



Progressive drought impact on *Eruca sativa* Mill.: a kinetic approach

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ABSTRACT

Plants facing drought stress in natural conditions are exposed to a progressive decrease in soil water content. The sequence of physiological events leading to plant response is important to depict in order to assess the timing of injury symptoms appearance. Plants of *Eruca sativa* Mill. were exposed for one month to a progressive decrease in soil moisture. Control plants were maintained at 80 % of field capacity while soil water content dropped to 43.5 % of field capacity in stressed one. All plants remained alive but 50 % of stressed plants encountered a premature flowering and inhibition of vegetative growth. Accumulations of proline and glycinebetaine were the first responses to water shortage reaching up to 51.2 and 38.3 $\mu\text{moles.g}^{-1}$ DW respectively after already one week of treatment. This was not sufficient to induce an immediate decrease in osmotic potential which dropped to -2.6 MPa after 4 weeks of treatment only. Water potential decreased before osmotic potential exhibiting values of -0.97 MPa after only two weeks of stress. An increase in total antioxidant activities was noticed already after one week of treatment but most endogenous antioxidant (glutathione, ascorbate, flavonoids and anthocyanin) accumulated after 3 weeks of treatment and did not avoid malondialdehyde and H_2O_2 accumulation. Photosynthesis inhibition was recorded after two weeks and attributable to a decrease in stomatal conductance ($188 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ in stressed plants versus 304 in control ones). Instantaneous water use efficiency, *Chla/Chlb* ratios and fluorescence-related parameters remained unaffected by water stress. During the 3 first weeks of treatment, water shortage had a limited impact on leaf water content but it strongly decreased the relative water content during the last week of treatment. It is concluded that *Eruca sativa* can trigger adaptive strategy at the beginning of the stress period but that some of them may be overwhelmed after a long-time exposure. This species presents an optimal compromise between drought escape and water-saving strategies.

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1. Introduction

Water stress is a major environmental constraint that compromises crop yields and ecosystem stability (Voltaire, 2018; Bandurska, 2022). With the prospect of climate change, drought episodes will become increasingly frequent and intense in the future (Rojas, 2020). It is therefore crucial to gain a better understanding of the effects of water stress on plants, in order to identify the selection criteria to be used to obtain better-performing plant material.

A deficit in water supply compromises all aspects of plant physiology. Water stress alters plant water status, reduces growth, accelerates organ senescence, reduces photosynthesis, affects

mineral supply, generates oxidative stress and modifies the plant's hormonal profile (Kabbadj et al., 2017; Takahashi et al., 2020; Razi and Muneer, 2021; Amnan et al., 2022; Bandurska, 2022; Sadiqi et al., 2022; Ahmad et al., 2024). In a natural field situation, drought implies a progressive reduction in the water content of the substrate (Voltaire, 2018; Sadiqi et al., 2022). The kinetics of this reduction depend on the precipitation regime, soil evaporation, substrate characteristics and the extent of root development at the time of stress onset (Tardieu et al., 2018; Bandurska 2022). This implies that the onset of stress injuries is not abrupt, and that the various symptoms do not appear simultaneously but rather according to a particular sequence, the nature of which depends on the species and the overall environmental situation encountered by the plant (Maseda and Fernandez, 2006; Gupta et al., 2020; Wu et al., 2022).

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The sequence in which symptoms appear is interesting to analyze because i) it enables us to identify the most sensitive physiological properties for which damage symptoms appear first, and ii) it unravels the relationships that may exist between modified physiological properties based on the order in which symptoms appear. For example, in isohydric species that rapidly close their stomata under stress conditions, the onset of oxidative stress linked to the production of free radicals usually occurs after the reduction in stomatal conductance and often appears as the consequence of a stimulation of photorespiration in C3 plants. Conversely anisohydric species keep their stomata open under stress conditions, and the appearance of ROS is often linked to partial desiccation altering electron transport chains in plants whose stomatal conductance reduction is late (Desikan et al., 2004; Flexas et al., 2006; Gallé et al., 2013).

Such a kinetic approach can also help to more accurately interpret plant behavior when the same response can be interpreted either as a symptom of damage, or as an attempt to adapt. For example, growth inhibition may be perceived as the negative consequence of reduced cell elongation linked to altered turgor, but may also be seen as an attempt by the plant to limit its water losses by reducing its evapotranspiring surface area (Gao et al., 2020; Huang et al., 2022; Wu et al., 2022). Many studies devoted to the impact of drought on plants analyze stress symptoms after a given period, reflecting a specific physiological situation, without being able to explain « why » and « how » these symptoms appeared.

Eruca sativa Mill. (arugula or wild rocket) is an annual herbaceous oil crop species belonging to the Brassicaceae family and native to the Mediterranean basin. It is widely cultivated in different regions, such as North Africa, Eastern Asia Middle East (Pagnotta et al., 2022). *Eruca sativa* is a moderately drought-resistant species which may be adequately integrated in sustainable cropping systems in arid regions after intercropping cultures allowing the maintenance of minimal residual soil moisture (Tanwar et al., 2023). According to Lee et al. (2024), a minimal substrate volumetric water content of $0.30 \text{ m}^{-3} \cdot \text{m}^{-3}$ is required to ensure a high water use efficiency while securing the growth and yield of arugula. Lower soil water content induced oxidative stress and reduced plant growth and seed yield (Khan et al., 2018; Yang et al., 2021; Ogran et al., 2021; Nikzad et al., 2023).

Extensive phenotypic and genetic variation were reported in *E. sativa* for numerous morphological traits and responses to biotic and abiotic stresses (Warwick et al., 2007; Ogran et al., 2021; Bajpai et al., 2022; Nikzad et al., 2023). The most drought-tolerant accessions were able to efficiently trigger antioxidative responses and osmotic adjustment through proline and glycinebetaine accumulation (Khan et al., 2018). Specific molecular targets were suspected to play a key role in *E. sativa* drought response. A transcriptomic analysis identified numerous differentially expressed genes between accessions exhibiting contrasting level of resistance to osmotic stress (Huang et al., 2019). These authors considered that up-regulation of proline, arginine and alpha-linolenic acid metabolism is crucial for drought tolerance. Mahawar and Shekhawat (2019) also reported that *EshO1* coding for heme oxygenase I played a key role in management of oxidative stress in arugula. These studies, however, were conducted in hydroponic system with nutrient solution containing high concentration of polyethylene-glycol (PEG) or various salts added in one step as osmotic stressors (Amiripour et al., 2016; Huang et al., 2019; Yang et al., 2021; Khan et al., 2022).

Obviously, a sudden exposure to stress has a different impact on plants than a progressive exposure such as it occurs in field conditions (Almansouri et al., 1999). The present comprehensive study was therefore undertaken to assess the kinetic appearance of water stress symptoms in a wild population of *Eruca sativa* exposed to a progressive decline in soil water content and to identify the first physiological parameters modified during water deficit.

2. Material and methods

2.1. Plant material and culture conditions

Seeds of *Eruca sativa* (wild accession) were provided by Agricultural Experimental Station of Tamanrasset (extreme south of Algeria). Surface-sterilized seeds were germinated for 24 h in the dark at 25 °C in Petri dishes containing two layers of filter paper (Whatman n° 2) moistened with 7 mL sterile water. The seedlings were then transplanted in square pots (11 cm × 12 cm; 20 cm height) filled with a mix of sand and loam (1:1, v/v) as substrate. Each pot contained 1.5 Kg of substrate with a soil gravimetric water content at field capacity of 9 %, a bulk density of $1.54 \text{ g} \cdot \text{cm}^{-3}$, an electrical conductivity of $1.74 \text{ dS} \cdot \text{m}^{-1}$ and a humus percentage of 13.8 %. The mineral content was estimated as (mg/100 g DW) K: 89; P 43; Mg 154; Ca 1023. The potted seedling were maintained in controlled greenhouse conditions (SEFY platform; UClouvain; Belgium) under a mean light intensity of $230 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ supplied by Philips HPIT 400 W lamps (Philips Lighting S.A., Brussels, Belgium) with a day/night temperature of 29/25 °C. Relative humidity fluctuated between 54 % during the night and 45 % during the day.

Every two days the plants were irrigated to 80 % field capacity (FC). The soil water content was measured using a ProCheck Operator's manual Version 5 (Decagon Devices, Wa, USA) soil moisture sensor type on the basis of changes in apparent dielectric constant. Recorded data were converted to volumetric water content based on preliminary calibration of the substrate. The soil water content (θ) was defined as $(W_w/DW_s) \times 100$ where W_w was the weight of the water contained in the soil sample and DW_s was the dry weight of the sample after incubation in oven at 60 °C. After four weeks, 100 uniform-sized plants were divided in two distinct lots of 50 plants: the first one remained irrigated in the same way (control) while the irrigation was withheld for the second batch (stress). The pots were randomly distributed in the greenhouse.

2.2. Plant growth and water status

Plant growth was estimated by measuring the plant height and leaf numbers of 20 plants per treatment every week. Shoot dry weight was determined on 5 harvested plants after 7, 14, 21 and 29 days of treatment. Leaves were harvested and incubated for 48 h at 70 °C to determine dry weight (DW). Leaf area was measured on 20 plants per treatment: leaves were scanned and the total leaf area was estimated using the ImageJ software.

For determination of relative water content (RWC), eight leaves from the middle part of the stem were removed from distinct plants for each treatment, weighed quickly and immediately enclosed in a Petri dish containing two layers of Whatman n° 2 filter paper to saturate them with water for the next 20 h at 4 °C. The turgid weight (TW) was then estimated and leaves were oven dried at 70 °C to determine DW. RWC was calculated according the following formula:

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$
 where FW was the fresh weight recorded at the time of harvest, TW was the turgid weight and DW was the oven dry weight.

