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Comparison of photosynthesis and antioxidative protection in *Sedum album* and *Sedum stoloniferum* (Crassulaceae) under water stress

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Abstract

Photosynthetic gas exchange, dry mass production, water relations and inducibility of crassulacean acid metabolism (CAM) pathway as well as antioxidative protection during the C_3-CAM shift were investigated in *Sedum album* and *Sedum stoloniferum* from Crassulaceae under water stress for 20 days. Leaf relative water content (RWC), leaf osmotic and water potential decreased with increasing water stress in both studied species. Significant reduction in dry matter production and leaf thickness was detected only in *S. stoloniferum* after 20-d water stress. Δtitratable acidity and phospho*enol*pyruvate carboxylase (PEPC) activity in *S. album* responded to drought at early stages of stress treatment, continued to increase throughout the entire stress period and reached levels 15 times higher than those in well-watered plants. In *S. stoloniferum,* however, both parameters responded later and after a transient increase declined again. In *S. stoloniferum*, in spite of increase by drought stress, net night-time CO_2 assimilation was negative resembling a C_3-like pattern of gas exchange. Catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) activities increased in plants subjected to mild water stress while declined as the stress became severe. Although malondialdehyde (MDA) content was higher in drought-stressed *S. stoloniferum*, the increase in the concentration of hydrogen peroxide (H_2O_2) that may act as a signal for C_3-CAM transition was higher in *S. album* compared with *S. stoloniferum*. In drought-stressed plants, SOD activity showed a clear diurnal fluctuation that was more steadily expressed in *S. album*. In addition, such pattern was observed for CAT only in *S. album*. We concluded that temporal and diurnal fluctuation patterns in the activity of antioxidant enzymes depended on duration of drought stress and was related to the mode of photosynthesis and degree of CAM induction. According to our results, *S. stoloniferum* developed a low degree of CAM activity, *e.g.* CAM-cycling metabolism, under drought conditions.

Additional key words: CAM-cycling; hydrogen peroxide; Δtitratable acidity.

Introduction

CAM is widespread within the plant kingdom across at least 343 genera in 35 plant families comprising ~6% of flowering plant species (Holtum *et al*. 2007). Plants that exhibit predominantly the CAM pathway are commonly known as obligate CAM plants (Silvera *et al*. 2010). However, C_3 species with an ability to switch their carbon metabolism to the CAM pathway have been also evidenced (Cushman *et al*. 2008, Winter *et al*. 2011). On the basis of gas-exchange measurements and day/night pattern of organic acid turnover, these species are categorized as C_3-CAM intermediate and CAM-cycling.

In C_3-CAM intermediate plants, the C_3-CAM transition is illustrated by a switch from daytime fixation to net uptake of CO_2 in the dark and accumulation/ breakdown of malic acid (Sayed 2001). The common ice plant, *Mesembryanthemum crystallinum* is a well studied example of C_3-CAM intermediate, facultative or inducible CAM under strict environmental conditions (Winter and Holtum 2007, Cushman *et al*. 2008). Inducible CAM species engage in CAM in response to environmental stimuli such as drought stress (Winter *et al*. 2008, 2011). *Sedum album* is an example of C_3-CAM intermediate in which CAM pathway is induced by drought (Castillo 1996).

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Abbreviations: APX – ascorbate peroxidase; CAM – crassulacean acid metabolism; CAT – catalase; DM – dry mass; E – transpiration rate; g_s – stomatal conductance; H_2O_2 – hydrogen peroxide; MDA – malondialdehyde; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; ROS – reactive oxygen species; RWC – relative water content; SOD – superoxide dismutase; Ψ_s – osmotic potential; Ψ_w – water potential.

CAM-cycling is characterized by CAM-like acid concentration fluctuations with C_3 gas-exchange pattern (Sayed 2001, Jones *et al*. 2003). The shift from C_3 photosynthesis to CAM-cycling has been documented in *Clusia aripoensis* (Borland *et al*. 1998) and *Sedum integrifolium* (Gravatt and Martin 1992). Other modes of CAM such as latent CAM are indicated by organic acid concentrations elevated above those normally present in C_3 plants, but without diel fluctuation that may represent a nascent C_3-CAM progression (Cushman and Borland 2002).

Stress factors such as drought trigger common reactions in plants and lead to cellular damages mediated by reactive oxygen species (ROS) (Boughalleb and Hajlaoui 2011). Oxidative stress refers to a serious imbalance between the production and removal of ROS. Antioxidative enzymes such as SOD, APX, and CAT play an important role against oxidative stress (Apel and Hirt 2004). It has been proposed that increase in carbon cycling during the C_3-CAM transition confer enough protection against photoinhibition under drought stress (Griffiths *et al*. 1989). Moreover, stress factors involved in the induction of CAM pathway are simultaneously responsible for increasing activity of antioxidative enzymes. Accordingly, it would be interesting to know the role of antioxidant defense system during the C_3-CAM shift. In recent years, the intermediate C_3-CAM plant *M. crystallinum* has become a very useful model for studying antioxidant response systems in both modes of photosynthesis: C_3 and CAM (Borland *et al*. 2006, Ślesak *et al*. 2007). Plants performing CAM have a strong diurnal rhythm in activity of some antioxidative enzymes such as catalase and MnSOD (Niewiadomska *et al*. 1999, Ślesak *et al*. 2002). However, diurnal fluctuation in the activity of mitochondrial MnSOD in *M. crystallinum* has been observed to be independent of its photosynthetic mode of CO_2 assimilation (Miszalski *et al*. 2007).

