www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/2: 15–24, 2011
DOI: 10.2478/v10182-011-0020-5

COMPARISON OF WATER STRESS AND UV RADIATION EFFECTS ON INDUCTION OF CAM AND ANTIOXIDATIVE DEFENSE IN THE SUCCULENT *ROSULARIA ELYMAITICA* (CRASSULACEAE)

GHADER HABIBI[1,2], and ROGHIEH HAJIBOLAND[1*]

[1]*Plant Science Department, University of Tabriz, 51666-14779 Tabriz, Iran*
[2]*Biology Department, Payame Noor University, 19395-4697 Tehran, Iran*

Received February 11, 2011; revision accepted October 30, 2011

Growth and photosynthetic characteristics, inducibility of the CAM pathway and the functioning of the antioxidant defense system were investigated in *Rosularia elymaitica* (Crassulaceae) under drought and UV stresses. Drought did not substantially affect the growth of the plants, but it significantly reduced leaf thickness as well as osmotic potential, water potential and relative water content. In contrast, UV radiation treatment affected neither growth nor the water relations of leaves. Water limitation for 12 days caused a significant increase in nighttime PEPC and NAD-MDH activity and an increase in Δtitratable acidity relative to well-watered plants. The nighttime CO_2 net assimilation rate increased significantly in drought-stressed plants but was still negative, resembling a C_3-like pattern of gas exchange. Twenty days of UV treatment, increased Δtitratable acidity slightly and increased only daytime PEPC activity, and did not affect other parameters of carbon metabolism. As judged by maintenance of membrane integrity and stable amounts of H_2O_2 under UV stress, the antioxidant defense system effectively protected the plants against UV radiation. In contrast, oxidative stress occurred under severe drought stress (20 days of withholding water). Except for higher daytime APX activity in the UV-treated plants, enzyme activity in the control and in the drought- and UV-stressed plants did not show any diurnal fluctuation during 24 h. Temporal changes in Δtitratable acidity and ΔPEPC activity coincided closely with those of antioxidant enzymes; both started to increase after 12 days of drought stress. These results indicate that drought stress but not UV radiation induced the CAM-cycling pathway in *R. elymaitica*.

Key words: CAM-cycling, net assimilation rate, oxidative stress, PEPC activity, titratable acidity.

INTRODUCTION

Crassulacean acid metabolism (CAM) is the second most common pathway of photosynthesis in vascular plants (Winter and Smith, 1996). In CAM-performing plants, stomata are closed during most of the day and opened at night. During the night, primary fixation of CO_2 results in the formation of malic acid stored in the vacuole. During the day, malate is decarboxylated to provide CO_2 for fixation by the Calvin cycle (Lüttge, 2004).

Plants that predominantly exhibit the CAM pathway are commonly known as obligate CAM plants and have a constitutive type of CAM. C_3 species with an ability to switch their carbon metabolism to the CAM pathway have also been found. Transition to CAM has been reported so far in 407 species from 23 angiosperm families (Sayed, 2001). Crassulaceae has the greatest number of species with the capability of CAM transition. Among genera of Crassulaceae, CAM inducibility has been documented for 26 *Sedum*, 23 *Cotyledon* and 13 *Kalanchoë* species (Sayed, 2001).

On the basis of gas exchange and the day/night pattern of organic acid turnover, CAM-inducible species are categorized as C_3-CAM intermediate and CAM-cycling species (Ting and Sipes, 1985). CAM-cycling is characterized by CAM-like acid concentration fluctuations with a C_3 gas exchange pattern (Sayed, 2001). 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). In species such as *Sedum telephium*, net CO_2 uptake could not be detected in the dark when shifted to CAM metabolism. In such cases of CAM-cycling, an

*e-mail: ehsan@tabrizu.ac.ir

PL ISSN 0001-5296

increase in phosphoenolpyruvate carboxylase (PEPC) activity may reveal the C_3-CAM transition (Groenhof et al., 1988). 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 a diel fluctuation, which may represent a nascent C_3-CAM progression (Cushman and Borland, 2002).

Various environmental stressors are capable inducing the CAM pathway in some plant species. *Sedum album* is an example of a C_3-CAM intermediate in which the CAM pathway is induced by drought (Castillo, 1996). CAM has facilitated the exploitation, by more than 7% of vascular plant species, of predominately hot and arid climates, semi-arid regions with seasonal water availability, or microclimates characterized by intermittent water availability (Smith and Winter, 1996).

Intense UV radiation in high mountains affects the plants growing at those elevations. The deleterious effects of UV radiation on growth, productivity and photosynthesis in higher plants have been studied extensively (Rybus-Zając and Kubiś, 2010; Skórska, 2011). UVB radiation impairs all major processes of photosynthesis, including photochemical reactions in thylakoid membranes (Skórska 2011), and causes oxidative damage and growth inhibition in higher plants (Rybus-Zając and Kubiś, 2010). UVA/blue light has been reported to induce a switch from the C_3 pathway to CAM in *Clusia minor* (Grams and Thiel, 2002).

Stress factors such as drought and UV radiation trigger common reactions in plants and lead to cell damage mediated by reactive oxygen species (ROS). The term oxidative stress refers to a serious imbalance between the production and removal of ROS. Antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) play an important role and protect against oxidative stress (Apel and Hirt, 2004). An increase in carbon cycling during the C_3-CAM transition has been suggested to confer enough protection against photoinhibition under drought stress (Griffiths et al., 1989), but other reports showed that antioxidative enzymes also make an important contribution to protection of plants during CAM induction (Ślesak et al., 2002). Moreover, stress factors involved in induction of the CAM pathway are simultaneously responsible for the increasing activity of antioxidative enzymes. In *M. crystallinum*, induction of CAM by water limitation was accompanied by increased antioxidant enzyme activity (Ślesak et al., 2002).

