Acta Biologica Turcica 30 (4) 146-151, 2017 Received 29 Oct. 2017; Accepted 4 Dec. 2017

ACTA BIOLOGICA TURCICA

E-ISSN: 2458-7893, http://www.actabiologicaturcica.com

The role of free proline and soluble carbonhydrates in serpentine stress on some serpetinophyte and serpentinovag plants

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Abstract: The aim of this study is to exhibit the roles of free proline and soluble carbonhydrates in serpentine stress. For this purpose, contents of free proline and soluble carbonhydrate were analyzed in serpentinophytes (*Paronychia angorensis* Chaudri) and serpentinovags [both serpentine and non-serpentine *Alyssum sibiricum* Willd., *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz, *Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet)]. While free proline content is low in serpentinophytes, soluble carbonhydrate ratio is high (Ch/Pr; 8.5). Free proline in the individuals of serpentinovags growing on serpentine soils is proportionally low, but soluble carbonhydrate ratio is high (Ch/Pr; 2.73, 2.57, 4.83). Soluble carbonhydrate ratio in the individuals of serpentinovags growing on non-serpentine soils increases (Ch/Pr: 7.95, 2.60, 1.96, 13.5) while free proline decreases. Although proline content is higher in some species growing on serpentine soils in comparison to those plants not under stress conditions this is not a general case. It is however observed that soluble carbonhydrate content in serpentine plants is commonly high.
Keywords: Serpentine stress, Serpentinophyte, Serpentinovag, Soluble carbonhydrate.

Introduction

Plants living in harsher habitats have to develop certain adaptations to survive. Adverse environmental conditions create extreme stresses on plants. Ecological specialization is rather common utilization of habitat in the nature (Futuyma and Moreno, 1988; Stevens, 1989; Brown, 1995; Gaston and Blackburn, 2000). Ecologic patterns play an important role in losses or gains of biological diversity in the evolutionary process. Nonetheless, evolutionary origin of adaptation to habitat has not been set forth completely, yet. Edaphic specialists are key players in the study of local adaptation and ecological speciation (Anacker, 2011; Crawford et al., 2014; Abdula et al., 2016).

Serpentine soils are rather harsh habitats for plants, and determination of how plants overcome such drawbacks has always been an interesting subject. Serpentine soils as a product of ultramafic rocks formed of ferromagnesian silicates are extremely rich in terms of floristic diversity, particularly of endemic and rare taxa. High content of Mg and Fe and low Ca in serpentine soils is not suitable for plants growth and development thereon. Serpentine soils, although rich in heavy metal such as Ni, Co, and Cr, are poor in certain basic nutrients such as N, P, and K (Avcı, 2005).

Serpentine systems are rather intriguing due to abundance of existing endemic plant species, adaptive morphologies of plants unique to serpentine, and the unique nature of serpentine bodies (Brady et al., 2005; Anacker, 2014). Such plants develop various adaptations unique to serpentine, such as schlerophyll and microphyll nature and spiny body structure (Iturralde, 2001). Specialist species on habitat survive by developing adaptations to such intense stress factors. Of those plants adapting to serpentine systems, those being essential serpentine plants, namely obligate ones, and not being able to go out of serpentine, are named as "*serpentinophyte*", and those able to grow facultatively under different edaphic conditions, both serpentine and non-serpentine, as "*serpentinovag*" (Kurt et al., 2013). Serpentine soils exist generally on rocky-substrate escarpments in low-humidity openings. Intense stress due

Table 1. Table of bioclimatic synthesis.

Station	Altitude (m)	P(mm)	M (^{o}C)	m (^{o}C)	Q	S	Precipitation regime	Bioclimate
Ankara	401	32.0	-1.7	62.8	41.3	1.96	S.W.A.S	Semi-arid lower cold Mediterranean climate

P: Average annual precipitation (mm); **M**: Average of maximum temperatures of the warmest month (^{o}C); **m:** Average of minimum temperatures of the coldest month (^{o}C); **Q**: The quotient ($Q=2000.P/M^2-m^2$); **S:** Arid index (S=PE/M).

to edaphic conditions of stress source causes speciation in serpentine soils and inverse speciation of serpentine endemism.

