48 a	67.11 a	16.03 a
40% FC	11.05 b	12.33 b	52.00 ab	9.00 b
70% FC	3.49 c	5.79 c	54.63 ab	6.30 b
Control	2.30 c	0.93 d	27.49 b	6.78 b
Kahak	6.17 b	11.73 a	42.69 a	8.78 a
Khalkhal	11.38 a	11.10 a	45.90 a	11.16 a
Semnan	7.41 b	9.83 a	62.33 a	8.64 a
S1P1	15.33 a	26.45 a	64.00 ab	14.20 a
S1P2	19.00 a	23.33 ab	72.00 ab	17.10 a
S1P3	15.00 a	23.66 ab	65.33 ab	16.80 a
S2P1	7.33 b	16.70 bc	50.67 ab	7.89 bc
S2P2	18.48 a	7.66 de	54.67 ab	12.32 ab
S2P3	7.33 bc	12.65 cd	50.67 ab	6.80 bc
S3P1	1.09 c	3.40 e	29.63 ab	6.11 bc
S3P2	6.06 b	12.30 cd	32.91 ab	7.22 bc
S3P3	3.33 bc	1.67 e	101.33 ab	5.58 c
S4P1	0.93 c	0.35 e	26.46 b	5.58 c
S4P2	2.00 c	1.10 e	24.00 b	8.01 bc
S4P3	3.99 bc	1.33 e	32.00 ab	5.40 c

Notes: Means in a column followed by the same letter are not significantly different at P≤0.05; S1 – 20% FC; S2 – 40% FC; S3 – 70% FC; S4 – Control; P1 – Kahak; P2 – Khalkhal; P3 – Semnan.

Table 3: Analysis of variance of the effect of drought stress and population on the measured traits

SOV	df	Mean Squares (MS)							
		Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Carotenoid	Proxidase	Proline	Protein	Soluble Sugar
Replication	2	0.006 ns	0.009 ns	0.031 ns	0.020*	6.574 ns	50.05 ns	1547.9ns	20.05 ns
Population	2	0.066*	0.020*	0.160 *	0.004 ns	89.093**	11.22 ns	1332.8ns	24.07 ns
Drought stress	3	0.064*	0.024 **	0.166 *	0.024 **	398.638**	936.14**	2474.2	181.50**
Drought Stress × Population	6	0.009 ns	0.003 ns	0.024 ns	0*	25.319**	52.55*	1235.1ns	5.52 ns
Error	22	0.018	0.004	0.0416	0.004	4.778	17.588	1375	10.753
CV %	-	19.48	23.48	21.15	17.34	24.25	23.51	23.7	23.39

Notes: ns – non significant; * – significant at P≤0.05; ** – significant at P≤0.01

Changes in contents of proline, total protein and soluble sugars

According to proline estimation (Table 2), the difference between proline content of three populations wasn't significant (P≤0.05). The Kahak population had the maximum proline content (11.73 mg g^{-1} fw), while Khalkhal and Semnan populations showed lower levels of proline – (11.10 mg g^{-1} fw) and (9.83 mg g^{-1} fw) respectively. On the other hand, well-water treated plants had the lowest content of proline (0.9 mg g^{-1} fw). Under mild drought stress, proline content was 5.79. In fact, with the increase of drought stress levels from 5.79 mg g^{-1} fw at 70% FC to 24.4 mg g^{-1} fw at 20% FC, proline content increased significantly (P≤0.05). The interactions between drought stress × population were significant (P≤0.05) (Table 3). Severe drought stress enhanced the proline content in Kahak, Khalkhal and Semnan populations 75.5-fold, 21.2-fold and 17.7-fold in comparison to well-water treated plants, respectively, this increasing role as an osmotic compatible and adjust osmotic potential which resulted in drought stress avoidance in Achillea. Proline accumulation is believed to play an adaptive role in plant stress tolerance. Accumulation of proline has been advocated as a parameter of selection for stress tolerance (Mafakheri et al., 2010).