Rosularia elymaitica (Crassulaceae) grows along an altitudinal gradient of increasing UV radiation in a mountainous area in northwest Iran. This species tends to occupy habitats that experience frequent low soil matric potentials, where periodic drought can occur. Since the CAM pathway contributes significantly to plants' survival under arid and semi-arid conditions (Cushman and Borland, 2002), the ability to shift from C_3 to CAM may be important in *R. elymaitica*'s adaptation and survival in its natural habitat. There are no published studies examining the CAM pathway in this species. Our previous work demonstrated that water deficit induces typical CAM in *Sedum album*, while *S. stoloniferum* and *R. elymaitica* exhibit CAM-cycling following drought stress (Habibi and Hajiboland, 2010).

Here we compare drought with UV radiation in terms of their ability to induce the CAM pathway in *R. elymaitica*. To find the starting time point of CAM induction, in addition to monitoring the dusk/dawn changes in titratable acidity (Δtitratable acidity) and PEPC activity (ΔPEPC activity), we examined the temporal changes of these parameters through 20 days of drought or UV radiation stress. To analyze the relationship between stress-induced CAM and the ability to cope with oxidative stress during the C_3-CAM shift, we studied the functioning of the antioxidant defense system: specifically, the diurnal pattern of antioxidative enzyme activity and temporal changes in the functioning of the antioxidant defense system at different time points after imposition of drought and UV stress.

MATERIALS AND METHODS

PLANT MATERIAL AND TREATMENTS

Rosularia elymaitica Boiss. & Hausskn. is an Irano-Turanian element, a perennial succulent with rosette leaves and pink flowers occurring in north, northwest and central Iran (Akhiani, 2000). Plants were collected from Mishou-Dagh, NW Iran, at 1890 m a.s.l. The plants were growing in rock crevices and on thin soil in the shade of boulders and shrubs. Collected plants were transferred to plastic pots containing washed sand and were grown for two months prior to the start of treatments. The plants were watered twice weekly with distilled water and received 50% modified Hoagland nutrient solution once weekly (Johnson, 1957). Following two months of acclimation, plants in separate pots were selected randomly and subjected to drought or UV radiation treatments and their respective controls.

Drought-stressed plants received no water for 20 days, a period defined as severe drought stress for *S. album* (Castillo, 1996), while control plants were watered twice a week with distilled water to field capacity. To minimize evaporation the exposed surface of each pot was covered with aluminum foil. For UV radiation treatments, in addition to photosynthetically available radiation (PAR, 400–700 nm) supplied by cool white fluorescent lamps throughout the daytime, UVAB fluorescent lamps (30 W, Hagen,

Japan) were used without filters for UVA+B, with a transparent plexiglas filter cutting wavelengths under 320 nm for UVA, and with a yellow plexiglas filter to cut wavelengths under 400 nm for control plants, with 6 h irradiance periods centered midway through the photoperiod. The photosynthetic photon flux density (PPFD) was 150 μmol m^{-2} s^{-1} for all three lighting treatments. The spectral outputs of the three lighting conditions were measured with a calibrated spectrophotometer (Shimadzu, UV-2450) and the biologically effective UV doses employed were 30 kJ m^{-2} d^{-1} calculated based on Caldwell's generalized plant damage action spectrum normalized to 300 nm (Caldwell, 1971).

Plants were grown under environmentally controlled conditions under fluorescent white light at ~150 μmol m^{-2} s^{-1} with an 18 h photoperiod from 5:00 a.m. to 8:00 p.m, 25/17°C day/night temperature and relative humidity 60/70%.

PLANT HARVESTING AND ANALYSIS OF GAS EXCHANGE

Twenty days after treatment the plants were harvested. Leaves were washed with distilled water, blotted dry on filter paper and after determination of fresh weight (FW) they were dried for 48 h at 70°C for determination of dry weight (DW). Gas exchange parameters were measured before harvesting. Net CO_2 fixation (*A*, μmol m^{-2} s^{-1}), transpiration rate (*E*, mmol m^{-2} s^{-1}) and stomatal conductance to water vapor (*gs*, mol m^{-2} s^{-1}) were measured with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) either 5 h into the light period (day samples) or 5 h into the dark period (night samples), sealed in the leaf chamber under PPFD 380 μmol m^{-2} s^{-1} for the day samples.

ANALYSIS OF WATER RELATIONS AND TITRATABLE ACIDITY

Leaf osmotic potential (ψ_s) was measured with an osmometer (Micro-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), all in the second youngest leaf harvested at 1 h after lights on in the growth chamber. Total titratable acidity was measured in the leaves at the end and beginning of the photoperiod according to the method described by Lara et al. (2003).

ASSAY OF ENZYME ACTIVITY AND RELATED METABOLITES

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was determined according to Groenhof et al. (1988). Samples were taken at 5 h into the light (day samples) or dark (night samples) period. Extraction and assay of NAD malate dehydrogenase (NAD-MDH, EC 1.1.1.37) and NAD malic enzyme (NAD-ME, EC 1.1.1.39) was performed according to the methods described by Holtum and Winter (1982) and Wang et al. (2007) respectively.

The activity of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) was determined as described by Habibi and Hajiboland (2010). Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid, and the H_2O_2 concentration was determined by the potassium titanium oxalate method (Hajiboland and Hasani, 2007).

Temporal changes in the measured parameters were studied by taking samples at 1, 4, 8, 12, 16 and 20 days after the start of treatments. Because the amount of plant material was limited, sampling for some analyses was confined to only some of these time intervals. Twenty days after the start of treatments, diurnal changes in enzyme activity were examined by sampling plants at different times of the day.

The experiments were done in a complete randomized block design with 4 replicates. Statistical analyses employed Sigma Stat ver. 3.02 with the Tukey test ($P<0.05$).

