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RESEARCH PAPER

Water stress drastically reduces root growth and inulin yield in *Cichorium intybus* (var. *sativum*) independently of photosynthesis

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Abstract

Root chicory (*Cichorium intybus* var. *sativum*) is a cash crop cultivated for inulin production in Western Europe. This plant can be exposed to severe water stress during the last 3 months of its 6-month growing period. The aim of this study was to quantify the effect of a progressive decline in water availability on plant growth, photosynthesis, and sugar metabolism and to determine its impact on inulin production. Water stress drastically decreased fresh and dry root weight, leaf number, total leaf area, and stomatal conductance. Stressed plants, however, increased their water-use efficiency and leaf soluble sugar concentration, decreased the shoot-to-root ratio and lowered their osmotic potential. Despite a decrease in photosynthetic pigments, the photosynthesis light phase remained unaffected under water stress. Water stress increased sucrose phosphate synthase activity in the leaves but not in the roots. Water stress inhibited sucrose:sucrose 1-fructosyltransferase and fructan:fructan 1 fructosyltransferase after 19 weeks of culture and slightly increased fructan 1-exohydrolase activity. The root inulin concentration, expressed on a dry-weight basis, and the mean degree of polymerization of the inulin chain remained unaffected by water stress. Root chicory displayed resistance to water stress, but that resistance was obtained at the expense of growth, which in turn led to a significant decrease in inulin production.

Key words: *Cichorium intybus*, drought, growth, inulin, photosynthesis, root chicory, sugar metabolism, water deficit, water stress.

Introduction

Cichorium intybus var. *sativum* is a biannual crop cultivated mainly in Western Europe and to a lesser extent in other parts of the world to produce inulin, which is a linear β(2,1)-type fructan that is widely used as a prebiotic agent with antioxidant properties (Stoyanova et al., 2011). Inulins selectively stimulate 'good bacteria' (such as *Bifidobacteria* and *Lactobacillae*) in the colon, contributing to overall good health and helping disease prevention (Roberfroid and Delzenne, 1998). During the growing season (first year), inulin is stored in the chicory tap roots as a reserve component.

Abbreviations: A, net CO_2 assimilation rate; DP, degree of polymerization; DW, dry weight; *E*, instantaneous transpiration rate; EDTA, ethylenediaminetetra-acetic acid; 1-FEH, fructan 1-exohydrolase; 1-FFT, fructan:fructan 1 fructosyltransferase; FW, fresh weight; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MDA, malondialdehyde; NPQ, non-photochemical quenching; φ_{PSII}, photosystem II efficiency; q_p, photochemical quenching; SPS, sucrose phosphate synthase; 1-SST, sucrose:sucrose 1-fructosyltransferase; SuSy, sucrose synthase.

Under natural conditions, this reserve allows chicory to survive winter and accomplish its reproductive cycle during the second year (Van Laere and Van den Ende, 2002). Fructans are also known to protect cells from desiccation during water stress, which is in line with their role as membrane stabilizers (Hendry, 1993; Valluru and Van den Ende, 2008). Water stress leads to an increased fructan content in numerous plant species (Spollen and Nelson, 1994; Volaire and Lelièvre, 1997; Kerepesi et al., 1998) and in bryophytes (Marschall et al., 1998). According to Pilon-Smits et al. (1995), transgenic tobacco containing fructans exhibits an improved resistance to water deficit compared with wild-type tobacco.

Inulin synthesis is initiated by sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99), which catalyses the transfer of a fructose moiety between two sucrose molecules to produce glucose and the trisaccharide 1-kestose. Fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100) transfers fructan moieties from and to 1-kestose or larger fructans, resulting in the formation of oligofructose and inulin with a higher degree of polymerization (DP). Conversely, inulin degradation involves a sequential removal of fructose units catalysed by fructan 1-exohydrolases (1-FEHs; EC 3.2.1.153) (Van Laere and Van den Ende, 2002). The mean DP of the inulin chain is an important component of root chicory production, with a higher DP being more suitable for industrial applications (Wilson et al., 2004).