Shoot Ψ_w was evaluated immediately after sampling by the pressure chamber method (PMS Instrument Co., Orlando, USA). For the measurement of osmotic potential (Ψ_s) leaves of 8 plants per treatment at each harvest time were cut into small segments quickly introduced in an Eppendorf tube perforated with four small holes. Eppendorf tubes were rapidly frozen in liquid nitrogen for 30 s and warmed at room temperature for 5 min in order to break the cellular membrane (Bajji et al., 2000). After 3 cycles of frozen/warmed, tubes were then encased in a second unperforated Eppendorf tube. Samples were centrifuged at 15,000 g for 15 min at 4 °C. The supernatant corresponding to the collected tissular sap was used to assess the

osmolarity (c) with a vapour pressure osmometer (Wescor 5520). Osmolarity was converted from mosmol Kg⁻¹ to MPa using the formula: Ψ_s (MPa) = $-c$ (mosmol kg⁻¹) $\times 2.58 \times 10^{-3}$ according to the Van't Hoff equation.

2.3. Photosynthesis-related parameters

Photosynthesis-related parameters (chlorophyll fluorescence and chlorophyll content, stomatal conductance and net photosynthesis) were all determined on leaves located at the middle portion of the main stem.

Net photosynthesis rate (A), intercellular CO₂ concentration (C_i), and instantaneous transpiration rate (E) were measured by IRGA (InfraRed Gas Analyzer, LCI-SD 4–100, ADC BioScientific Limited; Hertfordshire, UK) and an air supply unit (ASU 10.87) using a PLC Parkinson leaf cuvette for 1 min (25 records/min) and an airflow of 320 mL min⁻¹. Air taken from the external atmosphere was sent to a chamber into which a leaf portion was introduced. The net carbon assimilation rate (μ moles CO₂ m⁻² s⁻¹, A) was measured under constant photosynthetic photon flux. The intercellular CO₂ concentration (C_i; μ moles mol⁻¹) and the instantaneous transpiration (E; in mmoles H₂O m⁻² s⁻¹) were estimated. Leaf stomatal conductance (g_s) was determined on the abaxial surface of leaves on six plants per treatment, using an AP4 diffusion porometer (Delta-T Devices, Cambridge, England). All measurements were performed between 10:00 a.m. and 1:00 pm.

Chlorophyll fluorescence was analyzed on five plants per treatment using a Fluorescence Monitoring System (FMS II; Hansatech Instruments, Norfolk, United Kingdom). All measurements were performed on the upper side of the leaves. Leaf portions were acclimated to darkness for 30 min before illumination with a first pulse at 18,000 μ mol m⁻² s⁻¹ followed by a constant level of actinic light (660 μ mol m⁻² s⁻¹) for 2 min. A second saturating pulse of 18,000 μ mol m⁻² s⁻¹ was subsequently applied. The quantified parameters were the photosystem II efficiency (Φ_{PSII}), the photochemical quenching (q_p), the maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), and the non-photochemical quenching (NPQ) according to Maxwell and Johnson (2000) with slight modifications recommended by Swoczyna et al. (2022). Total chlorophyll and carotenoid concentrations were determined according to Lichtenthaler method (1987): 100 mg of fresh leaves were ground in 80 % cold acetone. The absorbance of the extracts was measured at 663.2, 646.8 and 470 nm using a Shimadzu UV-1800 spectrophotometer (Kyoto-Japan). Pigments concentrations were expressed on a dry weight basis (mg g⁻¹ DW).

2.4. Oxidative stress-related parameters

Oxidative-related parameters were estimated on five plants per treatment, considering leaves present in the middle portion of the stem. The level of lipid peroxidation in the control and stressed plants was assessed from the concentration of malondialdehyde (MDA) as determined by the thiobarbituric acid (TBA) reaction following the method of Heath and Packer (1968). Results were expressed in nmol g⁻¹ DW. Hydrogen peroxide levels were determined according to Alexieva et al. (2001). Leaf tissues (500 mg FW) were homogenized in an ice bath with 5 mL 0.1 % (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of supernatant was read at 390 nm. The content of H₂O₂ was estimated from a standard curve.

Total antioxidant capacity was quantified by two distinct methods as recommended by Thaipong et al. (2006). The first method is the ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996) and the second method is the 2,2 diphenyl-1-picrylhydrazyl (DPPH) according to Brand-Williams et al. (1995). Extraction was first

performed with methanol (10 mL per g FW) and the homogenates were kept at 4 °C during 8 h and then centrifuged at 10,000 g and 4 °C during 10 min. The supernatants were removed, stored at –20 °C until analyses and subsequently used for determination of antioxidant power of hydrophilic fraction (AOAM fraction) after aqueous extraction. The pellet was then dissolved with 20 mL dichloromethane, centrifuged at 12,000 g for 7 min and contained the lipophilic fraction (AOAD). The FRAP solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃·6H₂O solution) was warmed at 37 °C; 150 μ L of sample extracts and standards (0 – 800 mM Trolox) were allowed to react with 2850 μ L of the FRAP solution for 30 min in dark condition. After reaction with the FRAP solution, absorbance was read at 593 nm and results were expressed in μ M equivalent Trolox g⁻¹ FW. The DPPH stock solution consisted in 24 mg DPPH with 100 mL methanol and was stored at –20 °C until needed. 150 μ L of each fraction were allowed to react with 2850 μ L of the DPPH solution for 24 h in the dark. Then the absorbance was read at 515 nm. The standard curve was linear between 25 and 800 mM Trolox. Results are expressed in mM Trolox equivalent (TE) g⁻¹ dry weight.

2.5. Measurement of endogenous antioxidant concentration

For ascorbate extraction, the leaves (0.25 g FW) were ground in liquid nitrogen and homogenised with 5 % trichloroacetic acid, left 15 min on ice and centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was used for assays of total ascorbate and reductive-form contents (AsA + DHA). To determine the total ascorbate, oxidized ascorbate (DHA) was reduced to AsA with DL-dithiotreitol (DTT), and excess DTT was removed with NEM. For the determination of reduced ascorbate, 0.2 mL of the supernatant, 0.4 mL of 0.2 M phosphate buffer (pH 7.4), 0.4 mL of Milli-Q water, 1 mL of 10 % w/v TCA, 0.8 mL of 85 % H₃PO₄, 0.8 mL of 4 % dipyridyl, and 0.4 mL of 3 % FeCl₃ were used (Kampfenknel et al., 1995). The solutions to determine the reduced ascorbate and total ascorbate were incubated for 1 h at 37 °C, and the absorbance was read in a spectrophotometer at a wavelength of 525 nm. The determination of oxidized ascorbate (DHA) was estimated by subtracting the total minus reduced ascorbate values.

Glutathione was quantified according to Cereser et al. (2001). Total glutathione (GSH_t) was quantified after a reduction step of oxidized glutathione (GSSG) by DTT. The reduced form (GSH) and the oxidized form (GSSG) were also separately analyzed. To extract total glutathione (GSH_t), 200 mg fresh frozen tissue was incubated on ice in 500 μ L 0.1 M Tris buffer (pH 8.5) and 1 mM 25 mM DTT for 30 min. Then 8 mL of 6 % metaphosphoric acid were added before centrifugation at 10,000 g for 7 min at 4 °C. To extract GSH, 4 mL of 6 % metaphosphoric acid were added to 200 mg of fresh frozen tissue before centrifugation at 10,000 g for 7 min at 4 °C. Samples were injected onto a HPLC column with an acetonitrile-sodium acetate gradient system and detected fluorimetrically. Five μ L of sample were injected into a Shimadzu HPLC system (Shimadzu's-Hertogenbosch, The Netherlands) equipped with a Nucleodur C18 Pyramid column (125 $\times$ 4.6 mm internal diameter; 5 μ m particle size) (Macherey-Nagel, Düren, Germany). Derivatives were eluted in acetonitril gradient in a 50 mM sodium acetate buffer pH 6.2 at 30 °C at a flow rate of 0.7 mL min. Fluorimetric detection was performed with a spectra system Shimadzu RF-20A fluorescence detector at 420 nm after excitation at 340 nm. GSH was quantified on the basis of calibration curves with custom-made external standard solutions ranging from 0.0625 to 50 μ M and every ten injections, a check standard solution was used to confirm the calibration of the system. The recovery was determined using GSH as an internal standard. Then, GSSG concentration was obtained from subtraction of the GSH from the GSH_t values.

2.6. Analysis of phenolic compounds

Total phenolic compounds were extracted from frozen leaves samples (100 mg) with 2 mL of 80 % methanol. The mixture was centrifuged at 10,000 g for 10 min at 4 °C. Phenolic compounds were assayed using the Folin-Ciocalteu reagent, following Singleton's method (Singleton and Rossi, 1965). Aliquots (0.205 mL) of sample extracts were added to 3.5 mL of deionized water and 0.25 mL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 3 min, before adding 1 mL of 20 % Na₂CO₃ solution. After incubation for 40 min at 30 °C, the absorbance was read at 760 nm. Total phenolic content of plants was expressed as mg gallic acid equivalents (GAE) g⁻¹ calculated from a calibration curve with gallic acid. Total flavonoids were quantified by the aluminium chloride colorimetric method according to Lamaison and Carnet (1990). Approximately 100 mg of frozen leaves were homogenized in 2 mL of 80 % methanol. Methanolic extract (1 mL) was mixed with 1 mL of 2 % AlCl₃ and kept at room temperature for 10 min. The absorbance of the reaction mixture was measured at 440 nm. Quercetin was used as a calibration standard, and the results were calculated as quercetin equivalent (QE) per gram DW.

