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Antioxidative compounds, enzymes activity and nutrient elements in *Stachys byzantina* are altered by climate conditions not by soil parameters

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ABSTRACT

Environmental factors including climate factors and soil parameters affect growth, physiological processes and production of primary and secondary metabolites in plants. In addition, the responses of plants are widely varied to environmental factors. In the current study the impacts of rainfall, temperature and several soil factors such as soil texture, pH, electrical conductivity, organic matter, organic carbon, calcium carbonate and soil elements on nutrient elements, antioxidative compounds, activity, and isoenzymes patterns of peroxidase and superoxide dismutase in *Stachys byzantina* were investigated. *S. byzantina* as a valuable medicinal species was collected from three regions in the north of Iran with different altitudes and ecological conditions. The results showed that the content of nutrient elements (phosphorus, sodium, potassium, calcium), anti-oxidative compounds (phenols, flavonol, flavonoids, and anthocyanin) as well as the activity of peroxidase and superoxide dismutase enzymes along with intensity of their isoenzymes bands were positively correlated with temperature and rainfall, however, almost not correlated with soil parameters. Furthermore, the higher density of *S. byzantina* in the region with lower temperature and rainfall indicated greater adaptability of the plant in higher altitude areas, which is consistent with a decrease in the antioxidant compounds and activity of oxidative enzymes.

Keywords: Environmental factors, Oxidative enzymes, Phenolic compounds, Plant elements.
Article type: Research Article.

INTRODUCTION

Plants in general are influenced by environmental factors mostly climate factors and soil parameters and genetics as well (Nieto-Jacobo *et al*. 2017; Rodrigues *et al*. 2018). Plants are used as food and/or medicine especially due to their chemical composition, nutrient elements and anti-oxidative properties which differ due to several agents such as plant variety or cultivation, harvesting time, processing method, climate, soil and the interrelation of these various factors (Ozcan & Akgul 1995; Maiga *et al*. 2005; Alijanipoor *et al.* 2019; Omidipour *et al.* 2021; El Idrissi *et al.* 2021; Sautkin *et al.* 2021). In addition, different plants vary widely in the type of response to the same factor (Mittler 2006). Likewise, the response to environmental factors is complicated due to the intricate of their interactions with various molecular, biochemical and physiological processes affecting plant growth, development and function (Razmjoo *et al*. 2008). *Stachys byzantina* is a perennial herb belonging to the Lamiaceae family which is native to Turkey, Armenia and Iran (Rechinger & Hedge 1982; Ghahreman 1996). The genus *Stachys* consists of 200-300 species in the world (Rechinger & Hedge 1982) and Iran is a habitat of 34 species of this genus (Mozaffarian 2007). Phytochemical analysis of *S. byzantina* revealed the presence of saponin, terpenoids, steroids, flavonoids, alkaloids, tannins, and carbohydrates (Safarkar *et al*. 2017). The species of this genus is used in folk medicine as an anti-inflammatory agent, antioxidant (Erdemoglu *et al*. 2006; Morteza-semnani *et al*. 2006; Asnaashari *et al*. 2010), anti-microbial (Skaltsa *et al*. 2003; Ebrahimabadi & Batooli 2010), anti-fungal (Digrak

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et al. 2001), anti-nephritic (Hayashi *et al*. 1994), hypnagogic agent (Sindambiwe *et al*. 1999) and used to treat headaches, (Maleki *et al*. 2001; Khanavi *et al*. 2004), genital tumours, cough and angina, ulcers and also sclerosis of the spleen (Zargari 1990; Skaltsa *et al*. 2003). One of the considerable interests in the field of food chemistry and medicine is related to favourable biological effects of enzymatic and non-enzymatic antioxidants in plants (Raghavendra *et al*. 2010; Afsharnezhad *et al*. 2017). The enzymatic antioxidant system is included a number of enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and polyphenol oxidase (PPO) to eliminate the accumulated active oxygen species (AOS; Alici & Arabaci 2016). Polyphenolic compounds are produced through the phenylpropanoid pathway as non-enzymatic antioxidants with a wide range of chemical classes including phenolic acids, flavonoids, anthocyanins, flavons and flavonols as well as their glycosides (Wang & Zheng 2001; Manach *et al*. 2004). Climatic factors e.g., rainfall, relative humidity, temperature, and also altitude from sea level exhibit a significant impact on the plant growth, chemical composition, metabolism and quality of medicinal plants (Wheeler *et al*. 2000; Ncube *et al*. 2012). Increasing in the altitude from the sea level is accompanied by lowering the ambient temperature and moisture (Altshuler & Dudler 2006; Ruckstuhl *et al*. 2007). Antioxidant enzymes are sensitive to physiological changes of plants under different environmental condition (Xie *et al*. 2019) and their activities upraise under the influence of adverse climatic factors to protect plants from free radicals (Mohammadkhani & Heidari 2007). In addition, climatic factors such as temperature affects phenolic compounds in plant tissues due to the alteration in the activity of key enzyme phenylalanine ammonia-lyase (PAL) at different temperatures (Albert *et al*. 2009). Also, water availability can influence on the production of the phenolic compound by changing the photosynthesis system (Sampaio *et al*. 2011). Nutrient elements are very important for plant growth, development, production of secondary compounds and also are effective for the production of medicinal and aromatic plants (Maiga *et al*. 2005; Gilliam & Dick 2010; Butkute *et al*. 2016). The amount of elements that plants can absorb by the roots from the soil varies base on the type of plant, soil properties and climate factors (Baligar *et al*. 2001; Aqaei *et al*. 2020). In addition, organic matter as an agent in soil fertility (Jafari *et al*. 2011; Fageria 2012) can improve soil microbial activity, change in the water holding capacity and provide better access of plant to macro and microelements (Ladha *et al*. 2004; Dhaliwal *et al*. 2019). It has been revealed that soil pH affects phenolic compounds by alteration in the nutrient availability (Kraus *et al*. 2004; Min e*t al*. 2015). In addition, calcium carbonate content of the soils by creating a competition between the production of primary and secondary metabolisms in the plant affects the content of the phenolic compounds. Nitrogen, phosphorus and magnesium are considered as important parameters affecting the synthesis of phenolic due to the impact of these nutrients on the process of photosynthesis (Taiz & Zeiger 2004). On the other hand, the activity of antioxidant enzymes is also affected by the content of calcium and potassium (Moural *et al*. 2017). The wide distribution of *S. byzantina* in the north of Iran reveals a good adaptation of this plant to the ecological conditions of this region of Iran. Recognizing the effect of environmental factors on nutrient value and antioxidant property of medicinal plants has a high economic value and are beneficial in many aspects.

