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ORIGINAL ARTICLE

Changes of Enzymes Activity and Production of Secondary Metabolites of *Artemisia aucheri* in Different Altitudes and Its Relation to Adaptation

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KEYWORDS

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ABSTRACT: *Artemisia* plants are the most abundant plants species in Iran which contain strong antioxidant properties and as such, have medicinal and economic value. Despite wide distribution of *Artemisisa* species, ecophysiology of its adaptation to changes in altitude and soil property had not been investigated. In this study, the relationships between ecophysiological and adaptation capabilities of *A. aucheri* to altitude changes through measuring changes in the activity of its antioxidant enzymes and secondary metabolites *in situ* was investigated based on a completely randomized experiment. The enzyme activities of superoxide dismutase, catalase, peroxidase, and the amount of total phenolics, flavonoids, anthocyanins, malondialdehyde and chlorophylls A and B were measured in *A. aucheri plants* growing in three different altitudes at and above the 36° latitude on the southern slopes of Eastern Alborz Mountain ranges in triplicate 10*10 m quadrates. Statistical analysis of data showed that soil type was loamy significantly becoming more sandy-loam with lowering in altitude and the soil contained greater amounts of oxides of silicone, aluminum, magnesium, sodium, potassium and phosphorus in upper altitude except calcium which was present in greater quantity in lower altitude. With increasing altitude, activity of superoxide dismutase and quantities of chlorophylls and total phenols in leaves increased. Some biochemical factors in *A. aucheri* showed significant positive correlation($P \leq 0.05$) between them. Adaptation of *A. aucheri* to changes in altitude occurred through changing its antioxidant enzymes activity and production of secondary metabolites in response to factors related to the altitude including soil type and texture, moisture level, temperature and most importantly radiation

INTRODUCTION

Adaptation of plants to grow in different habitats requires specific abilities that differ among plant species. Production of reactive oxygen species (ROS) is one of the biochemical processes in plants that occurs as a result of living and non-living environmental stresses

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[1] and reduces plant performance through damaging plant parts and cell components such as cell membranes, proteins, lipids , pigments and DNA expression [2, 3]. Plants in order to accommodate more tolerance against environmental stresses have developed efficient physiological and biochemical enzymatic response mechanisms such as production of superoxide dismutase, catalase and peroxidases, and non-enzymatic antioxidant compounds such as phenolic compounds and flavonoids to rid themselves of free radicals [4-10]. Thirty four species of *Artemisia* (with English names worm wood and sage brush) are the main and most common prennial species in steppic and semi-steppic ecosystems of Iran [11]. Due to their distinctive features, *Artemisia* plants are highly resistant against extreme environmental conditions and very effective in stabilizing the habitat; have great forage value, are medicinal and exhibit strong antioxidant property via their phenolic compounds and have conservation and aesthetic values [12]. Despite wide distribution of *Artemisisa* species, ecophysiology of its adaptation to changes in altitude and soil propoerties has not been investigated under natural circumstances. In this study, the relationship between ecophysiological and adaptation of *A. aucheri* to altitude changes through measuring changes in the activity of antioxidant enzymes and secondary metabolites is investigated *in situ*.

MAREIALS AND METHODS

Sampling of soil and *A. aucheri* plants was done in triplicates in 3 altitudes at and above 36 °latitude line on the southern slopes of Alborz Mountains in Semnan Province, central Iran (Table 1) in a completely randomized design in late May, 2012. Samples were transported to the laboratory of Department of Plant Sciences, Tarbiat Modarres University for further analysis. Plant tissues (Roots, stems and leaves) were frozen in liquid nitrogen and were kept in - 80 °C freezer to be used for biochemical analysis. The reaction mixture without enzyme extract was used as controls. All materials used were prepared from Merck Company.