Total anthocyanins content was measured according to Rabino and Mancinelli (1986). Leaves anthocyanins were extracted from 100 mg of fresh samples with acidified methanol (methanol: HCl, 1:99, v/v). The absorption values were measured at 530 nm, and 657 nm for spectrophotometric quantification. The formula Absorbance (A) = (A₅₃₀ - 0.25 A₆₅₇) was employed to compensate for the contribution of chlorophyll and its by-products at 530 nm. The anthocyanin content was expressed as mg cyanidin-3-glucoside equivalent per gram DW (mg Cyd-3-glu.g⁻¹ DW) according to:

Total Anthocyanin

$$= \frac{\text{Absorbance (A)} \times 448.2 \times \text{Dilution factor} \times \text{Volume}}{29600 \times \text{Sample weight}}$$

2.7. Quantification of osmocompatible solutes

At each harvest, two leaves in the median portion of the stem were collected on 5 plants per treatment and quickly flash-frozen in liquid nitrogen. Proline was quantified according to Bates et al. (1973) on 200 mg of frozen fresh ground material. Proline was extracted in 3 % (w/v) sulfosalicylic acid with incubation during 30 min at 70 °C. After filtration (Whatman, 11 µm), 2 mL of ninhydrin solution (1.25 g of ninhydrin in 30 mL glacial acetic acid and 20 mL of H₃PO₄ 6 M) were added to the same volume of supernatant and kept at 100 °C for 1 h. Reaction was stopped on ice, then 2 mL toluene were added. Absorbance was read at 520 nm in a quartz cuvette after strong vortexing of the samples. Proline concentration was calculated on the basis of a standard curve established with L-proline (Sigma Aldrich chemicals). The glycinebetaine (GB) concentration was estimated by the method of Grieve and Grattan (1983). Samples were ground in the presence of liquid nitrogen until a fine powder was obtained. Powder was stirred in 20 mL distilled water and incubated for 24 h, filtered and stored at -80 °C. Extracts were mixed with 2 M H₂SO₄ (1:1) and aliquots (0.5 mL) were transferred to centrifuge tubes and cooled on ice for 1 h. KI-I₂ reagent was prepared by dissolving 15.7 g iodine and 20 g KI in 100 mL water. Cold KI-I₂ (200 µL) was added to centrifuge tubes containing sample extract and thoroughly vortexed. Samples were then incubated at 4 °C during 16 h and then centrifuged at 8000 g for 15 min at 4 °C. The obtained crystals were dissolved in 1,2-dichloroethane and the absorbance was measured at 365 nm. Standard curve was established using GB (SigmaAldrich) dissolved in 1 M H₂SO₄.

2.8. Statistical treatment of the data

Normality distribution and homoscedasticity were verified using the Shapiro–Wilk and Levene's tests, respectively, and data were transformed when required. When possible, two-way analysis of variance (ANOVA II) was used to assess the main effects of treatment, stress duration and their interaction. Comparisons between treatments were first analyzed using the Student's test, the permutation Student's *t*-test (if normality was not met), or the Wilcoxon test (if homoscedasticity was not met). For a given stress duration (harvest), comparisons between treatments were made using one-way analysis of variance (ANOVA I) using the permutation test (if normality was not met), or the Kruskal–Wallis test (if homoscedasticity was not met), followed by appropriate post-hoc tests. Data was analysed using one way Analysis of Variance (ANOVA) provided by Statistica 10.0 software (Statsoft. Inc.). The Duncan's multiple range test at the 0.05, 0.01 and 0.001 probability level, were performed in order to determine the significance of the differences between the means.

3. Results

3.1. Soil water content, plant growth and development

Soil water content of control treatment remained between 80 and 85 % of field capacity during the whole experiment (Fig. 1). Soil water content for stressed plants linearly decreased throughout the stress period and was significantly lower than soil water content of control from day 14, reaching minimal values at the end of the experiment (43.5 ± 0.7 %; *P* < 0.001). All plants remained alive until the end of the treatment including stressed plants. Stressed plants however exhibited premature flowering starting on day 14: 5 %, 15.1 % and 45.35 % of stressed plants flowered after 14, 21 and 29 days of stress, respectively. In contrast, most control plants remained at the vegetative stage since only 2 and 4 % of these plants exhibited flowering after 21 and 29 days.

Stem length remained rather low in control plants until day 21 and then abruptly increased reaching 35.4 ± 2 cm at the end of the treatment (Fig. 2A). Stressed plants did not encounter this elongation process and stem length remained lower than in controls (*P* < 0.001). The leaf number linearly increased in control plants reaching 10.83 ± 0.17 at the end of the experiment (Fig. 2B). The rate of leaf appearance started to decline after 21 days of stress exposure but difference recorded between control and stressed plants was significant

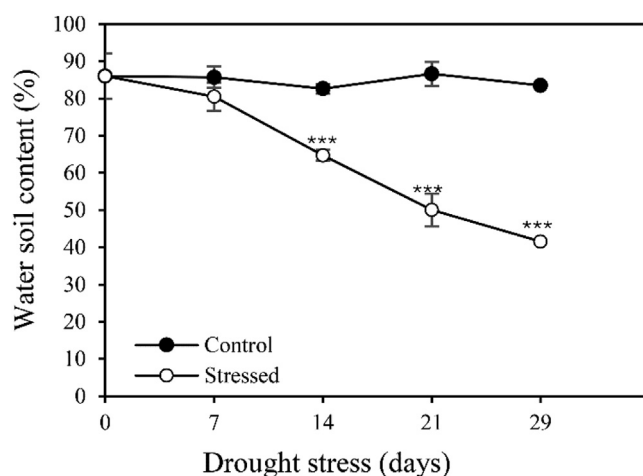


Fig. 1. Soil water content in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean (*n* = 5). Asterisks indicated significant difference with control at * *P* ≤ 0.05, ** *P* ≤ 0.01, *** *P* ≤ 0.001 by Duncan test.

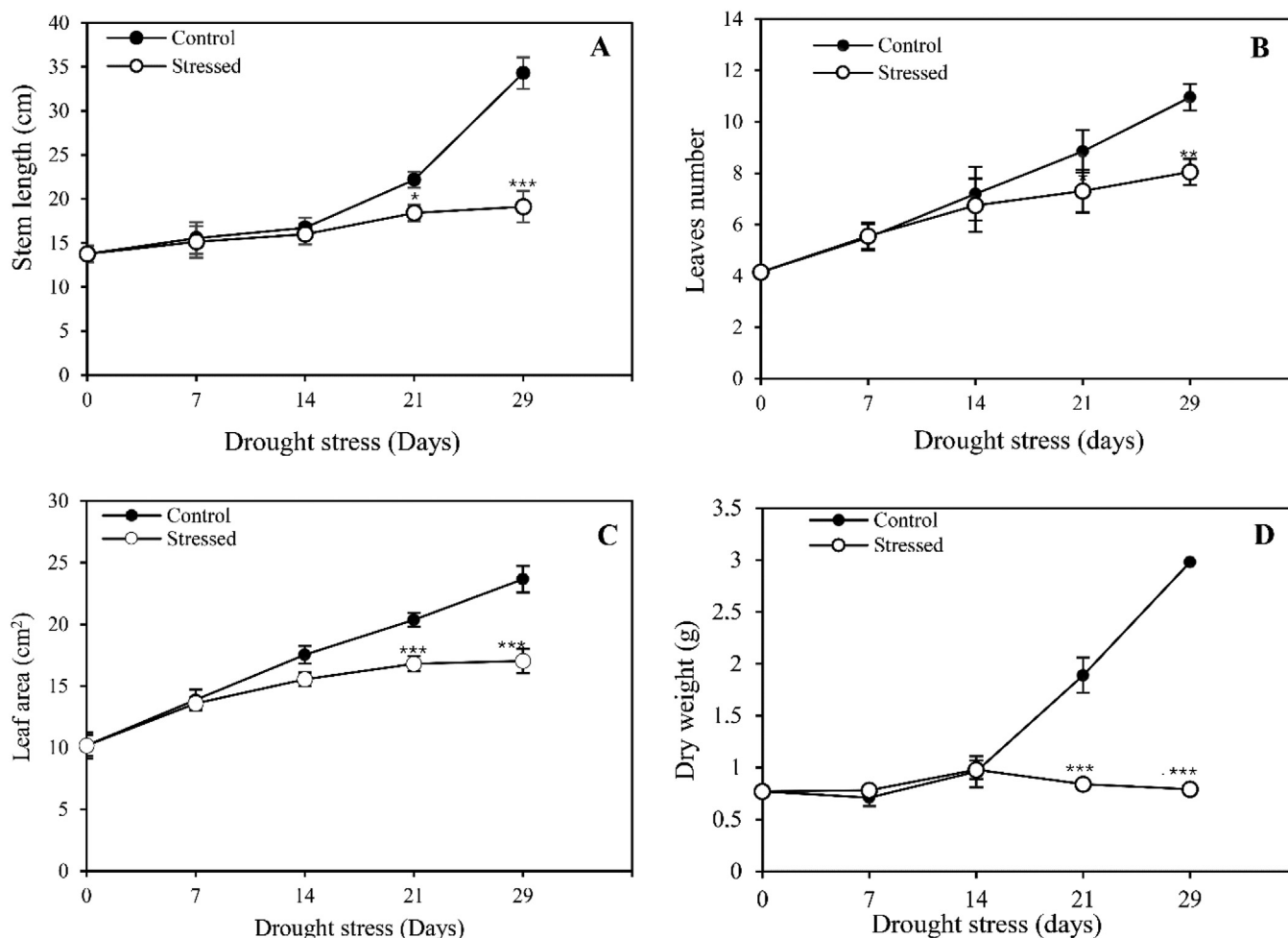


Fig. 2. Stem length (A), leaf number (B), total leaf area (C) and shoot dry weight (D) in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean ($n = 20$). Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Duncan test.

at the end of the stress period only. The total leaf area was lower in stressed plants from 14 days until the end of the treatment (Fig. 2C). The shoot total dry weight remained similar in control and stressed plants during the two first weeks of experiment and dry weight only slightly increased for both groups of plants during this period (Fig. 2D). From day 14 until the end of the treatment, shoot dry weight of control plants then started to strongly increase while the shoot DW of stressed plants remained constant and was significantly lower than in controls ($P < 0.001$).