S. stoloniferum is an unique *Sedum* characterized by its pink colored, star shaped flowers on trailing stems and often spread by means of stolons. Information on the mode of photosynthesis and responses of antioxidant defense system is still lacking for this species under environmental stresses such as drought. *S. album* and *S. stoloniferum* both tend to grow in habitats which experience frequent low soil matric potentials *e.g.* rock crevices or well-drained stony soils where periodic drought can develop rapidly. Accordingly, induction of change from C_3 to CAM or CAM-cycling may be an important means of survival for these species. CAM pathway contributes significantly in the plants survival in arid and semiarid regions of the world (Gravatt and Martin 1992, Cushman and Borland 2002, Holtum *et al*. 2007).

There is no published work concerning the expression of CAM in species from Crassulaceae belonging to flora of Iran. In addition, photosynthetic flexibility in a given species is highly dependent on environmental conditions and plant ecotype (Motomura *et al*. 2008). In this work, we examined the ability of *S. album* and *S. stoloniferum* to shift from C_3 to CAM under severe drought stress. *Sedum album* was included as a model species because of its known capability for CAM induction under drought stress (Castillo 1996). The present work aimed to: (*1*) characterize and provide experimental evidences for confirmation of the ability of *S. stoloniferum* to shift from C_3 to CAM under drought stress, and (*2*) study the relationship between stress-induced CAM and the ability to cope with oxidative stress during the C_3-CAM shift. We addressed the question of whether there is any interdependency between temporal and diurnal changes in the activity of antioxidant defense enzymes, photosynthetic mode**,** degree of CAM induction and duration of the imposed drought stress. In addition, temporal changes in the CO_2 exchange pattern, nocturnal acid accumulation and PEPC activity were examined in this work in order to find the starting time point of CAM induction through 20-d drought stress.

Materials and methods

Plant materials and treatments: Two species from Crassulaceae including *S. album* and *S. stoloniferum* were collected at juvenile stage from their natural habitats in East-Azerbaijan Province (NW Iran) in spring 2010. *S. album* L., a perennial succulent with decumbent main stems and vertical flowering stems occurs in North and NW Iran (Akhiani 2000). Whole plants were collected from Mishou-Dagh, near the town of Payam, NW Iran at an elevation of 1,890 m. *S. stoloniferum* S.G. Gmel, a perennial succulent with decumbent stems, occurs in north- (Guilan and Mazandaran) and northwest (Khalkhal, Astara) Iran (Akhiani 2000). The plants were collected from Anjerd, near the city of Ahar, NW Iran at an elevation of 2,010 m. Both species were found in crevices and thin soil on the shade of boulders and shrubs. After transportation to the lab, plants were transferred to plastic pots containing washed sand and were adapted to the environmentally controlled conditions under fluorescent white light at about 250 μmol m^{-2} s^{-1} (measured by a quantum sensor attached to the leaf chamber of the gas-exchange unit) with 18/6 h light/dark photoperiod and 25/17ºC day/night temperature and relative humidity of 60/70% for a period of two months prior to the start of experiments. Plants were watered with distilled water twice a week and received 50% modified Hoagland nutrient solution (Johnson *et al*. 1957) once a week. Following the two-month acclimation period, when the plants had developed 4–5 pairs of leaves, independent pots were selected randomly and assigned to two watering treatments. Control plants were watered twice a week with distilled water to field capacity, while drought-stressed plants received no water for

up to 20 d that has been defined as severe drought stress for *S. album* (Castillo 1996). To minimize evaporation, the exposed surface of each pot was covered with tin foil.

Harvests: Plants were harvested and analyzed in a temporal (on different days after imposition of drought stress) and diurnal (at different time of the day) manner. For daytime/night-time analysis of gas exchange and PEPC activity, leaves were harvested/analyzed either after 5-h light period (day samples) or after 5-h night period (night samples). For analysis without considering of diurnal variation, samples were taken during the light period mainly between 12:00 and 14:00 h. Diurnal samplings were undertaken at three time intervals including 09:00, 16:00 and 24:00 h. Total titratable acidity was measured at the end and beginning of the photoperiod. For determination of water-relation parameters, leaves were harvested 1 h after the light period started in the growth chamber. Fully expanded, mature and succulent leaves were used for gas-exchange and enzymatic analysis. For the latter analysis, leaf samples were frozen immediately in liquid N_2 and stored in it until assay.

Twenty days after treatments, plants were harvested. Leaves were washed with distilled water, blotted dry on filter paper and after determination of fresh mass (FM) dried for 48 h at 70°C for determination of dry mass (DM).

Gas-exchange analysis: Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance to water vapor (g_s) were measured with a calibrated portable gas-exchange system (*LCA4*, *ADC Bioscientific Ltd*., UK). All measurements were made under relative humidity of 70–80%, leaf temperature of 23–25°C and CO_2 concentration of 370–410 mg kg^{-1} and at a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} (for the day samples) provided in the leaf chamber of gas-exchange unit.

Determination of water relations and titratable acidity: Leaf osmotic potential (Ψ_s) was measured by an osmometer (*Heman Roebling MESSTECHNIK,* Germany), water potential (Ψ_w) was determined using a pressure chamber (*DTK-7000,* Japan) and relative water content (RWC) was measured and calculated according to Lara *et al.* (2003). For determination of titratable acidity, samples of a known mass were ground with liquid N_2, boiled for 10 min in distilled water and made up to 2 ml by distilled water. Titration was performed with 10 mM NaOH to pH 7.0 (Lara *et al*. 2003).

Assay of enzyme activities and related metabolites: PEPC (EC 4.1.1.31) activity was determined according to the method of Groenhof *et al*. (1988). Leaf tissues were extracted with 50 mM Tris-HCl buffer pH 8.2, containing 0.5 M sucrose and 2 mM DTT. The reaction mixture contained 50 mM Tris-HCl buffer pH 8.2, 15 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM phospho*enol*pyruvate (PEP), 0.15 mM NADH and 10 units of MDH. NADH oxidation was followed by measuring absorbance at 340 nm.