According to the Intergovernmental Panel on Climate Change, scenarios with drier summers are expected to occur in Western Europe in the next few decades (IPCC, 2007). In several parts of the world, root chicory is now cultivated under irrigation, but a better knowledge of its behaviour under water stress conditions should allow the minimal water requirements of this species to be defined and thus allow its culture to be extended to regions where irrigation is not technically feasible or economically justified. Although responses to low temperature have been extensively studied in this species, mainly with respect to vernalization (Dielen et al., 2005; Devacht et al., 2009; Mingeot et al., 2009), the impact of water stress has received less attention. De Roover et al. (2000) studied the impact of water stress at the seedling stage and demonstrated that water shortage increased glucose, fructose, and sucrose concentrations in the roots and leaves of stressed plants, leading to increased fructan concentrations in the roots. Using a field approach, Monti et al. (2005) demonstrated that water shortage had only a limited impact on yield, leaf photosynthetic capacity was poorly affected by water availability, and fructan chain length was not affected by the water regime. Only moderate water stress was applied in this study, and the activities of enzymes involved in inulin metabolism were not quantified. Recently, the severity and timing of drought were reported to strongly influence the sequence of plant reactions to water shortage (Muller et al., 2011; Skirycz et al., 2011).

Water stress that impacts photosynthesis in root chicory has been suggested to mainly be related to non-stomatal effects (Monti et al., 2005). Although data on the impact of drought on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) activity in this species are still missing, this enzyme was shown to be drastically affected in numerous plant species exposed to water shortages (Parry et al., 2002). Although a decline in Rubisco activity is expected to decrease sugar synthesis, water stress often paradoxically increases sucrose content through the stimulation of sucrose phosphate synthase (SPS; EC 2.4.1.14) (Fu et al., 2010). Therefore, the precise sugar status in water-stressed tissues could be considered to be the ultimate consequence of several interacting factors related to growth inhibition, CO_2 fixation, and enzyme activities involved in sucrose synthesis on the one hand (SPS), and sucrose breakdown (acid and neutral invertases; EC 3.2.1.26) on the other. Sucrose synthase (SuSy; EC 2.4.1.13) catalyses a reversible reaction, but it is usually involved in sucrose breakdown and generates UDP-glucose and fructose.

The soluble sugar concentration may directly influence inulin concentration in root chicory: sucrose was reported to increase the expression of the gene encoding 1-SST, leading to an increase in the corresponding enzyme activity (De Roover et al., 2000). According to Van Laere and Van den Ende (2002), vacuolar 1-SST may itself be involved in sink strength determination, thereby contributing to yield maintenance under stress conditions. Conversely, an increase in sucrose was reported to inhibit FEH activities, at least for some isoforms (Verhaest et al., 2007).

The present study was undertaken to evaluate the physiological and yield impacts of water stress on root chicory. Plant behaviour was analysed during and after a significant decrease in the soil water content. This kinetics approach aimed to identify the main morphophysiological parameters contributing to the deleterious impact of water stress on inulin production in relation to enzymatic activities involved in sugar metabolism.

Materials and methods

Plant material and growth culture

Seeds of *C. intybus* var. *sativum* (purified line issued from cultivar Crescendo and kindly provided by the Cosucra Group, Warcoing SA division, Chicoline, Belgium) were sown in columns that were 55 cm long and 25 cm in diameter. The 21 columns were filled with dry yellow sand, and 0.5 dm^3 of loam (loam for professionals; DCM N.V., Grobbendonk, Belgium) was added at the top. The mean temperature during the growing season was 23 °C during the 16-h day and 20 °C during the 8-h night. The mean relative humidity in the air was 75%, and the light intensity was 135 μmol m^{-2} s^{-1} (six Philips HPI-T lamps; 400 W) at the top of the canopy. Four days after germination only one plant was maintained in each column. Plants were provided with a nutrient solution of pH 5.4 and electrical conductivity of 963 μS cm^{-1} containing the following nutrient concentrations: 250 μM NH_4NO_3, 890 μM $Ca(NO_3)_2.4H_2O$, 990 μM KNO_3, 515 μM KH_2PO_4, 244 μM $MgSO_4.7H_2O$, 0.415 μM $MnSO_4.5H_2O$, 6.45 μM H_3BO_3, 0.161 μM $CuSO_4.5H_2O$, 0.0125 μM $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.697 μM $ZnSO_4.7H_2O$, and 10.12 μM Fe-EDDHA [ethylenediamine-*N*,*N*'-bis(2-hydroxyphenylacetic acid)]. To avoid evaporation from the soil surface, the top of each column was covered with a perforated Plexiglas plate, allowing only the basal part of the rosette to emerge.