3.2. Plant water status and photosynthesis-related parameters

The leaf RWC was lower in stressed plants than in controls already after 14 days of stress (Table 1). At day 29, it dropped to 36.98 % in stressed plants comparatively to 94.24 % in control ones ($P < 0.001$). The leaf water content remained unaffected until day 21 in stressed plants but was 20 % lower in drought-treated plants than in controls at the end of the experiment. The plant water potential (ψ_w ; Table 1) started to decline in stressed plants before the osmotic potential (ψ_s ; Table 1). A slight decrease in ψ_w was already recorded after 7 days of treatment in stressed plants but the difference was not significant. A significant difference in ψ_w ($P < 0.05$) between the two groups was recorded after 14 days of stress. The leaf osmotic potential (ψ_s ; Table 1) remained similar in stressed and control plants after 7 and 14 days and the recorded decrease in ψ_s after 21 days was not significant. At the end of

stress exposure, however, ψ_s was significantly lower in stressed plants comparatively to controls ($P < 0.01$).

Stomatal conductance (g_s) dropped to very low values in stressed plants while it remained constant in control ones (Fig. 3A). The recorded decrease was already significant after 14 days of treatment. Similarly, despite a slight increase at day 21, C_i values remained constant in control plants but significantly decreased in stressed ones ($P < 0.001$) after 21 and 29 days of treatment (Fig. 3B). Net photosynthesis (A) followed a similar trend with minimal values recorded in stressed plants at the end of the stress exposure. Instantaneous transpiration (E) remained constant during the two first weeks of treatment in stressed plants and slightly increased at day 14 in control ones (Fig. 3C and D). E values decreased during the third week in both control and stressed plants but remained always significantly lower in the latter than in the former. During the last week of treatment, E remained constant in control plants but it still decreased in stressed one reaching value lower than $0.5 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ at day 29.

Both chlorophyll a and b remained unaffected by water stress during the two first weeks of treatments and then significantly decreased during the following weeks in stressed plants (Table 2; $P < 0.05$). The Chl a/ Chl b ratio however remained constant throughout the duration of the culture and was unaffected by water stress. In contrast, carotenoids concentrations were higher in stressed plants than in controls at the end of the stress exposure ($P < 0.05$). Maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m)

Table 1

Relative water content (RWC; in %), water content (WC; in %), water potential (Ψ_w ; in MPa) and leaf osmotic potential (Ψ_s ; in MPa) in control (C) and stressed plants (S) of *Eruca sativa* Mill. during 29 days of water stress after withholding irrigation. Control plants were cultivated at 80 % field capacity. For a given parameter, means followed by the same letter are not significantly different at $P \leq 0.05$ according to the Duncan test.

Water stress	0 days	7 days	14 days	21 days	29 days
RWC (%)	94.56 $\pm$ 2.12 a	C 93.02 $\pm$ 1.04 a S 97.32 $\pm$ 1.64 a	C 94.51 $\pm$ 0.73 a S 84.49 $\pm$ 1.28 b	C 96.61 $\pm$ 1.57 a S 74.51 $\pm$ 2.88 c	C 94.24 $\pm$ 1.83 a S 36.98 $\pm$ 4.29 d
WC (%)	94.32 $\pm$ 0.01 a	C 93.85 $\pm$ 0.07 a S 94.62 $\pm$ 0.03 a	C 95.01 $\pm$ 0.04 a S 94.56 $\pm$ 0.01 a	C 95.90 $\pm$ 0.06 a S 88.67 $\pm$ 0.01 b	C 95.18 $\pm$ 0.04 a S 76.16 $\pm$ 0.03 c
Ψ_w (MPa)	−0.502 $\pm$ 0.143 a	C −0.47 $\pm$ 0.11 a S −0.68 $\pm$ 0.09 a	C −0.52 $\pm$ 0.14 a S −0.97 $\pm$ 0.08 b	C −0.56 $\pm$ 0.07 a S −1.27 $\pm$ 0.11 c	C −0.53 $\pm$ 0.11 a S −2.01 $\pm$ 0.14 d
Ψ_s (MPa)	−1.00 $\pm$ 0.04 a	C −1.10 $\pm$ 0.01 a S −1.13 $\pm$ 0.01 a	C −1.04 $\pm$ 0.11 a S −1.19 $\pm$ 0.08 a	C −1.18 $\pm$ 0.13 a S −1.35 $\pm$ 0.16 a	C −1.08 $\pm$ 0.05 a S −2.69 $\pm$ 0.09 b

slightly decreased at the end of stress exposure but the recorded decrease was not significant (Fig. 4A). No difference between stressed and control plants was recorded for Φ_{PSII} values (Fig. 4B). After 21 and 29 days of treatment, q_P values were slightly higher in stressed plants but once again, the difference was not significant (Fig. 4C). Similarly, drought had no impact on NPQ values (Fig. 4D).

3.3. Osmolyte synthesis and oxidative stress-related parameters

Proline and glycinebetaine quickly increased in stressed plants (Table 3). A significant increase in both compounds was already recorded after 7 days of treatment and their concentrations

culminated at the end of stress exposure. Malondialdehyde and H_2O_2 remained similar in control and stressed plants until day 14 (Table 3). MDA then progressively accumulated on day 21 and 29 while H_2O_2 increased at day 21 and thereafter remained constant. The DPPH and the FRAP assays provided contrasting data (Fig. 5). According to the DPPH measurements total antioxidant power of the AOAM fraction already increased by more than 600 % in stressed plants after 7 days of treatment ($P < 0.001$) and then remained constant until day 21 before exhibiting a slight decrease at the end of the stress exposure (Fig. 5A). The AOAD fraction increased by 17 % at day 14 ($P < 0.01$) and then increased by more than 100 % after 3 and 4 weeks of treatment (Fig. 5B). According to the FRAP measurement the AOAM

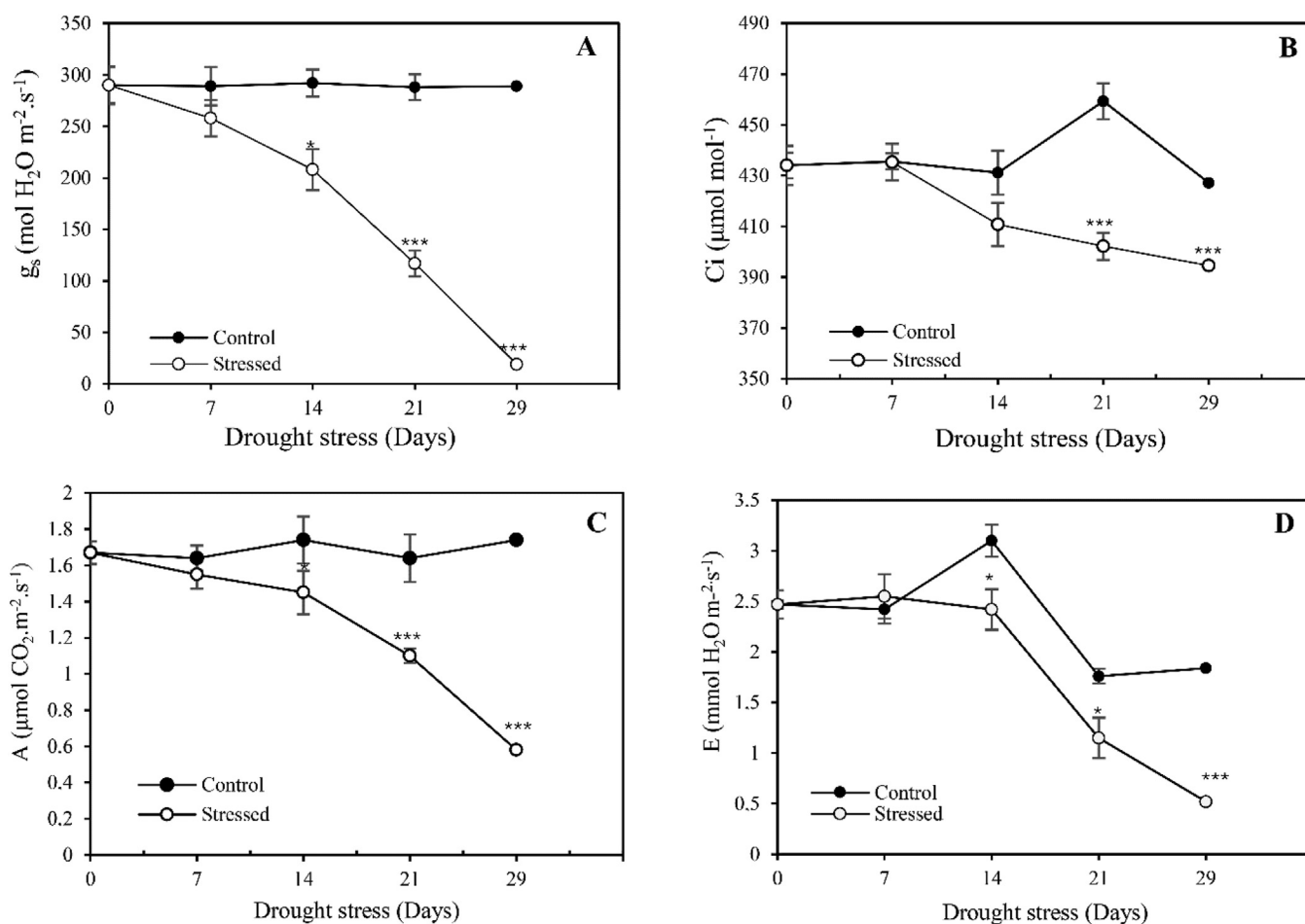


Fig. 3. Stomatal conductance (g_s ; A), intercellular CO_2 concentration (C_i ; B), net photosynthesis (A ; C) and instantaneous transpiration (E ; D) in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean ($n = 5$). Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Duncan test.

