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Article

The Role of Free Proline and Soluble Carbohydrates in Water Gypsum Stress on Some Gypsophyte and Gypsovag Plants

O Papel da Prolina Livre e dos Carboidratos Solúveis em Condições de Estresse Gypsum em Algumas Plantas Gypsophyte e Gypsovag

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ABSTRACT - The aim of this study is to identify the roles of free proline and soluble carbohydrates in water gypsum stress. This study is the first such study on gypsophyte and gypsovag plants. For this purpose, free proline and soluble carbohydrate contents in gypsophyteand gypsovag plants have been analyzed. It is known that proline increases under stress conditions and it is a nitrogen-containing compound with protective properties contributing to durability understress. Soluble carbohydrates accumulating under stress conditions, on the other hand, take on the protective task of regulating cell osmotic density. In gypsophytes, free proline is proportionally high (Ch/Pr:1.5 to 9.3) and the amount of soluble carbohydrates is low. In gypsovag individuals growing on gypsum, proline is proportionally low (Ch/Pr:25.5 to 9.2), but soluble carbohydrates are high. It is found that in gypsovag individuals growing on mediums other than gypsum, the amount of proline increases (Ch/Pr:11.6 to 8.5),but the proportion of soluble carbohydrate decreases. Accordingly, while gypsophytes adapt themselves to high proline amounts in response to water gypsum stress and gypsovags develop resistance to water gypsum stress with high amounts of soluble carbohydrates, it is observed that the Ch/Pr ratio in non-gypsum soils decreases.

Keywords: compatible solutes, gypsum, stress ecology.

RESUMO - O objetivo deste estudo é investigar o papel da prolina livre e dos carboidratos solúveis em condições de estresse gesso. Inicialmente, foram analisadas as prolinas e carboidratos contidos em gipsita e gipsovaque. Prolinas são compostos que contêm nitrogênio, produzidos sob condições de estresse, com propriedades de proteção e que contribuem para a durabilidade. Carboidratos solúveis se acumulam sob condições de estresse; por outro lado, assumem uma função protetora, regulando a densidade osmótica celular. Em gipsita, a quantidade de prolina livre é proporcionalmente alta (Ch/Pr; 1,5 para 9,3), e a de carboidrato solúvel é baixa. Nos indivíduos gipsovaques crescidos em solo gesso, a prolina é proporcionalmente baixa (Ch/Pr; 25,5 para 9,2), mas o carboidrato solúvel é alto. Foram encontrados indivíduos gipsovaques crescendo em outros meios além da gipsita, aumentando a quantidade de prolina (Ch/Pr; 11,6 para 8,5); no entanto, houve diminuição em proporção à quantidade de carboidrato solúvel. Consequentemente, enquanto as gipsitas se adaptam a uma quantidade elevada de prolinas contra o estresse do gesso, o estresse pelo desenvolvimento da resistência com a quantidade elevada do carboidrato solúvel é diminuído em solos não gesso, isto é, a relação Ch/Pr decresce proporcionalmente.

Palavras-chaves: solutos compatíveis, gesso, ecologia do estresse.

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INTRODUCTION

As known, taxa growing only in gypsiferous soils are named as gypsophytes and those plants growing in both gypsiferous and non-gypsiferous soils as gypsovags (Palacio et al., 2007; Cañadas et al., 2013; Bolukbasi et al., 2016). Gypsum is a physical and chemical stress factor for plant life in association with arid and semi-arid climatic conditions. Massive gypsum soils in semi-arid regions cause high amount of infiltration and surface flow of rain waters. Gypsum can hinder growing of seedlings and seeds by packing soil surface as a tight crust (Escudero et al., 2000). At the present time, studies are still conducted on the mechanism of factors affecting distribution and performances of gypsophile and gypsovag species (Escudero et al., 2015). It is long known that most gypsiferous soils are poor of organic matters, that more the gypsum content, less the cation exchange capacity, that cation exchange capacity mostly depends on organic matter content and texture of soil, that when Ca concentration is high Mg and K intake is blocked and Ca:Mg ratio in tissues increases in the relation between Ca, Mg, K macro nutrition elements (FAO, 1990). In addition, it is long confirmed that high calcium content due to existence of gypsum may cause to Ca-Mg antagonism. On the other hand, recent studies provided detailed results in such issues as soil fertility and heavy metal accumulation in gypsiferous soils and other soil types as well as formation and increase of toxic organic matters (Vereecken et al., 2016). 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).

