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Deciphering the mechanisms involved in *Portulaca oleracea* (C_4) response to drought: metabolic changes including crassulacean acid-like metabolism induction and reversal upon re-watering

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Portulaca oleracea is a C_4 plant; however, under drought it can change its carbon fixation metabolism into a crassulacean acid metabolism (CAM)-like one. While the C_3-CAM shift is well known, the C_4-CAM transition has only been described in *Portulaca*. Here, a CAM-like metabolism was induced in *P. oleracea* by drought and then reversed by re-watering. Physiological and biochemical approaches were undertaken to evaluate the drought and recovery responses. In CAM-like plants, chlorophyll fluorescence parameters were transitory affected and non-radiative energy dissipation mechanisms were induced. Induction of flavonoids, betalains and antioxidant machinery may be involved in photosynthetic machinery protection. Metabolic analysis highlights a clear metabolic shift, when a CAM-like metabolism is induced and then reversed. Increases in nitrogenous compounds like free amino acids and urea, and of pinitol could contribute to withstand drought. Reciprocal variations in arginase and urease in drought-stressed and in re-watered plants suggest urea synthesis is strictly regulated. Recovery of C_4 metabolism was accounted by CO_2 assimilation pattern and malate levels. Increases in glycerol and in polyamines would be of importance of re-watered plants. Collectively, in *P. oleracea* multiple strategies, from induction of several metabolites to the transitory development of a CAM-like metabolism, participate to enhance its adaptation to drought.

Introduction

Water deficit response and survival rely on a series of mechanisms that can be grouped in avoidance or tolerance responses. Plants respond to drought at physiological and molecular levels which leads to modifications in plant metabolome and transcriptome (Cushman and Bohnert 2000, Umezawa et al. 2006, Sreenivasulu et al. 2007, Claeys and Inzé 2013). On the one hand, model plants and crop species have been extensively used to decipher the response to drought (Seki et al. 2002, Guo et al. 2009, Campo et al.

Abbreviations – APX, ascorbate peroxidase; CAM, crassulacean acid metabolism; CAT, catalase; GC-MS, gas chromatography–mass spectrometry; GDH, glutamate dehydrogenase; GS, glutamine synthetase; PCA, principal component analysis; PEPC, phosphoenolpyruvate carboxylase; POD, peroxidase; qNP, non-photochemical quenching; qP, photochemical quenching; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase.

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2012). On the other hand, within the wide range of species adapted to diverse environments there might be different responses not only regarding morphology but also physiology. Given that in the world cultivable land is decreasing and that global climate changes are increasing the different stress that plants are exposed to, it results imperative to deeply explore the mechanism involved in the drought response and to search for new genetic resources (Texeira et al. 2010).

Portulaca oleracea, commonly known as Purslane or Verdolaga, grows well in a wide range of environments, from well-watered conditions of gardens and farms to very dry soils of roadsides and out-rocks. Because of its ability to withstand high temperatures combined with drought, high salt concentrations and even low nutrients concentrations, it is considered a very common world-wide distributed weed (Zimmerman 1976). On the other hand, *P. oleracea* has been widely used not only as an edible plant for humans and livestock but also as a traditional herbal medicine. It is a source of ω-3 fatty acids, antioxidant and other compounds of nutritional value (Simopoulos et al. 1992). Known as "vegetable for long life" it has been used as diuretic, antipyretic, antiseptic, spasm suppressor and anthelmintic (Xiang et al. 2005).

According to its photosynthetic metabolism, *P. oleracea* is a succulent plant classified as C_4 plant. The following reasons lead to investigate the possible occurrence of crassulacean acid metabolism (CAM) in this species (Koch and Kennedy 1980): (1) great geographical and environmental distribution; (2) presence of succulent leaves; (3) high water use efficiency and (4) occurrence within Portulacaceae which possess species displaying CAM, C_3 or C_4 photosynthesis (Lara and Andreo 2005). Under different stress conditions *P. oleracea* exhibited diurnal acid fluctuation, accounted by malic acid, accompanied by low nocturnal leaf resistance and net dark CO_2 uptake and day time CO_2 release, typical of CAM plants (Koch and Kennedy 1980, Kraybill and Martin 1996, Lara and Andreo 2005). After 23 days of drought the induction of a CAM-like metabolism was also observed. Photosynthesis was diminished as accounted by the decrease in the net CO_2 uptake and in the enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxykinase and NAD-malic enzyme that are involved in carbon fixation. In agreement with a nocturnal CO_2 uptake, modification in the activity during the light/dark periods of some of these enzymes was also described (Lara et al. 2004). Particularly, PEPC exhibited changes in its kinetic and regulatory properties; in well irrigated plants it exhibited characteristics of a C_4-type PEPC but in drought stressed plants the enzyme showed regulatory properties typical of a CAM-type PEPC (Lara et al. 2003).

CAM plants are adapted to withstand extreme drought conditions and are able to quickly recuperate upon re-watering (Ceusters et al. 2009). In contrast to the large number of studies on C_3-CAM transition, only few studies have explored the induction of CAM in C_4 species in Portulacaceae (Guralnick et al. 2002, Lara et al. 2003). Moreover, there is no published work exploring the recovery of these plants after re-watering. Given the plasticity of *P. oleracea* regarding its environments and its high drought and temperature tolerance (Yang et al. 2012), we hypothesized that besides CAM-like induction there might be other mechanisms that contribute to cope with severe drought in this species. In order to decipher the metabolic changes in *P. oleracea* during water stress and recovery, an integrated perspective using photosynthesis, pigment, polar compounds metabolic profiling and, central and antioxidant metabolism enzyme analysis was conducted. For this purpose, plants of *P. oleracea* were subjected to drought to induce a shift from C_4- to CAM-like metabolism (Lara et al. 2003) and after that plants were re-watered for another 21–23 days. Data presented demonstrate that *P. oleracea* deploys multiple strategies to cope with drought and that the CAM-like metabolism induction is a metabolic strategy of this C_4 species to maintain photosynthesis under conditions of drought.

Materials and methods

Plant material, growth conditions and sampling

Portulaca oleracea L. seedlings were developed from seeds in a compost : sand : perlite mixture (2:1:1 by volume) and were grown in a greenhouse with a 33/18°C day/night thermoperiod, a 12 h photoperiod (07:00 to 19:00 h) and a light fluency of 300 μmol m^{-2} s^{-1}. Six weeks old plants watered daily constituted the control group (C). For the stressed (S) group of plants water was withheld during 21–23 days. After that, plants were re-watered during another 21–23 days (re-watered group, R). At least 10 plants were used in each set of experiments. Samples were taken after 5 h into the light period at 12:00 h (L) or after 5 h into the night at 00:00 h (D), unless otherwise stated. For malate measurement, samples were collected at the end of the day or night periods (18:00 and 06:00 h, respectively). After collection, samples were immediately used or frozen in N_2(l) and stored at −80°C for further analysis.

Relative water content

The relative water content percent (%RWC) was determined as in Lara et al. (2003).

Gas exchange and chlorophyll fluorescence analyses

CO_2 exchange (μmol CO_2 h^{-1} g^{-1} FW) was estimated in detached leaves using an infrared gas analyzer (Qubit Systems Inc., Kingston, ON, Canada; Lara et al. 2003) and a LED source (PPD 333 μmol m^{-2} s^{-1}). Day and night temperatures were 30 and 18°C, respectively.

For chlorophyll fluorescence measurements a Chlorophyll Fluorescence Package containing a Fluorometer F1, from Qubit Systems Inc. was used as in Müller et al. (2010).

Pigment extraction and analysis

Flavonoids, chlorophyll, carotenoids, betaxanthins and betacyanins were measured as previously described (Casati et al. 2002, Gandía-Herrero et al. 2005, Müller et al. 2010).

Cell death

Cell integrity was assessed by Evans blue staining (Müller et al. 2010).

Ninhydrin soluble amino acid and ammonium quantification

Free amino acids were extracted from 100 mg of leaves (Funck et al. 2008). Free amino acids concentrations obtained in the toluenic fraction were calculated measuring absorbance at 520 nm and using a leucine standard curve from 0 to 10 m*M* treated in the same way as the samples. For each data point three independent replicates were analyzed in triplicate.

Ammonium was detected colorimetrically by using the phenol-hypochlorite method described below.

