Photosynthetic Characteristics and Antioxidative Responses in Three Species of Crassulaceae Following Drought Stress

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Abstract

Photosynthetic characteristics and induction of crassulacean acid metabolism (CAM) by drought stress were investigated in *Sedum album, Sedum stoloniferum* and *Rosularia elymaitica* from Crassulaceae. Titratable acidity, malate content, phosphoenolpyruvate carboxylase (PEPC) activity and gas exchange parameters were determined in plants at the end and beginning of the photoperiod. Results showed that, significant changes in dusk/dawn titratable acidity (ΔAcidity) and malate (ΔMalate) could be detected in all three studied species after 20 days drought stress. However, Δacidity and Δmalate in leaves of *Sedum stoloniferum* and *R. elymaitica* were less than values obtained for *S. album*. Although drought stress caused a significant increase in the activity of PEPC in all three studied species, difference between daytime and nighttime PEPC activity was significant only in *S. album*. In *S. stoloniferum* and *R. elymaitica,* despite of an increase by drought stress, net nighttime CO_2 assimilation was still negative resembling a C_3-like pattern of gas exchange. Comparison of responses of *S. album* with other two tested species showed that, drought stressed *S. stoloniferum* and *R. elymaitica* develop a low degree of CAM activity e.g. CAM-cycling metabolism, while *S. album* is capable to exhibit a typical CAM pathway under drought stress e.g. C_3-CAM intermediate. Activity of superoxide dismutase and catalase were decreased under drought stress, but that of peroxidase and ascorbate peroxidase did not change. The exception was an increase in peroxidase activity in drought stressed *R. elymaitica*, suggesting an important role for this enzyme in the protection against ROS in this species.

Keywords: Antioxidative enzymes; C_3-CAM shift; Phosphoenolpyruvate carboxylase (PEPC); Titratable acidity; Drought stress

Introduction

Most land plants can be categorized as C_3, C_4 and crassulacean acid metabolism (CAM) according to their photosynthetic pathway. In CAM plants, stomata are closed during most of the day and opened at night.

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During the night, the primary fixation of CO_2 is catalyzed by phosphoenolpyruvate carboxylase (PEPC), resulting in the formation of malic acid stored in vacuole. During the day, malate is decarboxylated to provide the CO_2 for the fixation by Calvin cycle [17,18].

Plants that exhibit predominantly the CAM pathway are commonly known as obligate CAM plants and have a constitutive type of CAM. However, C_3 species with an ability to switch their carbon metabolism to the CAM pathway have been also evidenced. 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 [25, 29].

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 [25]. *Sedum album* is an example of C_3-CAM intermediate in which CAM pathway is induced by drought [6]. CAM-cycling is characterized by CAM-like acid concentration fluctuations with C_3 gas exchange pattern [25]. The shift from C_3 photosynthesis to CAM-cycling has been documented in *Clusia aripoensis* [4] and *Sedum integrifolium* [10].

One of the biochemical changes in plants subjected to stress conditions is accumulation of reactive oxygen species (ROS). Oxidative stress refers to a serious imbalance between the production and removal of ROS. In C_3 plants, antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) play an important role against oxidative stress [2]. Although it has been proposed that, increase in carbon cycling during the C_3-CAM transition confer enough protection against photoinhibition under drought stress [11], other reports showed that antioxidative enzymes may also contribute to the protection of CAM-induced plants during CAM induction [6,27]. On the other hand, stress factors involve in the induction of CAM pathway are simultaneously responsible for increasing activity of antioxidative enzymes. Therefore, it would be interesting to know the role of antioxidant defense system during the C_3-CAM shift. In *Mesembryanthemum crystallinum* and *S. album* the induction of CAM by water limitation is accompanied by increased activities/capacities of antioxidant systems [6,23,28].

CAM transition has been reported so far (2001) in 407 species from 23 families of angiosperms [25]. Family Crassulaseae has the greatest number of species with capability for CAM transition. Among genera of Crassulaceae, CAM inducibility was documented for 26 *Sedum*, 23 *Cotyledon* and 13 *Kalanchoë* species [25].

However, 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 [22]. CAM pathway contributes significantly in the plants survival in arid and semi-arid regions of the world [8], therefore, verification of photosynthetic flexibility in the floral elements of Iran is important.