MATERIALS AND METHODS

The fresh plant of *S. byzantina* was collected in June 2017 from three locations including Khalkhil, Kiyasar and Filband in Mazandaran Province, the Northern Iran (Fig. 1).

Fig. 1. The sampling locations of *S. byzantina species* in north of Iran.

The geographical locations, altitude and climatic features of sampling locations have been depicted in Table 1. Khalkhil village in the suburbs of Sari City showed lower altitude (376 m above sea level) and higher temperature, rainfall and humidity than the other two sampling locations (Table 1). Temperature, rainfall and humidity in Kiyasar City by a altitude around 1612 m above sea level, were lower than those in Khalkhil (Table 1). The

Filband Mountains in the suburbs of Babol City by a highest altitude (2357 m above sea level) exhibited a lower temperature, rainfall and humidity (Table 1). The 5-year meteorological report exhibited that the average temperature, rainfall and humidity of Khalkhil were almost 1.8, 1.7 and 1.2 times higher than those in Filband, respectively (Table 1).

Table 1. Longitude, Latitude, altitude (m), temperature (°C), rainfall (mm) and humidity (%) in three plant sampling locations. Different letters indicate significant differences for each parameter ($p \leq 0.01$; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Plant sampling locations	Longitude	Latitude	Altitude (m)	Temperature (°C)	Rainfall (mm)	Humidity (%)
Khalkhil	36.3573821	53.2876436	378^{c}	18.3 ± 0.5^{a}	755 ± 86^{a}	77 ± 1.2^{a}
Kiyasar	36.2147823	53.6028113	1612^{b}	13.6 ± 0.6^{b}	465 ± 105^{b}	68.3 ± 1.5^{b}
Filband	36.1436258	52.5283649	2357^{a}	10 ± 0.4^{c}	448 ± 81^{b}	62.1 ± 1.1^{c}

The *S. byzantina* was collected in 5 replicates with 10 plants in each replicate. The plants were transferred to the laboratory at low temperature (4 °C). Then plant species were taxonomically identified by Flora Iranica (Rechinger 1988). Leaves, flowers and roots were separated, and the fresh weights were measured. For measurement of the chlorophyll a, b and carotenoids, protein, anthocyanin content, activity of peroxidase and superoxide dismutase and analysis of their isoenzymes patterns, plant samples were frozen immediately in liquid N_2 and stored at -80 °C. Also, several of plant samples were dried at room temperature (35 ± 2°C) to analyse the total phenols, flavonoids, flavanols and plant elements. Plant density has been calculated using randomly established five plots (2 m × 2 m) in each plant sampling location. The average annual temperature, rainfall and humidity were obtained using the nearest weather stations to the sampling locations from 2013-2017. Soil samplings were carried out by a collection of soil in 5 replicates from the side of each plant roots (10 plants) in each sampling location.

Soil analysis

The percentage of sand, silt and clay was evaluated by Bouyoucos method using a buffer solution containing sodium hexametaphosphate and sodium carbonate (Bouyoucos 1962). Air-dried and powdered soil samples were dissolved in distilled water (g mL^{-1}) and immersed for 12 h without movement, then pH and EC were measured by a multimeter portable device (AZ8603, Taiwan). The organic carbon and organic matters content were determined after oxidation of soil with $K_2Cr_2O_7$ (10 g mL^{-1}) and H_2SO_4 (10 g mL^{-1}). The ortho-penanthroline was used as an indicator and the solution was titrated with $(NH_4)_2Fe(SO_4)_2$ solution (Walkley & Black 1934). To measure calcium carbonate ($CaCO_3$), the soil sample was oxidized with HCL (10 g mL^{-1}). After adding Phenolphthalein as an indicator, the solution was titrated with NaOH (1 N; Ali Ehyaei & Behbahanizadeh 1993). The soil nitrogen content was determined by the Kjeldahl method (Kjeldahl 1883). The soil sample (1 g) digested with sulphuric acid (3 mL) and 1.1 g catalyst mixture (K_2SO_4: $CuSO_4$: Se; 100: 10: 1) at 420 °C. After cooling, the solution diluted with distilled water to a final volume of 25 mL. During this step the ammonium ions (NH_4^+) are converted into ammonia (NH_3) using sodium hydroxide solution (20 mL), then boric acid and Methyl red-bromocresol green indicator were added to the solution and finally the solution was titrated with sulfuric acid until the disappearance of green color. The soil phosphorus content was determined by Olsen method (Olsen 1954). The soil was extracted with sodium bicarbonate (0.5 M, pH 8.5). The suspension was shaken for 30 min and filtrated through a filter paper. After 60 min, the absorbance was measured at 720 nm. To determine sodium and potassium, the soil samples (5 g) were extracted in ammonium acetate (100 mL, 1 N) at pH 7.0. After 24 h, the concentration of sodium and potassium was measured by the flame photometric method at 589 and 766.5 nm, respectively. The content of these elements was calculated according to the standard curve (Ali Ehyaei & Behbahanizadeh 1993). To determine calcium and magnesium, the soil extract (1 mL) was added to 9 mL of lanthanum (0.1%) and the volume was made up to 25 mL using distilled water. Thereafter, their content was analysed using atomic absorption spectroscopy at 422.7 and 385.2 nm, respectively and calculated according to the standard curve (Ali Ehyaei & Behbahanizadeh 1993).