Quantification of carbohydrates and malate

Frozen leaves were ground (100 mg) and alcoholic soluble sugars were obtained by two consecutive extractions with 0.75 ml of 80% (v/v) ethanol at 70°C for 15 min, followed by concentration to dryness. Pellets were resuspended in 0.5 ml of water and used for reducing sugars and malate quantification. Reducing sugar determination was conducted by using the Somogyi–Nelson method.

Malate was measured spectrophotometrically according to Möellering (1974) in a media containing 100 m*M* glycine pH 10.0; 2 m*M* NAD; 50 m*M* glutamate pH 10.0 and 2 U glutamate-oxaloacetate transaminase (GOT). After measuring initial absorbance at 340 nm during 10 min 5.4 U of NAD-dependent malate dehydrogenase (NAD-MDH) were added. Final absorbance was recorded after the plateau of the reaction was achieved.

Starch was measured in the pellet obtained from alcoholic extraction as exactly described in Fahnenstich et al. (2007).

Metabolic profile by gas chromatography–mass spectrometry (GC-MS) analysis

Metabolite extraction, methoxyamination and silylation procedures were conducted as in Bohmert et al. (2000). An Autosystem XL Gas Chromatograph and a Turbo Mass Spectrometer (Perkin Elmer, Waltham, MA) from Facultad de Ciencias Bioquímicas y Farmacéuticas – UNR facilities was operated under electronic pressure control and equipped with a split/splitless capillary inlet. A 1 μl split injection (split ratio 1:40) was carried out. The injection temperature was set to 280°C. A VF-5ms column (Varian, Darmstadt, Germany) was used (30 m × 0.25 mm inner diameter and a 0.25 μm film). Helium was used as carrier gas with constant flow at 1 ml min^{-1}. The temperature program was 5 min at 70°C followed by a 5 min ramp to 310°C and final heating for 2 min at 310°C. The transfer line to the mass spectrometer was set to 280°C. Mass spectra were monitored in the mass range m/z = 70–600. Tuning and all other settings of the mass spectrometer were according to manufacturer's recommendations. Chromatograms were acquired with TURBOMASS 4.1 software (Perkin Elmer). Data were processed as described by Pedreschi et al. (2009).

Five independent determinations, composed by leaves of different plants in the same watering conditions, were performed to each sample analyzed. Data presented are normalized to the control leaves collected 5 h after the beginning of the light period (CL12) and expressed as log2 ratios using the MULTIEXPERIMENT VIEWER software (MEV v4.4.1, http://www.tm4.org/). Principal component analysis (PCA) was conducted on normalized data sets and Pearson correlation was applied with the aid of XLSTAT software package (Microsoft Excel).

Protein extraction

Total protein (200 mg) was extracted using 0.3 ml of buffer containing 50 m*M* KH_2PO_4, pH 7.5; 1 m*M* EDTA; 50 m*M* NaCl; 20 m*M* DTT, 1 m*M* phenylmethylsulfonylfluoride (PMSF) and 33 μg ml^{-1} protease inhibitor cocktail (Sigma, St. Louis, MO). The samples were ground completely in a cold mortar in the presence of insoluble polyvinyl polypyrrolidone (Sigma) and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was desalted in a cold Sephadex G-50 column pre-equilibrated with 25 m*M* KH_2PO_4, pH 7.5; 0.5 m*M* EDTA; 25 m*M* NaCl and used for urease and arginase determination.

For glutamate dehydrogenase (GDH), glutamine synthetase (GS) and antioxidant activities measurements, crude extracts were prepared in a buffer containing 50 m*M* KH_2PO_4, pH 7.0; 1 m*M* EDTA; 1 m*M* ascorbate; 20% (v/v) glycerol; 0.5% (w/v) Triton X-100; 10 m*M* β-mercaptoethanol; 5 m*M* $MgCl_2$; 1 m*M* PMSF and 33 μg ml^{-1} protease inhibitor cocktail (Sigma). After clarification extracts were used for protein quantitation or diluted in 0.25 *M* Tris–HCl, pH 6.8; 0.05% (v/v) bromophenol blue and 50% (v/v) glycerol for native activity gels.

Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and bovine serum albumin as standard.

Enzyme assays

Peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1) and ascorbate peroxidase (APX, EC 1.11.1.11) in gel antioxidant enzyme activity assays were carried out as in Casati et al. (2002). Bands were semi-quantified as described in Müller et al. (2013).

Urease (EC 3.5.1.5) activity was measured according to Witte and Medina-Escobar (2001) with modifications by measuring the production of NH_4^+ in a colorimetric method. Urease was activated by pre-incubation of crude extract at 50°C for 3 min. Reaction was started with urea at a final concentration of 66 m*M* and run at 50°C for 30 min. Before and after reaction, samples of 20 μl were taken and added to 200 μl of phenol reagent, followed by 200 μl of hypochlorite reagent and 480 μl of water for NH_4^+ quantification. Color reaction was conducted at 50°C for 20 min by triplicate. For the calibration curve, NH_4 was handled as above with preparations also including the crude extract.

Arginase (EC 3.5.3.1) activity was assayed as described by King and Gifford (1997) following urea generation. Arginase was activated by pre-incubating extracts for 30 min at 37°C in the presence of 1 m*M* $MnCl_2$. Five hundred microliters of standard assay medium contained 50 m*M* Tris–HCl, pH 9.6; 2 m*M* $MnCl_2$; 250 m*M* L-Arg (adjusted to pH 9.7 with KOH) and 30 μl of extract protein. Reaction was conducted for 30 min at 37°C and stopped with 4.5 μl H_2SO_4 98.8% (v/v). After neutralization, urea released was determined spectrophotometrically with the Urea UV kit (Wiener Lab, Rosario, Argentina) in a coupled assayed with urease and GDH. Endogenous urea levels, if any, determined at time zero, were subtracted from total urea levels to obtain a measure of urea evolved by arginase. One unit of activity is defined as the generation of 1 μmol of urea per min per mg of protein (IU mg^{-1}). Three independent replicates were analyzed in triplicate for each data point. To determine if any urease-mediated urea breakdown was occurring in the assay mixture, urease activity was tested under the same assay conditions and found negligible.

GDH (EC 1.4.1.2) was assayed in 100 m*M* KH_2PO_4, pH 8.0 containing 1 m*M* $CaCl_2$; 10 m*M* α-ketoglutarate; 100 mm NH_4Cl and 0.25 m*M* NADH as described by Mena-Petite et al. (2006). After pre-incubating at 30°C for 5 min, the reaction was started with NADH. NADH oxidation was monitored at 340 nm. Activity was corrected for endogenous NADH oxidation in the absence of NH_4Cl or α-ketoglutarate. One unit of GDH is expressed as the consumption of 1 μmol of NADH per min at 30°C.

GS (EC 6.3.1.2.) activity was measured according to Mena-Petite et al. (2006) with the following modifications: reaction was conducted in a final volume of 500 μl containing 70 μl of crude extract, buffer KH_2PO_4, pH 7.5 was used and activity was ended by the addition of 250 μl of ferric chloride reagent. Controls were conducted in the absence of glutamate or hydroxyalamine. GS activity was expressed as nanomoles per minute per milligram of protein (mIU mg^{-1}).

Statistics

One way analysis of variance (ANOVA), Bonferroni, Holm-Sidak, Dunett and Duncan tests ($\alpha = 0.05$) were employed by using the SIGMA STAT PACKAGE (Systat Software Inc., San Jose, CA).