RESULTS

Fresh weight of plants was not influenced by drought stress and dry weight was rather increased. However, the ratio of fresh to dry weight (FW/DW) and relative water content (RWC) decreased significantly due to water deficit (Tab. 1). Leaf osmotic and water potential also decreased in plants grown under water limitation. The leaf area of stressed plants increased slightly, while leaf thickness decreased significantly by ~33% versus the control. Unlike the drought treatment, UV radiation did not affect dry weight, fresh weight or the FW/DW ratio. Nor were water relation parameters and leaf morphology affected by the UV radiation treatment (Tab. 1).

Drought stress affected the daytime gas exchange parameters. The transpiration rate and stomatal conductance diminished slightly, but net CO_2 assimilation decreased significantly in drought-stressed plants during the light period. The nighttime transpiration rate and stomatal conductance showed a significant reduction relative to daytime values, but like daytime parameters they were not significantly influenced by drought stress. Under control conditions there was net CO_2 release during the night. Under drought stress the net CO_2 release was significantly lower than in control plants. The

TABLE 1. Fresh and dry weight (mg plant^{-1}) of leaves, ratio of fresh to dry weight (FW/DW), relative water content (RWC, %), leaf osmotic (ψ_s, MPa) and water potential (ψ_w, MPa), area (cm^2) and thickness (μm) in *R. elymaitica* grown for 20 days under drought or UV radiation treatments. Data for each parameter within treatment followed by the same letter do not differ significantly (P<0.05)

	Water stress treatment		UV treatment		
	Control	Drought	–UV	UVA	UVA+B
FW	384±42 a	382±28 a	381±30 a	382±19 a	362±17 a
DW	22.8±2.6 b	28.0±2.2 a	22.8±2.2 a	23.5±2.1 a	22.8±1.3 a
FW/DW	16.9±0.8 a	13.7±0.6 b	16.8±1.2 a	16.3±0.6 a	16.0±0.8 a
RWC	80.6±1.7 a	60.7±6.1 b	80.4±0.1 a	77.5±3.3 a	77.4±2.5 a
ψ_s	−0.56±0.04 a	−0.75±0.03 b	−0.61±0.02 a	−0.60±0.02 a	−0.60±0.03 a
ψ_w	−0.41±0.12 a	−0.63±0.21 b	−0.47±0.16 a	−0.50±0.22 a	−0.45±0.18 a
Leaf area	5.04±0.49 a	5.60±1.24 a	4.90±0.52 a	4.95±0.97 a	6.18±0.28 a
Thickness	825±50 a	550±57 b	800±81 a	850±129 a	855±120 a

TABLE 2. Net assimilation (*A*) and transpiration (*E*) rate and stomatal conductance to water vapor (*g*) in *R. elymaitica* grown for 20 days under drought or UV radiation treatments. Data for each parameter within treatment followed by the same letter do not differ significantly (P<0.05)

	A μmol m^{-2} s^{-1}		*E* mmol m^{-2} s^{-1}		g_s mol m^{-2} s^{-1}	
	Day	Night	Day	Night	Day	Night
Control	0.84±0.16 a	−1.11±0.12 c	1.07±0.26 a	0.35±0.05 b	0.45±0.13 a	0.09±0.03 b
Drought	0.32±0.26 b	−0.02±0.05 b	0.72±0.15 a	0.27±0.01 b	0.30±0.08 a	0.08±0.01 b
–UV	0.94±0.18 a	−0.66±0.41 b	0.96±0.21 a	0.47±0.09 b	0.37±0.07 a	0.13±0.03 b
UVA	0.90±0.15 a	−1.19±0.61 b	0.97±0.20 a	0.47±0.10 b	0.31±0.08 a	0.13±0.02 b
UVA+B	0.80±0.04 a	−0.91±0.60 b	0.96±0.12 a	0.46 ±0.09 b	0.36±0.08 a	0.14±0.01 b

TABLE 3. Daytime and nighttime PEPC activity (nmol NADH mg^{-1} protein min^{-1}) in *R. elymaitica* grown under drought or UV radiation treatments. Daytime and nighttime data within each treatment time followed by the same letter do not significantly differ (P<0.05).

Days of treatment	Treatment	Daytime activity	Nighttime activity
1	Control	76±7 a	97±15 a
	Drought	80±9 a	88±10 a
12	Control	80±9 b	89±5 b
	Drought	96±8 ab	123±27 a
20	Control	81±8 b	87±1 b
	Drought	104±6 ab	126±27 a
1	–UV	81±4 a	88±6 a
	UVA	80±5 a	80±3 a
	UVA+B	77±4 a	85±7 a
20	–UV	82±8 b	96±5 ab
	UVA	100±8 a	107±7 a
	UVA+B	84±7 b	87±8 b

TABLE 4. Activity of NAD-MDH and NAD-ME (nmol NADH mg^{-1} protein min^{-1}) 1 and 20 days after drought and UV treatment in *R. elymaitica*. Data for each parameter within treatment followed by the same letter do not differ significantly (P<0.05)

Treatment	NAD-MDH		NAD-ME	
	1 day	20 days	1 day	20 days
Control	150±22 b	149±24 b	5±2 a	6±3 a
Drought	141±16 b	290±37 a	4±1 a	5±2 a
–UV	130±10 a	128±20 a	6±3 a	5±2 a
UVA	141±28 a	125±10 a	6±2 a	6±3 a
UVA+B	113±12 a	106±7 a	6±2 a	6±3 a

UV radiation treatment did not affect any of the gas exchange parameters and, as expected, the transpiration rate, stomatal conductance and net CO_2 assimilation were significantly lower at night than during the day (Tab. 2).