Determination of the activity of antioxidant enzymes and concentration of related metabolites was undertaken according to the optimized protocols described previously (Habibi *et al*. 2010). Fresh samples were ground in the presence of liquid N_2 and measurements were undertaken using spectrophotometer (*Specord 200*, *Analytic Jena*, Jena, Germany). SOD (EC 1.15.1.1) activity was estimated according to the method of Giannopolitis and Ries (1977). Enzyme was extracted in 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture containing 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), and 13 µM riboflavin. One unit of SOD was defined as that being contained in the volume of extract that caused a 50% inhibition of the SOD-inhibitable fraction of the NBT reduction.

For determination of CAT (EC 1.11.1.6) activity, samples were homogenized with 50 mM phosphate buffer pH 7.0 and assayed spectrophotometrically by following degradation of H_2O_2 at 240 nm according to the method of Simon *et al*. (1974). Reaction medium contained 50 mM phosphate buffer pH 7.0 and 10 mM H_2O_2. APX (EC 1.11.1.11) activity was assayed by following reduction in absorbance at 290 nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The reaction mixture contained 50 mM phosphate buffer pH 7.0, 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg bovine serum albumin (BSA). Lipid peroxidation was estimated from the amount of MDA formed in a reaction mixture containing thiobarbituric acid (*Sigma*, USA) at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (*Sigma*) standard curve. The concentration of H_2O_2 was determined using potassium titanium-oxalate at 508 nm (Hajiboland and Hasani 2007). Soluble protein was estimated spectrophotometrically by the Bradford (1976) method.

Experiments were undertaken in complete randomized block design. Statistical analyses were carried out using *SigmaStat 3.5* (*Systat Software Inc.,* USA) with *Tukey* test (p<0.05). Correlation analyses were performed using *Spearman*'s Rank Order Correlation method.

Results

DM and leaf thickness were not influenced significantly by water stress in *S. album*. In contrast, a significant loss of DM and leaf thickness was observed in *S. stoloniferum* under severe water stress, *i.e.* 20 d after water withholding (Table 1). Ψ_s and Ψ_w of drought-stressed plants were decreased by about 13% and 26% in *S. album* and

Table 1. Leaf dry mass (DM), osmotic (Ψ_s)- and water (Ψ_w) potentials and thickness in *Sedum album* and *Sedum stoloniferum* grown for 20 d under drought treatment. Data of each parameter within each species followed by *the same letter* are not significantly different (p<0.05). Data are means ± SD (n = 8).

Parameters	*S. album*		*S. stoloniferum*	
	Control	Drought	Control	Drought
DM [mg plant^{-1}]	46.5 ± 4.1^a	44.5 ± 7.2^a	38.4 ± 5.8^a	28.3 ± 3.7^b
Ψ_s [MPa]	−0.56 ± 0.04^a	−0.65 ± 0.03^b	−0.45 ± 0.03^a	−0.54 ± 0.05^b
Ψ_w [MPa]	−0.42 ± 0.09^a	−0.57 ± 0.08^b	−0.51 ± 0.05^a	−0.63 ± 0.07^b
Thickness [mm]	2.12 ± 0.14^a	1.98 ± 0.27^a	0.77 ± 0.05^a	0.55 ± 0.06^b

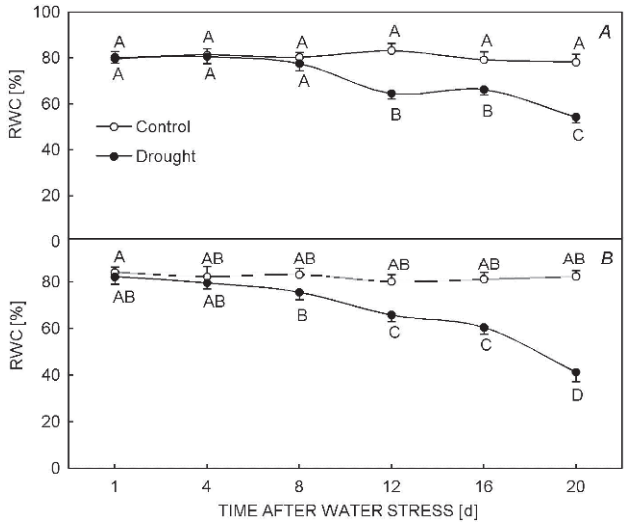

Fig. 1. Leaf relative water content (RWC) at different time intervals after drought treatment in *Sedum album* (*A*) and *Sedum stoloniferum* (*B*). Bars indicated with *the same letter* are not significantly different (p<0.05). Values are means ± SD (n = 6).

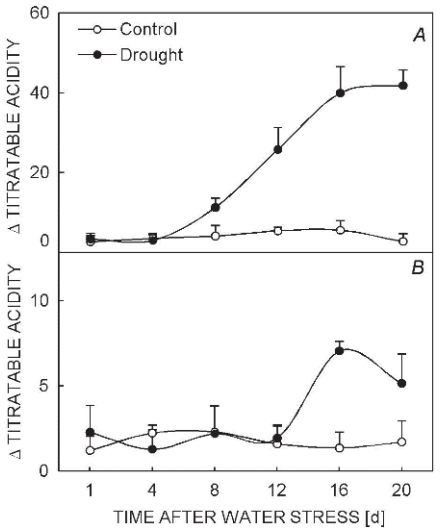

Fig. 2. Changes in Δtitratable acidity during 20-d drought treatment in *Sedum album* (*A*) and *Sedum stoloniferum* (*B*). Values are means ± SD (n = 6).

16% and 30% in *S. stoloniferum*, respectively, compared to their well-watered counterparts. RWC declined gradually during development of severe water stress in both species (Fig. 1*A,B*). At the end of experiment, lower RWC values were found in *S. stoloniferum* (41%) than in *S. album* (64%).