Results

Induction of a CAM-like metabolism in *P. oleracea* subjected to drought. Biochemical and physiological parameters

Well-watered plants were named as controls (C; Fig. 1A) and exhibited a typical C_4 CO_2 exchange pattern (Fig. 1B) and similar leaf malate contents at the end of the day and the night (Fig. 1C). To induce the CAM-like metabolism, plants were stressed (S) by withholding water (Fig. 1A), and samples were taken when gas exchange measurements showed nil CO_2 assimilation in the light (SL) and low CO_2 rates of uptake in the dark (SD) (Fig. 1B), which was typically achieved after 21–23 days (Lara et al. 2003). The establishment of a CAM-like metabolism was also verified by diurnal fluctuation in malic acid (Fig. 1C; Lara et al. 2003). After stress, plants were re-watered (R) and after 21–23 days samples were collected and analyzed (Fig. 1A). Gas exchange analysis revealed a drastic change in the diel pattern with net CO_2 uptake in the light (RL) and a net CO_2 release during the night (RD), similar to control plants (Fig. 1B). The recovery of plants was complete as determined

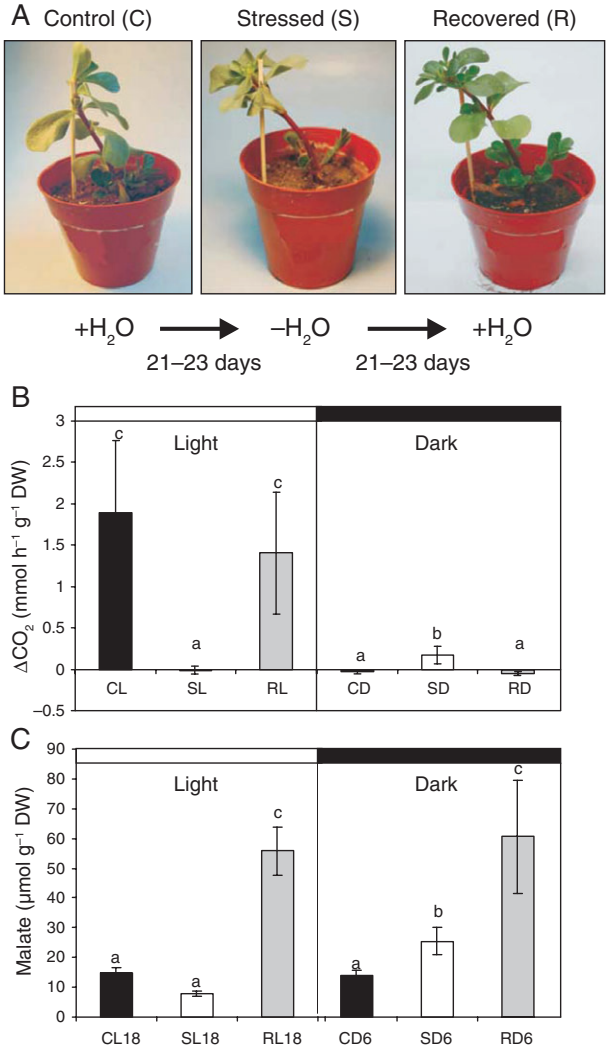

Fig. 1. Induction of CAM-like metabolism in *Portulaca oleracea* plants subjected to drought and recovery after re-watering. (A) Images of the same *P. oleracea* plant. Left: well irrigated 6 weeks old (C). Middle: the same plant after 23 days of withholding water (S). Right: image of the plant after 23 days of re-watering (R). (B) Diel gas exchange analysis. CO_2 gas exchange was evaluated in samples collected after 5 h of the beginning of the light period at 12:00 h (CL, SL and RL for control, stressed and re-watered plants, respectively) and measured under 150 μmol m^{-2} s^{-1} (left panel) and in leaves collected after 5 h into the night period at 00:00 h (CD, SD and RD for control, stressed and re-watered plants, respectively) and evaluated in the darkness (right panel). Values represent the mean ± SD. Positive and negative values represent CO_2 uptake and release, respectively. Values with the same letters are not significant different ($P < 0.05$). C. Accumulation of malate. Malate levels were determined spectrophotometrically at the end of the light period at 18:00 h (CL18, SL18 and RL18, left panel) and at the end of the dark period at 06:00 h (CD6, SD6 and RD6, right panel) in leaves from well-watered (C), drought-stressed (S) and re-watered (R) plants. Values represent the mean ± SD. Values with the same letters are not significant different ($P < 0.05$).

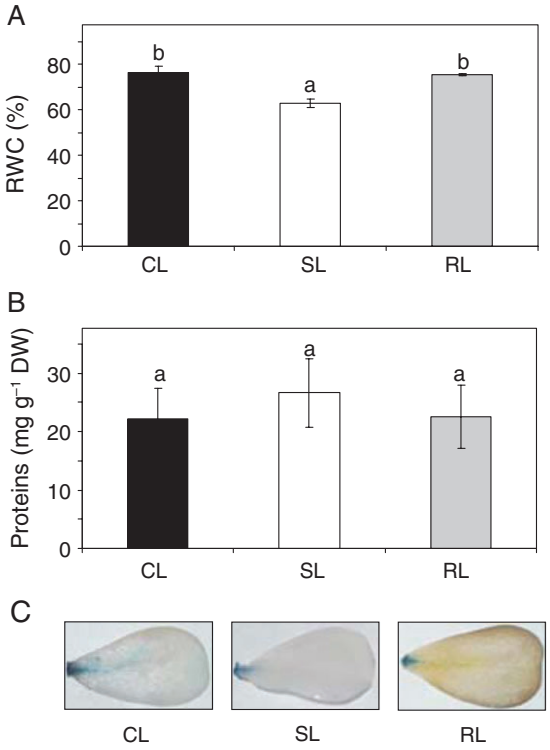

Fig. 2. Relative water content (%) (A) and total soluble proteins (B) of leaves of *Portulaca oleracea* plants. (C) Images of Evans blue stained leaves from well-watered (CL), drought-stressed (SL) and re-watered (RL) plants collected after 5 h into the light period. A typical image representative of all leaves analyzed is shown.

by visual inspection (Fig. 1A), CO_2 assimilation and malate levels (Fig. 1B, C). Besides malate was no longer accumulated during the night (RD), it was greater than in control and stressed plants, irrespectively of the time of analysis (Fig. 1C).

The RWC of *P. oleracea* control leaves decreased an 18% in stressed plants indicating that withholding water effectively affected the plant physiology. After 21–23 days of re-watering the RWC was completely restored. Because leaf fresh weight varied due to changes in leaf water content (Fig. 2A) results obtained are expressed on a dry weight basis (g DW).

The total protein amount remained unchanged in drought-stressed leaves (Fig. 2B). Evans blue staining, indicator of cell death, revealed no differences among samples (Fig. 2C).

Chlorophyll fluorescence parameters and pigment composition

The functionality of the photosynthetic apparatus was evaluated. Maximum quantum yield of PSII (Fv/Fm) was not affected after withholding water. In contrast,

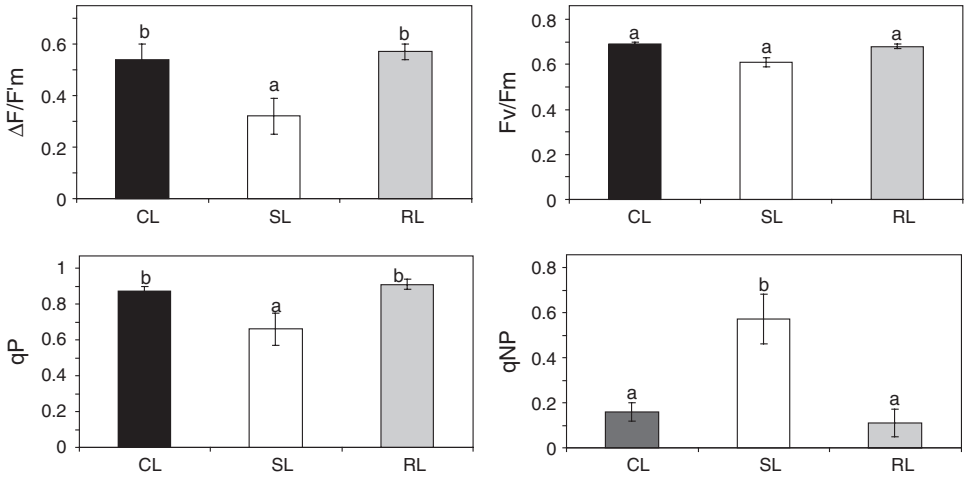

Fig. 3. Chlorophyll fluorescence analysis. ΔF/Fm′, PSII effective quantum yield; Fv/Fm, PSII maximum quantum yield; qP, photochemical quenching coefficient and qNP, non-photochemical quenching coefficient. Mean values ± SD of three individual determinations (n = 6) using different plants are shown. For each parameter, values with the same letters are not significantly different ($P < 0.05$). Samples analyzed are described in Fig. 1B.

the effective quantum yield of PSII (ΔF/Fm′) and the excitation capture efficiency of open centers (Fv′/Fm′, not shown) were decreased in water stressed plants (SL; Fig. 3). The photochemical quenching coefficient (qP) which provides a measure for the oxidative state of the primary acceptor of PSII, was reduced by 24% in leaves performing CAM-like metabolism (SL) with respect to controls (CL). In contrast, an increase of 3.5-fold in the non-photochemical quenching (qNP) was observed in drought-stressed plants (Fig. 3). After re-watering and a recovery period, all the parameters returned back to the levels of C_4-control plants (Fig. 3).