During the experiment, four harvests were performed at 7, 13, 19, and 25 weeks after sowing. Three plants per treatment were collected at each harvest. The drought stress started 12 weeks after

sowing. At this time, plants were distributed into two groups: control and drought-stressed plants. Water was supplied to the control plants to maintain the volumetric water content (θ_v) between 11 and 19% (close to field capacity: $\theta_v = 15\%$) throughout the culture period, whereas one-fifth of this volume was supplied to the stressed plants, which were permanently exposed to a substrate with less than 3% of its volumetric water content (close to the wilting point: $\theta_v = 2\%$) (Fig. 1).

The total leaf area was measured at each harvest for three plants per treatment using a Leaf Area Meter AM300 (ADC Bioscientific, Hoddesdon, Hertfordshire, UK). The mean duration required for the macroscopic appearance of a new leaf was calculated by dividing the number of days between two measurements by the number of leaves appearing between the measurement times.

Plant water status

The water contents of leaves and roots were measured after drying the samples in an oven for 72 h at 70 °C. The leaf water potential (ψ_w) was measured at each harvest at mid-day (between 12 a.m. and 2 p.m.) on the fifth and sixth unfolded leaves using a Scholander pressure chamber, considering the youngest unfolded leaf of the rosette as no. 1. The osmotic potential (ψs_{raw}) was measured on a portion of the second fully unfolded leaf with a Vapour Pressure Osmometer 5520 (Wescor, Logan, CT, USA) and adjusted to the water content of the control plants according to Lefèvre et al. (2009): $\psi s = \psi s_{raw} * (WC_s/WC_{control})$, where WC_s corresponds to the water content of the stressed plants and $WC_{control}$ to the water content of the control plants.

The stomatal conductance (g_s) was measured using an AP4 system (Delta-T Devices, Cambridge, UK) on the first unfolded leaf and four other randomly selected leaves halfway through the photoperiod.

Photosynthesis-related parameters

Chlorophyll fluorescence-related parameters were measured for five plants per treatment by the Fluorescence Monitoring System II (Hansatech Instruments, Norfolk, UK) on the second and third unfolded leaves after dark-adaption for 30 min. After a saturating pulse (18 000 μmol m^{-2} s^{-1}) was sent to the leaf, the leaf was exposed to a constant intensity of actinic light (600 μmol m^{-2} s^{-1}) for 3 min, followed by a second saturating pulse of 18 000 μmol m^{-2} s^{-1}. Photosystem II efficiency (φ_{PSII}), non-photochemical quenching (NPQ), and photochemical quenching (q_p) were estimated according to Maxwell and Johnson (2000).

Chlorophyll (Chl a and Chl b) and total carotenoid (xanthophylls + β-carotene) concentrations were quantified for three plants per treatment on the sixth fully unfolded leaf in the rosette. Samples [~150 mg fresh weight (FW)] were ground in the dark in 8 ml of 80% acetone and centrifuged at 1000 *g* for 10 min at 4 °C. The absorbance of the sample was read at three different wavelengths (663.2, 646.8, and 470 nm) using a spectrophotometer (DU 640, Beckman Coulter, South Pasadana, CA, USA). Each measurement was repeated three times. The pigment concentrations were calculated according to Lichtenthaler (1987).

Gas exchange was recorded with an infrared gas analyser (LCA4 8.7; ADC Bioscientific, Hoddesdon, Hertfordshire, UK) using a PLC Parkinson leaf cuvette on intact leaves for 1 min (20 records min^{-1}) and an air flow of 300 ml min^{-1}. Air taken in the greenhouses was sent to a chamber into which a leaf portion of 6.25 cm^2 was introduced. The net CO_2 assimilation rate (*A*) and instantaneous transpiration rate (*E*) were estimated on the second and third fully unfolded leaves. Five plants were measured for each treatment, and all measurements were performed around midday (between 12 a.m. and 2 p.m.).