In this work we studied ability of *Sedum stoloniferum* and *Rosularia elymaitica* to shift from C_3 to CAM under severe drought stress. *Sedum album* was also included as a model species because of its known capability for CAM induction under drought stress [6]. In addition of monitoring the dusk/dawn changes in titratable acidity, accumulation/breakdown of malic acid, activity of phosphoenolpyruvate carboxylase (PEPC) and gas exchange pattern, we examined the effect of severe water stress on the antioxidant enzymes during CAM induction.

Materials and Methods

Plant Materials and Treatments

Three species from Crassulaceae including *Sedum album*, *Sedum stoloniferum* and *Rosularia elymaitica* collected from their habitats in East-Azerbaijan Province (NW Iran) were studied in this work.

Sedum album L. a perennial succulent with decumbent main stems and vertical flowering stems occurs in North and NW Iran [1]. Whole plants were collected from Mishou-Dagh, near the town of Payam, NW Iran at an elevation of 1890 m. Plants were restricted to shallow soils under the shade of boulders and shrubs.

Sedum stoloniferum S.G. Gmel. a perennial succulent with decumbent stems occurs in North (Guilan and Mazandaran) and North West (Khalkhal, Astara) Iran [1]. Whole plants were collected from Anjerd, near the city of Ahar, NW Iran at an elevation of 2010 m. Plants were growing in crevices and thin soil on the shade of boulders and shrubs.

Rosularia elymaitica Boiss. & Hausskn. an Irano-Turanian element, perennial succulent with rosette leaves and pink flowers occurs in North, North West and Central Iran [1]. Plants were collected from Mishou-Dagh, near the town of Payam, NW Iran at an elevation of 1890 m. Plants were growing 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 150

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 25/17°C day/night temperature and relative humidity of 70/60% for a period of two months prior to the start of experiments. Plants were watered twice weekly with distilled water and received once weekly 50% modified Hoagland nutrient solution [14]. Following the two months acclimation period, independent plant pots were selected randomly and subjected to control and drought 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 days that has been defined as severe drought stress for *S. album* [6]. To minimize evaporation, the exposed surface of each pot was covered with tin foil.

After 20 days treatment, gas exchange measure-ments were undertaken and sampling was done for biochemical determinations. For an estimation of the time course of CAM induction, leaf samples were taken for determination of titratable acidity 3, 4, 8, 12, 16 and 20 days after starting drought treatment. The highest amount for Δtitratable acidity was obtained from the 16th day of treatment onward. Therefore, all determinations were made on plants after 20 days treatment.

Gas Exchange Analysis

Net CO_2 fixation (A, μmol m^{-2} s^{-1}) and transpiration rate (E, mmol m^{-2} s^{-1}) were measured with a calibrated portable gas exchange system (LCA-4, ADC Bioscien-tific Ltd., UK). Leaves were detached either after 5 h into the light period (day samples) or after 5 h into the night period (night samples) and sealed in the leaf chamber under a photon flux density of 380 μmol m^{-2} s^{-1} for the day samples.

Determination of Titratable Acidity and Malate Concentration

Total titratable acidity and malate concentration were measured in leaves of control and treated plants at the end and beginning of the photoperiod. For determination of titratable acidity, samples of known weight were ground with liquid N_2, then boiled for 10 min in distilled water. Additional distilled water was added to bring to a final volume of 2 ml. Titration was performed with 10 mM NaOH to pH 7.0 [15]. For determination of malate concentration, samples were ground with liquid N_2 and deproteinized with perchloric acid. The reaction medium contained 340 mM hydrazine, 430 mM glycine pH 9.0, 2.75 mM NAD and

10 U of MDH. Malate was measured spectrophoto-metrically at 30°C by monitoring NADH oxidation at 340 nm in a reaction with NAD-MDH [13].

Assay of Enzymes Activity

Activity of enzymes was determined in leaves harvested in the middle of the day. For determination of nighttime PEPC activity, leaves were sampled after 5 h into the dark period.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was determined according the method of Groenhof et al. [12]. 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 phosphoenolpyruvate (PEP), 0.15 mM NADH and 10 units of MDH. NADH oxidation was followed by measuring absorbance at 340 nm.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated according to the method of Giannopolitis and Ries [9]. Enzyme was extracted in 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture contained 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 μM nitroblue tetrazolium chloride (NBT), 13 μM riboflavin. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under assay conditions.