Chlorophylls showed a differential response to drought (Table 1). While chlorophyll *a* decreased a 26% in stressed leaves (S) and remained low in re-watered plants (R); chlorophyll *b* content was not affected by drought (S) but significantly declined (76%) after recovery (R).

Carotenoids decreased a 21% in stressed leaves with respect to controls and were partially restored upon re-watering (R). In contrast, flavonoids and two betalains, betacyanins and betaxanthins, were increased in stressed leaves (2-, 3.5- and 2.3-fold, respectively) with respect to control leaves, and reduced thereafter (Table 1).

Antioxidant metabolism

Because in plants performing CAM-like metabolism stomata are closed during the day and chlorophyll fluorescence parameters are affected, the antioxidant metabolism was monitored.

Drought produced a 2.2-fold increase in APX activity (Fig. 4) by inducing the expression of an isoform which was not present in the controls (see Fig. S1, band indicated by arrowhead), and a 1.8-fold increase in peroxidase (POD) activity, both with respect to C_4-control plants. No differences were observed for the other H_2O_2-scavenging enzyme analyzed, CAT, in leaves performing CAM-like metabolism (Fig. 4). SOD activity was not modified, indicating that the capacity of this enzyme is enough to catalyze the dismutation of superoxide generated under drought stress conditions (Fig. 4).

Upon re-watering, a strong increase (fivefold with respect to control plants) in POD activity was noticed

Table 1. Pigment quantitation of *Portulaca oleracea* leaves under drought stress and recovery. For each pigment, different letters indicate statistically significant differences.

Pigment	Control	Stressed	Re-watered
Chlorophyll *a* (mg g^{-1} DW)	378.02 ± 9.87^a	278.97 ± 64.93^b	224.00 ± 58.20^b
Chlorophyll *b* (mg g^{-1} DW)	84.66 ± 22.18^a	87.66 ± 21.93^a	22.98 ± 14.74^b
Carotenoids (mg g^{-1} DW)	133.60 ± 1.34^a	105.99 ± 13.66^b	101.63 ± 11.48^b
Flavonoids (relative to control)	0.47 ± 0.04^a	1.00 ± 0.04^b	0.35 ± 0.08^a
Betacyanins (μmol g^{-1} DW)	0.33 ± 0.12^a	1.15 ± 0.36^b	0.44 ± 0.18^a
Betaxanthins (μmol g^{-1} DW)	0.24 ± 0.14^a	0.55 ± 0.16^b	0.25 ± 0.10^a

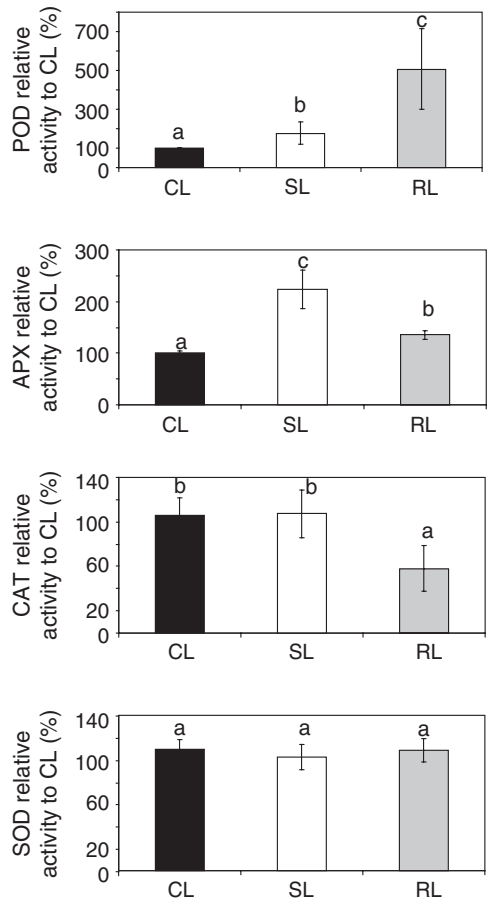

Fig. 4. Antioxidant activity. Densitometric analyses of POD, CAT, SOD and APX activities on native PAGEs. Representative gels are shown in Fig. S1. For each enzyme analyzed, bars with the same letters are not statistically different ($P < 0.05$). Samples analyzed are described in Fig. 1B.

(Fig. 4). Although the band of APX induced by drought was not observed after recovery, the activity of APX was a 30% higher than in C_4 control leaves (Fig. 4). In contrast, the activity of CAT decreased a 55% with respect to control and stressed plants (Fig. 4). Again, no changes in the activity of SOD were observed in recovered plants (Fig. 4).

Metabolic profiling of *P. oleracea* leaves under different watering conditions

Representative leaves samples were collected at different times during the day (12:00 and 17:00 h) and night (00:00 and 05:00 h) from C_4-control (C) plants, from drought-induced CAM-like metabolism leaves (S) and after recovery (R). By GC-MS analysis, 24 metabolites were undoubtedly identified and their levels were expressed relative control leaves at noon (CD12; see Table S1). Free amino acids, ammonia, reducing sugars, starch and enzymes activity were also measured.

Principal component and correlation analyses

Significant changes in the metabolite profile were observed when water was withheld for 21–23 days (see Fig. S2). The data set obtained was examined by PCA (Fig. 5), with three principal components representing 75.5% of the overall variance of the metabolic data (32.2, 23.8 and 19.5% for PC1, PC2 and PC3, respectively). This analysis denotes an important change in the metabolome, when a CAM-like metabolism is induced and then reversed. Samples from day control plants (CL12 and CL17) were grouped together and separately from those collected at night (CD0 and CD5). Regarding CAM-like induced plants, based on PC1 and PC2, samples collected during the night (SD0 and SD5) were placed closer to control plants collected during the day (CL12 and CL17) than those collected during the night (CD0 and CD5) (Fig. 5). Interestingly, re-watered samples were placed together, irrespectively of the sampling period of the day, and separately from the control and drought-stress samples (Fig. 5). Again, PC3 revealed clear differences among control, CAM-like induced and re-watered samples (Fig. 5).

The contribution of metabolites to each principal component is shown in Table S2. Glucose; hexadecenoic acid; *myo*-inositol; sucrose are the variables that contribute the most to PC1. The main contributors to PC2 are glycerol; putrescine; fructose and Ala. Finally, citrate; malate; Glu and xylitol are the main variables contributing to PC3.

A total of 325 pairs of metabolites were analyzed by correlation analysis, from which 48 were statistically significant ($P < 0.05$), of these 42 were positive and 6 were negative (Fig. 6). The pairs with the highest positive correlations coefficients were malate-citrate; Phe-urea; hexadecenoic acid with *myo*-inositol; glucose-sucrose and *myo*-inositol-glucose. Sugars were strongly and positively associated within each other (glucose with sucrose and melibiose; fructose with melibiose; galactose with fructose) and with alcohols (glucose positively correlated with *myo*-inositol; fructose with glycerol and sucrose with *myo*-inositol, xylitol and sorbitol). The evaluation of the behavior of the metabolite network also revealed that polyamines were positively associated with each other (putrescine with cadaverine and spermidine); with phosphate (putrescine and spermidine), with glycerol (cadaverine and putrescine) and with octadecanoic acid (putrescine and spermidine). Ala positively correlated with cadaverine; fructose and glycerol. In contrast, galactose correlated negatively with Phe, pinitol and urea;