It was suggested long ago that among the responses of plants exposed to stress is to contribute embryonic development of cell by accumulating certain soluble matters such as proline, carbonhydrate and glycinebetaine in cell cytoplasm and organelles; that proline accumulated particularly in arid and saline environments acts as osmo-regulator and plays a role in prevention of nitrogen amount and energy in post-stress period. In the literature at the present time, there are studies in this regard and supportive results explaining its role (Signorelli, 2016).

Soluble carbonhydrates accumulated under stress conditions take a role in regulating osmotic cell density acting as a protection and preventing cell dehydration. Having sufficient amount of soluble carbonhydrates in plant leaves prevent proline oxidation (Choi et al., 2016). It is assumed that soluble carbonhydrates act as precursors enabling free proline synthesis. When plants got stressed, they accumulate carbonhydrates such as glucose, fructose, and sucrose for performing the maintenance of osmotic equilibriums, C accumulation, etc. (Sami et al., 2016). Proline is thought of as one factor for plants to drought stressful conditions, but cannot be used to distinguish potato genotypes for their stress tolerance to osmotic stress in vitro conditions (Bündig et al., 2016).

This study addresses exchanges of only two variables, free proline, and soluble carbonhydrate that are specified as closely related to the subject. Serpentine soils exist generally on rocky-substrate escarpments in low-humidity openings, and the vegetation acclimatized themselves to these habitats are under osmotic stress in a substantial part of their life cycles. This study is intended for testing the hypothesis claiming that in order to tolerate osmotic stress, *serpentinophyte* and *serpentinovag* plants must have high concentrations of compounds with osmotic effects such as free proline and soluble carbonhydrates. To our knowledge, this is the first study to test this hypothesis in *serpentinophyte* and *serpentinovag* plants.

Materials and Methods

Brief description of the study area: The study area is located in the east part of Central Anatolia. The altitudinal range of the area varies between 850 and 1400 m. (Location of study area has given in Table 2 as coordinate). The climate of the region in which the steppe vegetation is dominant is characterised by cold winters, often with frost, and hot summers with drought periods (Fig. 1). That indicates the prevalence of semi-arid lower cold and semi-arid upper very cold variants of Mediterranean climate. The steppe vegetation developing under xeric conditions is characterised by xerophytic species of Irano-Turanian origin. The data of the meteorological stations of Ankara was used to determine the type of the climate in the region Ankara is under the effective control of very cold, upper semiarid variant of Mediterranean type, particularly predominant in east part of central Anatolia (Akman, 2010). Bioclimatic data show that the study area is located in a semi-arid upper and lower zone effected of Mediterranean climate in Central Anatolia (Table 1).

Study material is composed of some plant specimens collected from serpentine bodies as well as those collected from another field of non-serpentine soil for comparison purposes during the vegetation period in 2015 and 2016 around Elmadag (Ankara) (Table 2). Dividing the study area into grids of 1x1 km^2, so many plant specimens were collected in different directions, elevations and gradients representing the study area. After brought to the laboratory, plant specimens collected as whole were rinsed with distilled water, its dwarf shoots (leaves) were picked, and leaf specimens were dried in a drying oven for 12 hours at 60°C.