Plant analysis

To determine the content of chlorophyll a, b and carotenoids, frozen plant samples were extracted in 80% acetone (10 g mL^{-1}). After centrifugation (30000 g, 20 min), the absorbance was read spectrophotometrically at 663, 647,

and 470 nm for analyses of the chlorophyll a and b and carotenoids, respectively and finally calculated according to Lichtenthaler (1987). The total nitrogen content was determined by the Kjeldahl method (Kjeldahl 1883). Oven-dried plant material (0.3 g) was digested in sulphuric acid and hydrogen peroxide at 280 °C. Afterward, sodium hydroxide (2 mL; 10 N), boric acid (15 mL) and Methyl red-bromocresol green indicator were added to the extraction to collect NH_3^+ by boric acid. In the final step, the amount of the ammonia was titrated with sulfuric acid (0.01 N) continuing until the disappearance of green colour. Phosphorus content was determined based on vanadate-molybdate reagent (Emami 1996). Oven-dried plant material (1 g) was extracted by 5 mL hydrochloric acid (2 N) and 1 ml Barton reagent. The absorbance of the solution was measured at 470 nm. The concentration of phosphorus in plant samples was calculated based on mg g^{-1} DW. To determine the content of potassium, sodium, calcium and magnesium, oven-dried plant sample (1 g) was extracted with 5 mL hydrochloric acid (2 N). Thereafter, the volume was made up to 100 mL with distilled water. Then the absorbance of the plant extract was measured using flame photometer at 766.5 and 589 nm for determination of potassium and sodium content, respectively (Ali Ehyaei 1997). In the case of measuring calcium and magnesium, plant extract (1 mL) was mixed with lanthanum oxide (9 mL) and made up to 20 ml with distilled water, then the absorbance was recorded using the atomic absorption method at 422.7 and 385.2 nm for measuring calcium and magnesium, respectively. In the case of providing plant extract for analysing anti-oxidative compounds, air-dried leaves and flowers (0.4 g) were extracted by 70% methanol then sonicated for 2 h using ultrasound followed by centrifuging for 20 min at 6000 rpm. Then the supernatant was used for measurement of the total phenols, flavonoids and flavanol content (Thygesen *et al*. 2007). Total content of phenols content was measured based on a colorimetric oxidant/ reduction reaction (Singleton *et al*. 1999). Folin-ciocalteu reagent was used as an oxidizing agent. 125 µL plant extracts were made up to 500 µL with distilled water, mixed thoroughly with 2.5 mL 10% folin for 6 min, followed by the addition of 2 mL sodium carbonate (7.5%; w/v). After 90 min incubation at 25 °C, the absorbance was measured at 765 nm. The total phenols content was calculated based on the calibration curve of Gallic acid. Total content of flavonoid was assayed according to the Akkol method (2008) based on the flavonoid-aluminium complex. Plant extracts (2 mL) were mixed with distilled water (2.8 mL), aluminium chloride solution (100 µL) and potassium acetate (100 µL). The mixture was kept in the dark for 30 min at room temperature and absorbance of the mixture was measured at 415 nm. The total content of flavonoid was calculated based on calibration curve of Rutin.

Total content of flavonol was determined according to the Akkol method (2008). Plant extract (1 mL), was mixed with aluminium chloride solution (1 mL) and sodium acetate (3 mL) followed by incubating at 25°C for 2.5 h and measuring the absorbance at 440 nm. The total content of flavonol was calculated based on the calibration curve of Rutin. The measurement of anthocyanin was performed according to the procedure given by Masukasu *et al*. (2003). Plant fresh weight (0.2 g) was homogenized with 3 mL methanol- HCl (V/V HCl 1%) for one min, then filtered through Whatman No.1 filter paper. The extract was centrifuged at 6000 rpm for 25 min at room temperature. The supernatant was stored at 4 °C in dark for 24 h. The absorbance of the solution was recorded at 550 nm and expressed as µM g^{-1} FW. The DPPH radical-scavenging activity was assayed by the procedure described by Thygesen *et al*. (2007). The plant sample (0.4 g) was homogenized in methanol (70%). The mixture was centrifuged at 90 rpm for 2 h. Then, the fresh DPPH solution (1 mL) was added to a plant extract (1 mL) to start the radical antioxidant reaction at a final concentration of 0.004% $DPPH^{\bullet}$. The reaction mixture was kept in the dark for 30 min when DPPH reacted with an antioxidant compound (the colour of the solution changed from deep violet to light yellow) and the absorbance was measured at 517 nm. To provide plant extract for analysing activity of anti-oxidative enzymes and their isoenzyme patterns, frozen plant samples were extracted in 0.1 M potassium phosphate buffer with pH 7.0 (1 g fresh weight per 10 mL) at 4 ºC and filtered through one layer of Miracloth. The filtered extract was centrifuged at 16,000 g at 4 ºC for 25 min. The soluble protein content was determined by Bradford's method using bovine serum albumin as a standard (Bradford 1976). Peroxidase (POD, EC.1.11.1.7) activity was assayed according to the procedure of Nakano & Asada (1981). The reaction mixture contained 200 µL plant extract, 2.5 mL potassium phosphate buffer (0.1 M; pH 7.0), 80 µL guaiacol reagent (20 mM) and 250 µL hydrogen peroxide (40 mM) in a total volume of 3 mL. After 1 min the absorbance was recorded at 470 nm. The enzyme activity was expressed as U mg^{-1} protein. Peroxidase isoenzymes were detected by incubating the polyacrylamide gel (12.5%) in a reaction mixture containing 50 mM sodium acetate buffer (pH 4.5) and 2 mM benzidine hydrochloride which initiating the reaction by the addition of H_2O_2 (Abeles & Biles 1991). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured according to a method described by Beauchmp & Fridovich (1971). The reaction mixture contained 0.1 M potassium phosphate (PH

7.8), 13 mM L-Methionine, 0.1 mM EDTA, 10 µM Riboflavin, 75 µM nitrobluetetrazolium (NBT) and 400 µL plant extract. Then the reaction mixture was illuminated with a fluorescence lamp for 10 min and the absorbance read at 560 nm. The enzyme activity was expressed as U mg^{-1} protein. Superoxide dismutase isoenzymes were detected on the gels by the method of Beauchmp & Fridovich (1971). The sodium dodecyl sulphate-polyacrylamide gels (10%) were incubated for 60 min in dark in a staining buffer (0.24 mM NBT, 28 µM riboflavin, 28 mM TEMED, 0.5 M EDTA and 50 mM Monosodium phosphate). Then the gel was illuminated by two fluorescent lamps (20 W each) to promote the photoreactive staining and continued until the bands became visible.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed and the treatment means were compared using Tukey's HSD all-pairwise comparisons at the $p \leq 0.01$ level as a post-hoc test and the correlations between the variables were determined by Pearson's correlation coefficient.