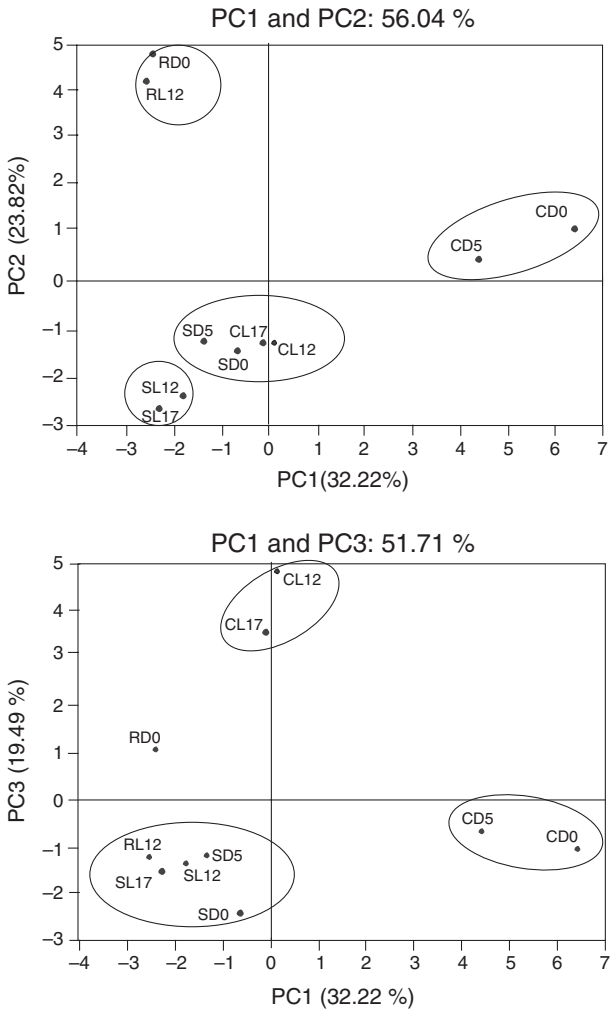

Fig. 5. PCA of 24 identified metabolites by GC-MS data. *Portulaca oleracea* leaves samples collected from C_4 control plants (C), withholding water for 21–23 days (S) and re-watered for 21–23 days (R) were analyzed during the light (CL, SL and RL) and dark (CD, SD and RD) periods. Leaves were collected at 00:00 h (CD0, SD0 and RD0) and 05:00 h (CD5, SD5 and RD5) during the night, and at 12:00 h (CL12, SL12 and RL12) and 17:00 h (CL17, SL17 and RL17) during the day. The variance associated to each component (%) is shown in each axis.

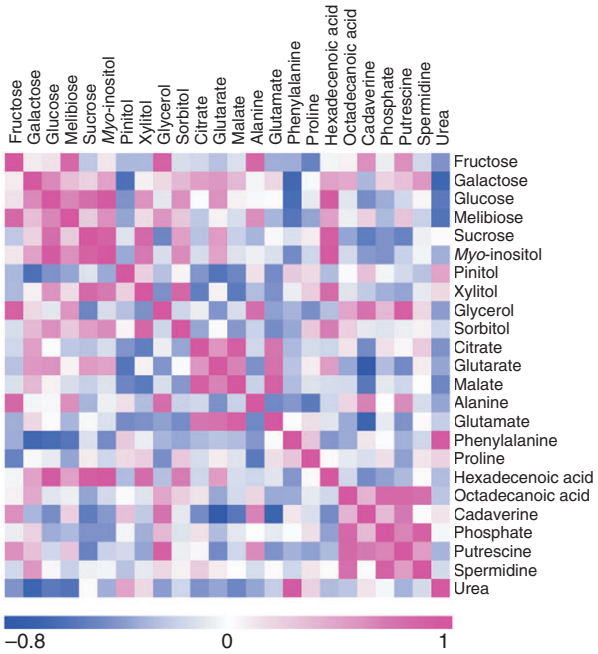

Fig. 6. Visualization of metabolite–metabolite correlations during CAM-like induced metabolism by drought and after recovery. Pearson correlation was used. A total of 325 pairs of metabolites was tested from which 48 pairs exhibited statistically significant coefficients ($P < 0.05$). Among them, 42 were positive and 6 negative. Color scale used is shown at the bottom.

phenylalanine with glucose; while cadaverine displayed negative associations with Glu and glutarate (Fig. 6).

Sugar and sugar-alcohol analyses

Glucose and fructose displayed a surprising behavior; they exhibited greater relative levels at night than during the day in both C_4 control and CAM-like leaves (Fig. 7). After withholding water, they decreased to about a 20% (in the case of glucose and fructose) of values in control leaves when comparing the same sampling moment (Fig. 7). When plants were recovered, fructose displayed significant increases of 7.5- and 1.3-fold with respect to control leaves during the day and night, respectively. Glucose, which was slightly increased during the day in recovered plants with respect to controls, decreased fivefold in the night (Fig. 7). Even though galactose exhibited fluctuations over the day-night period in C_4-control and in CAM-like stressed plants, it was greater in C_4 than in CAM-like leaves (Fig. 7). In recovered plants galactose was similar to controls. Melibiose followed the trend of fructose (see Fig. S2).

Total reducing sugars were quantified at noon (CL12, SL12 and RL12) and at midnight (CD0, SD0 and RD0). In C_4 plants, the pattern of total reducing sugar match with those of the individual monosaccharides, with the amount of the night (CL) doubling that of the day (CD). In contrast, no differences were observed in the night/day relations of the other samples (see Fig. S3).

Sucrose followed a similar trend as glucose; in C_4 control plants it was also higher at night than at day (Fig. 7), with a sixfold increase detected at CD5 than at CL12 (Fig. 7). In contrast, after induction of a CAM-like metabolism sucrose was similar in both photoperiods, thus its relative content resulted greater during the day and smaller at night in comparison with control plants. After re-watering, sucrose decreased with respect to both

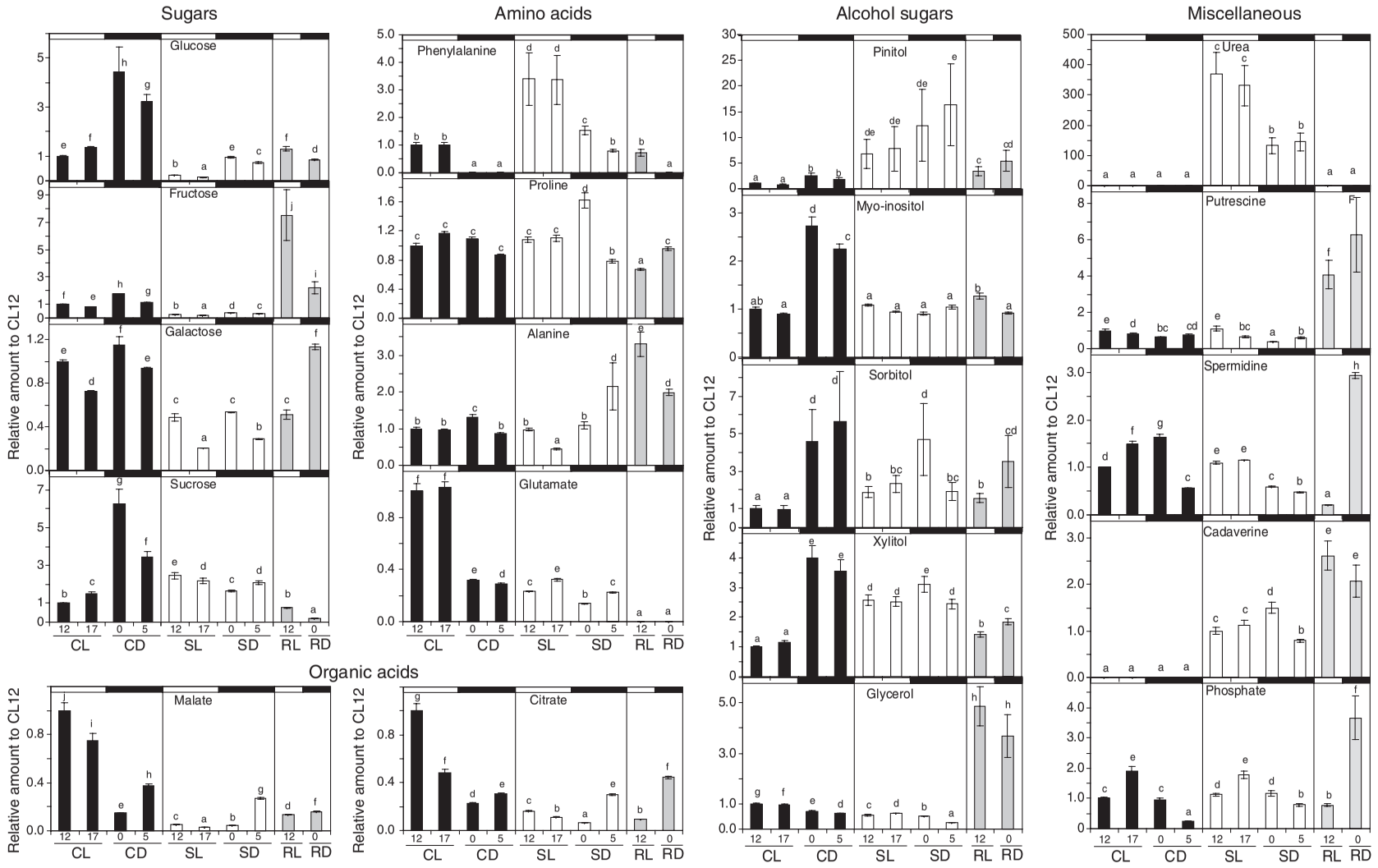

Fig. 7. Metabolite profiles of leaves of *Portulaca oleracea* plants under different watering conditions. The ratio between the amounts in each sample relative to the levels in leaves of C_4-control plants collected at noon (CL12) is indicated. Sampling is as described in Fig. 5. Table S1 shows the relative values of each metabolite. Values not labeled with an identical letter are statistically significantly different ($P < 0.05$).