Table 2

Chlorophyll a, chlorophyll b, carotenoids concentration, and chlorophyll a/chlorophyll b ratio in control (C) and stressed plants (S) of *Eruca sativa* Mill. during 29 days of water stress after withholding irrigation. Control plants were cultivated at 80 % field capacity. For a given parameter, means followed by the same letter are not significantly different at $P \leq 0.05$ according to the Duncan test.

Water stress	0 days	7 days	14 days	21 days	29 days
Chlorophyll a (mg.g ⁻¹ DW)	6.2 ± 0.94 a	C 6.24 ± 0.15 a S 6.39 ± 0.29 a	C 5.81 ± 0.12 a S 5.33 ± 0.15 a	C 6.54 ± 0.24 a S 4.82 ± 0.62 b	C 6.64 ± 0.09 a S 4.21 ± 0.42 b
Chlorophyll b (mg.g ⁻¹ DW)	2.15 ± 0.43 a	C 2.28 ± 0.03 a S 2.50 ± 0.07 a	C 2.21 ± 0.03 a S 2.19 ± 0.13 a	C 2.37 ± 0.02 a S 1.78 ± 0.04 b	C 2.35 ± 0.05 a S 1.41 ± 0.11 b
Carotenoids (mg.g ⁻¹ DW)	1.16 ± 0.04 a	C 1.46 ± 0.12 a S 1.48 ± 0.06 a	C 1.51 ± 0.16 a S 1.68 ± 0.05 a	C 1.55 ± 0.18 a S 1.99 ± 0.29 a	C 1.59 ± 0.20 a S 2.23 ± 0.22 b
Chla/ Chlb (mg.g ⁻¹ DW)	2.88 ± 0.02 a	C 2.73 ± 0.18 a S 2.55 ± 0.02 a	C 2.62 ± 0.09 a S 2.43 ± 0.24 a	C 2.75 ± 0.05 a S 2.70 ± 0.08 a	C 2.82 ± 0.31 a S 2.98 ± 0.12 a

fraction did not increase during the two first weeks of stress while the AOAD fraction already increased by more than 100 % after 7 days of stress comparatively to controls (Fig. 5C and D). The AOAM fraction then increased by c.a. 30 % after 3 and 4 weeks of treatment.

3.4. Endogenous antioxidant and phenolic compounds concentrations

Endogenous concentrations of glutathione and ascorbate are indicated in Fig. 6. The total glutathione remained similar in control and

stressed plants until day 21. After 3 and 4 weeks of stress, total glutathione was significantly higher in stressed plants than in controls. Reduced (GSH) and oxidized (GSSG) glutathione however exhibited contrasting trends (Fig. 6B and C). Indeed, GSH already increased after 14 days of stress and a very high level of GSH accumulation was recorded in stressed plants comparatively to controls after 29 days of drought exposure. In contrast, GSSG remained similar in control and stressed plants until day 21; it then decreased and reached quite low values in stressed plants at the end of drought exposure. Total

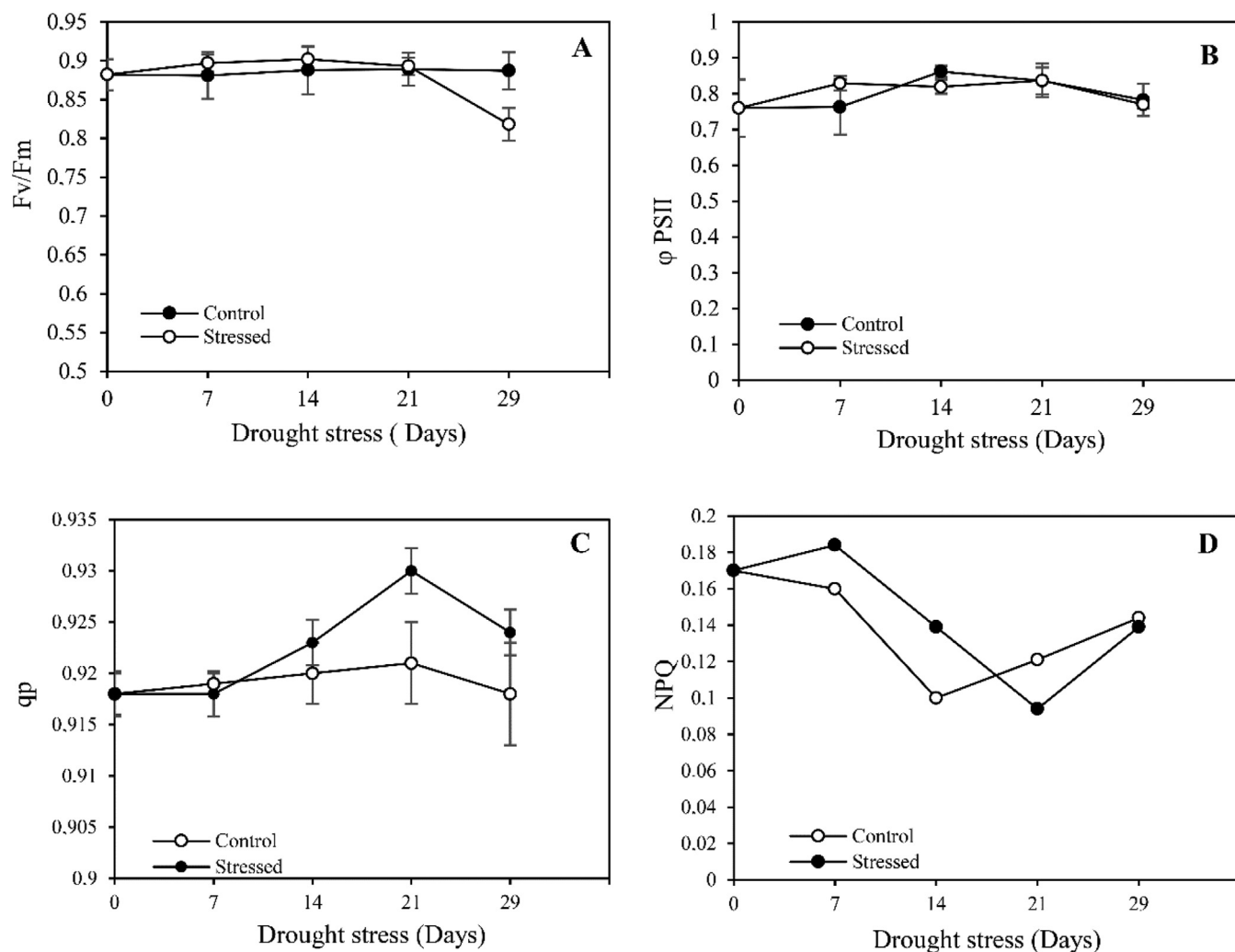


Fig. 4. Maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m ; A), operational efficiency of PSII photochemistry (Φ_{PSII} ; B), photochemical quenching (q_p ; C) and non-photochemical quenching (NPQ; D) in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean ($n = 5$). Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Duncan test.

Table 3

Proline, glycinebetaine, malondialdehyde (MDA) and H_2O_2 concentration in control (C) and stressed plants (S) of *Eruca sativa* Mill. during 29 days of water stress after withholding irrigation. Control plants were cultivated at 80 % field capacity. For a given parameter, means followed by the same letter are not significantly different at $P \leq 0.05$ according to the Duncan test.

Water stress	0 days	7 days	14 days	21 days	29 days
Proline ($\mu\text{moles. g}^{-1}$ DW)	32.31 $\pm$ 4.98 a	C 35.44 $\pm$ 6.49 a S 51.27 $\pm$ 6.16 b	C 33.73 $\pm$ 1.40 a S 91.23 $\pm$ 5.34 c	C 39.10 $\pm$ 3.53 a S 112.71 $\pm$ 3.81 cd	C 36.53 $\pm$ 1.76 a S 122.25 $\pm$ 5.29 d
Glycinebetaine ($\mu\text{moles. g}^{-1}$ DW)	15.37 $\pm$ 0.87 a	C 17.14 $\pm$ 0.21 a S 38.33 $\pm$ 3.45 b	C 16.17 $\pm$ 0.47 a S 40.26 $\pm$ 0.21 b	C 18.65 $\pm$ 1.11 a S 42.36 $\pm$ 0.47 b	C 17.56 $\pm$ 0.88 a S 57.14 $\pm$ 1.07 c
MDA (mmole.g ⁻¹ DW)	0.34 $\pm$ 0.04 a	C 0.35 $\pm$ 0.03 a S 0.33 $\pm$ 0.03 a	C 0.38 $\pm$ 0.00 a S 0.38 $\pm$ 0.02 a	C 0.37 $\pm$ 0.02 a S 0.52 $\pm$ 0.02 b	C 0.39 $\pm$ 0.02 a S 0.71 $\pm$ 0.03 c
H_2O_2 ($\mu\text{moles. g}^{-1}$ DW)	52.65 $\pm$ 7.54 a	C 53.43 $\pm$ 0.84 a S 55.54 $\pm$ 3.30 a	C 49.42 $\pm$ 3.56 a S 58.65 $\pm$ 1.29 a	C 52.90 $\pm$ 1.81 a S 97.57 $\pm$ 4.73 b	C 51.85 $\pm$ 4.90 a S 98.86 $\pm$ 2.96 b

ascorbate (Fig. 6D), reduced ascorbate (Fig. 6E) and oxidized ascorbate (Fig. 6F) did not differ in control and stressed plants after 7 and 14 days of treatment. After 21 and 29 days of treatment, all forms of ascorbate were higher in stressed comparatively to control plants, differences being higher after 21 than after 29 days of treatment. The total polyphenol concentrations significantly increased in stressed plants after 14 days of drought ($P < 0.05$), reached a maximal value at day 21 and then remained constant until day 29 (Fig. 7A). Total flavonoids concentration was two times higher in stressed plants than in controls after 7, 14 and 21 days of treatment, and it then strongly increased by more than 400 % at the end of stress exposure (Fig. 7B). In contrast, total anthocyanin remained similar in control and stressed plants after 21 days of treatment and reached maximal values at the end of stress exposure (Fig. 7C).