For the determination of catalase (CAT, EC 1.11.1.6) activity, samples were homogenized with 50 mM phosphate buffer pH 7.0 and assayed spectrophoto-metrically by following the degradation of H_2O_2 at 240 nm according to the method of Simon et al. [26]. Reaction medium contained 50 mM phosphate buffer pH 7 and 10 mM H_2O_2.

Peroxidase (POD, EC 1.11.1.7) activity was deter-mined using the guaiacol test at 470 nm [7]. The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol.

Ascorbate peroxidase (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 [3]. The reaction mixture contained 50 mM phosphate buffer pH 7, 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 μg bovine serum albumin (BSA).

Soluble protein was estimated spectrophoto-metrically by the Bradford method [5]. All experiments were conducted using 4 independent replications. Statistical analyses were carried out using Sigma stat (2.01) with Tukey test at $p < 0.05$.

Results

Nocturnal titratable acidity of leaf extract was increased by drought stress up to 70% in *S. album* (Table 1). In contrast, drought stress did not cause any significant change for leaf titratable acidity of night samples in *S. stoloniferum* and *R. elymaitica*. Diurnal titratable acidity was affected by drought stress not only in *S. album*, but also in *S. stoloniferum*. This parameter, however, was not influenced under drought conditions in *R. elymaitica*. Difference between control and drought stressed plants in the Δtitratable acidity, an indication of that CAM pathway occurring, was significant in all three studied species. However, species differed in the magnitude of difference between well-watered and drought stressed plants, e.g. Δtitratable acidity increased up to 14.2 times in drought-stressed *S. album* compared with control, while the corresponding values for *S. stoloniferum* and *R. elymaitica* were 3.2 and 1.9 respectively (Table 1).

After drought treatment, accumulation of malic acid in leaf extracts during night was not significantly different from control plants in all studied species (Table 2). In contrast, malate content in day time leaf samples in plants subjected to drought stress was significantly higher than control ones. Increase in the malate concentration of leaves reached up to 280%, 68% and 38% compared with well watered plants in *S. album, S. stoloniferum* and *R. elymaitica* respectively. Therefore, greater changes in malate concentration during day time were observed in *S. album*, followed by *S. stoloniferum* and *R. elymaitica*. Differences between nocturnal and diurnal malate concentration (Δmalate) differed significantly between control and drought-stressed plants for all three studied species. As expected, greater difference in Δmalate between control and stressed plants was recorded for *S. album* (18.4 times), followed by *S. stoloniferum* (5.2 times) and *R. elymaitica* (2.6 times) (Table 2).

Activity of PEPC increased in response to drought stress in all tested species during day as well as night with the exception of day time PEPC activity in *R. elymaitica* (Table 3). Difference between day time and night time PEPC activity (ΔPEPC activity), however, was influenced significantly by drought stress only in *S. album*. Absolute amounts of PEPC activity, irrespective to the treatments and time of sampling, were considerably (3-6 times) greater in *S. album* than *S. stoloniferum* and *R. elymaitica* (Table 3).

In well watered plants, gas exchange parameters showed typical pattern of C_3-photosynthesis with a negative values for net CO_2 uptake during night in all three studied species (Table 4). Drought stress influenced strongly net assimilation rate during light period in all three tested species. After drought treatment, a positive value for nighttime net CO_2 uptake was observed in *S. album* which was even two times higher than daytime net CO_2 uptake of stressed plants. In *S. stoloniferum* and *R. elymaitica,* however, though a reduction in negative values, nighttime net CO_2 uptake was not positive. Day amounts of transpiration rate were also altered by drought stress, decreased up to 67%, 57% and 82% in *S. album*, *S. stoloniferum* and *R. elymaitica* respectively. In contrast, transpiration rate during night was not different between control and drought stressed plants in all three tested species. Concentration of leaf intercellular CO_2 (Ci) was affected significantly neither by drought stress nor during the dark-light transition in *S. stoloniferum* and *R. elymaitica*. In *S. album*, in contrast, a significant reduction of Ci in drought stressed plants was detected during night time gas exchange measurements (Table 4).

Water stress caused a significant reduction of SOD and CAT activity in all studied species (Fig. 1). Change of SOD activity was greater for *S. album* with up to 55% reduction, while for *S. stoloniferum* and *R. elymaitica* reduction of SOD activity due to drought stress were 39% and 42% compared with the well watered plants respectively. APX and POD activity did not change significantly by drought stress with the exception of significantly higher POD activity in drought-stressed *R. elymaitica* compared with control plants (Fig. 1).