C_4 control and CAM-like plants, with greater relative levels detected during the day (RL12) than at night (RD5) (Fig. 7).

Starch was evaluated enzymatically and significantly reduced in drought-stressed leaves (five- and threefold decrease in day and night samples, respectively, with respect to C_4-control leaves). After re-watering, starch reached the levels of controls, paralleling the increase in the CO_2 uptake (see Fig. S3). No statistically significant changes were recorded in the diel pattern among samples from well watered plants collected at midday or midnight.

Concerning polyols, pinitol largely increased (between 5- and 10-fold) in drought-stressed vs control plants when compared at the same sampling time (Fig. 7). After recovery, pinitol decreased but it was still greater than in control plants. On the other hand, while *myo*-inositol decreased during the night in CAM-like leaves and after recovery (Fig. 7), xylitol and sorbitol increased about twofold in stressed plants during the day with respect to control plants in the light and displayed similar or lower levels in the night with respect to control plants in the dark (Fig. 7). Following recovery, xylitol and sorbitol decreased but their relative levels during the day were still greater than in control plants. Finally, glycerol displayed a remarkable fivefold increase in re-watered plants with respect to both control and drought stressed leaves during the day and night periods.

Organic acids

Three organic acids were detected by GC-MS: malate, citrate and glutarate (Fig. S1, Table S1). Drought significantly decreased glutarate between 3- and 10-fold with respect to well-watered control plants. Following watering, it was not detected.

Interestingly, even though citrate decreased in CAM-like leaves at 00:00 h with respect to C_4-control plants, it was accumulated during the night in stressed leaves [it increased 2.6-fold at the end of the night (SD5) with respect to the end of the day (SD17)]. This trend was maintained after re-watering (Fig. 7).

Regarding malate, a similar time-course profile as that shown in Fig. 2, was observed. That is, it was greater during the day than at the night in C_4-control plants and it was accumulated toward the end of the night in drought-stressed leaves (Fig. 7). After re-watering, samples were collected only at midday and at midnight and no changes were detected, in agreement with the maintenance in the malate amount at the end of both light and night periods (Fig. 2). Differences in the relative amounts were detected between the two methods of analyses.

Fatty acids

Hexadecenoic and octadecanoic acids displayed a significant increase during the night both in control and stressed leaves, but the magnitude of the increase was smaller in drought-stressed plants. While after recovery, no changes were observed in hexadecenoic acid in samples collected, the relative level of octadecanoic acid was largely increased in RD5 with respect to the rest of the samples (Fig. 7).

Metabolism of nitrogen-containing compounds

Regarding amino acids, Pro did not exhibit significant changes, except for a 1.5-fold increase in CAM-like leaves at 05:00 h in comparison with C_4-leaves (CD5). In contrast, Phe was not detected during the night in control leaves and it was largely increased in CAM-like leaves (3.4-fold increase during the day with respect to controls). After re-watering, Phe returned to the levels of C_4-control leaves (Fig. 7). Regarding Glu, a diel fluctuation was observed in control plants, with greater levels during the day than at the night. After drought, Glu significantly decreased in both day and night samples and no longer detected after re-watering (Fig. 7). Regarding Ala, the main differences were observed in recovered plants which showed greater levels with respect to both control and stressed leaves (Fig. 7).

Because only four amino acids were detected, total free amino acid content was assessed (Fig. 8A). It was significantly increased in CAM-like leaves (S) with respect to control and recovered (R) plants.

Urea was barely detected in C_4-control plants only during the day (Fig. 7). Nevertheless, it was largely increased in CAM-like leaves (370-fold at noon). After re-watering, it decreased being undetectable (Fig. 7). To get more insight into this urea variation, the activity of two enzymes involved in its metabolism was measured (Fig. 8B). Arginase, which catalyzes urea synthesis, was doubled in CAM-like leaves with respect to C_4-control plants; while urease, catabolizing urea, was remarkably decreased (30-fold) with respect to C_4-plants. After a 21–23-day recovery period the activity of both enzymes returned to the levels in control leaves (Fig. 8B). Ammonium, the initial substrate for urea synthesis and also produced by urea degradation, was also quantified (Fig. 8A). Drought stress induced a threefold in ammonium. After recovery, ammonium was decreased but it was not statistically significant.

The activities of GS and of GDH involved in ammonium assimilation and metabolism were also measured. While no changes in GS were observed among samples, fluctuations in GDH were recorded (Fig. 8B). GDH activity increased a 30% in SL with respect to control plants.

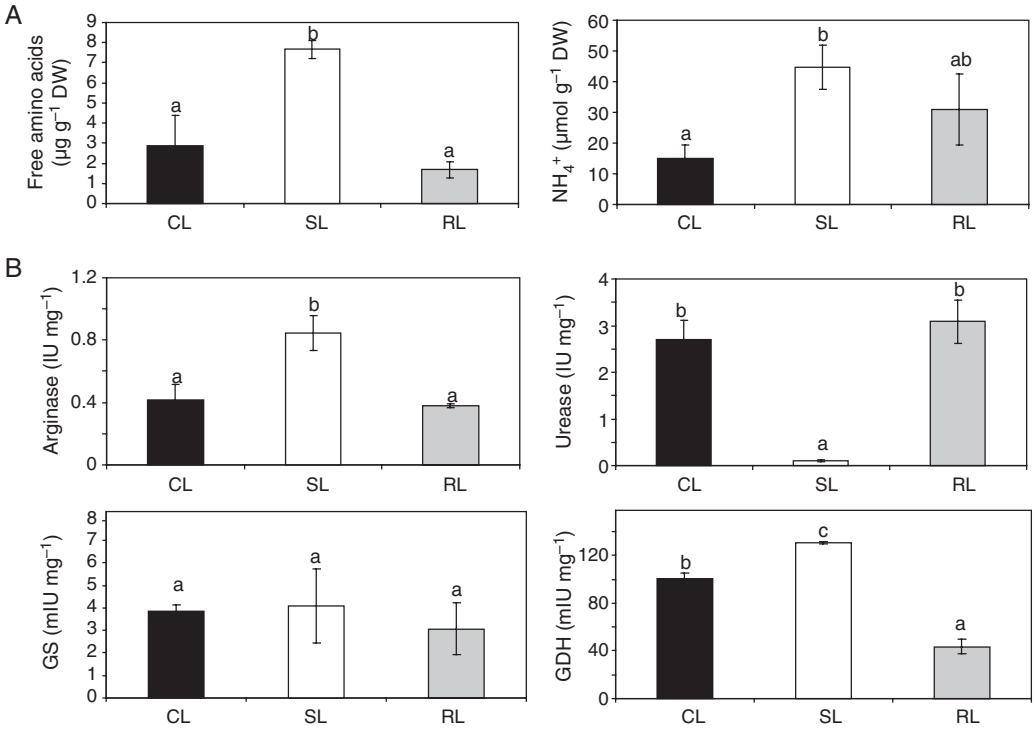

Fig. 8. Nitrogenous containing compounds metabolism. (A) Free amino acid content and ammonium in leaves of *Portulaca oleracea*. (B) Determination of urease, arginase, glutamate dehydrogenase and glutamine synthetase. Activities were measured in crude extracts using leaves from control (CL), drought-stressed (SL) and re-watered plants (RL) collected after 5 h into the light period. Specific activity is expressed in milli-International Units per milligram of protein. Values represent the mean ± SD of at least six determinations using different plants. For each parameter, values with the same letters are not significantly different ($P < 0.05$).