Difference between control and drought-stressed plants in the Δtitratable acidity, an indicator of CAM pathway, was significant in *S. album* after 8-d water stress (Fig. 2*A*). In contrast, withholding water for 12 d did not influence Δtitratable acidity in *S. stoloniferum*. In this species, from day 16 onwards, an increase in Δtitratable acidity was detectable in drought-stressed plants. Two examined species differed also in both the magnitude of difference between well-watered and drought-stressed plants and in the constitutive amount of Δtitratable acidity. Δtitratable acidity was 6 times higher in drought-stressed *S. album* compared with *S. stoloniferum*. Changes in Δtitratable acidity were well correlated with RWC values (r = 0.72, p<0.01) only in water-stressed *S. album* (Fig. 3).

After 12-d water stress, a positive value for night-time net CO_2 uptake was recorded for *S. album* (Table 2).

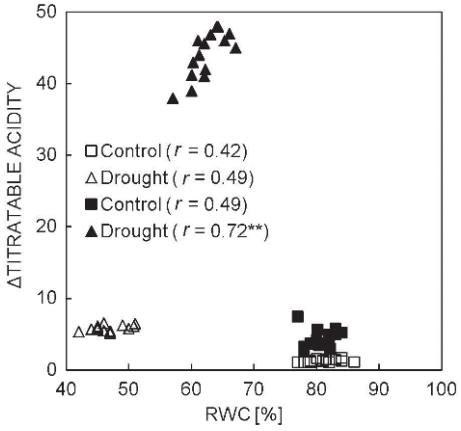

Fig. 3. Correlations between leaf relative water content (RWC) and Δtitratable acidity in *S. album* (*closed symbols*) and *S. stoloniferum* (*open symbols*) grown for 20 d under drought treatment. ns – nonsignificant; * and ** – significant at the 5% and 1% levels of probability, respectively.

In *S. stoloniferum*, however, though substantial increase in the nighttime net CO_2 uptake, it remained negative even after 20-d drought stress (Table 3). Night-time

Table 2. Net photosynthetic (P_N)- and transpiration (E) rates and stomatal conductance to water vapor (g_s) in *Sedum album* grown under drought treatment. Data of each parameter within each harvest interval followed by *the same letter* are not significantly different ($p<0.05$). Data are means ± SD ($n = 4$).

Time after water stress [d]		P_N [μmol(CO_2) m^{-2} s^{-1}]		E [mmol(H_2O) m^{-2} s^{-1}]		g_s [mol m^{-2} s^{-1}]	
		Day	Night	Day	Night	Day	Night
1	Control	3.97 ± 0.12^{a}	-0.16 ± 0.06^{b}	0.66 ± 0.06^{a}	0.19 ± 0.05^{b}	0.26 ± 0.04^{a}	0.06 ± 0.02^{b}
	Drought	3.17 ± 0.93^{a}	-0.24 ± 0.15^{b}	0.69 ± 0.17^{a}	0.20 ± 0.04^{b}	0.23 ± 0.11^{a}	0.06 ± 0.01^{b}
12	Control	5.29 ± 0.52^{a}	-0.05 ± 0.02^{c}	0.96 ± 0.32^{a}	0.30 ± 0.02^{bc}	0.83 ± 0.54^{a}	0.09 ± 0.02^{b}
	Drought	1.26 ± 0.44^{b}	1.02 ± 0.63^{bc}	0.13 ± 0.08^{c}	0.61 ± 0.04^{b}	0.03 ± 0.02^{b}	0.19 ± 0.02^{b}
16	Control	3.81 ± 0.19^{a}	-0.01 ± 0.01^{d}	0.52 ± 0.03^{b}	0.24 ± 0.07^{c}	0.15 ± 0.01^{b}	0.06 ± 0.01^{b}
	Drought	0.88 ± 0.16^{c}	3.41 ± 0.12^{b}	0.14 ± 0.06^{c}	0.81 ± 0.13^{a}	0.04 ± 0.02^{b}	0.71 ± 0.37^{a}
20	Control	4.12 ± 0.82^{a}	-0.05 ± 0.09^{c}	0.90 ± 0.05^{a}	0.22 ± 0.11^{c}	0.16 ± 0.03^{a}	0.06 ± 0.03^{b}
	Drought	0.78 ± 0.02^{c}	2.46 ± 0.57^{b}	0.11 ± 0.06^{c}	0.50 ± 0.10^{b}	0.06 ± 0.02^{b}	0.18 ± 0.02^{a}

Table 3. Net photosynthetic (P_N)- and transpiration (E) rates and stomatal conductance to water vapor (g_s) in *Sedum stoloniferum* grown under drought treatment. Data of each parameter within each harvest interval followed by *the same letter* are not significantly different ($p<0.05$). Data are means ± SD ($n = 4$).

Time after water stress [d]		P_N [μmol(CO_2) m^{-2} s^{-1}]		E [mmol(H_2O) m^{-2} s^{-1}]		g_s [mol m^{-2} s^{-1}]	
		Day	Night	Day	Night	Day	Night
1	Control	3.03 ± 0.41^{a}	-0.46 ± 0.40^{b}	0.54 ± 0.02^{a}	0.20 ± 0.17^{b}	0.16 ± 0.01^{a}	0.01 ± 0.00^{b}
	Drought	2.63 ± 0.69^{a}	-1.19 ± 0.43^{b}	0.53 ± 0.04^{a}	0.07 ± 0.01^{b}	0.23 ± 0.08^{a}	0.02 ± 0.01^{b}
12	Control	2.91 ± 0.94^{a}	-0.73 ± 0.21^{b}	0.82 ± 0.18^{a}	0.07 ± 0.01^{b}	0.32 ± 0.13^{a}	0.02 ± 0.01^{b}
	Drought	2.05 ± 0.45^{a}	-0.96 ± 0.08^{b}	0.48 ± 0.32^{a}	0.07 ± 0.01^{b}	0.06 ± 0.04^{b}	0.02 ± 0.01^{b}
16	Control	2.68 ± 0.06^{a}	-0.69 ± 0.15^{c}	0.96 ± 0.01^{a}	0.06 ± 0.04^{bc}	0.44 ± 0.01^{a}	0.01 ± 0.01^{c}
	Drought	0.99 ± 0.34^{b}	-0.24 ± 0.15^{c}	0.14 ± 0.06^{b}	0.04 ± 0.03^{c}	0.40 ± 0.03^{b}	0.02 ± 0.01^{c}
20	Control	2.77 ± 0.66^{a}	-0.64 ± 0.08^{c}	0.80 ± 0.22^{a}	0.06 ± 0.01^{b}	0.31 ± 0.14^{a}	0.01 ± 0.01^{b}
	Drought	0.75 ± 0.32^{b}	-0.70 ± 0.07^{c}	0.13 ± 0.07^{b}	0.07 ± 0.03^{b}	0.04 ± 0.02^{b}	0.01 ± 0.01^{b}