Water limitation for 8 days did not affect the fluctuation of total titratable acidity. From day 12 onwards, however, Δtitratable acidity increased in drought-stressed plants versus control leaves. Following 20 days of drought stress, leaves presented an almost fivefold increase in Δtitratable acidity as compared with well watered plants. UVA treat-

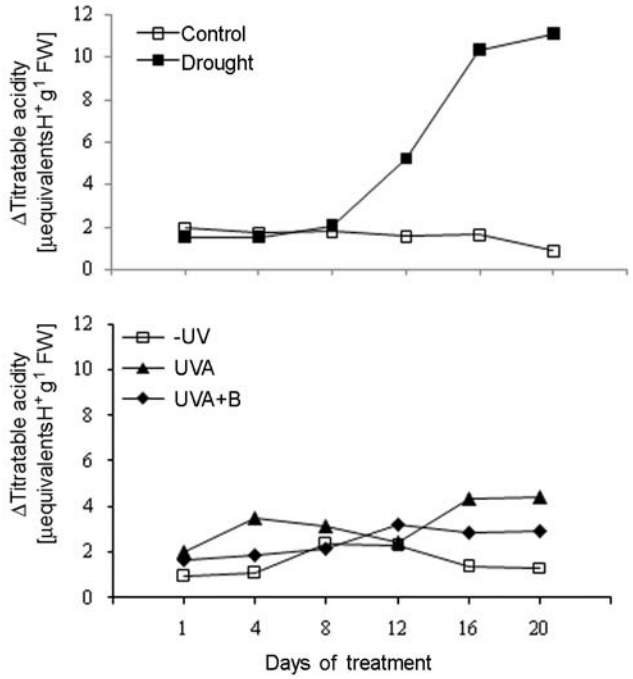

Fig. 1. Changes in Δtitratable acidity during 20-day drought and UV radiation treatments in *R. elymaitica*.

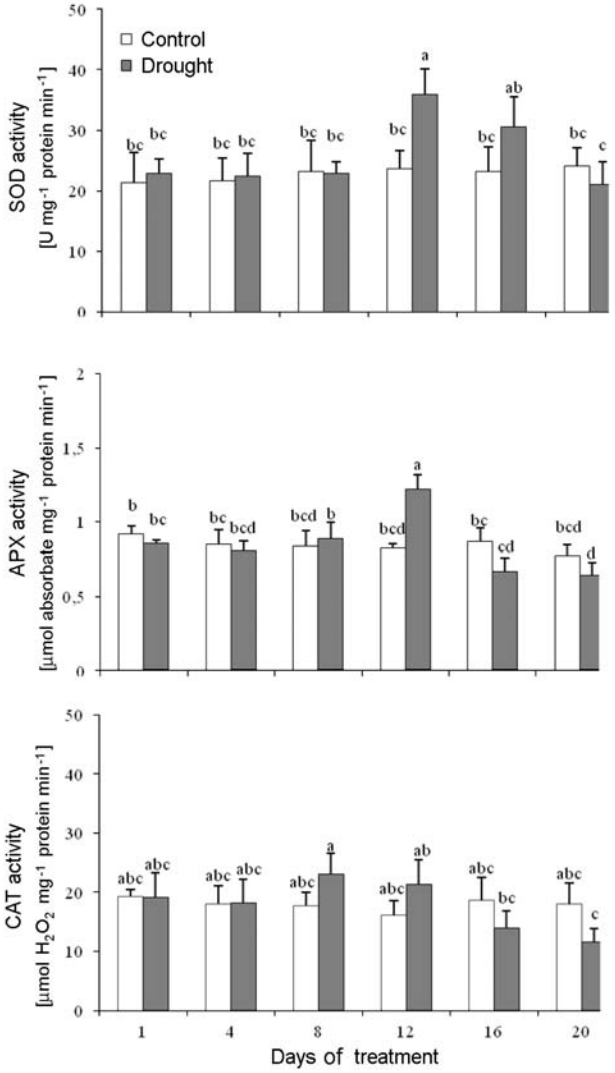

Fig. 2. Specific activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) at different intervals of drought treatment in *R. elymaitica*. Bars with the same letter do not differ significantly ($P<0.05$).

ment elevated the fluctuation of titratable acidity only slightly (Fig. 1).

Significant increases of nighttime PEPC activity were noted after 12 and 20 days of drought stress. ΔPEPC activity (nighttime PEPC activity/daytime PEPC activity) reached 27 at day 12 of drought treatment. At day 20, daytime PEPC activity was stimulated significantly by UVA but not by UVA+B radiation (Tab. 3). Drought stress for 20 days caused a significant increase in NAD-MDH activity, up to 48%, and had no effect on NAD-ME. UV radiation treatment influenced neither NAD-MDH nor NAD-ME after 20 days of treatment (Tab. 4).

Antioxidant enzyme activity was affected by drought stress. Twelve days of drought stress significantly increased SOD and APX activity. Before day 12, SOD, CAT and APX activity did not differ between water-stressed plants and well watered ones. Continuation of the water stress for more than 12 days caused a significant reduction of the activity of all analyzed antioxidant enzymes. After 20 days of stress the activity of APX and CAT was even lower than at the beginning of treatment. Unlike in drought-stressed plants, in well watered plants the activity of antioxidant enzymes remained quite stable over the 20-day experimental period (Fig. 2).

In UV-treated plants, SOD activity was affected only slightly. APX activity was increased by UVA and that of CAT was increased by both the UVA and the UVA+B treatments. UV treatment affected enzyme activity after relatively long exposure: at day 16 for APX activity and at days 12–16 for CAT activity (Fig. 3).

In general, the activity of APX, SOD and CAT did not show any significant 24-hour fluctuation in the control or in drought-stressed plants, but we did observe a slight rise in SOD and APX activity in the samples taken at 16:00, irrespective of the watering regime (Tab. 5). In the control plants of the UV experiment, no significant change in diurnal enzyme activity was observed. In the UV-treated plants, SOD and CAT activity also remained unchanged through the day. APX activity in the samples taken at 24:00 was slightly or significantly lower than in the samples taken at 9:00 and 16:00 (Tab. 6).