Plants accumulate proline for adaptation to such environmental factors as drought, brackishness, high temperature, nutritional deficiency, exposure to heavy metals, high acidity (Oncel et al., 2000). Increase in metals such as Cu, Zn, and Pb increases proline concentration in leaves. In many plants exposed to heavy metals, free proline accumulation is observed in response to stress. In case of a risk of toxic heavy metal concentrations, many plants accumulate proline at high concentrations. The main role of proline is to protect enzymes against to dehydration and salt deposition by not diminishing likely osmotic potential (Thomas, 1990).

The osmotic adaptation is also a capability of accumulating certain inorganic ions (Na, K, etc.) or certain organic matters (sucrose, proline, betaine, etc.). The statement that osmotic stress in potato genotypes induces proline increase but the increase rate does nit explain the tolerance level (Bundig et al., 2017) supports the complex relations emphasized above. It is known that proline increases under stress conditions, participates in detoxification of free O_2 radicals, and is a nitrogen-containing compound of protective property contributing to durability to stress conditions. Increase in proline concentration inhibits protein synthesis meanwhile promoting proline oxidation. The statement that a rapid increase of proline in plants provides protection against to damages due to oxygen radicals in the adaptation of plants is supported by assertion that accumulation of proline, an important osmolite, by plants under abiotic stress conditions provides protection of turgescence and proteins in the process of dehydration (Rontein et al., 2002). Many researchers agree on that proline accumulation in plants is an injury symptom non-tolerant to metal and other stresses. Another important development observed in various stress types in plants is the exposure to lipid peroxidation causing dysfunctions and adverse effects on membrane functions. High amount of proline accumulation under drought and saline conditions is a key to explain the endurance mechanism.

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, sucrose, and starch for performing the maintenance of osmotic equilibriums, C accumulation, etc. (Sami et al., 2016).

Plant stress physiology and ecophysiology are among current and popular research interest and studied in wide range of aspects; the literature shows also the complexity of the subject.

This study addresses exchanges of only two variables, free proline, and soluble carbonhydrate that are specified as closely related to the subject.

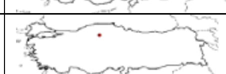
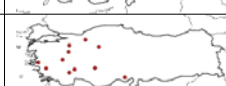
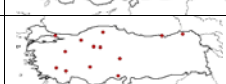
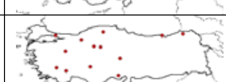
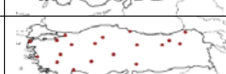
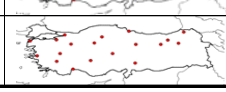
Gypsiferous soils are common in arid and semi-arid regions, and the vegetation acclimatized themselves to these habitats are under osmotic stress in a substantial part of their life cycles. This study is intended for testing the hypothesis claiming that in order to tolerate osmotic stress, gypsophyte or gypsovag 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 gypsophyte and gypsovag plants.

MATERIALS AND METHODS

Plant specimens were collected from non-gypsiferous and gypsiferous soils in central Anatoliaat the end of 2015 and 2016 vegetation periods (Table 1). 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. 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 (Akman, 1982). 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 ^{o}C. Analyses of proline and soluble carbonhydrate in the specimens pertaining to various populations collected from Beypazari and Sivrihisar regions 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 Analysis (ANOVA).

Free Proline - 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 ^{o}C for 1 hour and cooled down, and 4 mL of cold toluen added on and stirred. Absorbency of toluen phase at 520 nm was measured and the