stomatal conductance did not respond to water stress in *S. stoloniferum* while increased significantly in *S. album*.

Drought stress caused reduction of daytime gas-exchange parameters in both species tested (Tables 2,3). Interestingly, response of gas-exchange parameters to drought stress was obvious from day 12 onwards in *S. album* while in *S. stoloniferum* it could be detected later, on day 16 onwards. P_N, E, and g_s decreased significantly in drought-stressed plants during the light period.

Activity of PEPC increased in response to drought stress in *S. album* during the day as well as night-time when water stress period exceeded 12 d. Similar response was observed for *S. stoloniferum,* however, this response was detected 16 d after water stress and like Δtitratable acidity, declined again towards the end of experiment. In addition, constitutive amounts of PEPC activity were considerably (2–2.5 times) higher in *S. album* than *S. stoloniferum* irrespective to the treatment and sampling time (Table 4).

Responses of antioxidant defense system to drought: Activity of all analyzed enzymes, was quite stable over 20-d experimental period in well-watered plants (Figs. 4,5). Drought stress, however, influenced activity of enzymes significantly. In both species, activity of all three antioxidant enzymes increased under mild water stress while it was later decreased as water stress became severe. After 20-d water stress, activity of all three studied enzymes in *S. stoloniferum* and that of CAT in *S. album* was even lower than that in well-watered plants. For CAT, the rise and fall of the activity was observed within 4–12 d after water-stress imposition, while it occurred later for SOD and APX *e.g.* between 8–16 d after starting water stress. In addition, for SOD and APX, drought-stress-induced activity in *S. album* was observed prior to that in *S. stoloniferum*.

In control plants of both species, except of a slight rise in the activity of enzymes in the samples taken at 16:00 h, significant diurnal change was not observed (Tables 5,6). In drought-stressed plants, in contrast, SOD activity showed significant fluctuation during a 24-h period in both species (Table 5). Interestingly, such diurnal pattern disappeared in the *S. album* plants after 20-d drought stress (Table 6). In *S. album*, in contrast to *S. stoloniferum*, diurnal pattern of changes was also found in the activity of CAT 12 d (but not 20 d) after drought treatment (Tables 5,6).

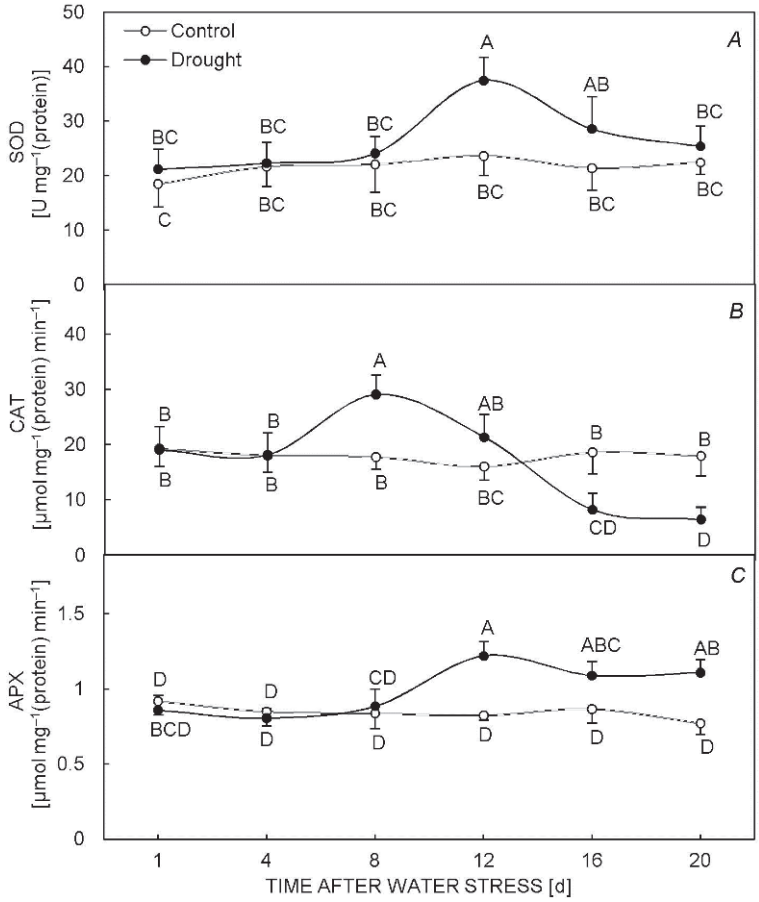

Fig. 4. Specific activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) at different time intervals after drought treatment in *Sedum album*. Bars indicated with *the same letter* are not significantly different (p<0.05). Values are means ± SD (n = 4).