4. Discussion

All plants remaining alive until the end of the treatment: this suggests that *Eruca sativa* was able to cope with water progressive water shortage. However, *E. sativa* clearly displayed a drought escape strategy which implies premature flowering and a shorter vegetative growth. Early flowering time is a strategy adopted by numerous plants to maximize the chance of reproduction under adverse environmental conditions (Kazan and Lyons, 2016). In the present work, plant dry weight and total leaf area strongly increased in control plants after 14 days of treatment but remained rather low in stressed ones. Transition from vegetative to reproductive growth requires reallocation of biochemical and metabolic resources throughout the plant (Shavrukov et al., 2017; Chen et al., 2023) and it is therefore

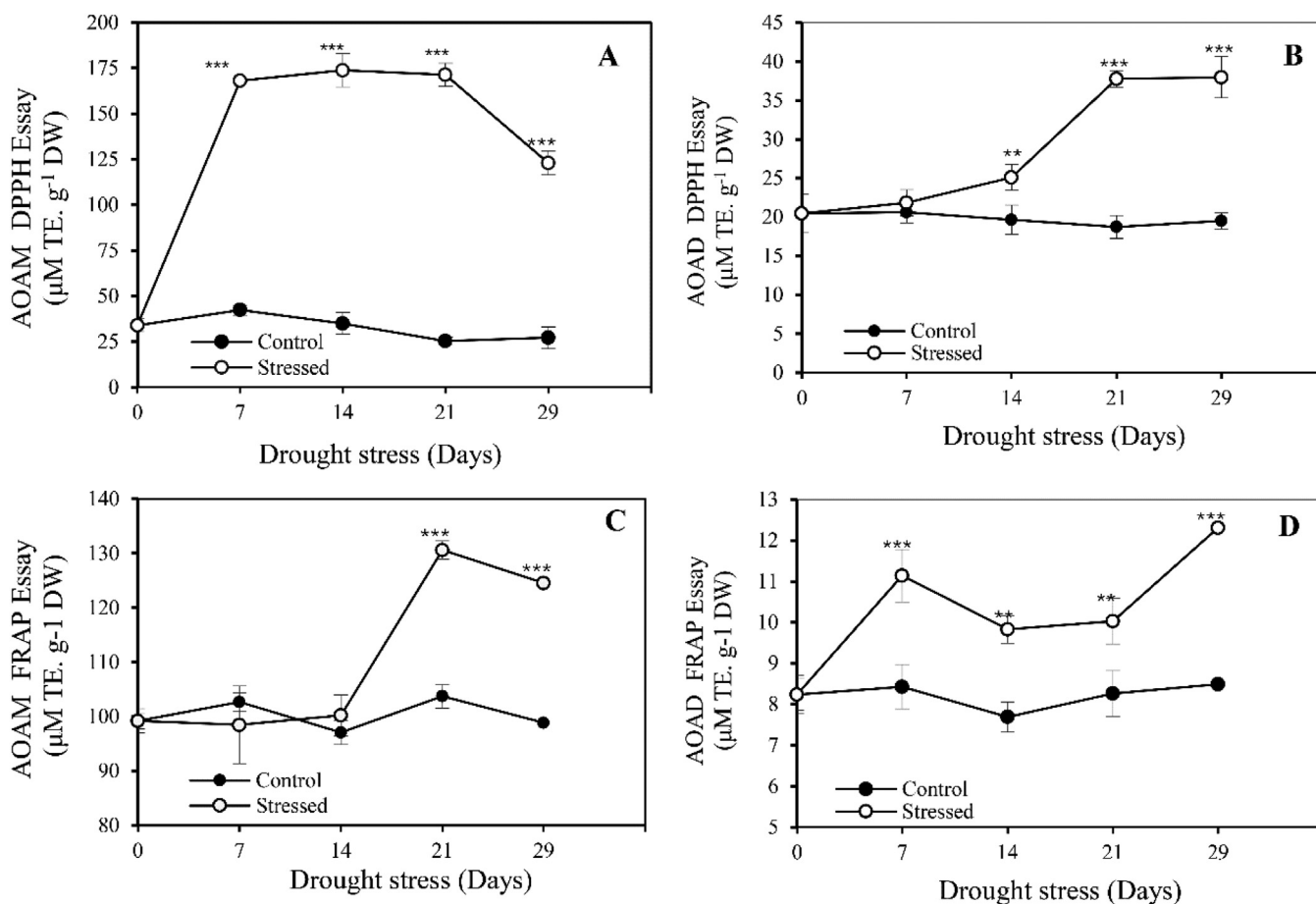


Fig. 5. Activity of hydrophilic (AOAM; A) and hydrophobic (AOAD; B) antioxidants by DPPH assay and activity of hydrophilic (AOAM; C) and hydrophobic (AOAD; D) antioxidants by FRAP assay in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean ($n = 5$). Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Duncan test.

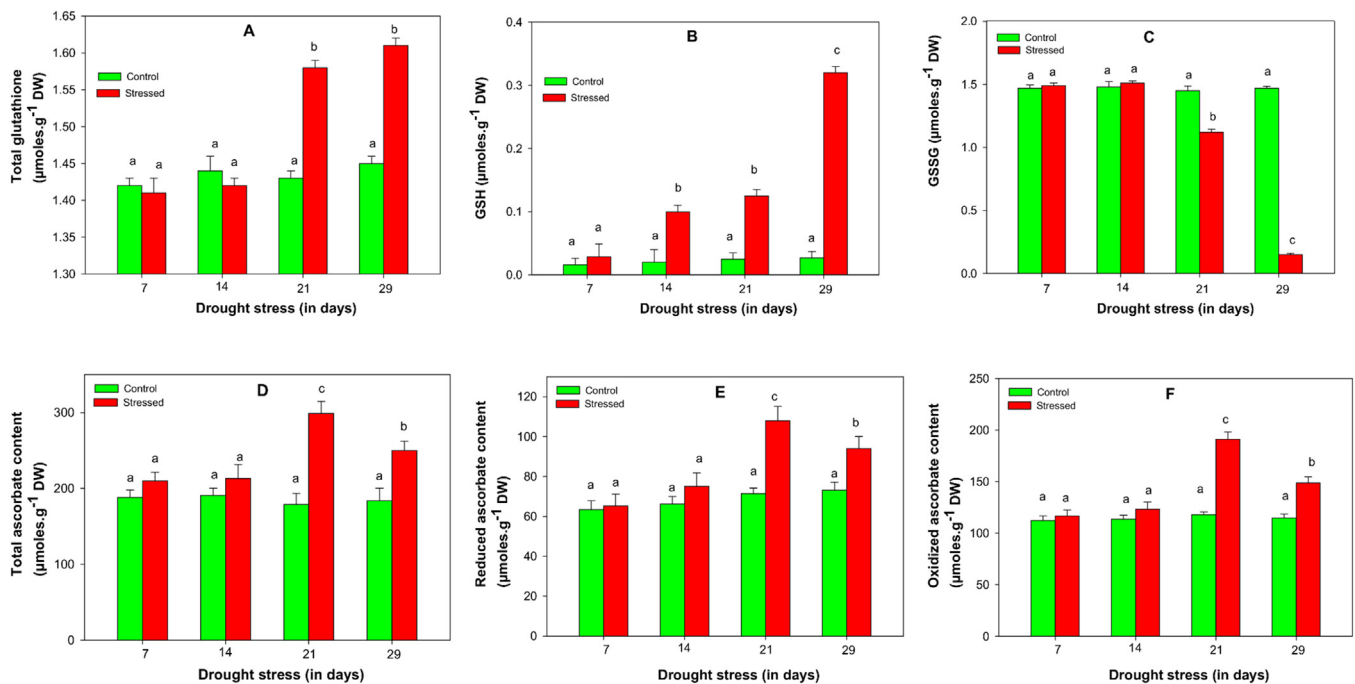


Fig. 6. Total, reduced (GSH) and oxidative (GSSG) forms of glutathione (A; B and C); total, reduced and oxidative forms of ascorbate (D; E and F) in control and drought stressed plants of *Eruca sativa* Mill. Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Bars represent standard error of mean ($n = 5$). Means with the same letter are not significantly different at $P \leq 0.05$ according to the Duncan test.