Analyses of proline and soluble carbohydrate in the specimens pertaining to various populations collected from Elmadag region were carried out in three repetitions. Analysis results were calculated as mean values for each species. Degree of diversity between species of different living strategies was determined by means of variance

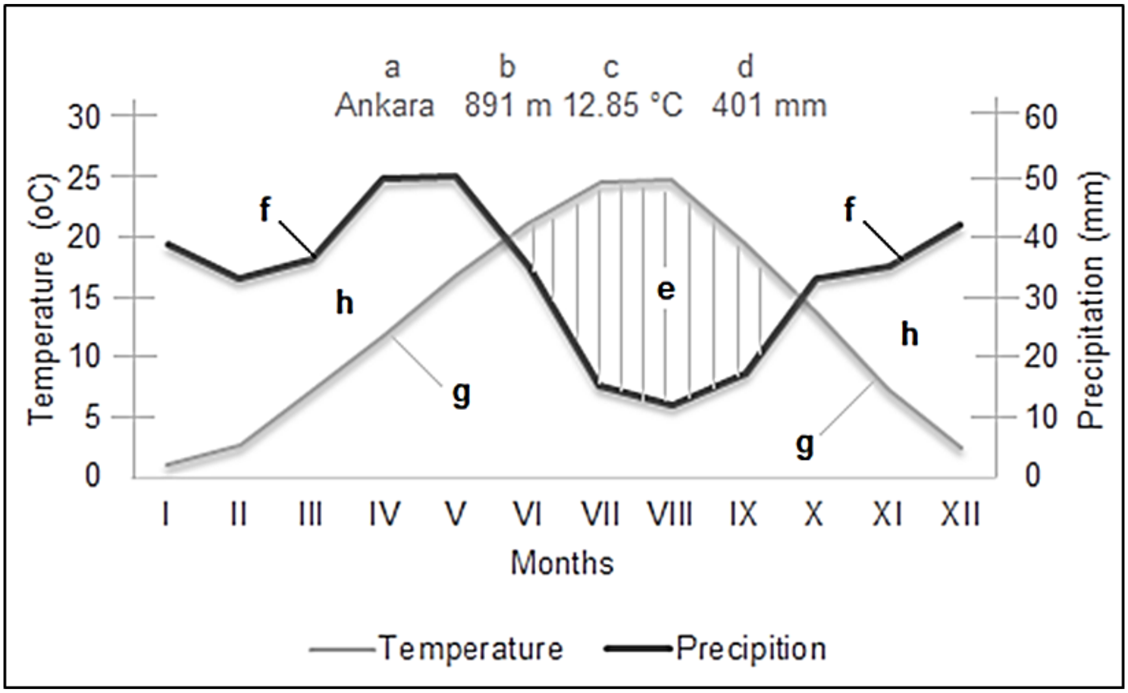
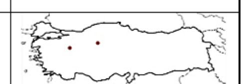
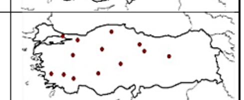
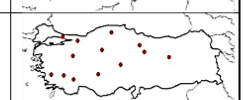
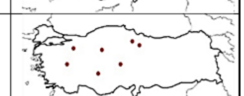
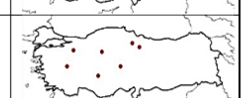
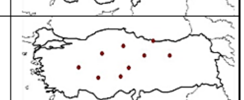
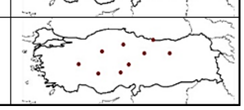


Figure 1. Ombrothermic diagram of Ankara station (a. Station name b. Altitude c. Average of annual temperature (°C) d. Average of annual precipitation (mm) e. Arid period f. Precipitation curve g. Temperature curve h. Rainy period).

Table 2. Serpentine and non-serpentine plants.