MDA content did not change significantly even after 20-d exposure to water stress in *S. album* (Fig. 6*A*), while in *S. stoloniferum* MDA content increased significantly in the samples taken 20 d after drought (Fig. 6*B*). H_2O_2 content increased up to 50% in water-stressed *S. album* but the corresponding value in *S. stoloniferum* was only 35% (Fig. 6*C*,*D*).

Discussion

Contrastive responses to drought: Reduction of dry matter production by drought stress has been observed for many drought-tolerant species (Liu and Stützel 2004, Degu *et al*. 2008). In this work, exposure of plants to severe water stress defined by the RWC values lower than 60% (Castillo 1996), influenced dry matter production only in *S. stoloniferum*. Accordingly, though reduction of RWC and considerable water loss, tolerance of *S. album* to water scarcity was considerably higher than *S. stoloniferum*. Maintenance of dry matter production during drought has been considered a general measure of drought tolerance (Tschaplinski *et al*. 2006).

In contrast to a known behavior of leaf morphological parameters such as succulence and increase in the palisade and spongy parenchyma in drought-resistant species (Ennajeh *et al*. 2010), leaf thickness in *S. stoloniferum* was influenced rather negatively by exposure to severe water stress. On the other hand, RWC of *S. stoloniferum* leaves decreased more than that in *S. album* under water deficit. Significant correlation between RWC and Δtitratable acidity in the latter species suggested also that elevated stomatal limitation by drought was one of the major mechanisms involved not only in the increase of water-use efficiency and RWC (Wahbi *et al*. 2005) but also probably in the stimulation of acid fluctuations in the leaves of *S. album* in this work.

A remarkable reduction in the leaf RWC and Ψ_w in drought-stressed *S. stoloniferum* and *S. album* was associated with significant reduction of daytime P_N. Reduction of g_s due to decreased Ψ_w that inhibits supply of CO_2 and consequently reduces CO_2 assimilation is a well known phenomenon in drought-stressed plants (Bacelar *et al*. 2006, Ben Ahmed *et al*. 2009). In addition, mesophyll limitation of photosynthesis during drought stress is involved in various sites of inhibition including Rubisco activity and supply of reducing equivalents (*e.g.* NADPH) through photosynthetic electron transport (Lawlor and Cornic 2002).

Table 4. Daytime and night-time phospho*enol*pyruvate carboxylase (PEPC) activity in plants grown under drought treatment. Data within each harvest interval and species followed by *the same letter* are not significantly different ($p<0.05$). Data are means ± SD ($n = 4$).

Time after water stress [d]		PEPC [nmol(NADH) mg^{-1} (protein) min^{-1}] *Sedum album*		*Sedum stoloniferum*	
		Day	Night	Day	Night
1	Control	170 ± 17^{a}	200 ± 25^{a}	73 ± 7^{b}	95 ± 12^{a}
	Drought	180 ± 29^{a}	208 ± 40^{a}	70 ± 6^{b}	84 ± 12^{ab}
12	Control	176 ± 27^{c}	189 ± 35^{bc}	86 ± 7^{b}	99 ± 15^{ab}
	Drought	280 ± 59^{ab}	323 ± 47^{a}	102 ± 19^{ab}	133 ± 27^{a}
16	Control	210 ± 19^{c}	287 ± 31^{bc}	80 ± 8^{c}	88 ± 3^{bc}
	Drought	316 ± 58^{b}	526 ± 37^{a}	112 ± 4^{b}	142 ± 22^{a}
20	Control	221 ± 68^{c}	297 ± 45^{c}	81 ± 5^{ab}	97 ± 15^{a}
	Drought	420 ± 26^{b}	558 ± 50^{a}	74 ± 6^{b}	88 ± 10^{ab}

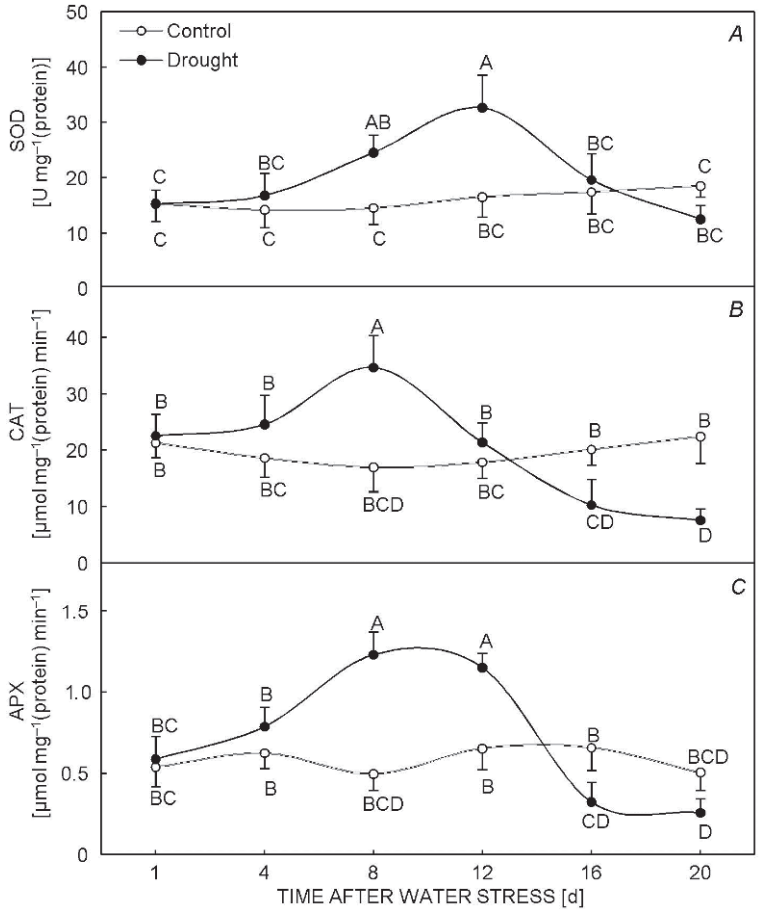

Fig. 5. Specific activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) at different time intervals after drought treatment in *Sedum stoloniferum*. Bars indicated with *the same letter* are not significantly different ($p<0.05$). Values are means ± SD ($n = 4$).