RESULTS

Soil analyses

The soil texture in Filband (sandy loam) was different from those in Khalkhil and Kiyasar (clay loam). Soil pH exhibited a marked difference among different sampling locations while the electrical conductivity (EC) in Kiyasar was higher than those in Khalkhil and Filband (Table 2). The organic matter and organic carbon content in the soil of Kiyasar were approximately 4 and 2 times higher than those in Khalkhil and Filband, respectively (Table 2), while the calcium carbonate content in the Khalkhil was almost 3 and 8-fold higher than those in Kiyasar and Filband, respectively (Table 2). The results showed that total nitrogen content in Khalkhil was 3 and 7-fold higher than that in Kiyasar and Filband, respectively, while the phosphorous content was remarkably altered among different sampling locations (Table 2). The highest amount of sodium (0.284 g kg^{-1}) and potassium (0.506 g kg^{-1}) were observed in Kiyasar. The sodium and potassium content in Kiyasar were approximately 1.5 and 2.5 times higher than those in the other two locations, respectively (Table 2). Also, the calcium content in the samples of Khalkhil and Kiyasar were almost 1.5 times higher than that in Filband, respectively, while, magnesium in Khalkhil was approximately 1.8 and 2.5 times higher than in Kiyasar and Filband, respectively (Table 2).

Table 2. Texture, electrical conductivity (dS m^{-1}), pH, organic matter (g kg^{-1}), organic carbon (g kg^{-1}), calcium carbonate (g kg^{-1}) and the soil elements content (nitrogen, phosphorous, sodium, potassium, calcium and magnesium) in three plant sampling locations of Northern Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five replicates (± SD) with 10 soil samples in each replicate. Different letters indicate significant difference for each parameter ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Plant sampling locations	Khalkhil	Kiyasar	Filband
Texture	clay loam	clay loam	sandy loam
Electrical conductivity (dS m^{-1})	0.47 ± 0.004^{c}	0.7 ± 0.01a	0.61 ± 0.005^{b}
pH	7.8 ± 0.02^{a}	7.6 ± 0.01^{a}	7.2 ± 0.4^{a}
Organic matter (g kg^{-1})	11 ± 0.4^{c}	45 ± 0.2^{a}	19 ± 0.3^{b}
Organic carbon (g kg^{-1})	6.6 ± 0.14^{c}	26.2 ± 0.13^{a}	11 ± 0.3^{b}
Calcium carbonate (g kg^{-1})	179 ± 7^{a}	55 ± 10^{b}	23 ± 7^{c}
Nitrogen (g kg^{-1})	9 ± 0.1^{a}	3 ± 0.12^{b}	1.3 ± 0.2^{c}
Phosphorous (mg kg^{-1})	3.4 ± 0.1^{a}	2.9 ± 0.3^{a}	3.4 ± 0.2^{a}
Sodium (g kg^{-1})	0.165 ± 0.01^{b}	0.25 ± 0.007^{a}	0.17 ± 0.01^{b}
Potassium (g kg^{-1})	0.17 ± 0.003^{b}	0.5 ± 0.006^{a}	0.16 ± 0.007^{b}
Calcium (g kg^{-1})	6.5 ± 0.1^{b}	7.4 ± 0.08^{a}	4.9 ± 0.1^{c}
Magnesium (g kg^{-1})	0.51 ± 0.1^{a}	0.28 ± 0.01^{b}	0.22 ± 0.01^{c}

Plant analysis

The plant density was different among three sampling locations. The higher plant density was observed in Filband which was approximately 2 and 5.5 times higher than that of the Kiyasar and Khalkhil, respectively (Table 3).

Table 3. Plant density (Plants m^{-2}) in three plant sampling locations in northern Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five plots (replicates; ± SD). Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Plant sampling locations	**Khalkhil**	**Kiyasar**	**Filband**
Density (Plants m^{-2} ± SD)	8 ± 5^{c}	20 ± 3^{b}	45 ± 10^{a}

The results showed that the content of chlorophyll a and b in the plant leaves collected from Filband was approximately 2.5 and 2 times higher than those from Khalkhil and Kiyasar (Fig. 2). This incremental trend was also true for carotenoids. So that, the carotenoids content was approximately 1.8 and 1.3 times higher in the plant leaves collected from Khalkhil than those from Khalkhil and Kiyasar, respectively (Fig. 2). In addition, the ratio of chlorophyll a to b was remarkably altered in the plant leaves collected from different sampling locations meaning that its content changed identically in different sampling locations (Fig. 2). The total nitrogen content in the plant leaves and flowers collected from Khalkhil was 1.1 and 1.3 times higher than those from Kiyasar and Filband, respectively (Fig. 3). Similarly, the total nitrogen content in the plant roots was higher in Khalkhil, while there was no significant difference between those collected from Kiyasar and Filband (Fig. 3). In addition, the phosphorus content in the plant leaves, flowers and roots collected from Kiyasar was slightly lower than those from Kiyasar and Filband (Fig. 3). Calcium content of the plant leaves, flowers and roots in Khalkhil was significantly higher than those in Kiyasar and Filband. The calcium content in the plant leaves collected from Khalkhil was approximately 1.5 and 2 times higher than those from Kiyasar and Filband, respectively, while its content in the plant roots from Khalkhil was 1.5 and 2.5 times higher than those from Kiyasar and Filband, respectively. Similarly, its content in the flowers from Khalkhil was higher than those from other two locations. Likewise, the highest content of sodium was observed in the plant leaves, flowers and roots grown in Khalkhil (Fig. 3).

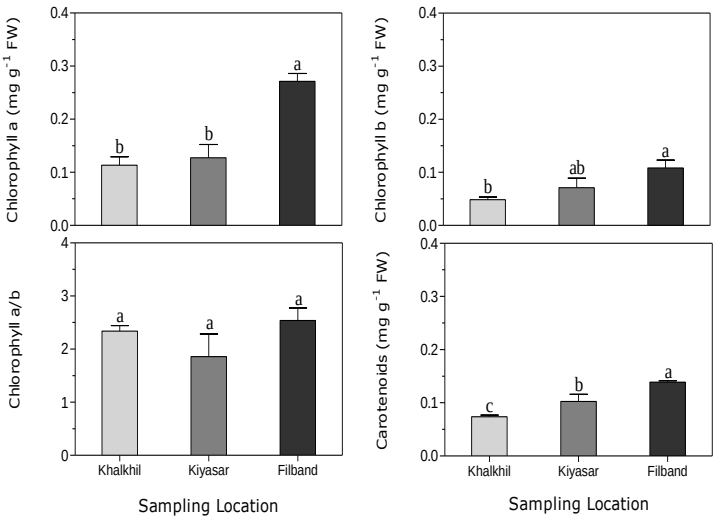

Fig. 2. Chlorophyll a, b and carotenoids content (mg g^{-1} FW) of the plant leaves collected from the three sampling locations in Northern Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five replicates (±S D) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Potassium content in the leaves collected from Khalkhil was 1.4 and 1.8 times higher than that of the plant leaves collected from Kiyasar and Filband, respectively. Similarly, the highest amounts of potassium was observed in the plant roots and flowers collected from Khalkhil (Fig. 3). Result showed that, the content of magnesium in the plant leaves collected from Khalkhil was almost 1.4 times higher than those collected from Kiyasar and Filband. In addition, there was no significant difference between the magnesium content in the plant flowers collected from the Khalkhil and Filband, while its content in the flowers collected from these two locations was approximately 1.4 times higher than that in Kiyasar. In addition, its content in the roots from Khalkhil was approximately 1.4 times higher than those from Kiyasar and Filband (Fig. 3).