For determination of Rubisco (EC 4.1.1.39), fresh matter was collected on the third, fourth, and fifth unfolded leaves for three plants per treatment, quickly frozen in liquid nitrogen and homogenized in 4 ml of extraction buffer [100 mM Tris/HCl, pH 7.8, containing 0.4 mM ATP, 10 mM $MgCl_2$, 1 mM ethylenediaminetetra-acetic acid (EDTA), 0.1% (v/v) Triton X-100, 12.5% (w/v) glycerol, 15 mM mercaptoethanol, and 30 mg polyvinyl polypyrrolidone]. The extract was centrifuged for 30 s at 10 000 *g* and 4 °C. To measure the initial activity, 30 μl of this extract was rapidly added to 970 μl of a reaction buffer consisting of 50 mM Hepes/KOH, pH 8.0, 20 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 5 mM DTT, 2.5 mM ATP, 5 mM phosphocreatine, 0.2 mM NADH, 0.6 mM ribulose 1,5-bisphosphate, 10 mM $NaHCO_3$, 6 U ml^{-1} phosphoglycerate kinase (Sigma ALdrich, St Louis, MO, USA), 6 U l^{-1} glycerate 3-phosphate dehydrogenase (Sigma), and 20 U ml^{-1} phosphocreatine kinase (Sigma). The oxidation of NADH was determined during a 3 min period at 25 °C by measuring the difference between the absorbances at 340 and 400 nm. The total activity was quantified according to the same procedure after an activation period of 10 min in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$. The activation ratio was

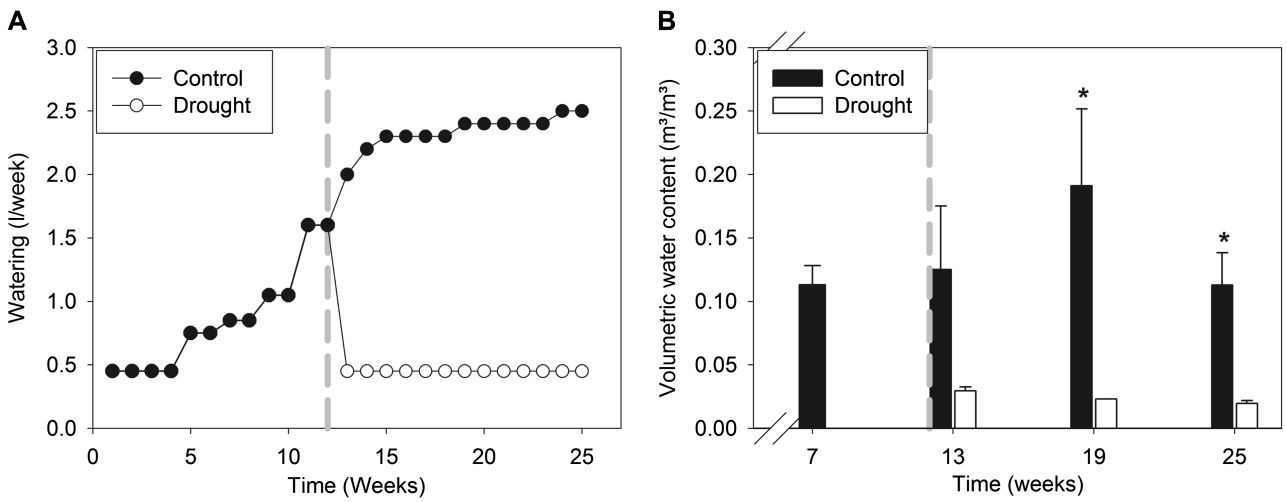

Fig. 1. Volume of nutrient solution supplied to each column (in l $week^{-1}$) (A) and soil volumetric water content (B) during the 25 weeks of plant culture for *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Filled symbols indicate control plants and open symbols indicate drought-stressed plants. In Fig. 1B, each value is the mean of 3 replicates and vertical bars are the standard errors. The stars indicate the presence of a statistical difference between the treatments (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.001$).

calculated by dividing the initial activity by the total activity according to Du et al. (1996). Two replicates were made for each sample.