Code	Family	Species	Soil	Life Strategy	Life Form *	Phytogeo graphy**	Location	IUCN	Distribution
S1	*Illecebraceae*	*Paronychia angorensis* Chaudri	Serpentine	Narrow Serpentinophyte	H	Ir.-Tur. End.	Ankara (Elmadag) N 39° 47' 55.0" E 033° 12' 66.7"	VU	
S2-A	*Brassicaceae*	*Alyssum sibiricum* Willd.	Serpentine	Serpentinovag	H	-	Ankara (Beynam Forest) N 39° 41' 33.4" E 032°58' 44.1"	-	
S2-B	*Brassicaceae*	*Alyssum sibiricum* Willd.	Non-serpentine	Serpentinovag	H	-	Ankara (Polatlı) N 39° 56' 51.5" E 031° 56' 54.6"	-	
S3-A	*Asteraceae*	*Centaurea urvillei* DC. subsp. *stepposa Wagenitz*	Serpentine	Serpentinovag	H	Ir.-Tur.	Ankara (Beynam Forest) N 39° 41' 33.4" E 032°58' 44.1"	LC	
S3-B	*Asteraceae*	*Centaurea urvillei* DC. subsp. *stepposa Wagenitz*	Non-serpentine	Serpentinovag	H	Ir.-Tur.	Ankara N 40° 09' 34.4" E 032°46' 15.5"	LC	
S4-A	*Lamiaceae*	*Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet	Serpentine	Serpentinovag	H	Ir.-Tur. End.	Ankara (Beynam Forest) N 39° 41' 33.4" E 032°58' 44.1"	LC	
S4-B	*Lamiaceae*	*Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet	Non-serpentine	Serpentinovag	H	Ir.-Tur. End.	Ankara N 40° 09' 27.7" E 032°45' 36.5"	LC	

*Life form: H: Hemicryptophyte **Phytogeography: Ir.-Tur.: Irano-Turanian End.: Endemic ***IUCN: VU: Vulnerable LC: Least concern.

analysis (ANOVA).

Free Proline Content: Of the dried specimens, 0.2 gram was crushed and extracted in 10 ml of 3% 5-sulfasalicylic acid in a mortar. 2 ml of ninhydrin and 2 ml of glacial acetic acid were added on 2 ml of the extract; the solution was incubated at 100°C for 1 hour and cooled down, and

Table 3. Free proline and soluble carbonhydrate content of species.

Species	Life Strategy	Free Proline		Soluble Carbonhydrate		Ch/Pr
		μmol g^{-1}	±SS	mg g^{-1} KA	SD	
S1 *Paronychia angorensis* Chaudri	Serpentinophyte	5.6	1.6	47.6	3.6	8.5
S2-A *Alyssum sibiricum* Willd.	Serpentinovag (on serpentine)	23.9	1.7	65.3	2.3	2.73
S2-B *Alyssum sibiricum* Willd.	Serpentinovag (on non serpentine)	6.4	1.3	50.9	4.0	7.95
S3-A *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz	Serpentinovag (on serpentine)	14.9	3.4	38.3	2.8	2.57
S3-B *Centaurea urvillei* DC. subsp. *stepposa* Wagenitz	Serpentinovag (on non serpentine)	19.0	1.0	49.5	5.2	2.60
S4-A *Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet	Serpentinovag (on serpentine)	14.8	2.2	71.5	2.4	4.83
S4-B *Salvia absconditiflora* (Montbret & Aucher ex Bentham) Greuter & Burdet	Serpentinovag (on non serpentine)	29.9	3.3	58.6	3.2	1.96

4 ml of cold toluene added on and stirred. Absorbency of toluene phase at 520 nm was measured and the proline amount was determined from the curve created using the proline standard (Bates et al., 1973).

Soluble Carbonhydrate Content: For glucose+sucrose extraction, 0.2 g of dried specimen was crushed in 80% ethyl alcohol in a mortar, centrifuged in 10.000 g for 10 minutes, and the supernatant was diluted at 1/100 ratio. On 1 ml of the extract, 2 ml of anthrone reactive was added, cooled after incubation at 100°C for 5 minutes, and the absorbency was determined at 620 nm. The glucose+ sucrose amount was calculated from the curve created using the glucose standard (Halhoul and Kleinberg, 1972).

Results

In *Paronychia angorensis*, growing only on serpentine soils, and whose life strategy is a serpentinophyte, the amount of free proline is 5.6 μmol g^{-1} and soluble carbonhydrate is 47.6 mg g^{-1} KA, and soluble carbonhydrate is 8.5 times of free proline (Ch/Pr).