Table 1. Nocturnal and diurnal titratable acidity (μequivalents (H^+) g^{-1} FW) under control and drought conditions in two species of *Sedum* and one species of *Rosularia*. Data of absolute or Δacidity were compared within each species. Values with the same letter within each species are not significantly different (P<0.05). Data are the mean ± SD (n=4)

	Titratable Acidity		
Plant species	Diurnal	Nocturnal	ΔAcidity
S. album			
Control	8.46±0.49[c]	11.37±0.71[bc]	2.90±1.16[b]
Drought	14.38±1.39[b]	55.63±5.01[a]	41.25±5.56[a]
S. stoloniferum			
Control	12.73±1.15[c]	15.96±0.75[b]	3.23±1.77[b]
Drought	11.29±0.70[c]	21.66±0.45[a]	10.37±0.69[a]
R. elymaitica			
Control	13.59±1.72[b]	19.71±1.47[a]	6.11±1.86[b]
Drought	11.90±2.34[b]	23.47±1.63[a]	11.57±2.27[a]

Discussion

One hallmark of CAM plants is the remarkable plasticity of the basic metabolic framework. Environmental factors such as water availability influence the proportion of CO_2 taken up at night via PEPC or during the day by Rubisco. Facultative CAM plants exhibit predominantly C_3 photosynthesis when well watered, but develop CAM when water stressed, thereby reduce water lost through transpiration [18].

According to our results, *S. album* plants subjected to drought stress showed a significant daily fluctuation in titratable acidity and malate concentration as well as relatively high level of PEPC activity, all typical characteristic of CAM. Differences between drought stressed and well-watered plants in the Δacidity and Δmalate were also more prominent in *S. album* than two other tested species. Induction of CAM in this species has been also reported by Castillo [6] under drought stress. However, the value for Δtitratable acidity observed in the present work is by about 25% lower than that reported by this author (~ 70).

During C_3-CAM transition, CO_2 uptake in the dark is not always evident and depending on water availability, some species do not show CO_2 net uptake in the dark when shifted to CAM metabolism. In such cases i.e. CAM cycling, the re-fixation of respiratory CO_2 in the dark is associated with day-to-night fluctuations of titratable acidity [8]. In our work, fluctuations in titratable acidity and malic acid in *S. stoloniferum* and *R. elymaitica* were lower than values obtained for *S. album* and in contrast to *S. album,* net CO_2 uptake during the night was still negative in drought-stressed *S. stoloniferum* and *R. elymaitica*. On the other hand, increase in the activity of PEPC in drought-stressed *S. stoloniferum* and *R. elymaitica* were considerably lower than that of drought-stressed *S. album*. In *S. stoloniferum* and *R. elymaitica*, though the activity of PEPC was significantly higher in drought compared with well watered plants similar with *S. album*, the difference in these species between nighttime and daytime values was not significant and much lower than that in *S. album*. Regarding above mentioned differences between *S. album* with *S. stoloniferum* and *R. elymaitica*, latter species could be considered CAM–cycling species. CAM-cycling, as an initial stage of CAM, is characterized by CAM-like acid concentration fluctuations but C_3 gas exchange patterns [29]. CAM cycling has been reported for *Clusia aripoensis* [4] and *Sedum integrifolium* [10] under drought stress with similar amounts for Δtitratable acidity with our work. 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 [19], experienced by plants growing in thin soils or in rock outcrops, typical for habitats of our studied species.

Some authors suggested that, during C_3-CAM transition and in CAM-cycling plants, CO_2 released during the day from organic acid decarboxylation contribute to the photoprotection in these plants [11]. In contrast, in other works calculations showed that the CO_2 obtained from decarboxylation may have been insufficient to maintain sink demand for electron transport and photoinhibition occur also in CAM-

Table 2. Nocturnal and diurnal malic acid concentration ($\mu mol\ g^{-1}$ FW) under control and drought conditions in two species of *Sedum* and one species of *Rosularia*. Data of absolute or Δ malic acid were compared within each species. Values with the same letter within each species are not significantly different (P<0.05). Data are the mean ± SD (n=4)

| Plant species	Concentration of Malic Acid		
	Diurnal	Nocturnal	Δ Malate
S. album			
Control	8.63±0.95[b]	10.16±1.17 [b]	1.53±0.26 [b]
Drought	10.38±0.25[b]	38.57±1.60 [a]	28.19±1.36 [a]
S. stoloniferum			
Control	9.24±0.59[c]	11.01±0.52 [b]	1.77±0.27 [b]
Drought	8.90±0.22[c]	18.48±0.20 [a]	9.57±0.14 [a]
R. elymaitica			
Control	8.22±0.62[c]	11.43±0.38 [b]	3.21±0.50 [b]
Drought	7.30±0.59[c]	15.80±0.58 [a]	8.50±0.42 [a]