After re-watering, GDH decreased 3-fold with respect to stressed samples (SL) and 2.3-fold with respect to controls (Fig. 8B).

Regarding polyamines, putrescine, spermidine and cadaverine were detected in *P. oleracea* leaves (Fig. 7). No changes in the patterns of putrescine and spermidine over the light/night periods were observed in leaves of control and stressed plants. In contrast, after re-watering, both compounds were significantly increased during the night with respect to control and stressed plants (Fig. 7). Putrescine was also increased during the day in recovered plants; whereas, spermidine was decreased (Fig. 7). Cadaverine was not detected in control leaves, but increased upon drought and it was further enlarged after re-watering (Fig. 7).

Finally, phosphate was highest during the night in drought-recovered plants (Fig. 7).

Discussion

Portulaca oleracea responds uniquely to drought

CAM is a photosynthetic type that can be induced in response to abiotic and biotic stresses and which leads to the development of the CAM phenotype under drought or CO_2-limited conditions (Borland et al. 2006). The fundamental features that describe CAM are abilities to take carbon at night and to accumulate it in the vacuole as an organic acid (Silvera et al. 2010). Thus, the succulence of the photosynthetic tissue (leaves and/or stems) is associated with this type of carbon fixation metabolism (Borland et al. 2011). *Portulaca oleracea* subjected to drought stress showed significant daily malate fluctuation (night : day ratio 3.2), net CO_2 uptake in the dark (Fig. 1), daily changes in the relative activity and regulatory properties of PEPC and daily fluctuation in titratable acidity (Lara et al. 2003, 2004), all typical characteristics of CAM. In addition to malate, citrate would also contribute to nocturnal acidification like in other CAM plants such as *Kalanchoe pinnata* and *Clusia minor* (Borland and Griffiths 1997, Chen et al. 2002). Modifications in enzymes involved in glycolysis and carbon fixation were also reported (Lara et al. 2004).

Because *P. oleracea* displays Kranz anatomy, the term "CAM-like" has been used to refer to *P. oleracea* case (Lara et al. 2003). In addition to the bundle sheath and mesophyll cells, the leaves of *P. oleracea* present

large and vacuolated water storage cells contributing to the succulence of the leaves (Lara et al. 2003). The dark CO_2 fixation under drought represents approximately the 10% of the daytime assimilation in C_4-control plants (Fig. 1), indicating a very little contribution to plant biomass production. Nevertheless, this low carbon uptake during the night and/or recycling of respiratory CO_2 may contribute to the maintenance of a positive carbon uptake (or the avoidance of a net CO_2 drain) under water deficit (Herrera 2009).

The induction of CAM-like metabolism in *P. oleracea* has been also reported under natural environmental conditions (Koch and Kennedy 1980, 1982, Kraybill and Martin 1996). The species belonging to *Portulaca* constitute a line in the Portulacaceae family that has evolved from CAM ancestors (Guralnick and Jackson 2001). In the course of evolution, it is probable that over an existing CAM tissue the C_4 photosynthesis somehow occurred in *Portulaca* and that CAM was loosened and now has a role only in particular stress situations such as drought (Sage 2002).

The recovery of the magnitude and the diel pattern of CO_2 assimilation rates upon re-watering (Fig. 1) indicate that drought did not permanently modify the photosynthetic apparatus and that the change in the pattern of carbon acquisition was transitory and reversible. In spite of having measured high levels of malate in re-watered plants, its night:day ratio was 1.1 as in C_4-control plants (0.9; Fig. 1). The operation of a CAM-like metabolism in C_4 *P. oleracea* under drought would serve as a mechanism for water saving, evading carbon losses due to respiration and prolonging the life of the cell, as it has been proposed for C_3-CAM facultative plants (Herrera 2009).

Portulaca oleracea photosynthetic machinery is quite resistant to water deficits

Photosynthesis is one of the first metabolisms influenced by drought, exhibiting decreases as a consequence of the closure of the stomata and of other pore-independent responses (Lawlor and Cornic 2002). In *P. oleracea*, the change in the diel pattern of CO_2 exchange and the decrease in the net CO_2 assimilation (Fig. 1) due to drought may surely lead to an excess in electrons captured with respect to those required for carbon fixation, which ultimately would lead to an over-excitation and then photoinhibition. Plant photosynthetic capabilities were explored revealing a decrease in the quantum yield of PSII upon drought and an increase of non-radiative energy dissipation mechanisms, accounted by increases in qNP and decreases in Fv′/Fm′ during stress. Therefore, by these means photodamage may be prevented through dynamic photoinhibition. Because CAM-like induced plants showed closed stomata during the day (Lara et al. 2003) and thus, reduced water losses by transpiration, heat dissipation by this latter means could be affected and it is then reasonable that leaves present more optimized mechanism for energy dissipation, as accounted by increased qNP. The impairment to photosynthesis was transitory, because upon re-watering the assimilation rates (Fig. 1) as well as the PSII parameters (Fig. 3) were recovered, denoting that the *P. oleracea* photosynthetic machinery is quite resistant to water deficits. Moreover, under drought or salt stress conditions CAM induction has been proposed to mitigate the oxidative load during the day by providing CO_2 from malate and by avoiding the over-reduction of the transporters (Borland et al. 2006).

Diminishments in effective quantum yield of PSII and in qP indicate an over-excitation of the photochemical systems. If so, accumulation of reduced electron acceptors may probably lead to the generation of reactive radicals. Water deficit induced the levels of flavonoids, betalains (Table 1), Phe (Fig. 7) and the antioxidant enzymatic machinery (Fig. 4) in *P. oleracea* leaves, which may prevent cellular damage due to ROS production. Kaempferol, apigenin, myricetin, quercetin and luteolin are major flavonoids in this species (Xu et al. 2006). Rather than anthocyanins, *P. oleracea* contains a type of nitrogenous water-soluble pigments called betalains (betacyanins and betaxanthins), which are potent antioxidants and antimutagenics (Strack et al. 2003, Stintzing and Carle 2004). Betacyanins and betaxanthins are derived from tyrosine (Bianco-Colomas 1980). Here, the increase in Phe (Fig. 7) detected after withholding water may support the increased synthesis of flavonoids through the phenylalanine ammonia lyase route as well as of betalains by providing Tyr.

Salinity and high temperature tolerances of *P. oleracea* were related to the increased capacity of antioxidative system (Yazici et al. 2007, Yang et al. 2012). Moreover, *P. oleracea* polysaccharides were demonstrated as scavengers of superoxide and hydroxyl radicals (Guo et al. 2009). Thus, the great antioxidant capacity of this plant surely contributes to cope with abiotic stresses.

Concerted changes in C and N metabolism in *P. oleracea* under drought and re-watering

Several metabolic changes were also evidenced in CAM-like induced plants and after recovery.

In agreement with a decrease in the net CO_2 fixation (Fig. 1) and in the RuBisCO immunoreactive protein (Lara et al. 2004), monosaccharides such as glucose, fructose and galactose (Fig. 7), and starch (see Fig. S3)

were reduced in drought-stressed leaves. On the other hand, the increase in sucrose during the day (2.5- and 1.5-fold increase at 12:00 and 17:00 h, respectively) may be related to a decrease in the export of sugar from the leaves. Thus, the lower net CO_2 fixation under water restriction may be compensated by a lower export activity. The starch hydrolysis, which may also provide substrates for the oxidative pentose pathway, and the accumulation of reducing sugars in leaves under drought (see Fig. S3) may protect against oxidative damage. On the other hand, starch and sugar degradation accompanied by changes in some enzymes of glycolysis (Lara et al. 2004) would satisfy the demand for substrate for PEPC.

In C_4-control plants glucose, fructose, galactose and sucrose show a peak at midnight (CD0; Fig. 7), which is also evidenced in the reducing sugar measurements (see Fig. S3). This is a remarkable feature for a C_4 species, especially the great increase in sucrose at midnight, and to our knowledge it has not been described before and it suggests that *P. oleracea* could use soluble sugars as storage carbohydrates; as it has been described for some CAM species like *K. pinnata*, *K. daigremontiana* and *A. comosus* (Carnal and Black 1989, Chen et al. 2002).