Table 1 - Gypsophyte and gypsovag plant species

Code	Family	Species	Soil	Life Strategy	Life Form[1]	Phytogeography[2]	Location	IUCN	Distribution of species
J1	*Plumbaginaceae*	*Acantholimon riyatguelii* Yıldırım	Gypsum	Narrow Gypsophyte	Ch	- End.	Sivrihisar N 39º 22' 10.0" E 031º 29' 09.1"	CR	
J2	*Lamiaceae*	*Thymus leucostomus* Hausskn. & Velen var. *gypsaceus*	Gypsum	Narrow Gypsophyte	Ch	Ir.-Tur. End.	Polatlı N 39º 34' 36.9" E 031º 55' 09.7"	CR	
J3	*Scrophulariaceae*	*Verbascum gypsicola* Vural & Aydoğdu	Gypsum	Narrow Gypsophyte	H	Ir.-Tur. End.	Beypazarı N 40º 06' 26.5" E 031º 42' 54.4"	EN	
J4-A	*Fabaceae*	*Astragalus lydius* Boiss.	Gypsum	Gypsovag (on gypsum)	H	Ir.-Tur. End.	Sivrihisar N 39º 35' 44.4" E 031º 47' 02.9"	LC	
J4-B	*Fabaceae*	*Astragalus lydius* Boiss.	Non-Gypsum	Gypsovag (on non gypsum)	H	Ir.-Tur. End.	Polatlı N 39º 37' 51.5" E 031º 56' 54.6"	LC	
J5-A	*Cistaceae*	*Fumana procumbens* (Dunal) Gren. &Godr.	Gypsum	Gypsovag (on gypsum)	H	-	Çankırı N 40º 07' 30.5" E 032º 00' 24.8"	-	
J5-B	*Cistaceae*	*Fumana procumbens* (Dunal) Gren. &Godr.	Non-Gypsum	Gypsovag (on non gypsum)	H	-	Polatlı N 39º 37' 51.5" E 031º 56' 54.6"	-	
J6-A	*Fabaceae*	*Onobrychis oxyodonta* var. *armena* (Boiss. & Huet) Aktoklu	Gypsum	Gypsovag (on gypsum)	H	-	Polatlı N 39º 34' 36.9" E 031º 55' 09.7"	LC	
J6-B	*Fabaceae*	*Onobrychis oxyodonta* var. *armena* (Boiss. & Huet) Aktoklu	Non-Gypsum	Gypsovag (on non gypsum)	H	-	Polatlı N 39º 37' 51.5" E 031º 56' 54.6"	LC	

* Life form: Ch: Chamaephyte H: Hemicryptophyte ** Phytogeography: Ir.-Tur.: Irano-Turanian End.: Endemic *** IUCN: CR: Critically endangered EN: Endangered LC: Least concern.

proline amount was determined from the curve created using the proline standard (Bates et al., 1973).

Soluble Carbonhydrate - 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 AND DISCUSSION

In *A. riyatguelii*, growing only on gypsiferous soils, and whose life strategy is a gypsophyte, the amount of free proline is 19.8 µmol g^{-1} and soluble carbonhydrate is 30.0 mg g^{-1} KA, and soluble carbonhydrate is 1.5 times of free proline (Ch/Pr). In *T. leucostomus* var. *gypsaceus*, another gypsophyte, the amount of free proline is 5.7 µmol g^{-1} and soluble carbonhydrate is 25.4 mg g^{-1} KA, and the ratio of Ch/Pr is 4.4. In *V. gypsicola*, another gypsophyte, the amount of free proline is 0.8 µmol g^{-1} and soluble carbonhydrate is 100.9 mg g^{-1} KA, and the ratio of Ch/Pr is 9.3. In *F. procumbens*, growing both on gypsum and no-gypsum soils and called as gypsovag, the amount of free proline is 1.1 µmol g^{-1} and soluble carbonhydrate is 28.1 mg g^{-1} KA in the individuals growing on gypsum soils, and the ratio of Ch/Pr is 25. In *F. procumbens*, the amount of free proline is 4.5 µmol g^{-1} and soluble carbonhydrate is 52.2 mg g^{-1} KA in the individuals growing on non-gypsum soils, and the ratio of Ch/Pr is 11.6. Likewise, in *O. armena,* another gypsovag, the amount of free proline is 3.3 µmol g^{-1} and soluble carbonhydrate is 50.9 mg g^{-1} KA in the individuals growing on gypsum soils, and the ratio of Ch/Pr is 15.5. In *O. armena,* the amount of free proline is 8.1 µmol g^{-1} and soluble carbonhydrate is 58.4 mg g^{-1} KA in the individuals growing on non-gypsum soils, and the ratio of Ch/Pr is 7.2. In *A. lydius,* another gypsovag, the amount of free proline is 8.8 µmol g^{-1} and soluble carbonhydrate is 80.8 mg g^{-1} KA in the individuals growing on gypsum soils, and the ratio of Ch/Pr is 9.2. In *O. armena*, the amount of free proline is 6.7 µmol g^{-1} and soluble carbonhydrate is 56.7 mg g^{-1} KA in the individuals growing on non-gypsum soils, and the ratio of Ch/Pr is 8.5 (Table 2; Figure 1).