TABLE 5. Daily fluctuations of the specific activity of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), ascorbate peroxidase (APX, μmol ascorbate mg^{-1} protein min^{-1}) and catalase (CAT, μmol H_2O_2 mg^{-1} protein min^{-1}) in *R. elymaitica* grown for 20 days under drought treatment. Data for each parameter within treatment followed by the same letter do not differ significantly (P<0.05)

	Time of day	Control	Drought
SOD	9:00	23±4 bc	31±4.9 ab
	16:00	23±4 bc	33±3.5 a
	24:00	21±4 c	27±4.9 abc
APX	9:00	0.83±0.01 ab	0.64±0.03 c
	16:00	0.87±0.09 a	0.67±0.09 bc
	24:00	0.79±0.04 ab	0.62±0.09 c
CAT	9:00	21±3 a	16±1.7 ab
	16:00	19±4 ab	14±3.0 b
	24:00	19±3 ab	15±1.6 ab

MDA content remained unchanged up to day 16 of drought stress, but was slightly higher than in the corresponding control plants in samples taken at day 20. Content of H_2O_2 in these samples was lower than in well watered plants, however (Fig. 4). In contrast to drought stress, UV treatment did not influence MDA of H_2O_2 content even after 20 days of exposure to radiation (Fig. 5).

DISCUSSION

In contrast to the known behavior of leaf morphological parameters, such as succulence and increases in palisade and spongy parenchyma in drought-resistant species (Ennajeh et al., 2010), severe water stress rather negatively affected leaf thickness in *Rosularia elymaitica*. Its lack of increasing leaf succulence in response to drought is similar to that observed in the succulent *Portulaca oleracea* (Lara et al., 2003).

Exposing *R. elymaitica* plants to severe water stress, defined as RWC lower than 60% (Castillo, 1996), did not reduce their dry matter production. This indicates high tolerance to drought stress.

The remarkable reduction in leaf RWC and ψ_w in drought-stressed plants was associated with significant reduction of net CO_2 assimilation. Reduced stomatal conductance (gs) due to decreased leaf water potential, which inhibits CO_2 supply and consequently reduces CO_2 assimilation (*A*), is a well known phenomenon in drought-stressed plants (Ben Ahmed et al., 2009). In our work, however, a slight reduction of g_s (33%) was associated with significant impairment of *A* (up to 62%) in drought-stressed plants. This probably indicates a reduction of Rubisco activity and supply of reducing equivalents (e.g., NADPH) through photosynthetic electron transport (Lawlor and Cornic, 2002).

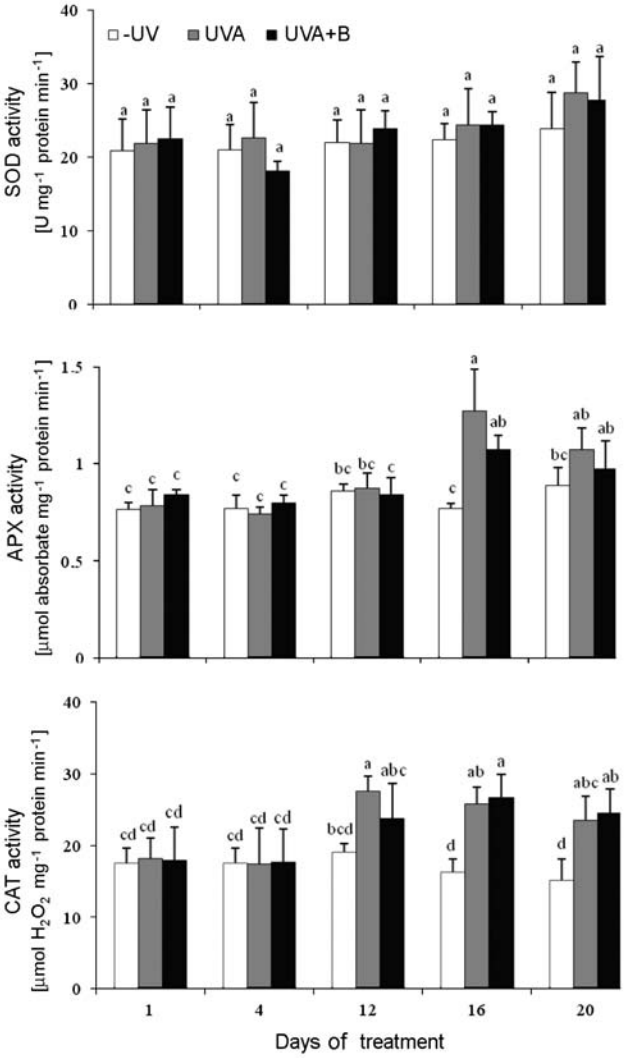

Fig. 3. Specific activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) at different intervals of UV treatment in *R. elymaitica*. Bars with the same letter do not differ significantly (P<0.05).

Facultative CAM plants predominantly exhibit C_3 photosynthesis when well watered but develop CAM when water-stressed, thereby reducing water loss through transpiration (Lüttge, 2004). After a period of at least 12 days without watering, *R. elymaitica* plants showed significant daily Δtitratable acidity and ΔPEPC activity, both characteristics typical of CAM (Herrera et al., 2010). Continuation of drought conditions further increased Δtitratable acidity, so that by day 20 of drought stress it was 5–6 times higher than in the respective control plants; however, it was considerably lower than that reported for C_3-CAM intermediate plants such as

TABLE 6. Daily fluctuations of the specific activity of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), ascorbate peroxidase (APX, µmol ascorbate mg^{-1} protein min^{-1}) and catalase (CAT, µmol H_2O_2 mg^{-1} protein min^{-1}) in *R. elymaitica* grown for 20 days under UV treatment. Data for each parameter within treatment followed by the same letter do not differ significantly (P<0.05)