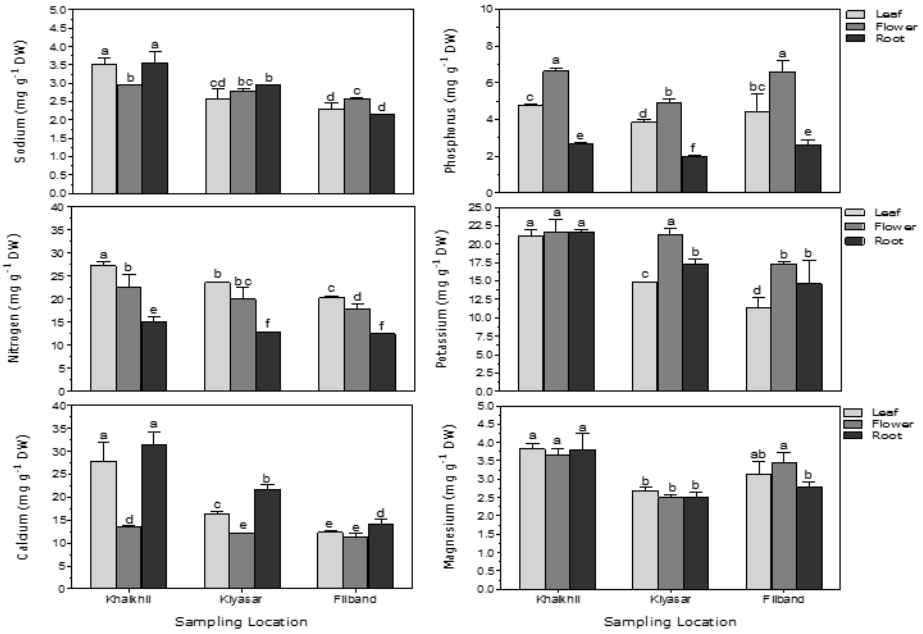

Fig. 3. Total nitrogen, phosphorus, sodium, potassium, calcium and magnesium content (mg g^{-1} DW) of the plant leaf, flower and root collected from the three sampling locations in Northern Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five replicates (± SD) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

The results showed that the content of phenols in the plant leaves grown in Khalkhil was approximately 1.4 times higher than those collected from the two other locations (Fig. 4), while, its content in flowers did not show any significant difference among various sampling locations (Fig. 4). In addition, flavonoids content in the plant leaves collected from Khalkhil was almost 1.4 times higher than those from the two other locations. Similarly, their content in the plant flowers from Khalkhil was significantly higher than those from Kiyasar and Filband, respectively (Fig. 4). The flavonol content in the leaves from Khalkhil and Kiyasar was almost 1.3 times higher than that in Filband (Fig. 4), while the flavonol content in the flowers collected from Kiyasar was 1.1 and 1.3 times higher than those in Khalkhil and Filband, respectively (Fig. 4). Moreover, the anthocyanin content in the leaves collected from Khalkhil was almost 1.4 times higher than the two other locations. Similarly, its content in the flowers from Khalkhil was significantly higher than those from Kiyasar and Filband, respectively (Fig. 4).

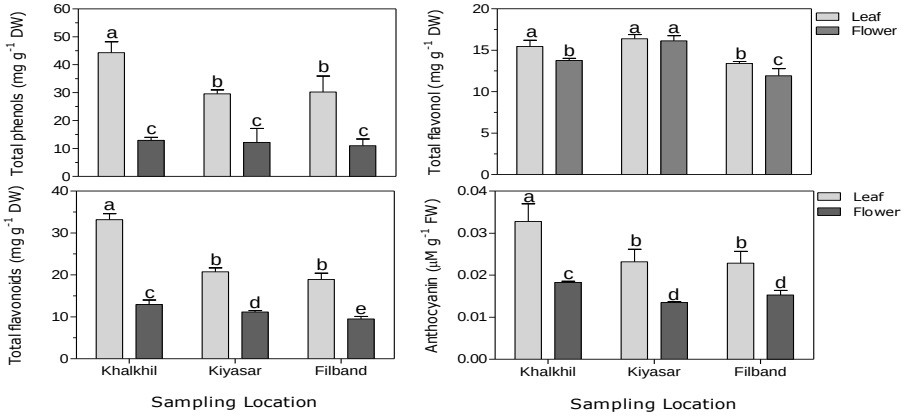

Fig. 4. Total Phenols (mg g^{-1} DW), flavonoids (mg g^{-1} DW), flavonol (mg g^{-1} DW) and anthocyanin (µg g^{-1} FW) content of the plant leaf and flower collected from the three sampling locations in north of Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five replicates (± SD) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

The result showed that the free radical DPPH in the leaves and flowers was not statistically different between Kiyasar and Filband. Its content in the both flowers and leaves from Kiyasar and Filband was higher than that from halkhil, by almost 20 and 14 %, respectively (Fig. 5).

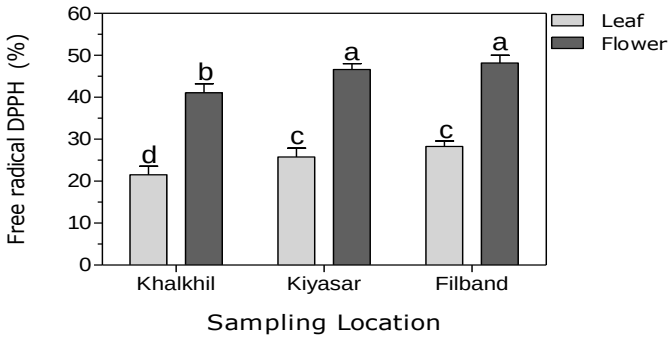

Fig. 5. Free radical DPPH (%) of the plant leaf and flower collected from three sampling locations in northern Iran including Khalkhil, Kiyasar and Filband. Data represent the mean of five replicates (± SD) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Peroxidase activity in both leaves and flowers collected from Khalkhil was almost 60% higher than that grown in the two other locations (Fig. 6A), while its activity in both leaves and flowers grown in Kiyasar and Filband exhibited no significant differences (Fig. 6A). The peroxidase isoenzyme patterns showed 3 bands (a, b and c) in both leaves and flowers (Fig. 6B). The intensities of all three bands were higher in leaves collected from Khalkhil (Fig. 6B). Likewise, the intensities of bands a and b in flowers from Khalkhil and Kiyasar was higher than those collected from Filband (Fig. 6B).