Leaf malondialdehyde concentration

The level of lipid peroxidation was measured as the 2-thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (MDA), following the modified method of Heath and Packer (1968). Leaf portions (250 mg FW) were ground in liquid nitrogen with 5 ml of a solution of 5% trichloracetic acid and 1.25% glycerol. The homogenate was centrifuged at 6700 *g* for 10 min at 4 °C and then filtered through Whatman paper no. 1 filter paper. Then 2 ml of the supernatant was mixed with 2 ml 0.67% thiobarbituric acid. The samples were incubated at 100 °C for 30 min and immediately transferred to ice for 5 min, followed by centrifugation at 6700 *g* for 1 min at 4 °C. The supernatant absorbance was read at 532 nm, and values corresponding to non-specific absorption (600 nm) were subtracted. MDA concentration was calculated using its molar extinction coefficient ($\epsilon = 155\ mM^{-1}\ cm^{-1}$).

Sugar concentration

Portions of the third, fourth, and fifth unfolded leaves (~300 mg FW) collected from three plants per treatment were ground in liquid nitrogen, mixed with 7 ml of 70% ethanol (w/v) for 5 min on ice and centrifuged at 6700 *g* for 10 min at 4 °C. After reacting 200 µl of the supernatant with 1 ml of an anthrone solution (0.5 g anthrone, 250 ml 95% H_2SO_4, and 12.5 ml distilled water), the absorbance was read at 625 nm (spectrophotometer UV-1800, Shimadzu, Kyoto, Japan) to quantify the total soluble sugars according to Yemm and Willis (1954). A calibration curve was established using glucose as the standard.

The remaining supernatants were dried in a thermomixer using nitrogen (45 min at 50 °C), and sugars were derivatized using 200 µl of an oxymation solution (hydroxylamine chlorhydrate dissolved in a pyridine solution): 100 µl of hexamethyldisilazane and 10 µl of trifluoroacetic acid were added to the samples. Mono-, di-, and trisaccharide concentrations were specifically determined by gas chromatography (Autosystem XL, MN Optima-5 Accent, Perkin Elmer, Waltham, MA, USA; 30 m×0.32 mm internal diameter×0.25 µm) using helium as a carrier gas at a flow rate of 1 ml min^{-1}. The injector and flame ionization detector temperatures were 270 and 310 °C, respectively. The oven temperature was held at 120 °C for 3 min and then programmed to 230 °C at 3 °C min^{-1}. It was kept at this temperature for 12 min, then re-programmed to 300 °C at 20 °C min^{-1} and held for 15 min.

To extract sugars and enzymes from the roots, 30 g of root material was ground in 30 ml of 50 mM Hepes/KOH, pH 7.5, containing 100 mM KCl, 30 mM $MgCl_2$, 10 mM $NaHSO_3$, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1 mM mercaptoethanol, and 0.1% Polyclar (Serva, Heidelberg, Germany). Then, 2 ml was incubated at 90 °C for 15 min. After cooling at room temperature, the extract was centrifuged at 3000 *g* for 5 min, and 700 µl of the supernatant were purified on a column with two ion-exchange resins (1 ml Dowex 50 H^+ and 1 ml Dowex 1-acetate). Finally, 25 µl of the neutralized fraction was analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex, Sunnyvale, CA, USA) to determine the sugar content after separation on a CarboPac® PA1 anion exchange column and detection by a pulsed anperometric detector equipped with a gold electrode (potentials: E_1: + 0.05 V; E_2: +0.6 V; E_3: –0.8 V). The flow rate was 1 $ml.min^{-1}$. The elution conditions were 90 mM NaOH over a 60-min period. The column was regenerated with 1 M NaOH for 10 min and equilibrated for 20 min after each run. Quantification was performed on the peak areas using the external standards methods for glucose, fructose, sucrose, 1-kestose and 1,1-nystose (Van den Ende et al., 1996). In parallel, 25 µl of the neutralized fraction was mixed with 6 N HCl to completely depolymerize all of the inulin into its single components (glucose and fructose). The extract was analysed using the same HPAEC-PAD technique.