	Time of day	UV treatment		
		–UV	UVA	UVA+B
SOD	9:00	22±5 a	29±4.18 a	28±6 a
	16:00	23±4 a	25±3.19 a	26±4 a
	24:00	19±2 a	25±3.76 a	24±4 a
APX	9:00	0.82±0.01 cd	0.96±0.08 bc	1.26±0.19 a
	16:00	0.89±0.09 cd	1.10±0.11 b	0.97±0.14 bc
	24:00	0.75±0.03 d	0.86±0.04 cd	0.88±0.03 cd
CAT	9:00	19±2 ab	25±5 a	22±3 ab
	16:00	16±2 b	24±3 ab	25±3 a
	24:00	19±2 ab	21±3 ab	22±4 ab

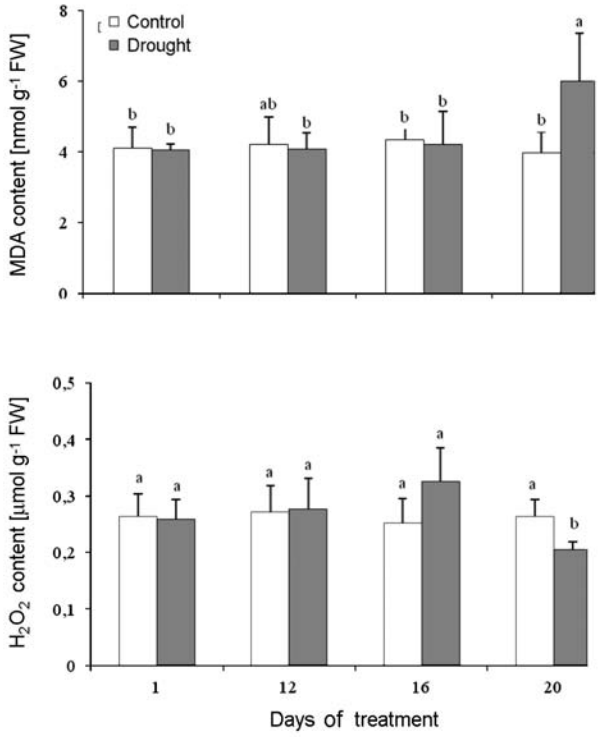

Fig. 4. Leaf content of malondoialdehyde (MDA) and hydrogen peroxide (H_2O_2) in *R. elymaitica* at different intervals of drought treatment in *R. elymaitica*. Bars with the same letter do not differ significantly (P<0.05).

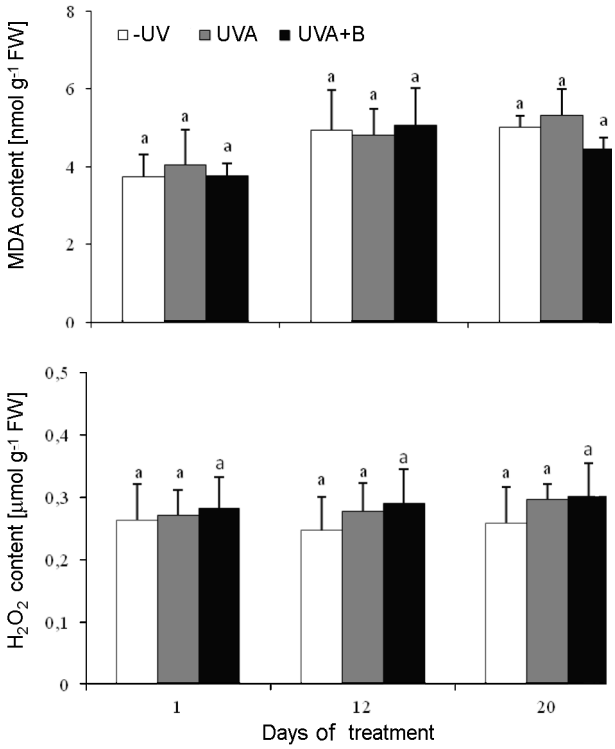

Fig. 5. Leaf content of malondoialdehyde (MDA) and hydrogen peroxide (H_2O_2) of *R. elymaitica* at different intervals of UV radiation at 30 kJ m^{-2} d^{-1}. Bars with the same letter do not differ significantly (P<0.05).

Sedum album (Castillo, 1996). This probably indicates performance of weak CAM-cycling metabolism. Although there was a significant increase in the nighttime net CO_2 assimilation rate, it was still negative after 20 days of drought stress. This response is similar to that reported in CAM-cycling plants such as *Clusia aripoensis* (Ting and Sipes, 1985; Borland et al., 1998) and *Sedum integrifolium* (Gravatt and Martin, 1992), where the amounts of Δtitratable acidity under drought stress were similar to what we noted.

CAM inducibility under increased irradiance or UVA/blue light radiation has been reported in some species such as *Clusia minor* (Grams and Thiel,

2002). In our work, UV treatment elevated Δtitratable acidity only slightly and did not affect other parameters such as net CO_2 exchange, nighttime PEPC and NAD-ME activity. The UV radiation dose we applied was in the range used by authors who reported effective induction of CAM in UV treatment, but photosynthetic photon flux density was much lower than in their work (Grams and Thiel, 2002). The difference in applied light intensity may explain the divergence between our results and theirs, so we cannot rule out inducibility of CAM by UV radiation stress in *R. elymaitic*, but we point out that *R. elymaitic* does not experience higher light intensities in its natural habitats because it grows in the shade of boulders and shrubs and in other semi-shaded to shaded sites. In *Clusia minor* it has been reported that exposure to high-intensity light rather suppresses CAM activity under well watered conditions (Kornas et al., 2010).

The significant increase in daytime PEPC activity observed after 20 days of UVA radiation treatment may be explained as a common response to UVA radiation without any relationship to changes in photosynthetic carbon metabolism. Although PEPC plays a vital role in primary carbon fixation in leaves of C_4 and CAM plants, it is also highly regulated by several other external factors such as light or inorganic nutrients (Murmu et al., 2003).