tempting to speculate that vegetative growth inhibition recorded in stressed plants might be, at least partly, related to plant adaptation related to drought escape rather than to deleterious symptom of stress injury. It is well established that water stress modifies the plant phytohormonal profile and plant growth regulators may assume key functions in premature flowering (Tran, 2023). Nevertheless, it is frequently considered that early flowering related to drought escape shows benefits only in conditions of strong terminal drought events, at a time when plant biomass is sufficient to ensure enough photosynthate production to sustain flower development (Kazan and Lyons, 2016; Shavrukov et al., 2017; Chen et al., 2023). The scenario was somewhat different in the present study since plants experienced a progressive exposure to mild drought. Under these circumstances, some plants such as the model plant species *Arabidopsis thaliana* presented high water use efficiency and late flowering leading to drought avoidance rather than drought escape, the plant remaining able to survive and eventually to recover from temporary stress (Kenney et al., 2014; Schmalenbach et al., 2014). According to our data, the considered population of *E. sativa* did not fit with this model: instantaneous WUE (estimated on the basis of A/E ratio) did not strongly differ between control and stressed plants (mean value of 0.78 ± 0.04 and $0.83 \pm 0.06 \mu\text{moles CO}_2.\text{mmoles}^{-1} \text{H}_2\text{O}$ for control and stressed plants, respectively ($P > 0.05$)) and almost half of the stressed plants flowered at the end of the stress period. This suggest that the low biomass of stressed plants and their lower photosynthesis were sufficient to allow flowering, although we did not analyze the efficiency of the post-anthesis processes in the present work.

Table 4 summarize the kinetic of appearance of stress-induced physiological modifications. Proline and glycinebetaine (GB) accumulations were among the first traits exhibiting a significant modification after already 7 days of treatment (Tables 3 and 4). At this time, neither soil water content (Fig. 1) nor leaf WC (Table 1) were significantly affected by water stress. Moreover, these osmocompatible solutes are thought to play a key role in osmotic adjustment, but leaf ψ_s remained unaffected by water stress until day 29, despite the early-occurring accumulation of proline and GB. These data suggest that

signal transduction processes leading to proline and GB accumulation were quite efficiently triggered in response to imperceptible environmental modification in *E. sativa*. Numerous transcriptional factors are regulating proline synthesis and genes coding for enzymes involved in proline accumulation exhibit numerous *cis*-regulatory elements which could be recognized by HD-HOX, AP2/EREBP, MYB, C2C2-Dof and bZIP transcription factor (Yan et al., 2025). These authors reported that ABA-dependent mechanisms leading to proline biosynthesis, SnRK2 protein kinases pathways leading to transcription factor phosphorylation, calcium-dependent protein kinases pathway and mitogen-activated protein kinase may all occur very quickly in plants, even after a few hours of stress exposure.

The fact that proline and GB accumulation occurred a long time before osmotic adjustment suggests either that their level of accumulation at the beginning of the treatment was not sufficient to induce a significant ψ_s decrease and/or that these compounds accumulated in specific intracellular compartments. Glycinebetaine is often considered to exhibit a chloroplastic accumulation since choline mono-oxygenase (EC 2.4.15.7) and betaine aldehyde dehydrogenase (EC 1.2.1.8) involved in the two steps synthesis from choline are encoded by the chloroplastic genome (Annunziata et al., 2019; Huang et al., 2020). Proline is thought to accumulate in cytosol and in specific organelles (mainly mitochondria and chloroplast) (Zheng et al., 2021; Hoermiller et al., 2022). Alternatively, both compounds may assume other functions than osmotic adjustment. Although ψ_s was modified by 160 % between day 14 and day 29, proline and GB concentration increased only by 34 and 24 %, respectively, during the same period therefore suggesting that other compounds may be involved in osmotic adjustment. However, the fact that both compounds increased during the last week of treatment confirmed that stressed plants remained alive and metabolically active. Indeed, proline and GB concentration in Table 3 were expressed on a DW basis and not on a FW basis: hence, an increase in concentration should not be regarded as a passive process resulting from a decrease in water content but rather as an active process linked to compound synthesis. Proline may protect cellular structures and stabilize enzyme

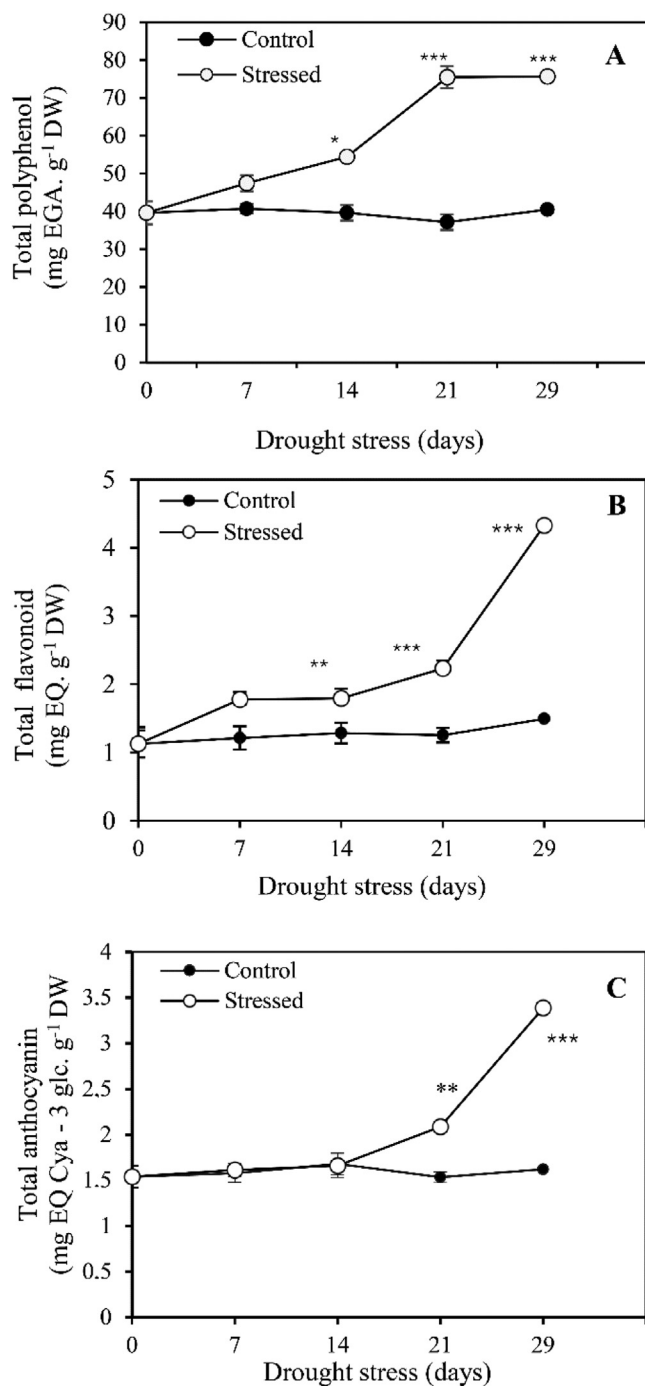


Fig. 7. Total polyphenol (A), total flavonoid (B), and total anthocyanin (C) concentrations in control and drought stressed plants of *Eruca sativa* Mill. Bars represent standard error of mean ($n = 5$). Control plants were maintained at 80 % field capacity while stressed plants were exposed to progressive drought during 29 days. Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Duncan test.

conformation. It may act as a signaling molecule and as a reducing equivalent shuttle (Zheng et al., 2021; Alvarez et al., 2022; Ghosh et al., 2022; Raza et al., 2023). Several lines of evidence clearly demonstrate that proline may assume key functions in the management of oxidative stress in plants (Zulfiqar and Ashraf, 2023). Similarly, the protective role of GB on PSII tridimensional structure is well established. Indeed, GB is able to interact with both hydrophilic and hydrophobic domains of macromolecules: GB stabilizes the intact structure of PSII to ensure its normal active state and it especially protects the

vulnerable D1 protein which play a key role in the photochemistry of PSII (Annunziata et al., 2019; Huang et al., 2020). Hence, the early synthesis of proline and GB might explain the recorded increase in AOAM total antioxidant activity already recorded after 7 days by the DPPH assay, as well as the absence of any deleterious impact of water stress on chlorophyll fluorescence-related parameters (Fig. 4).

Photosynthesis is a complex physiological property which relies on several interacting parameters. The alteration of one single parameter may be sufficient to impair the whole photosynthetic process. It is frequently considered that photosynthesis inhibition induced by abiotic stresses may be related to non-stomatal and/or stomatal causes (Chauchan et al., 2023). Since the light phase of photosynthesis remained unaltered by water shortage in *Eruca sativa*, drought-induced inhibition of photosynthesis was mainly attributable to stomatal closure (Table 4). Intrinsic water use efficiency (A/g_s) remained constant in control plants with a mean value of $0.1536 \mu\text{moles CO}_2 \text{ moles}^{-1} \text{ H}_2\text{O}$ while the value already dropped to $0.0064 \mu\text{moles CO}_2 \text{ moles}^{-1} \text{ H}_2\text{O}$ after only 7 days of treatment in stressed plants although it re-increased to 0.305 at the end of stress exposure. The carboxylation efficiency (A/C_i) also remained constant in control plants ($3.9 \text{ mmol.m}^{-2}.\text{s}^{-1}$) but linearly decreased in stressed plants from 3.6 to $1.5 \text{ mmol.m}^{-2}.\text{s}^{-1}$. The fact that C_i decreased during stress exposure once again confirmed that plants remained alive at the end of the treatment. Nevertheless, the lowest carboxylation efficiency recorded for plants exhibiting the lowest conductance and the lowest C_i value is in accordance with the work of Fleixas et al. (2006) who stated that decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. It is also noteworthy that despite a decrease in E values in stressed plants, NPQ did not increase and this could be related to the maintenance in carotenoid concentration allowing dissipation of an excess of energy through conversion of violaxanthine to zeaxanthine via the xanthophyll cycle (Maxwell and Johnson, 2000; Bandurska, 2022; Swoczyna et al., 2022). Chlorophyll a and chlorophyll b decreased only slightly after 14 days of stress exposure and Chla/Chlb ratio remained constant: chlorophyll decrease was therefore not considered as the cause of photosynthesis inhibition at the beginning of drought treatment.