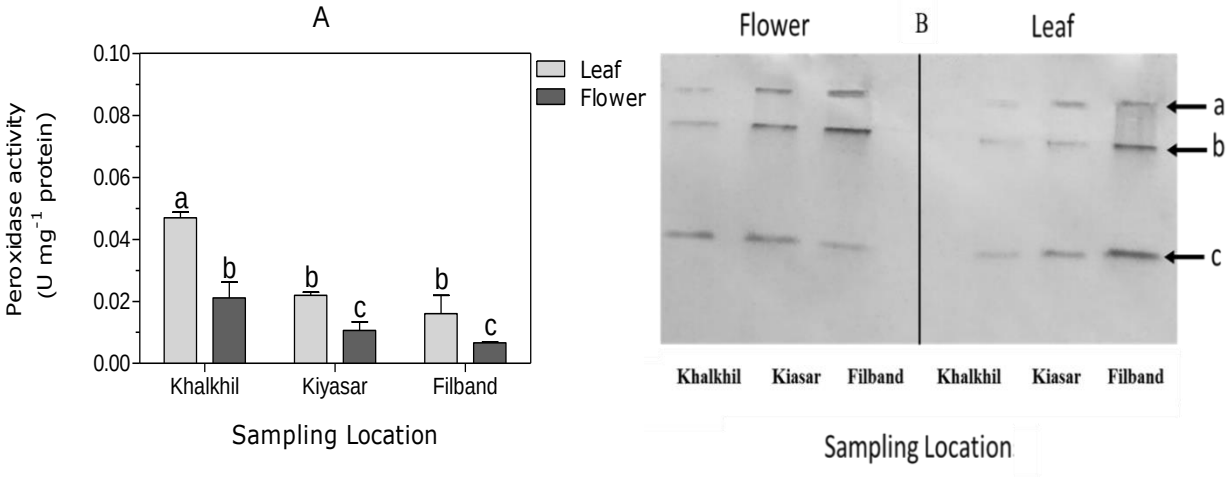

Fig. 6. Peroxidase activity (A; U mg^{-1} protein) and peroxidase isoenzyme pattern (B) of the plant leaf and flower collected from three sampling locations in northern Iran including Khalkhil, Kiyasar and Filband. Data for Peroxidase activity represent the mean of five replicates (± SD) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-Way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Superoxide dismutase activity in the plant leaves grown in Khalkhil was almost 30 and 60 % higher than those collected from Kiyasar and Filband, respectively (Fig. 7A). Also, its activity in flowers from Khalkhil was approximately 25 and 45 % higher than those grown in Kiyasar and Filband, respectively (Fig. 7A). The superoxide dismutase isoenzyme pattern showed four bands (a, b, c and d) in the leaves and six bands (a, b, c, d, e and f) in the flower (Fig. 7B). The intensity of band a in the leaves collected from all three sampling locations was almost the same, while, the intensities of bands b, c and d in the leaves from Khalkhil were higher than the bands from the two other sampling locations (Fig. 7B).

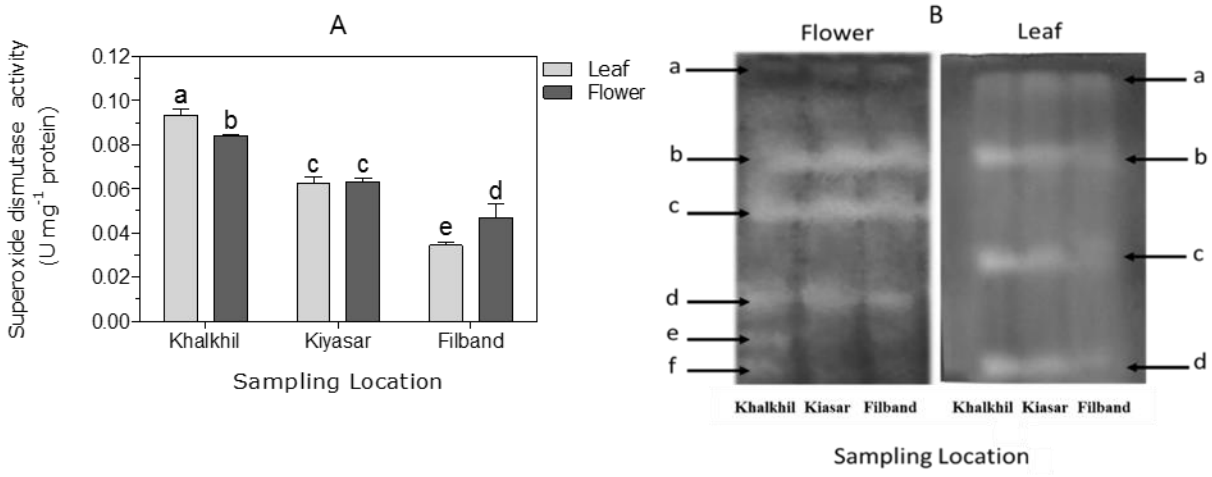

Fig. 7. Superoxide dismutase activity (A; U mg^{-1} protein) and superoxide dismutase isoenzyme pattern (B) of the leaf and flower of the plants collected from three sampling locations in north of Iran including Khalkhil, Kiyasar and Filband. Data for Superoxide dismutase activity represent the mean of five replicates (±SD) with 10 plants in each replicate. Different letters indicate significant difference ($p \leq 0.01$; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