Table 1. Geographical coordinates and other properties of sampling stations

Station	Altitude(m)	Geographical Coordinates	coordinates between stations (m)	Slope (percent)
E1	2338	N:35,59,55.2 E:53,35,46.5	E1- E2 3325.89	E1-E2 0.110
E2	2009	N:35,58,41.3 E:53,29,0.97	E2- E3 8059.76	E2-E3 0.031
E3	1783	N:36,02,16.6 E:53,24,48.9	E1- E3 41173.8	E1-E3 1.497

Soil Analysis

Soil samples were air dried for 72 hours. Soil subsamples (3g of 2 mm mesh) were analyzed via XRF method in Geology Laboratory of Tarbiat Modares University. Soil texture was determined using a hydrometer after soaking 200 g of soil for 24 h. Soil pH was determined using potentiometric method and EC was measured using an EC meter [13,14].

Enzyme Assays

Total protein content was determined by the Bradford (1976) method. The standard curve was developed using a solution of 0.5 mg ml bovine serum albumin (BSA) and a solution of 0.15 mM NaCl with spectrophotometry at 595 nm. The concentration of protein in the plant extract was calculated as per mg protein per g of fresh tissue [15].

Catalase activity (CAT) was measured by the method of Cakmak and Horst (1991) using 0.2 g thawed sample in 3 ml of 25 mM sodium phosphate buffer, pH 6.8. Absorption at a wavelength of 240 nm was used. Activities of the absorption changes in fresh weight were expressed as mg protein per minute [16, 17].Peroxidase activity (POD) was determined using method of Chance and Maehly (1955). Absorption at a wavelength of 470 nm was used. Enzyme activity changes were expressed as per mg protein per minute [18-20].Superoxide dismutase activity (SOD) was determined using method of Giannopolitis and Ries (1997) on 0.2 g frozen sample in 3 ml of HEPES-KOH buffer, pH 7.8 containing EDTA 0.1 mM at absorbance of 560 nm. One unit of SOD activity was defined as the amount of enzyme which resulted in 50% inhibition of nitro blue tetrazulium at 560 nm [21].Flavonoids were measured according to the method of Chang et al. (2003) on 0.4 g plant tissue pulverized with and centrifuged in 4 ml of methanol at absorbance of 415 nm. Using a standard solution and linear equation routines, the concentration of flavonoids in extracts of plant samples were expressed in terms of mg per g of plant fresh tissue [22]. Total anthocyanins were measured according to the method of Krizek et al. (1993) on 0.1 g of plant tissue pulverized with and centrifuged in 3 ml of acidified methanol containing hydrochloric acid and methanol in the ratio of 99 to 1 at absorbance of 550 nm. To calculate the concentration, the extinction coefficient of 33000 cm^{-2} mol^{-6} was used. The anthocyanin concentration was expressed as mg per g of plant fresh tissue [23, 24].

Phenols content was calculated using a standard curve based on asolution of gallic acid as mg per g of plant fresh tissue [25, 26]. Total phenolics were determined using the method of Miliauskas et al., (26) on 0.1 g of plant tissue pulverized in 3 mL of methanol at absorbance of 730 nm.

Measurement of lipid peroxidation (LPO) was carried out according to the method of Heath and Packer (1968) [27], using measurements of malondialdehyde (MDA), as a final product of membrane lipid peroxidation on 0.2 g frozen plant material mixed in 3 ml of 10% TCA (Trichloroacetic acid) at absorbance of 532, 440 and 600 nm. MDA content was calculated using a constant extinction coefficient (ε=155 mM-1cm-1) applied by De vos et al., (1991) [28]. Leaf chlorophyll content was measured using method of Arnon (29) on 0.5 g fresh leaf tissue in 25 ml of acetone at absorbance of 663 and 645 nm wavelengths. Contents of chlorophylls A, B and total chlorophyll per mg /g wet weight were calculated [29].Statistical analyses of data were performed with three independent replicates using Excel and SPSS and significant differences were determined via analysis of variance (ANOVA) and comparisons were tested with Duncan's multiple range test, $P \leq 0.05$.

RESULTS

Statistical analysis of soil samples showed that soil type was loamy significantly becoming more sandy- loam with decrease in altitude (Table 2). Silt content was greater in the lowest altitude. pH and EC did not differ ($P < 0.05$) amongst stations. Soil samples were slightly alkaline and haline. Soil elemental analysis showed presence of oxides of silica, aluminum, magnesium, sodium, potassium and phosphorus in upper altitudes except calcium which was present in greater content in the lowest altitude. Oxide of silicone (SiO_2) occurred in

greater quantities in all in all stations. Such a trend was not observed for iron and sulfur.