Total antioxidant activities (Fig. 5) were also quickly modified in stressed plants already after 7 days of treatment concomitantly with proline and GB accumulation (Table 4). FRAP and DPPH assays however provided contrasting results since antioxidant power of the hydrophilic fraction rapidly increased in the latter while lipophilic fraction increased in the former. Investigation of antioxidant capacity is complex and no single method is capable to fully depict the natural reaction occurring in vivo. Numerous authors therefore recommend to use several methods to encompass the total antioxidant behavior of biological tissues (Thaipong et al., 2006; Müller et al., 2011; Sethi et al., 2020; Rumpf et al., 2023). In the present study, extraction procedures were similar for both assays implying that discrepancies between FRAP and DPPH assays should result from difference of sensitivity towards extracted antioxidants but not to differences in the presence of antioxidants in the extracts. FRAP and DPPH assays rely on distinct mechanisms: the FRAP method implies a single electron transfer leading to reduction of ferric ion, while the DPPH method also involves an additional hydrogen atom transfer which is relatively slow comparatively to electron transfer (Rumpf et al., 2023). The FRAP assay has the disadvantage of non-physiological low pH (3.6) during measurement which is required to maintain iron solubility (Müller et al., 2011; Sethi et al., 2020).

Proline, ascorbic acid, glutathione and most phenolics (including anthocyanins) are mainly present in the hydrophilic fraction and the early increase recorded by DPPH assay for AOAM fraction may be ascribed to proline and GB, but not to glutathione and ascorbate whose concentrations increase started latter on day 21 (Fig. 6; Table 4). The increase of AOAM fraction in the FRAP assay was

Table 4

Summary table showing levels of significance of the impact of water stress on morpho-physiological parameters recorded in plants of *Eruca sativa* L. during 29 days of progressive water stress resulting from irrigation withholding. Control plants were cultivated at 80 % field capacity. Asterisks indicated significant difference with control at * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, Not significant (NS) by Duncan test.

Water stress	0 days	7 days	14 days	21 days	29 days
Water soil content	NS	NS	**	***	***
RWC	NS	NS	NS	**	***
Ψ_w	NS	NS	*	***	***
Ψ_s	NS	NS	NS	NS	**
Shoot high	NS	NS	NS	**	***
Leaves number	NS	NS	NS	NS	**
Leaf area	NS	NS	NS	***	***
Dry weight	NS	NS	NS	***	***
Flowering %	NS	NS	NS	***	***
g_s	NS	NS	*	**	***
C_i	NS	NS	NS	***	***
A	NS	NS	*	***	***
E	NS	NS	*	*	***
F_v/F_m	NS	NS	NS	NS	NS
Φ_{PSII}	NS	NS	NS	NS	NS
q_p	NS	NS	NS	NS	NS
NPQ	NS	NS	NS	NS	NS
Chl a	NS	NS	NS	*	*
Chl b	NS	NS	NS	*	NS
Carotenoids	NS	NS	NS	NS	*
Chl a/Chl b	NS	NS	NS	NS	NS
Proline	NS	***	***	***	***
Glycinebetaine	NS	*	**	**	***
MDA	NS	NS	NS	*	**
H_2O_2	NS	NS	NS	***	***
AOAM FRAP	NS	NS	NS	***	***
AOAD FRAP	NS	***	**	**	***
AOAM DPPH	NS	***	***	***	***
AOAD DPPH	NS	NS	***	***	***
Total ascorbate	NS	NS	NS	***	***
Oxydized ascorbate	NS	NS	NS	***	***
Reduced ascorbate	NS	NS	NS	***	***
Total Glutathione	NS	NS	NS	***	**
GSH	NS	NS	*	**	***
GSSG	NS	NS	NS	***	**
Total polyphenols	NS	NS	*	***	***
Total flavonoids	NS	NS	**	**	***
Total anthocyanins	NS	NS	NS	**	***

significant only at day 21 and 29 and may thus reflect the recorded increase in total GSH and ascorbate. However, it was also strongly correlated with anthocyanin accumulation (Fig. 7). These water-soluble compounds assume key functions as antioxidants but may also be stored in vacuoles and remain stable at low pH close to the pH value used during FRAP assay (Dabrowski and Isayenkov, 2023). Carotenoid and α -tocopherol are lipophilic antioxidant and should therefore be present in AOAD fraction. According to Müller et al. (2011), DPPH could hardly detect physiological concentrations of carotenoids mainly due to steric hindrance in cyclic carotenoids. This implies that the recorded increase in AOAD fraction recorded in DPPH and in FRAP assays at day 7, 14 and 21 might be due to an increase in α -tocopherol, which was not specifically quantified in the present work. As far as the FRAP assay is concerned, the recorded increase in AOAD fraction occurring between days 21 and 29 might be partly explained by the recorded increase in carotenoid during the last week of stress exposure (Table 2). Moreover, GB is an amphoteric compound and its molecular features allow it to interact with both hydrophilic and hydrophobic domain of macromolecules (Huang et al., 2020). Its presence in both the AOAM and AOAD fractions could therefore not be ruled out.

Beside the drought escape strategy, *Eruca sativa* also displayed a water-saving strategy under progressive drought conditions since it closed its stomata to prevent water losses by transpiration. This

strategy allowed stressed plants to maintain their water content almost until the end of the treatment: even at the end of stress period, leaf water content remained compatible with the maintenance of essential metabolic functions. However, the relative water content was quite low at the end of stress exposure and dropped by more than 50 % between 21 and 29 days of treatment. Such a decrease could not be explained solely by a decrease in WC but rather by an obvious increase in turgid weight (TW; detailed data not shown) and suggests that the capacity of leaf segments to rehydrate strongly increased in a short time when stress intensity increased. In natural conditions, water stress is not necessarily permanent and may occur transiently (Bandurska, 2022; Wu et al., 2022). Hence, the fitness of the plant does not only depend on its capacity to cope with drought events, but also on its ability to quickly recover and rehydrate after the stress relief (Maseda and Fernandez, 2006; Gupta et al., 2020; Huang et al., 2022). It has been demonstrated that plant cells may to some extent anticipate the recovery period and set up strategies allowing them to cope with a brutal rehydration when water starts to be available again (Bajji et al., 2000). However, if water availability itself is also transient or limited from a quantitative point of view, rehydration process should occur rapidly and this is challenging for the plants because a massive rehydration of protoplasm preliminary adapted to a partial desiccation may induce cellular disorders and constitute itself a stress event (Tardieu et al., 2018). Cell wall is an important compartment interacting with water. Strongly bound water may assume some roles in drought tolerance and persists in tissue during dehydration (Rascio et al., 1998, 1999). However, chemical and physical modifications of the cell wall may occur during drought stress and may have an impact on interaction between cell wall and water during subsequent rehydration in relation to the porous structure of cell with inner capillary forces leading to accumulation of free water with strong mobility or to pectin synthesis able to store water (Ding et al., 2021; Maloney et al., 2023). This strategy could have the advantage to “buffer” rehydration, allowing strong rehydration of the cell wall at the time of water availability but progressive rehydration of protoplasm through aquaporin regulation (Boaneres et al., 2024). Proline, which accumulated before RWC decrease (Table 4) may also modulate the stability and integrity of cell wall structure and has an impact on its architecture which could lead to a higher capacity of water storage (Lin et al., 2024).

5. Conclusions

A kinetic study of *Eruca sativa* behavior during progressive soil dehydration allows to depict the sequence of physiological events occurring during the first month after the onset of a drought period. It confirms that argula may be regarded as moderately resistant to water shortage. The first responses detected at the plant level after 7 days may indeed be regarded as tolerance processes in relation to osmoprotectant accumulation (proline and GB) and total antioxidant activities which occurred before injury symptoms appearance. These responses were already detected for marginal decreases in soil and leaf water content. The plant found an optimal compromise between drought escape and water-saving strategies. It reduced vegetative growth and exhibited premature flowering in response to stress. Photosynthesis inhibition occurred after 14 days as a consequence of stomatal closure while chlorophyll fluorescence-related parameters and Chl a/Chl b ratios remained unaltered throughout the stress period suggesting an efficient protection of PSII tridimensional structure. The water potential (Ψ_w) decreased after 14 days before significant decrease in osmotic potential (Ψ_w) suggesting that the early accumulation of osmoprotectant was not sufficient to allow osmotic adjustment. Most of specific antioxidants (ascorbate, glutathione, polyphenols, flavonoids, anthocyanins and carotenoids) started to significantly accumulate after 3 weeks. Their contribution to

antioxidative response was however not sufficient to avoid MDA and H₂O₂ accumulation at the end of stress exposure.

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Data availability

Data sets analysed during the study are available from authors upon personal request

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Selma Barris: Writing – review & editing, Visualization, Investigation, Formal analysis. **Aicha Belkhebir:** Writing – review & editing, Supervision, Conceptualization. **Brigitte Vanpee:** Validation, Methodology, Investigation. **Mohamed Toumi:** Formal analysis. **Fatiha Aïd:** Writing – review & editing, Supervision. **Stanley Lutts:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Conceptualization.

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