UV radiation stress did not affect the plants' dry matter production, a response characteristic for plants growing at high elevations (Filella and Penuelas, 1999). Plants are known to react to UV radiation by increasing their free radical scavenging capacity and/or synthesis and accumulation of leaf pigments (Jacobs et al., 2007). We did not analyze leaf carotenoids and flavonoids, but evidence from enzyme activity suggests that the antioxidant defense system effectively protected the plants against UV radiation. The amounts of MDA and H_2O_2 remained unchanged under UV stress, presumably as a result of efficient scavenging following significant enhancement of CAT and APX activity.

The responses of antioxidant enzymes to drought depend on the plant's species and developmental stage, and on the intensity and duration of the imposed stress (Apel and Hirt, 2004). In our work the activity of antioxidant enzymes increased after 12 days of water stress, that is, mild stress, but decreased again during the following days under stress. This seems to indicate that the antioxidant defense system protected the plants under mild stress conditions but that under severe stress the imbalance between ROS production and scavenging produced oxidative stress, as judged by accumulation of MDA. We did not study the isozyme profiles of the antioxidant enzymes, so the contribution of each individual isozyme to the defense mechanism cannot be pinpointed. Different diurnal patterns of change in the activity of various SOD isozymes have been observed in some C_3-CAM species (Kornas et al., 2007), suggesting that the role of individual isozymes varies depending on the time of day.

Temporal changes in Δtitratable acidity and ΔPEPC activity coincided with those of the antioxidant enzymes, and both began to increase from day 12 onwards. This suggests a role for antioxidant enzymes in the signaling pathway that shifts C_3 to CAM-cycling. On the other hand, ROS such as H_2O_2, with lower reactivity and a greater ability to diffuse from the production site to initiate intracellular and systemic signaling as compared with other ROS (Mullineaux et al., 2006), may play a role in stress signaling (Neill et al., 2002). Also, H_2O_2 provides essential information on the cell redox state, and regulates gene expression associated with biotic and abiotic stress responses to optimize defense and survival (Hong-Bo Shao et al., 2008). Oscillations of H_2O_2 content as a consequence of its production or scavenging by enzymes may determine the final response of plants to this signal. As in our work, a strong diurnal rhythm in the activity of some antioxidative enzymes has been observed in C_3-CAM intermediate plants (Ślesak et al., 2002; Niewiadomska, 2004; Miszalski et al., 2007; Kornas et al., 2007; Kuźniak et al., 2011).

In this study we showed induction of CAM cycling by drought and effective protection against UV radiation by antioxidant enzymes in *Rosularia elymaitica*. These metabolic responses are clearly adaptive, suiting the plant to the environment in which it grows: in thin soils of rock crevices in high-altitude habitats characterized by extended dry periods and elevated UV radiation.

REFERENCES

Akhiani K. 2000. Crassulaceae. In: Assadi M, Khatamsaz M, Maassoumi AA, Babakhanlou P, Zehzad B [eds.], *Flora of Iran*, vol. 32, 19–57. Research Institute of Forests and Rangelands, Iran.

Apel K, and Hirt H. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55: 373–399.

Ben Ahmed C, Ben Rouinab B, Sensoyc S, Boukhrisa M, and Ben Abdallah F. 2009. Changes in gas exchange, proline accumulation and antioxidative enzyme activities in three olive cultivars under contrasting water availability regimes. *Environmental and Experimental Botany* 67: 345–352.

Borland AM, Tecsi LI, Leegood RC, and Walker RP. 1998. Inducibility of Crassulacean acid metabolism (CAM) in *Clusia* species: Physiological/biochemical characterization and intercellular localization of carboxylation and decarboxylation processes in three species which exhibit different degrees of CAM. *Planta* 205: 342–351.

Caldwell MM. 1971. Solar UV irradiation and the growth and development of higher plants. In: Giese AC [ed.],

Photophysiology, 131–137. Academic Press, New York, USA.

Castillo FJ. 1996. Antioxidative protection in the inducible CAM plant *Sedum album* L. following the imposition of severe water stress and recovery. *Oecologia* 107: 469–477.

Cushman JC, and Borland AM. 2002. Induction of crassulacean acid metabolism by water limitation. *Plant, Cell and Environment* 25: 295–310.

Ennajeh M, Vadel AM, Cochard H, and Khemira H. 2010. Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar. *Journal of Horticultural Science and Biotechnology* 85: 289–294.

Filella I, and Penuelas J. 1999. Altitudinal differences in UV absorbance, UV reflectance and related morphological traits of *Quercus ilex* and *Rhododendron ferrugineum* in the Mediterranean region. *Plant Ecology* 145: 157–165.

Grams TEE, and Thiel S. 2002. High light-induced switch from C_3-photosynthesis to crassulacean acid metabolism is mediated by UV-A/blue light. *Journal of Experimental Botany* 53: 1475–1483.

Grams TEE, Herzog B, and Lüttge U. 1998. Are there species in the genus *Clusia* with obligate C_3-Photosynthesis? *Journal of Plant Physiology* 152: 1–9.

Gravatt DA, and Martin CE. 1992. Comparative ecophysiology of five species of *Sedum* (Crassulaceae) under well-watered and drought-stressed conditions. *Oecologica* 92: 532–541.

Griffiths H, Ong BL, Avadhani PN, and Goh CJ. 1989. Recycling of respiratory CO_2 during Crassulacean acid Metabolism: alleviation of photoinhibition in *Pyrrosia piloselloides*. *Planta* 179: 115–122.

Groenhof AC, Bryant JA, and Etherington JR. 1988. Photosynthetic changes in the inducible CAM plant *Sedum telephium* L. following the imposition of water stress. II. Changes in the activity of phosphoenolpyruvate carboxylase. *Annals of Botany* 62: 187–192.

Habibi G, and Hajiboland R. 2010. Photosynthetic characteristics and antioxidative responses in three species of Crassulaceae following drought stress. *Journal of Sciences*, I.R. Iran 21: 205–212.