It is well known that proline contents in leaves of many plants are enhanced by several stresses including drought stress. Thus, we monitored the proline levels in leaves of Achillea populations during drought stress period. Our results on the drought-induced dramatic increase in proline contents in leaves of Achillea agree with earlier reports of accumulation of proline as a compatible osmolyte during the drought exposure. The increased accumulation of proline in Achillea might be due to the decreased activity of proline dehydrogenase, a catabolic enzyme of proline. Thus, it appears that the increase in proline contents during drought induction is an adaptive mechanism in Achillea (Parida et al., 2007). Thus, proline was high enough to be considered the principal solute that may allow plants to overcome drought effect through osmotic adjustment, and serves as storage forms of nitrogen and carbon for future use under less stressful conditions. A function of proline as non-protein amino acid in osmo-adjustment has been proposed, although there may be no cause and effect relationship between proline accumulation and osmo-regulation in plants grown under drought conditions and responses of plants suggested by differences in proline concentrations and responses of plants species to drought. However, the accumulation of proline during drought may have other functions, such as enzyme protection and stabilization of biological membranes, and the degradation of proline may improve the energy status of cells recovering from water deficit (Parida et al., 2007).

As for the total protein content, it is varied widely (Table 2). There was not a significant difference (P≤0.05) between protein content of three populations. The Semnan population had the highest protein content (62.3 μ mol g^{-1} fw) compared with Khalkhal (45.9 μ mol g^{-1} fw) and Kahak population (42.69 μ mol g^{-1} fw). The control plants had the least amount of protein (27.49 μ mol g^{-1} fw), but the enhancement of protein content observed in mild drought-stressed plants (54.63 μ mol g^{-1} fw). Severe drought stress increased protein content to (67.11 μ mol g^{-1} fw). The interactions between drought stress × population were not significant (P≤0.05) (Table 3). The Semnan population under mild drought stress increased 3.1-fold, Khalkhal and Kahak populations under severe drought stress increased 3-fold and 2.4-fold, respectively compared with well-water treated plants.

The marginal change in protein contents in Achillea suggests that protein synthesis or proteolysis is affected minimally by drought stress in this plant. Several reports of alteration of protein synthesis or degradation of protein in various plant species in response to drought support our results. A drought induced decrease in total soluble protein has also been reported in safflower (*Carthamus mareoticus L.*) by Abdel-Nasser and Abdel-Aal (2002). Moreover, the degradation of a 23 kDa polypeptide in the non-secreting mangrove *B. parviflora* in response to high salinity has been reported (Parida et al., 2005). However, in two cultivars of tall fescue (*Festuca arundinacea L.*), the levels of 20 and 29 kDa polypeptides were increased during the drought stress, and a 35 kDa polypeptide was noted in both cultivars only when subjected to drought stress either with or without abscisic acid treatment (Jiang and Huang, 2002). Our results on Achillea are in agreement with increasing evidences of drought-induced accumulation of proteins and physiological adaptations to water limitation (Parida et al., 2007).

The changes observed in total protein, free amino acid and proline contents of several drought-stressed plant species have been attributed to a reduction in the rates of protein synthesis and an increase in proteolytic activity, both of which tend to cause an increase in the total soluble

nitrogen (Shen et al., 1990). In the present study, drought stress resulted in a marginal increase in total protein. These results would suggest that the increase in the protein contents cannot be related to the decrease in amino acids, but could be due to the slight reduction in protein synthesis rather than the initiation of proteolysis as previously shown in *Brassica napus* (Good and Zaplachinski, 1994) and wheat seedlings (Mattioni et al., 1997).

Regarding the soluble sugar content in leaves, the drought stress induction enhanced soluble sugar content (Table 2). The amount of soluble sugars in the Khalkhal population (11.16 mg g^{-1} fw) was higher than that of in the Kahak (8.78 mg g^{-1} fw) and the Semnan populations (8.64 mg g^{-1} fw). The soluble sugar content of well-watered plants was 6.78 mg g^{-1} fw, but it was enhanced significantly ($P \leq 0.05$) to 16 mg g^{-1} fw at severe drought-stressed plants (20% FC). The interactions between drought stress × population had a significant ($P \leq 0.05$) effect on this trait (Table 3). In severe drought induced plants of Khalkhal and Semnan population, the total soluble sugar content was increased 2.1-fold and 3.1-fold, respectively, in comparison to well-watered plants of these populations. In fact, the drought stress induction had a significant ($P \leq 0.05$) effect on the soluble sugar content.

Like other cellular constituents, sugar levels are also affected by stress. In three Achillea populations, we observed an increase in the total soluble sugar content by drought stress. There are also contradictory results on the effect of water and salt stress on sugar accumulation. Some studies have reported sugar contents rose, while others have found sugar contents decreased or remained constant during stress conditions (Parida et al., 2007).

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