In *Alyssum sibiricum,* growing both on serpentine and non-serpentine soils and called as serpentinovag, the amount of free proline is 23.9 μmol g^{-1} and soluble carbonhydrate is 65.3 mg g^{-1} KA in the individuals growing on serpentine soils, and the ratio of Ch/Pr is 2.73. In *A. sibiricum,* the amount of free proline is 6.4 μmol g^{-1} and soluble carbonhydrate is 50.9 mg g^{-1} KA in the individuals growing on non-serpentine soils, and the ratio of Ch/Pr is 7.95. Likewise, in *Centaurea urvillei* subsp. *stepposa,* another serpentinovag, the amount of free proline is 14.9 μmol g^{-1} and soluble carbonhydrate is 38.3 mg g^{-1} KA in the individuals growing on non-serpentine soils, and the ratio of Ch/Pr is 2.57. In *C. urvillei* subsp. *stepposa,* the amount of free proline is 19.0 μmol g^{-1} and soluble carbonhydrate is 49.5 mg g^{-1} KA in the individuals growing on non-serpentine soils, and the ratio of Ch/Pr is 2.6. In *Salvia absconditiflora,* another serpentinovag, the amount of free proline is 14.8 μmol g^{-1} and soluble carbonhydrate is 71.5 mg g^{-1} KA in the individuals growing on non-serpentine soils, and the ratio of Ch/Pr is 4.83. In *S. absconditiflora,* the amount of free proline is 29.9 μmol g^{-1} and soluble carbonhydrate is 58.6 mg g^{-1} KA in the individuals growing on non-serpentine soils, and the ratio of Ch/Pr is 1.96 (Table 3).

Discussion

Plants developed on serpentine are exposed to secondary water stress due to being exposed to high concentrations of heavy metals such as Ni, Co, and Cr. High concentrations of heavy metals may damage roots leading to blocking water intake from soils. Growth of plants developed on serpentine soils is limited both by heavy metal toxicity and water stress as well as deficiency in nutrient elements. High concentrations of osmoprotectants, such as proline and soluble carbonhydrate should be expected in plants growing under such conditions.

Findings of the present study indicate that soluble carbonhydrates are substantially high in quantity in all of the species examined. Although free proline concentration is substantially high in comparison to plants without water stress, there are great variations between species. High heavy metal content of serpentine soils cause secondary water stress in plants and the increase of free proline and soluble carbonhydrate levels. Although proline values in steppe plants not under stress in their natural environments are approximately 5 μmol g^{-1} DW in average, it is average 16 μmol g^{-1} DW in plants developed in the Alpine zone (Öncel et al., 2004). Average proline content in serpentine plants analyzed in the present study is identified as 16.4. While soluble carbonhydrate levels in six wheat types not under stress are around 20 mg g^{-1}, average value was measured as 40 mg g^{-1} under stress (Keleş and Öncel, 2004).

While free proline is proportionally low in serpentinophytes, amount of soluble carbonhydrates is high (Ch/Pr; 8.5). In those individuals of serpentinovags growing on serpentine, proline is proportionally low (Ch/Pr; 2.73, 2.57, and 4.83) but soluble carbonhydrate is high. In those individuals of serpentinovags growing on non-serpentine soils, proline amount decreases (Ch/Pr; 7.95, 2.6, and 1.96) but free carbonhydrate ratio increases. Although proline content is higher in some species growing on serpentine soils in comparison to those plants not under stress conditions this is not a general case. It is however observed that soluble carbonhydrate content in serpentine plants is commonly high.

In conclusion, there are substantial differences between species in terms of concentrations of soluble matter. Some species have higher concentrations of soluble carbonhydrates while some have high content of free proline. This may be due to that the species studied utilize different mechanisms in drought tolerance. Nevertheless, it is observed that *serpentinophyte* and *serpentinovag* species do not have common properties in terms of capacity of accumulating soluble matter under serpentine-stress conditions.

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