Table 2. Comparison of means by Duncan's test at 5% probability level and standard deviation of soil physical factors sampled at three heights

Stations (Mean ± SD)			Characteristics
Altitude1800m	**Altitude2000m**	**Altitude2300m**	
(E3)	**(E2)**	**(E1)**	
76.4^{a}±1.632	70.4^{c}±1.41	72.4^{b} ±1.63*	Sand
5.6 ^{a} ±0.00	7.35 ^{a} ±0.5	5.6 ^{a} ±0	Clay
18 ^{a} ±1.632	22.25 ^{b} ±1.25	22 ^{b} ±1.63	Silt
8.0775^{a}±0.026	8.0475^{a}±0.05	8.13^{a}±0.073	Soil acidity (pH)
1.1875^{a}±0.0629	1.175^{a}±0.05	1.1^{a}±0.081	Electrical conductivity (EC)

Different letters in each row indicate significant differences between treatments at the 5% level

Biochemical analysis of Artemisia tissues

With increasing altitude, the amount of superoxide dismutase activity in roots and shoots increased and this difference was significant compared to the station located at 36 ° latitude (Table 3). The amount of enzyme activity in leaves showed no significant difference between the three stations. The lowest peroxidase activity and the most catalase activity of *Artemisia* plant roots occurred in the station located at 36 ° latitude with statistically significant differences. Comparison of catalase activity in *Artemisia* stems showed that with increasing altitude activity of this enzyme significantly decreased.

The highest amount of total flavonoids in Artemisia occurred in plants growing in the station located at 36 ° latitude with a significant difference with that of flavonoids in upper altitude. Amount of flavonoids in the roots showed no significant differences between different altitudes but flavonoid contents in stems were significantly different between the plants growing in the lowest and the highest altitudes. The amount of total phenolics in *A. aucheri* significantly increased with increasing altitude. The amount of anthocyanin in roots of plants, but not in leaves and stems, in all stations showed significant difference between plants growing in higher altitudes to those plants growing at 36 °latitude. Content of malondialdehyde in *A. aucheri*, as an end-product of lipid peroxidation reaction, decreased with increasing altitude (Table 2).Contents of chlorophylls a and b in leaves of *A. aucheri* showed significant differences between the plants growing in the highest altitude and plants of other stations. Also, at each station, the amount of chlorophyll a was significantly greater than the amount of chlorophyll b (Figure 1).

The results showed that there was positive correlation between root superoxide dismutase activity with the activity of catalase and chlorophyll b content of leaves. Also, the amount of anthocyanins correlated moderately with contents of flavonoids and malondialdehyde of *Artemisia* vegetative organs.

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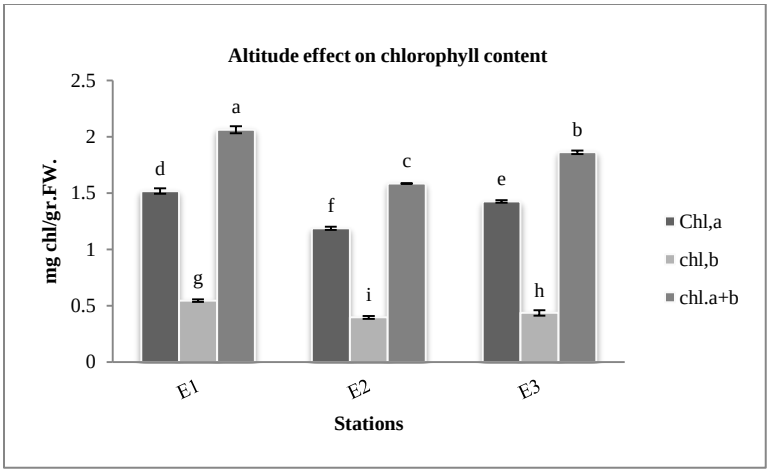

Figure 1. Effect of altitude on the *Artemisia aucheri* plant chlorophyll content