Table 3. Daytime and Nighttime PEPC activity (nmol mg^{-1} protein min^{-1}) under control and drought conditions in two species of *Sedum* and one species of *Rosularia*. Data of enzyme activity were compared within each species. Values with the same letter within each species are not significantly different (P<0.05). Data are the mean ± SD (n=4)

Plant species	PEPC Activity		
	Daytime	Nighttime	Δ PEPC Activity
S. album			
Control	183±10 [c]	211±8 [c]	28±11 [b]
Drought	459±40 [b]	588±61 [a]	130±44 [a]
S. stoloniferum			
Control	59±6 [b]	66±5 [b]	7±5 [a]
Drought	80±6 [a]	92±7 [a]	11±5 [a]
R. elymaitica			
Control	89±7 [b]	103±15 [b]	20±10 [a]
Drought	120±19 [ab]	139±20 [a]	27±14 [a]

Table 4. Photosynthesis rate (A, μmol m^{-2} s^{-1}), transpiration rate (E, mmol m^{-2} s^{-1}) and intercellular CO_2 concentration (Ci, μmol mol^{-1}) under control and drought conditions in two species of *Sedum* and one species of *Rosularia*. Data of each parameter were compared within each species. Values with the same letter within each species are not significantly different (P<0.05). Data are the mean ± SD (n=4)