Photosynthetic pathways under drought stress: The essential criteria that define CAM are abilities to fix CO_2 during the dark and to store the carbon fixed in the vacuole as an organic acid (Silvera *et al*. 2010). According to our results, *S. album* subjected to drought stress showed significant daily fluctuation in titratable acidity, net CO_2 uptake in the dark as well as relatively high level of PEPC activity, all typical characteristics of CAM. Thus, *S. album* can be classified as a C_3-CAM intermediate plant. Induction of CAM in this species has been also reported by Castillo (1996) under drought stress. However, the value for Δtitratable acidity observed in the present work (~41) is by about 35% lower than that reported by this author (~70). Leaf thickness in *S. album*, irrespective to the treatment and time of sampling, was considerably (2–2.5 times) higher than that in *S. stoloniferum*. A tight correlation between greater tissue succulence and increased magnitude of CAM has been observed within the Crassulaceae (Kluge *et al*. 1993) and in other diverse CAM families (Nelson *et al*. 2005, Nelson and Sage 2008).

Table 5. Daily fluctuations in the specific activity of superoxide dismutase (SOD) and catalase (CAT) in *Sedum album* and *Sedum stoloniferum* grown for 12 d under drought treatment. Data of each enzyme followed by *the same letter* are not significantly different (p<0.05). Data are means ± SD (n = 4).

Time of day [h]	Treatment	SOD [U mg^{-1}(protein)]		CAT [µmol(H_2O_2) mg^{-1}(protein) min^{-1}]	
		S. album	*S. stoloniferum*	*S. album*	*S. stoloniferum*
09:00	Control	18 ± 2.3^{b}	13 ± 2.2^{b}	14 ± 3.1^{ab}	21 ± 2.3^{a}
	Drought	19 ± 3.7^{b}	14 ± 3.4^{b}	14 ± 4.3^{ab}	19 ± 3.7^{a}
16:00	Control	23 ± 3.6^{b}	16 ± 3.7^{b}	16 ± 2.1^{ab}	17 ± 2.9^{a}
	Drought	37 ± 4.2^{a}	32 ± 5.9^{a}	21 ± 4.2^{a}	21 ± 3.5^{a}
24:00	Control	15 ± 3.5^{b}	9 ± 1.5^{b}	13 ± 3.0^{b}	18 ± 3.0^{a}
	Drought	16 ± 4.9^{b}	8 ± 4.9^{b}	10 ± 1.6^{b}	16 ± 1.6^{a}

Table 6. Daily fluctuations in the specific activity of superoxide dismutase (SOD) and catalase (CAT) in *Sedum album* and *Sedum stoloniferum* grown for 20 d under drought treatment. Data of each enzyme followed by *the same letter* are not significantly different (p<0.05). Data are means ± SD (n = 4).

Time of day [h]	Treatment	SOD [U mg^{-1}(protein)]		CAT [µmol(H_2O_2) mg^{-1}(protein) min^{-1}]	
		S. album	*S. stoloniferum*	*S. album*	*S. stoloniferum*
09:00	Control	15 ± 2.3^{bc}	12 ± 3.1^{ab}	15 ± 3.2^{a}	19 ± 2.8^{a}
	Drought	17 ± 3.7^{b}	7.5 ± 2.4^{bc}	5.9 ± 3.1^{b}	6.4 ± 2.4^{b}
16:00	Control	22 ± 2.2^{ab}	18 ± 2.9^{a}	18 ± 3.6^{a}	22 ± 4.8^{a}
	Drought	25 ± 3.7^{a}	12 ± 4.5^{ab}	6.5 ± 2.3^{b}	7.6 ± 2.1^{b}
24:00	Control	17 ± 3.5^{b}	11 ± 3.5^{abc}	16 ± 3.0^{a}	17 ± 3.0^{a}
	Drought	8.3 ± 3.9^{c}	4.5 ± 2.9^{c}	7.8 ± 1.6^{b}	5.3 ± 1.6^{b}

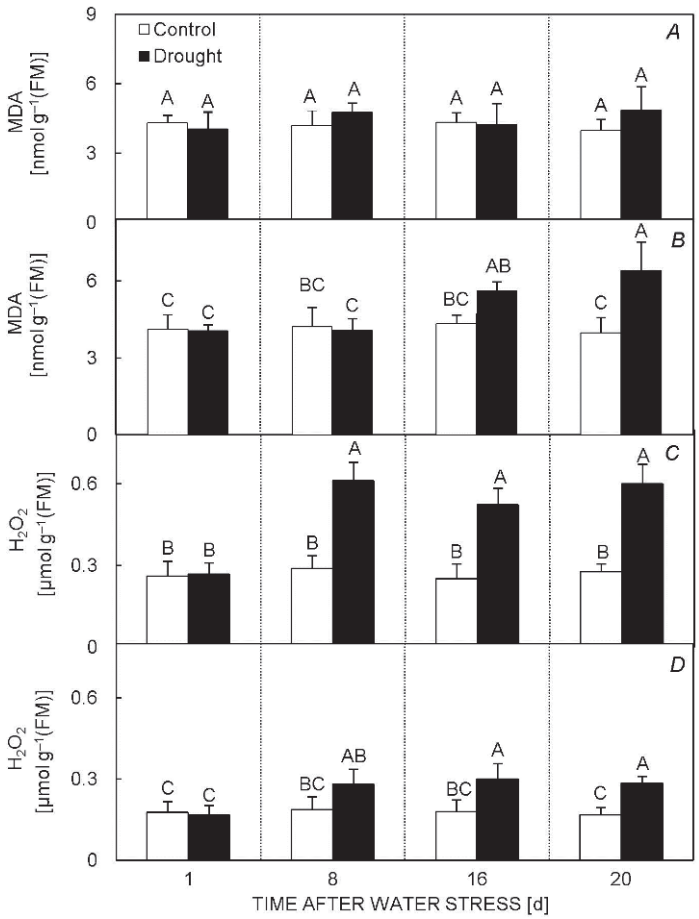

Fig. 6. Leaf content of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in *Sedum album* (*A*,*C*) and *Sedum stoloniferum* (*B*,*D*) at different time intervals after drought treatment. Bars indicated with *the same letter* are not significantly different (p<0.05). Values are means ± SD (n = 4).