DISCUSSION

Generally, the soil nutrient production and accumulation especially soil carbon and nitrogen which are the main sources of soil organic carbon and soil organic matter, depends largely on the vegetation type and density (Prescott 2002; Rees *et al*. 2005; Thomas *et al*. 2007; Shedayi *et al*. 2016). Furthermore, vegetation density is decreased in forest and pasture at higher elevations due to the short growing period (Rees *et al*. 2005; Thomas *et al*. 2007). In addition, clay soils contain more elements than sandy soils due to low permeability (Nouri *et al*. 2001), and also the presence of negative charges on the surfaces of clay particles may lead to attracting more cations element than sandy soil (El-Ghani *et al*. 2003). Similar to the clay soil, organic matter has a negative charge and it possesses the ability to absorb and hold cations such as K^+, Na^+ and Ca^{2+} (Brady & Weil 2007). Therefore, Filband with a higher altitude than the other sampling locations may exhibit rather a low amount of soil nutrient elements such as nitrogen, potassium, calcium, and magnesium, due to its sandy-loam soil texture, low soil organic carbon and soil organic matter. The quantity and quality of medicinal plants are influenced by genetics, climate factors, soil parameters and their interactions (Abdalla & El-Khoshiban 2007). All plants require elements to complete their life cycle (Singh & Schulze 2015). Balance of elements in plant tissues is of great importance in plant growth and is directly and indirectly affected by the climate conditions, soil substrate, geographical location and plant taxonomy (Ordonez *et al*. 2009; Zhang *et al*. 2012). Our study displayed that the nutrient elements content in plant organs were not mostly in line with that in the soil, along with the soil organic carbon and organic matter (Table 2; Fig. 3). In addition, pH was remarkably altered among different sampling locations (Table 2). In addition, electrical conductivity (EC) in all three sampling locations was lower than 1 ds m^{-1} meaning that the plant grows in a non-saline area (Table 2). In addition, it seems that a slight difference in EC has led to almost the same effect on plant growth. Indeed, there is not always a significant relationship between the amount of nutrients in the soil and plant (Govasmark *et al*. 2005). Availability of the soil nutrient and interactions between them affect the content of the plant nutrient elements (Broadley *et al*. 2001; Ordonez *et al*. 2009), and concentration of an ion in the soil affects the penetration rate of the same ion inside the cell (Limeneh *et al*. 2020). It seems that the content of cation elements in plant depends not only on the absolute quantities of the cations in the soil solution but also on their activities relative to one another. For example, the inhibitory effect of calcium on potassium absorption have been attributed to an effect of calcium in decreasing the permeability of cellular membranes (Elzam & Hodges 1967). In addition, many studies also indicate that an antagonistic relationship between the uptake of potassium and calcium (Wakeel 2013; Rietra *et al*. 2017) may due to ion competition at the site of absorption (Lombin & Fayemi 1976). A significant positive correlation among climate factors including temperature, rainfall and humidity has been observed in the three different plant sampling locations (Table 4).

Table 4. Correlation of environmental factors with each other in the three different sampling locations including Khalkhil, Kiyasar, and Filband, Mazandaran Province, northern f Iran. Values are present as Pearson correlation coefficient (Pearson r) and the sign of two stars (**) was considered as significant statistical sign at the probability levels of 1%.

		Altitude	Rainfall	Humidity	Temperature
Altitude	Pearson Correlation	1			
	Sig. (2-tailed)	0			
	N	15			
Rainfall	Pearson Correlation	-.906**	1		
	Sig. (2-tailed)	0			
	N	15	15		
Humidity	Pearson Correlation	-.999**	.882**	1	
	Sig. (2-tailed)	0	0		
	N	15	15	15	
Temperature	Pearson Correlation	-.994**	.948**	986**	1
	Sig. (2-tailed)	0	0	0	
	N	15	15	15	

In the current study, the most nutrient elements content in the different plant tissues was in line with climate factors in the three different sampling locations (Table 5, Fig. 3). In addition, the content of many elements in the plants was positively correlated with temperature (Table 5). In general, soil temperature around the roots is directly affected by the air temperature with a difference of around ± 2 °C (Pregitzer *et al*. 2000). Likewise, increase in soil temperature leads to enhance in the rate of nutrient uptake capacity via the energy consuming transport system associated with root respiration (Hussain & Maqsood 2011; Boczulak *et al*. 2014), and affects chemical reaction speed, the amount of water and elements transportation in the soil (Pregitzer & King 2005). In addition, rainfall leads to increase in soil water and subsequently increase in the rate of mobility, diffusion and absorbing of nutrient elements by the root (Brouder & Volenec 2008; Bista *et al*. 2018). A positive correlation between rainfall with the content of sodium, potassium, calcium and nitrogen in leaves and flowers of *S. byzantine* (Table 5) indicates that accessibility to water is an important factor in uptake of elements by the plant. Anti-oxidative compounds and enzymes are valuable groups of plant metabolites and enzymes that are widely distributed throughout plants. In addition, they can be used as chemical markers in plant against environmental variables (Kováčik *et al*. 2007; Stockham *et al*. 2013; Awasthi *et al*. 2015). Several studies have demonstrated an enhancement in the concentration of phenolic compounds with altitude, as a protective response to the high UV radiation at elevated altitudes (Spitaler *et al.* 2006; Jaakola *et al.* 2004; Spitaler *et al.* 2006). However, this trend has not been observed in the current study. So that, the content of phenolic compounds and also the activity of anti-oxidative enzymes were declined by increasing altitude. The hypothesis that plants from higher altitudes contain higher amounts of radical scavenging compounds cannot be affirmed in general (Markham *et al.* 1998; Spitaler *et al*. 2006; Rieger *et al.* 2008; Bernal *et al.* 2013). It has been suggested that the reduction of flavonoids might be a consequence of increased synthesis of other defensive compounds like wax that seems to be biosynthetically preferred at higher altitudes (Shepherd & Griffiths 2006; Bernal *et al*. 2013). Since both, cuticular waxes and flavonoids, are derived from acetyl-CoA (Kunst & Samuels, 2003). Furthermore, in the current study it has been observed that the impact of climatic factors on enzymatic and non-enzymatic antioxidants was higher than soil factors. The response of plant to temperature is changed based on the plant species (Hasanuzzaman *et al*. 2012). As the density of *S. byzantina* in Filband was almost 6 times higher than that in Khalkhil (Table 3), it seems that *S. byzantina* is a cold-adapted plant. It has been shown that an elevation in the number of mitochondria in the cells of cold-adapted plant makes them sensitive to the relatively warm temperatures and thereby alter the normal homeostasis of plant cells (Miroslavov & Kravkina, 1991; Noctor *et al*. 2007; Korner *et al*. 2016). In addition, temperature higher than need in cold-adapted plants increases respiration, sometimes above the rate of photosynthesis. Thus, photosynthesis products are used faster than their production and this has a negative effect on carbon balance and the plant will be under stress (Körner & Larcher 1988).