Amounts of free proline and soluble carbonhydrate show great variations in the species studied. However, tests of variance analysis (ANOVA) indicate that average free proline and soluble carbonhydrate values of gypsophyte (on gypsum), and gypsovag (non gypsum) groups do not show great differences.

It is reported in many studies that osmotic stress tolerance of compatible solutes such as free proline and soluble carbonhydrates is the most important indicator. Soluble matter accumulations may contribute to osmotic regulation under direct stress, such as drought, or indirect water stress, such as low temperature, brackishness, and heavy metal stress (Ingram and Bartels, 1996). In case of water stress, proline accumulation starts increasing in a few hours at the beginning of discoloration. Radioactive carbon trials have shown that proline accumulation is due to new proline synthesis (Boggess et al., 1976). Thomas (1990), studying

Table 2 **-** Free proline and soluble carbonhydrate contents and soluble carbonhydrate / free proline ratio of species

Species	Life strategy	Free proline		Soluble carbonhydrate		Ch/Pr
		µmol g^{-1}	± SS	mg g^{-1} KA	SD	
J1 *Acantholimon riyatguelii*	Gypsophyte	19.8	5.3	30.0	1.2	1.5
J2 *Thymus leucostomus*	Gypsophyte	5.7	1.1	25.4	1.2	4.4
J3 *Verbascum gypsicola*	Gypsophyte	0.8	0.5	100.9	2.8	9.3
J4-A *Astragalus lydius*	Gypsovag (on gypsum)	8.8	1.6	80.8	7.2	9.2
J4-B *Astragalus lydius*	Gypsovag (on non gypsum)	6.7	1.9	56.7	7.7	8.5
J5-A *Fumana procumbens*	Gypsovag (on gypsum)	1.1	0.6	28.1	5.8	25.5
J5-B *Fumana procumbens*	Gypsovag (on non gypsum)	4.5	0.6	52.2	8.9	11.6
J6-A *Onobrychis oxyodonta* var. *armena*	Gypsovag (on gypsum)	3.3	0.8	50.9	4.8	15.5
J6-B *Onobrychis oxyodonta* var. *armena*	Gypsovag (on non gypsum)	8.1	2.6	58.4	9.0	7.2

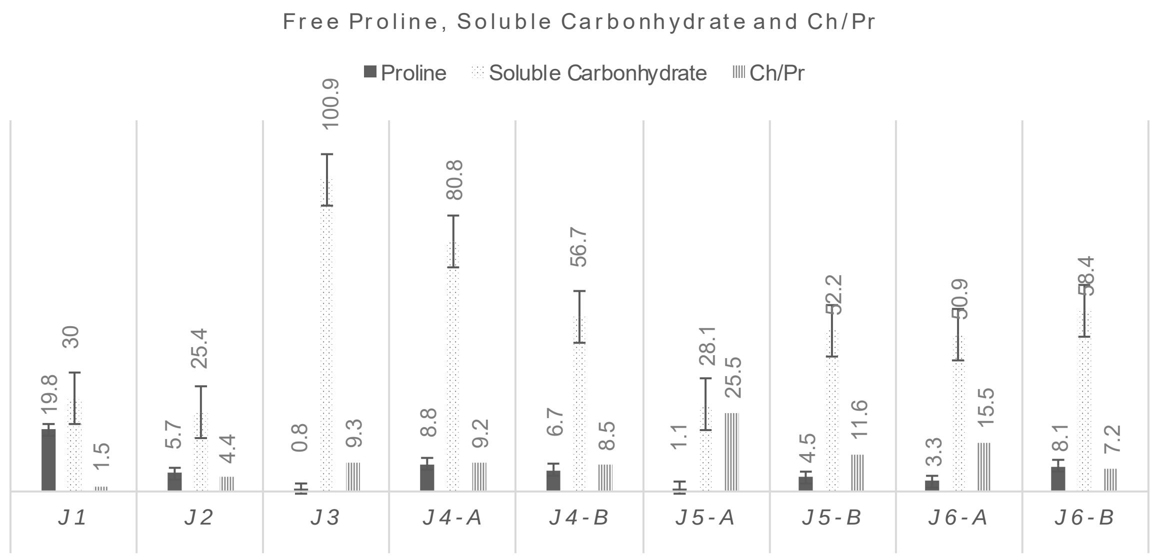


Figure 1 - Free proline and soluble carbonhydrate contents and soluble carbonhydrate/free proline ratio of species J1: ***Acantholimon riyatguelii*** (Gypsophyte), J2: ***Thymus leucostomus*** (Gypsophyte) J3: ***Verbascum gypsicola*** (Gypsophyte) J4-A: ***Astragalus lydius*** [Gypsovag (on gypsum)] J4-B: ***Astragalus lydius*** [Gypsovag (on non gypsum)] J5-A: ***Fumana procumbens*** [Gypsovag (on gypsum)] J5-B: ***Fumana procumbens*** [Gypsovag (on non gypsum)] J6-A: ***Onobrychis oxyodontavar****. armena* [Gypsovag (on gypsum)] J6-B: ***Onobrychis oxyodontavar****. armena* [Gypsovag (on non gypsum)].