Data represent the mean of three replicates and vertical bars are standard deviations. In each group, non-identical letters indicate significant differences at the level of $p \leq 0.05$. E1: 2300 meters, E2: 2000 m, E3: altitude of 1800 m

DISCUSSION

The results of this study showed that with increasing altitude, the amount of superoxide dismutase activity, total phenolic and chlorophyll contents increased and membrane lipid peroxidation decreased in mountain sagebrush (*A. aucheri*). Plants differences in physiological response of sagebrush plants in different altitudes are expected, however factors contributing to these differences merits detailed consideration. The fact that soil type in all stations was sandy - loamy and only contents of sand and silt differing amongst stations makes effectiveness of physical and chemical properties of soil in determining ecophysiological changes and adaptive capabilities of the sagebrush plants arguable. This could be further analyzed either by increasing the difference in altitude between stations and or selectively sampling plant populations with distinct differences in their soil types. But still, one cannot rule out the importance of soil texture and quality on plant growth. Other contributing factors associated with altitude in mountainous habitats are changes in UV radiation, temperature and humidity due to precipitation which potentially and effectively could affect levels of oxidative stress and type and quantity of antioxidants in *A. aucheri* plants.

Oxidative stress resulting from ultraviolet radiation leads to the generation of ROS in plants. ROSs is also produced during normal metabolic processes and under various biotic and abiotic stresses [30, 31]. Superoxide radicals are formed in cells under oxidative stress [32] and cause aging in plants [33]. Thus, different species, in order to improve tolerance against environmental stresses have developed complex and effective biochemical mechanisms to detoxify antioxidants [34]. In fact, higher plants possess a number of enzymatic and non-enzymatic brooms to eliminate ROSs and water and fat soluble oxidants in different parts of the cell. Antioxidants are able to transfer of a hydrogen atom to free radicals and thus prevent damaging oxidation reactions [35] and triggering mechanisms of resistance to stress [36]. Higher peroxidase activity and lower peroxidation (lower MDA) in higher altitudes signified the importance of adaptive response of *A. aucheri* to maintain root growth, a finding that supported the findings of Nematy Corym (1999) [37].

The powerful antioxidant properties of *Artemisia* are attributed to chlorogenic acid, a phenolic compound that resembles the strong antioxidant, ascorbic acid which protects cells from oxidative damage [38, 39]. The primary role of superoxide dismutase (SOD) is to protect against ROS which can directly determine the concentration of intracellular O^{-}_{2} and H_2O_2 [19] via removal of superoxide to prevent production of hydroxyl radical [40, 41]. In this study, a significant increase in the activity of SOD in root and stem tissues reflected an increased rate of (O^{-}_{2}) as a result of SOD dissmutation of ROSs with increasing altitude. Hydrogen peroxide is stored in peroxysomes as well as glyoxysomes and broken down by SOD and or UV light in presence of oxygen. Furthermore, peroxidases, available in cytosol, vacuoles and other cell organels, also show a greater affinity towards breaking down hydrogen peroxide than catalase which requires 2 molecules of H_2O_2 to occupy its active site [42]. Greater peroxidase and lower catalase activity in *A. aucheri* in higher altitudes confirms earlier findings [43, 44].

Peroxidation of cell membrane phospholipids leads to the production of free radicals initiated by ROSs and cell lipooxygenases and mediated by hydroxyl groups [45-48]. It is well known that phenolic compounds are important components of plant defense mechanisms and play significant role in stress responses of plants to biotic and abiotic stresses [8]. Antioxidant properties of phenolic compounds are attributed to the presence of hydroxyl groups in their structure [49, 50]. Plants such as *A. aucheri*, which contain greater phenolic compounds have the potential to detoxify free radicals to a greater extent [51-53].

The quality and quantity of light affects total chlorophyll content as well as photosynthetic ability of plants. In this study, the amount of chlorophylls A and B was significantly higher in upper altitudes. Increased, chlorophyll contents along with increased contents of anthocyanins and total phenolic compounds indicated the adaptive capability of *A. aucheri* to increased radiation, particularly UV radiation in upper altitudes. Although, in this investigation, the effect of UV radiation on plant performance and adaptive response was not directly measured, and since geographical location affects the amount of light reception by plants, the subject merits detailed investigation [54]. On the other hand, increased photosynthesis increases the production of soluble sugars, accommodates structural polysaccharides and maintains stabilized osmotic potential, characteristics essential to preserve and maintain basic metabolic processes during stress conditions.