Plant species	A μmol m^{-2} s^{-1}		E mmol m^{-2} s^{-1}		Ci μmol mol^{-1}	
	Daytime	Nighttime	Daytime	Nighttime	Daytime	Nighttime
S. album						
Control	1.69 ± 0.04 [a]	− 1.45 ± 0.10 [d]	0.64 ± 0.22 [a]	0.08 ± 0.02 [b]	379 ± 2 [b]	426 ± 2 [a]
Drought	0.34 ± 0.06 [c]	0.68 ± 0.21 [b]	0.21 ± 0.14 [b]	0.11 ± 0.07 [b]	378 ± 20 [b]	372 ± 14 [b]
S. stoloniferum						
Control	2.42 ± 0.30 [a]	− 0.86 ± 0.15 [d]	1.41 ± 0.52 [a]	0.05 ± 0.02 [b]	378 ± 4 [a]	431± 3 [a]
Drought	1.48 ± 0.10 [b]	− 0.18 ± 0.02 [c]	0.60 ± 0.10 [b]	0.06 ± 0.03 [b]	381 ± 2 [a]	438 ± 13 [a]
R. elymaitica						
Control	3.47 ± 0.30 [a]	− 0.97 ± 0.10 [d]	1.22 ± 0.40 [a]	0.08 ± 0.01 [b]	370 ± 11 [a]	434 ± 7 [a]
Drought	0.35 ± 0.20 [b]	− 0.27 ± 0.01 [c]	0.22 ± 0.08 [b]	0.09 ± 0.05 [b]	390 ± 4 [a]	439 ± 14 [a]
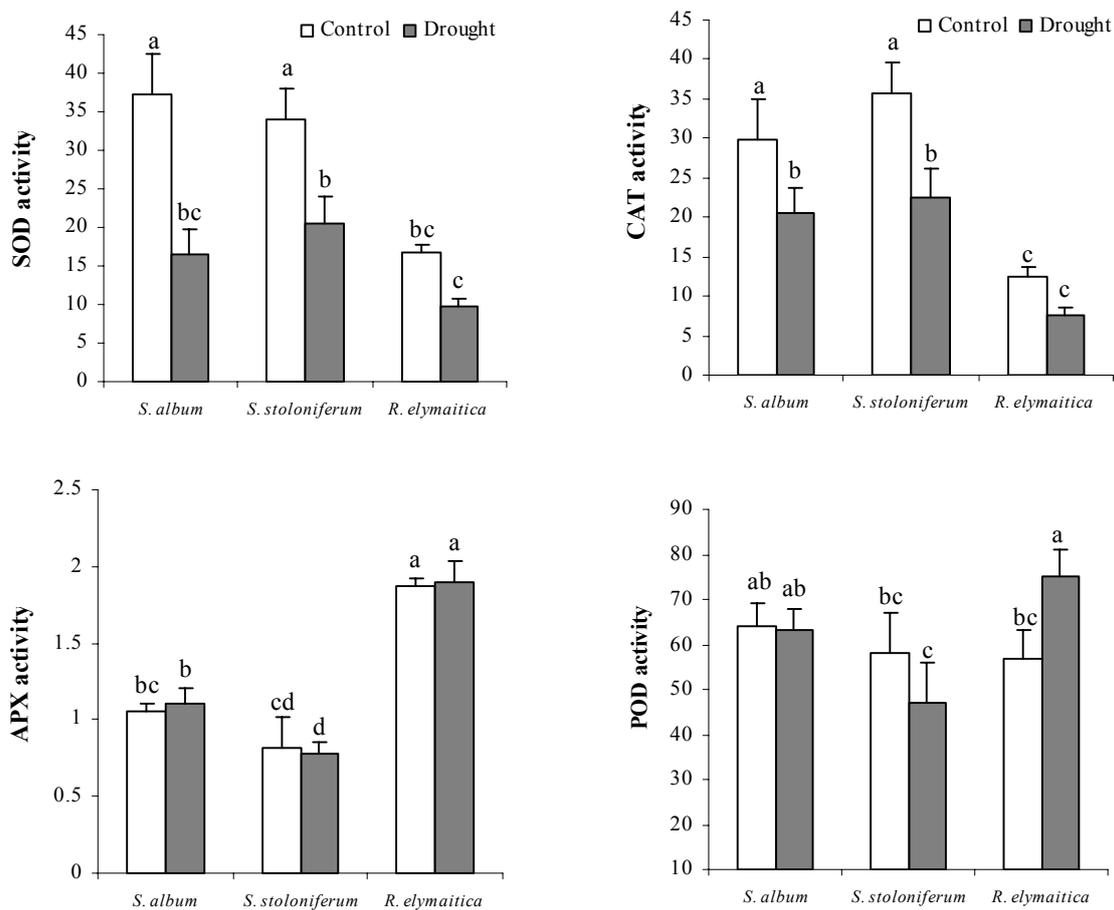
Figure 1. Specific activity of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), ascorbate peroxidase (APX, μmol ascorbate mg^{-1} protein min^{-1}), catalase (CAT, μmol H_2O_2 mg^{-1} protein min^{-1}) and peroxidase (POD, μmol tetraguaiacol mg^{-1} protein min^{-1}) in control and drought stressed plants. Values are the mean ± SD (n=4). Bars indicated with the same letter are not significantly different (P<0.05).

droughted plants [6]. Therefore, similar with C$_3$ plants subjected to drought stress, an alternative protection in CAM plants could be performed by an increased activity of antioxidant enzymes. An increase in the transcript levels of different isoforms of SOD and APX was reported during salinity treatments in *Mesembryanthemum crystallinum* a C$_3$-CAM intermediate plant [20]. In the present work, activity of CAT and SOD exhibited a decline in all three studied species subjected to drought stress. Activity of other two tested enzymes, APX and POD, remained mainly unchanged under drought stress. For *Sedum album*, response of antioxidant defense enzymes depends on the water status of the plants, with greater activity under mild and lower activity under severe water stress [27]. Under drought stress, SOD activity was found to be increased in pea [21] and tobacco [30], decreased in sunflower [24], but was not affected in maize [16]. It was also reported that, SOD and CAT activity increased or remained unchanged in wheat during the early phase of drought but decreased with prolonged water stress [31,32].

Under severe drought stress, other enzymes such as glutathione reductase (GR) are important in the protection against ROS [6]. We did not determine GR activity, but in contrast to other species, activity of POD in *R. elymitica* increased under drought stress which is likely important in the protection against ROS in this species. On the other hand, we performed enzyme assays only at the end of experiment, therefore, a transient increase in the enzymes activity at the early period of water withholding period could not be excluded. Responses of antioxidative enzymes depend on plant species, developmental stage of the plant, the intensity and duration of the imposed drought stress [2].

This work is the first report on the induction of CAM photosynthesis pathway in *S. stoloniferum* and *R. elymaitica*. Although there are reports on some species of *Sedum*, experimental evidence had not yet been published on the induction of CAM in any species of *Rosularia*.

Acknowledgment

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