proline accumulation *Lolium perenne* under drought and low temperature, has found that proline accumulation is important more under drought conditions. It is determined that proline accumulation has an important role in drought and saline tolerance of wheat plants, however, it was noted that there are substantial differences between species and orders (Keles and Oncel, 2004). In comparison of plants growing in Alpine and steppe ecosystems in terms of proline accumulation, proline content in steppe plants have been indicated as lower than Alpine plants (Oncel et al., 2004).

Water stress in those plants growing on gypsum is widely obsevred. In case of high amount of gypsum in soil, water distribution in soil at depths where plant roots are is irregular. Therefore, water stress in gypsiferous soils is an important limiting factor (Llinares et al., 2015). In a study conducted on osmolite levels in gypsophyte and gypsovag species growing in arid regions of Spain, it was suggested that there are substantial differences between species in terms of proline accumulation; moreover, proline accumulation shows positive correlation with drought and salinity while being affected in a limited amount from the amount of gypsum in soil (Boscaiu et al., 2013).

Englmaier (1987), studying carbonhydrate metabolism in saline-tolerant *Puccinelliapeisonis*, stated that soluble carbonhydrate accumulation eliminate ion excess contributing to maintenance of osmotic potential. Kuznetsov and Shevyakova (1997), studying interaction of high temperature and saline tolerance in tobacco plants stated that soluble carbonhydrate content decreases at high temperatures, but increases with saltiness. Increase in respiration with high temperature may reduce soluble carbonhydrate levels. Although excessive ion accumulation in plants growing in saline soils inhibits enzyme activities, osmotic regulation provided with accumulation of soluble organic matters does not block enzyme activities.

These effects caused by drought and salinity retard the yield of crops up to 50% (FAO, 2008). To overcome this issue, an understanding of the physiological, biochemical, and molecular responses of plants naturally tolerant to drought and salinity is important in order to engineer stress tolerant crops (Sekmen Esen et al., 2012).

Llinares et al. (2015) suggested that no positive correlation exists between soluble sucrose levels ans stress level in plants growing in gypsiferous soils. However, in the species without high proline accumulation, soluble carbonhydrate accumulation may provide osmotic regulation. Present study indicates that gypsophyte plants may have a lower soluble carbonhydrate/free proline ration.

According to the findings of this study, in gypsophytes, proline is proportionally high (Ch/Pr; 1.5 to 9.3) and free carbonhydrate amount is low. In those individuals of gypsovags growing on gypsum, proline is proportionally low (Ch/Pr; 25.5 to 9.2) but soluble carbonhydrate is high. In those individuals of gypsovags growing on non-gypsiferous soils, proline amount increases (Ch/Pr; 11.6 to 8.5) but free carbonhydrate amount decreases. In the evolutionary process, gypsophytes have developed a “specialist model” as their life strategies and adapted to water gypsum stress by utilization of high amounts of proline. On the other hand, although gypsovags develops resistance to water gypsum stress by means of high rate of soluble carbonhydrate in the “refuge model” developed in the evolutionary process, this ratio of Ch/Pr in non-gypsum soils is lower. Özbey et al. (2017) reported that the importance of free proline and soluble carbonhydrate ratio in drought stress. They reported that the ratio of free proline and soluble carbonhydrate is low in serpentinophytes, whereas serpentinovag that grow outside the serpentine also stated that the soluble carbonhydrate is 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 gypsophyte and gypsovag species do not have common properties in terms of capacity of accumulating soluble matter under gypsum-stress conditions. It is extremely important to reveal the resistance mechanisms of the gypsophyte and gypsovag species to drought. The ability of taking up the crystallized water within gypsum by gypsophyte and gypsovag plants is important for arid land reclamation such as dry gypsiferous areas and exobiology of the world, i.e. Mars (Palacio et al., 2014).

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