After a period of at least 16 d without watering, *S. stoloniferum* showed a significant rise of Δtitratable acidity and PEPC activity, both characteristics typical of CAM (Herrera *et al*. 2010). However, both parameters declined again towards the end of the stress period. In addition, observed Δtitratable acidity (~7) was only 5 times higher in the stressed- than in the control plants and was considerably lower than that observed for *S. album* in this work or reported by other authors (Castillo 1996).

Although there was a significant increase in the night-time P_N, it remained negative after 16 d drought stress in *S. stoloniferum*. This response was similar to other reports on CAM-cycling plants such as *Clusia aripoensis* (Borland *et al*. 1998), *Sedum integrifolium* (Gravatt and Martin 1992) and some weak CAM species (Silvera *et al*. 2005, Holtum *et al*. 2007) with equal amounts for Δtitratable acidity with our work. CAM-cycling, as an initial stage of CAM, is characterized by CAM-like acid concentration fluctuations but C_3 gas-exchange patterns (Ting and Sipes 1985). Accordingly, *S. stoloniferum* can be categorized as a C_3 plant with a small capacity for CAM-cycling. The presence or absence of the CAM cycle in this species must be confirmed at molecular level using markers such as CAM-specific PEPC isogenes (Taybi *et al*. 2004, Gehrig *et al*. 2005). In CAM-cycling plants, the recapture of respiratory CO_2 at night allows the maintenance of a positive carbon balance during frequent episodes of drought (Martin 1996), experienced by plants growing in thin soils or in rock outcrops, typical for habitats of our studied species. In conclusion, induction of CAM by drought and protection of plants against water stress by antioxidant enzymes could be regarded as ecological advantages for our studied species growing on thin soils of rock crevices and extended dry periods.

Responses of antioxidant defense system during CAM induction: Response of antioxidative enzymes to water stress was highly dependent on plant species and the intensity of the imposed water stress. It is widely accepted that function of antioxidant defense system depends on plant species, developmental stage of the plant, and the intensity and duration of the imposed stress (Apel and Hirt 2004). In C_3-CAM intermediate *S. album*, amounts of MDA remained unchanged after 20-d water stress obviously because of an efficient scavenging of ROS following elevated activity of antioxidant enzymes. According to our results, each enzyme was effective during a given time interval of water-stress period. During early stages of drought, CAT and in the later stages SOD and APX were effective in scavenging ROS.

In *S. album*, H_2O_2 concentration during daytime was greater in *S. album* than in *S. stoloniferum*. Li *et al*. (2001) compared the leaf concentration of H_2O_2 in several species of C_3, C_4, and CAM plants and showed that the average concentration of H_2O_2 in CAM plants is about two-folds higher than that in C_3. H_2O_2 acts as a signaling molecule and affects the gene expression of various enzymes (Desikan *et al*. 2001, Ślesak *et al*. 2007). According to our results, as CAT activity started to reduce, H_2O_2 accumulated that may act as a signal for C_3-CAM transition as observed by other authors (Lüttge 2004, Ślesak *et al*. 2007, Niewiadomska and Borland 2008). Moreover, experiments with *M. crystallinum* suggested that CAM induction causes lower ROS production in plants exposed to extended periods of salinity (Borland *et al*. 2006). Nevertheless, the question of how H_2O_2/ROS are involved in the CAM mode of photosynthesis needs more detailed studies.

In this work, temporal changes in the Δtitratable acidity and ΔPEPC activity coincided well with those of antioxidant enzymes and both started to increase from day 12 onwards. This may suggest a role for antioxidant enzymes in the induction of a signaling pathway resulting in a shift from C_3 to CAM-cycling pathway.

It was also shown that CAM plants have a strong diurnal rhythm in activity of some antioxidative enzymes in comparison to C_3 plants (Ślesak *et al*. 2002, Niewiadomska 2004, Kuźniak *et al*. 2011). In our experiment, well-watered plants of *S. album* and *S. stoloniferum* did not exhibit diurnal rhythms for SOD and CAT activity in C_3 mode of photosynthesis, while in *M. crystallinum* plants performing C_3 photosynthesis diel oscillations in CAT and MnSOD activity has been observed (Kornas *et al*. 2007). Under 12-d drought stress, however, activity of SOD was higher during the evening hours than in the morning and night hours in both species. This diurnal pattern continued for the entire period of water stress in *S. album* while disappeared in *S. stoloniferm*. In contrast to *S. album*, in *S. stoloniferum* CAT activity did not exhibit diurnal rhythm. Even in *S. album*, CAT activity did not continue to fluctuate diurnally after 20-d water stress suggesting that severe water stress may inhibit diurnal fluctuation of CAT activity.

In conclusion, our experiment revealed that diurnal fluctuation of CAT and SOD activity occurred more pronouncedly in *S. album* than in *S. stoloniferum*. These results suggested that diurnal fluctuation pattern of antioxidant enzymes is likely associated with the degree of CAM induction. Further comparative studies of CAM-cycling and C_3-CAM intermediate plants are necessary to elucidate antioxidant strategies that are engaged as essential components for the operation of different levels of CAM photosynthesis under drought conditions.

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