Hajiboland R, and Hasani BD. 2007. Responses of antioxidant defense capacity and photosynthesis of bean (*Phaseolus vulgaris* L.) plants to copper and manganese toxicity under different light intensities. *Acta Biologica Szegediensis* 51: 93–106.

Herrera A, Martin CE, Tezara W, Ballestrini C, and Medina E. 2010. Induction by drought of crassulacean acid metabolism in the terrestrial bromeliad, *Puya floccose*. *Photosynthetica* 48(3): 383–388.

Holtum JAM, and Winter K. 1982. Activity of enzymes of carbon metabolism during the induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum* L. *Planta* 155: 8–16.

Jacobs JF, Koper GJM, Ursemb WNJ. 2007. UV protective coatings: A botanical approach. *Progress in Organic Coatings* 58: 166–171.

Johnson CM, Stout PR, Broyer TC, and Carlton AB. 1957. Comparative chlorine requirements of different plant species. *Plant and Soil* 8: 337–353.

Kornaś A, Ślesak I, Gawronska K, Fischer-Schliebs E, Miszalski Z. 2007. Daily rhythm of MnSOD in the C_3-CAM intermediate *Clusia fluminensis* Planch. et Triana. *Acta Physiologiae Plantarum* 29: 369–374.

Kornaś A, Miszalski Z, Surówka E, Fischer-Schliebs E, and Lüttge U. 2010. Light stress is not effective to enhanced crassulacean acid metabolism. *Zeitschrift für Naturforschung C, Journal of Biosciences* 65: 79–86.

Kuzniak E, Gabara B, Skłodowska M, Libik-Konieczny M, and Miszalski Z. 2011. Effects of NaCl on the response of *Mesembryanthemum crystallinum* callus to *Botrytis cinerea* infection. *Biologia Plantarum* 55: 423–430.

Lara MV, Disante KB, Podesta FE, Andreo C, and Drincovich MF. 2003. Induction of a Crassulacean acid-like metabolism in the C_4 succulent plant, *Portulaca oleracea* L.: physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. *Photosynthesis Research* 77: 241–254.

Lawlor DW, and Cornic G. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell and Environment* 25: 275–294.

Lüttge U. 2004. Ecophysiology of crassulacean acid metabolism (CAM). *Annals of Botany* 93: 629–652.

Miszalski Z, Kornaś A, Gawronska K, Ślesak I, Niewiadomska E, Kruk J, Christian AL, Fischer-Schliebs E, Krisch R, and Lüttge U. 2007. Superoxide dismutase activity in C_3 and C_3/CAM intermediate species of *Clusia*. *Biologia Plantarum* 51: 86–92.

Mullineaux, PM, Karpinski S, and Baker NR. 2006. Spatial dependence for hydrogen peroxide-directed signaling in light-stressed plants. *Plant Physiology* 141: 346–350.

Murmu J, Chinthapalli B, and Raghavendra AS. 2003. Phosphoenolpyruvate carboxylase from leaves of C_4 plants. Biochemistry and molecular biology of regulation. *Indian Journal of Plant Physiology* 1: 164–73.

Neill SJ, Desikan R, Clarke A, Hurst RD, and Hancock JT. 2002. Hydrogen peroxide and nitric oxide as signaling molecules in plants. *Journal of Experimental Botany* 53: 1237–1247.

Niewiadomska E, Karpinska B, Romanowska E, Ślesak I, and Karpinski S. 2004. A salinity-induced C_3-CAM transition increases energy conservation in the halophyte *Mesembryanthemum crystallinum* L. *Plant and Cell Physiology* 45: 789–794.

Rybus-Zając M, and Kubiś J. 2010. Effect of UV-B radiation on antioxidative enzyme activity in cucumber cotyledons. *Acta Biologica Cracoviensia Series Botanica* 52: 97–102.

Sayed OH. 2001. Crassulacean acid metabolism 1975–2000, a check list. *Photosynthetica* 39: 339–352.

Skórska E. 2011. Comparison of chlorophyll fluorescence parameters of *Cucumis sativus* and *Mentha piperita* leaves exposed to short-term UV-B irradiation. *Acta Biologica Cracoviensia Series Botanica* 53: 16–19.

Ślesak I, Miszalski Z, Karpinska B, Niewiadomska E, Ratajczak R, and Karpinski S. 2002. Redox control of oxidative stress responses in the C_3-CAM intermediate plant *Mesembryanthemum crystallinum*. *Plant Physiology and Biochemistry* 40: 669–677.

Shao H-B, Chu L-Y, Lu Z-H, and Kang C-M. 2008. Primary antioxidant free radical scavenging and redox signaling

pathways in higher plant cells. *International Journal of Biological Sciences* 4: 8–14.

SMITH JAC, and WINTER K. 1996. Taxonomic distribution of crassulacean acid metabolism. In: Winter K and Smith JAG [eds.], *Crassulacean Acid Metabolism: Biochemistry, Ecophysiology and Evolution*, vol. 114, 427–436. Springer-Verlag, Berlin.

TING IP, and SIPES D. 1985. Metabolic modifications of Crassulacean acid metabolism in CAM-idling and CAM-cycling. In: Luden PW and Burris JE [eds.], *Night Fixation and CO_2 Metabolism*, 371–378. Elsevier, Amsterdam.

WANG J, TAN H, and ZHAO Z. 2007. Over-expression, purification, and characterization of recombinant NAD-malic enzyme from *Escherichia coli* K12. *Protein Expression and Purification* 53: 97–103.

WINTER K, and SMITH JAC. 1996. An introduction to crassulacean acid metabolism. Biochemical principles and ecological diversity. In Winter K and Smith JAC [eds.], *Crassulacean Acid Metabolism*, 1–13. Springer-Verlag, Berlin, Germany.