Greater content of some soil elements such as silica, aluminum, magnesium, potassium, sodium and phosphorus along with height imply occurrence of less washing because of lower precipitation, and consequently, indicating the adaptive ability of *A. aucheri* to maintain itself under alkaline soil pH and slightly moderate salinity. It is hereby, concluded that *A. aucheri* avoids harmful effects of UV radiation in higher altitudes via production of strong antioxidants [38, 39]. On the other hand, altitude, direction of sunlight and degree of slop affect environmental factors such as temperature fluctuations, humidity levels and nutrient availability [55].

CONCLUSION

Findings of this research, although corroborated findings of earlier studies (56, 57) in regards to the effects of topography and the 1800 m elevation above sea level on the distribution of sagebrush (*A. aucheri*) species, reports on the ability of this plant growing at lower elevations as well. Therefore, it is suggested a more detailed quantitative and qualitative investigation of antioxidant production by *Artemisia* species *in situ* and *in vitro* circumstances be undertaken both with conservation and exploitation objectives.

Table 3. Comparison of mean and standard deviation (SD) activity of antioxidant systems in three high mountain sagebrush vegetative organs by Duncan test at 5% probability level

Stations	Treatment	Catalase (ΔAbc240/mg protein)	Peroxidase (ΔAbc470/mg protein)	Superoxide dismutase (unite/mg protein)	Flavonoids total (mg/g F.W)	Anthocyanins (mg/g F.W)	Phenol (mg GA/g F.W)	MDA (µ mol/g FW)
Altitude 2,300 m (E1)	Root	4.386^{bcd} ±0.340	53.787^{b*} ±2.843	189.988^{b} ±10.22	11.288^{e} ±0.30	34.204^{f} ±2.23	3.905^{c} ±0.03	1.807^{e} ±0.08
	Stem	3.606^{cd} ±0.503	63.442^{a} ±7.019	228.271^{a} ±21.76	21.699^{d} ±0.69	42.312^{f} ±3.37	4.506^{b} ±0.19	2.088^{dc} ±0.06
	Leaf	7.248^{a} ±0.900	26.979^{c} ±1.271	183.047^{bc} ±3.89	46.267^{b} ±3.46	97.254^{c} ±9.51	5.024^{a} ±0.03	2.583^{c} ±0.23
Altitude 2000 m (E2)	Root	7.493^{a} ±0.852	32.148^{c} ±1.622	144.379^{d} ±11.78	12.399^{e} ±0.92	60.090^{de} ±4.68	3.183^{d} ±0.31	2.445^{cd} ±0.04
	Stem	3.351^{d} ±0.423	32.502^{c} ±3.817	189.611^{b} ±17.54	24.635^{cd}±2.44	44.747^{f} ±3.98	4.402^{b}±0.02	2.849^{bc}±0.18
	Leaf	4.742^{bc} ±0.622	14.869^{d} ±0.441	172.010^{bcd}±19.01	49.812^{b} ±4.83	118.095^{b} ±11.36	4.948^{a} ±0.36	4.591^{a} ±0.28
Altitude 1800 m (E3	Root	6.753^{a} ±1.022	50.617^{b} ±5.635	151.454^{cd} ±6.02	13.424^{e} ±0.73	64.333^{d} ±5.63	2.240^{e} ±0.09	2.901^{bc} ±0.24
	Stem	5.538^{b} ±0.590	69.406^{a} ±7.481	199.357^{ab} ±27.24	28.702^{c} ±2.52	47.716^{ef} ±4.18	4.205^{bc} ±0.28	3.270^{b}±0.33
	Leaf	5.019^{b} ±0.598	33.339^{c} ±4.934	195.275^{b} ±25.15	66.212^{a} ±3.52	136.702^{a} ±13.54	4.377^{b}±0.29	4.829^{a} ±0.48

* Different letters in columns indicate significant differences between treatments at the 5% level

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