Table 5. Correlation of climate parameters with plant elements (nitrogen, phosphorus, sodium, potassium, calcium and magnesium) in three different sampling locations including Khalkhil, Kiyasar, and Filband at Mazandaran province in north of Iran. Values are present as Pearson correlation coefficient (Pearson r) and the sign of two stars (**) was considered as significant statistical sign at the probability levels of 1%.

		Nitrogen (Leaf)	Nitrogen (Flower)	Nitrogen (Root)	Phosphorous (Leaf)	Phosphorous (Flower)	Phosphorous (Root)	Calcium (Leaf)	Calcium (Flower)	Calcium (Root)	Sodium (leaf)	Sodium (Flower)	Sodium (Root)	Potassium (Leaf)	Potassium (Flower)	Potassium (Root)	Magnesium (Leaf)	Magnesium (flower)	Magnesium (Root)
Altitude	Pearson Correlation	-0.97**	-0.70**	-0.85**	-0.306	-0.168	-0.169	-0.94**	-0.91**	-0.922**	-0.937**	-0.935**	-0.973**	-0.980**	-0.726**	-0.997**	-0.403	-0.431	-0.813**
	Sig. (2-tailed)	0	0.004	0	0.267	0.548	0.548	0	0	0	0	0.000	0	0	0.002	0	0.136	0.109	0
	N	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Rainfall	Pearson Correlation	0.827**	0.536**	0.864**	0.203	0.536	0.536	0.898**	0.861**	0.868**	0.904**	0.769**	0.784**	0.890**	0.438**	0.671**	0.526	0.514	0.988**
	Sig. (2-tailed)	0	0.039	0.0	0.057	0.039	0.039	0	0	0	0	0.001	0.001	0	0.103	0.006	0.044	0.05	0
	N	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Humidity	Pearson Correlation	0.978**	0.699**	0.836**	0.278	0.121	0.121	0.934**	0.907**	10.00**	0.929**	0.944**	0.984**	0.978**	0.752**	0.941**	0.383	0.415	0.800**
	Sig. (2-tailed)	0	0.004	0	0.315	0.668	0.668	0	0	0	0	0	0	0	0.001	0	0.159	0.124	0
	N	15	15	15	15	15	12	15	15	15	15	15	15	15	15	15	15	15	15
Temperature	Pearson Correlation	0.952**	0.685**	0.869**	0.364	0.27	0.27	0.947**	0.916**	0.981**	0.946**	0.908**	0.941**	0.974**	0.663**	0.872**	0.443	0.461	0.888**
	Sig. (2-tailed)	0	0.005	0	0.182	0.331	0.331	0	0	0	0	0	0	0	0.007	0	0.098	0.084	0
	N	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15

Indeed the overproduction of reactive oxygen species (ROS) and subsequently increasing the activity of antioxidant enzymes in plants is the result of temperature-induced stress (Padda & Picha 2008). This may explain an upraise in the superoxide dismutase and peroxidase activity and the intensity of their isozymes bands in the warmer sampling location (Khalkhil) in the current study. The higher production of phenolic compounds under the influence of higher temperature probably is due to the increased activity of PAL enzyme activity, an important enzyme in the biogenesis of various phenolic compounds, for protection of plant tissue damage (Padda & Picha 2008). Rainfall higher than the plant tolerance may cause hypoxia directly in the root and indirectly in the aerial parts of the plant (Drew 1983). In this situation, all systems involved in the generation of energy, respiration and photosynthesis are suppressed and the functional relationships between roots and shoots are disturbed (Vartapetian & Jackson 1997; Aqaei *et al*. 2020). As a result, any perturbation in respiration and photosynthesis can cause the formation of ROS (Yordanova *et al*. 2004). An imbalance between the formation of ROS and antioxidant scavenging capacity is resulted in the creating of oxidative stress in the plants. Therefore, an increase in the activity of antioxidant enzymes and intensity of their isoenzymes bands, as well as increase in the content of phenolic compounds in Khalkhil with higher rainfall than two other locations, may be expected to control the level of ROS and to protect the cell. Arslan & Ozcan (2011) in a study on the phenolic profile and antioxidant properties of olive fruits in different locations, tended to similar results with our study. They observed that the olive fruits collected from locations that had the highest average rainfall compared to the other sampling locations was contained the highest phenolic compounds (Arslan & Ozcan 2011). Among soil parameters, only the content of soil nitrogen and magnesium was in line with the content of antioxidative compounds and the activity of antioxidative enzymes (Figs. 3, 6 and 7). Nitrogen as a macronutrient is necessary for the plant growth and synthesis of phenolic compounds (Zahedzadeh *et al*. 2015) which is probably due to stimulation of the PAL activity as a key enzyme in the biosynthesis of phenolic antioxidant compounds (Olsen *et al*. 2009), by providing the amino acid phenylalanine as a substrate for biosynthesis of phenolic components (Sun *et al*. 2012). Also, sufficient nitrogen availability enhances the activity of antioxidant enzymes and upregulates the gene expression of the antioxidant enzymes and protect the plant against environmental stressful conditions (Zhang *et al*. 2013; Movludi *et al*. 2014; Ahmad *et al*. 2019). Magnesium as another important nutrient is essential for the maintenance of the activity of enzymes such as kinases, polymerases and homeostasis of reactive oxygen species (Hermans & Verbruggen 2005; Sreedhara & Cowan 2002) and activity of 4-coumaryl CoA ligase and glutathion S-transferase enzymes which are involved in biosynthesis and translocation of anthocyanin (Alfenito *et al*. 1998).

CONCLUSION

In conclusion, nutrient elements in the shoot and root of *S. byzantina* were significantly affected by temperature and rainfall and less affected by soil parameters except for the content of nitrogen and magnesium which was positively correlated with their content in the soil. Likewise, the content of most anti-oxidative compounds as well as the activity of peroxidase and superoxide dismutase enzymes and intensity of their isoenzyme bands were in line with temperature and rainfall but not with soil parameters. Furthermore, it seems that cold-adapted plant such as *S. byzantina* is rather sensitive to relative elevation in temperature and rainfall.

Conflict of interest

We declare that we have no conflict of interest.

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