Significant increases in sugar-alcohols were observed in drought-stressed leaves during the day (xylitol and sorbitol) or both day and night periods (pinitol) (Fig. 7). Accumulation of polyols has been shown to cope with water deficit or high salt conditions (Popp et al. 1985, Popp and Smirnoff 1995). Their hydroxyls groups substitute those of water in the H-bonds and keep proteins and membranes in their native form, they accumulate in the cytosol and balance the high osmotic potential of the vacuole and some of them could detoxify highly reactive hydroxyl radicals that lack enzymes for their scavenging (Chaves et al. 2003, Smirnoff 1993, Yancey 2005). During the C_3-CAM transition of *M. crystallinum* pinitol synthesis is induced but as part of a stress response which is distinct from the CAM induction (Vernon and Bohnert 1992).

Urea is the metabolite that showed the greatest increase in drought-stressed leaves (Fig. 7). Urea accumulation is usually related to enhanced protein degradation, which is not the case (Fig. 2B). On the contrary, de novo synthesis in specific amino acids would account for the increase in free amino acids (Fig. 8A). Urea is an osmolyte that accumulates at high levels in mammals' kidneys and urine, and it is the main organic osmolyte of elasmobranches (Yancey 2005). On the other hand, at high levels urea disturbs protein folding and binding (Laxson et al. 2011). Nevertheless, methylamine compounds (like glycine-betaine) are synthesized in fish to neutralize the negative consequences of this perturbant on proteins (Yancey 2005). In plants, urea is synthesized in the mitochondria but it can be accumulated in the vacuole (Witte 2010). In *P. oleracea* high levels of urea in the vacuole, where its negative effects would be reduced, may contribute to osmolarity maintenance. Moreover, the increased polyamine cadaverine could help to counteract urea under drought (Fig. 7). Reciprocal fluctuations in arginase and urease (Fig. 8B) in control vs drought-stressed leaves indicate that urea is strictly controlled, denoting its relevance in drought-stressed leaves. On the other hand, considering that cell death or signs of senescence were not observed (Figs 2 and 8A) it is unlikely that urea comes from purine turn-over.

On the basis of the increased GDH activity (Fig. 8) and the pronounced decrease of Glu (Fig. 7) under drought, the anaplerotic deamination of Glu would function to funnel the C skeletons into the tricarboxylic acid cycle during conditions of reduced net CO_2 uptake, as it has been proposed for plants under sugar starvation (Melo-Oliveira et al. 1996). Increased GDH activity would also contribute to NH_4 pool in CAM-like leaves (Fig. 8). In addition, increased NH_4 would also modulate PEPC (Murmu and Raghavendra 2005). On the other hand, the absence of increases in GS, a senescence marker (Kamachi et al. 1991) in either stressed or re-watered plants (Fig. 8B), together with other data presented, suggest senesce is not induced in *P. oleracea* subjected to drought.

Upon re-watering, although the CO_2 rates of C_4 control leaves were recovered, the metabolome was greatly modified (Figs 5 and S2). The fixed carbon would be used to synthesize sugars like fructose (Fig. 7) and starch (see Fig. S3). Sucrose relative levels at midday that were recovered would be rapidly exported to other tissues. This export would restore growth, which was slowed or even stopped during drought (Fig. 7). Urease (Figs 7 and 8B) would act providing NH_4 and CO_2, which would be re-fixed to reestablished growth, as in the case of rapidly growing tissue (Todd et al. 2001). Photosynthesis would also support the great increase in glycerol in recovered plants (Fig. 7). At least in yeast, glycerol accumulation would regulate the osmotic potential and would decrease the concentration of NADH, and thus it would improve mitochondrial performance and it would decrease the generation of reactive species (Shen et al. 1999). In the green algae *Dunaliella salina* the remarkable halotolerance is mediated by the massive de novo synthesis of the glycerol (Alkayal et al. 2010, Chen et al. 2012). In *P. oleracea*, glycerol was not modified during drought but after re-watering (Fig. 7), suggesting that it may be of importance during the recovery phase. Moreover, the rise in the activity of POD upon re-watering denotes that ROS are also enhanced at this state (Fig. 4).

Water deficit is associated with early senescence and leaf abscission. Plant manipulation avoiding the set-up of senescence conducted to plants that better cope with prolonged drought (Rivero et al. 2007). In this respect, the great increases in polyamines measured in re-watered *P. oleracea* plants may be related to their proposed role in delaying senescence, mediating the recovery of the drought-stressed plants (Fig. 7).

Concluding remarks

On the basis of the results presented, *P. oleracea* engages multiple mechanisms that converge to cope with water deficit. A CAM-like metabolism is induced, in which the nocturnal CO_2 uptake helps to maintain the carbon balance and the fixation of the CO_2 released from malate and citrate during the day, contributes to curtail the production of radical oxygen species and photodamage. Photosynthetic machinery protection is also related to the increased capacity of the antioxidative system, which includes enzymes, pigments, sugars and other compounds, to scavenge reactive oxygen species. On the other hand, the induction of osmolytes such as pinitol and other nitrogenous containing compounds such as urea, allowed *P. oleracea* leaves to maintain a cellular homeostasis under drought. Considering the great plasticity of *P. oleracea* it is highly probable that the induction of these metabolites is independent of CAM-like induction (Vernon and Bohnert 1992). Upon re-watering, diurnal CO_2 fixation and photosynthetic parameters were recovered and whole metabolism rearranged, in agreement with the great plasticity of this species.

Reduced water accessibility and diminishment in cultivable soils limit agriculture. On the other hand, there is a growing need for harvested products. Therefore, it is mandatory to search for new genetic resources that can deal in a better way with stresses. As well as to increase the knowledge of the mechanisms contributing to adaptation to stresses. *Portulaca oleracea* is more nutritive than the 20 major crops used for human feeding. This and previous studies (Simopoulos et al. 1992, Liu et al. 2000, Yazici et al. 2007, Ren et al. 2011), show that *P. oleracea* grows well under diverse stressful environments and it is generally more drought tolerant than most crops. Therefore, this edible plant could be used as a nutritive food in areas of reduced water availability and/or to reduce soil water losses due to food production. In addition, deciphering the mechanisms involved in *P. oleracea* response to drought will facilitate the generation of other species of economically importance with improved stress tolerance (Ren et al. 2011).

Author contributions

C. S. A. and M. V. L. conceived the project. R. M. D. performed the experiments. C. S. A., R. M. D. and M. V. L. analyzed the data. M. V. L. wrote the article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Relative levels of 24 metabolites analyzed by GC-MS in leaves of *Portulaca oleracea* under different watering conditions and types of carbon assimilation metabolisms.

Table S2. Variable contribution to the principal components (%).

Fig. S1. In-gel activity peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) from *Portulaca oleracea* leaves. Forty microgram of protein were loaded in each lane for APX and CAT activities. For POD and SOD native PAGEs, 30 and 35 μg were downloaded, respectively. Representative images of at least three gels conducted with different extracts are shown. The band of APX induced in SL is indicated by an arrowhead.

Fig. S2. Overlay heat map of metabolite profiles in leaves of *Portulaca oleracea* collected at different times during the day and night. Well watered control plants performing C_4 photosynthesis, plants in which CAM-like metabolism has been induced due to drought stress and drought-stressed plants recovered after re-watering were analyzed. Each square represents the relative level of each metabolite to its amount found in control leaves collected 5 h after the beginning of the light period (CL12). Normalized values are shown on a color scale (shown at the bottom of the figure), which is proportional to the content of each identified metabolite. Mean values of five independent determinations for each time of collection were expressed as the ratio between log2 and CL12. Gray color indicates not determined value. Heat map was created using MULTIEXPERIMENT VIEWER version 4.1. (MeV). Relative values for each metabolite peak area are provided in Table S1.

Fig. S3. Total reducing sugars (A) and starch content (B). Measurements were conducted using leaves from control (C), drought-stressed (S) and re-watered plants (R) collected during the day (CL, SL and RL) and during the night (CD, SD and RD). Values represent the mean ± SD of at least six determinations using different plants. For each parameter, values with the same letters are not significantly different ($P < 0.05$).

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