The mean DP is calculated according to a formula adapted from Baert (1997):

$$DP_{mean} = \frac{F_{hydrol} - F_{free} - S_{free}}{G_{hydrol} - G_{free} - S_{free}} + 1$$

where F_{hydrol} and G_{hydrol} are the concentrations of fructose (*F*) and glucose (*G*) after hydrolysis with HCl, and F_{free}, G_{free}, and S_{free} are the concentrations of free fructose, glucose, and sucrose (*S*).

The percentage of inulin in the root was estimated using a formula adapted from Baert (1997):

$$Percentage_{inul} = \frac{\left(F_{hydrol} - F_{free} - S_{free} + G_{hydrol} - G_{free} - S_{free}\right) * \left(180 - \frac{18*(DP_{mean}-1)}{DP_{mean}}\right)}{1000 * (1 - WC)}$$

considering that each addition of one fructose unit to the inulin chain induces the loss of one molecule of water (18 g mol^{-1}).

Sugar-metabolizing enzymes

Root 1-SST (EC 2.4.1.99), 1-FFT (EC 2.4.1.100), and 1-FEH (EC 3.2.1.153) activities were determined according to Van den Ende et al. (1996) for three plants per treatment. After 5 ml of the extraction buffer containing the extract was centrifuged for 2 min at 10 000 *g*, two aliquots (2 ml each) were mixed with 4 ml of saturated ammonium sulphate. After 30 minutes at 0 °C, the pellet was centrifuged at 40 000 *g* for 15 min and washed with ammonium sulphate 80% (w/v). The pellet was centrifuged again under the same conditions, suspended in 500 µl of 50 mM sodium acetate buffer, pH 5.0, and then centrifuged for 2 min at 10 000 *g*. The enzymes 1-SST and 1-FEH were incubated with their substrate at 30 °C (100 mM sucrose for 1-SST and 1% inulin for 1-FEH) for 0, 20, 40, and 60 min for 1-SST and 0, 40, 80, and 120 min for 1-FEH. The 1-FFT enzyme was incubated with 10 mM 1-kestose on ice for 0, 20, 40, and 60 min. The reactions were stopped by incubating the samples at 95 °C for 5 min. The enzyme activities were determined by product quantification (1-kestose for 1-SST, 1,1-kestotetraose for 1-FFT, and fructose for 1-FEH) with HPAEC-PAD.

SPS (EC 2.4.1.14), SuSy (EC 2.4.1.13), and invertase (acid and neutral; EC 3.2.1.26) activities were determined in the leaves and roots. For this purpose, 700 mg of material was combined with 3.5 ml of extraction buffer (50 mM Hepes/KOH, pH 7.5, containing 100 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHSO_3$, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 1 mM mercaptoethanol, and 0.1% Polyclar) for leaves, and 5 g of material was combined with 5 ml of the extraction buffer for roots. The extract was then centrifuged at 10 000 *g* for 5 min at 4 °C. Two aliquots (1.5 ml each) were mixed with 6 ml of saturated ammonium sulphate. After 30 min at 0 °C, the pellet was centrifuged at 40 000 *g* for 15 min, washed with 80% (w/v) ammonium sulphate, and centrifuged as before. The obtained pellet was then resuspended in 3 ml of 50 mM Hepes/KOH buffer, pH 7.5, containing 100 mM KCl, 20 mM $MgCl_2$, and 2 mM EDTA. SPS activity was determined according to Huber et al. (1991). SuSy and invertase activities were assessed according to King et al. (1997), with slight modifications involving the use of a 3,5-dinitrosalicylic acid solution (45 mM 3,5-dinitrosalicylic acid, 10% NaOH, and 1 M potassium sodium tartrate) to stop the reaction after incubation.

The protein concentration in the extract was estimated according to Bradford (1976) using bovine serum albumin as standard.

Statistical treatment of the data: At each harvest, three plants per treatment were analysed, except in cases of non-destructive physiological analysis, for which five plants per treatment were used. Each experiment was repeated twice and exhibited similar trends. Data presented hereafter are from one representative experiment. The statistical analysis was performed using SAS

software (SAS System for Windows, version 9.1). The normal distributions of the data were analysed using a Shapiro–Wilk test. When needed, the data were transformed to have a normal distribution.

Data were then subjected to an analysis of variance (ANOVA I) considering the presence or absence of stress as a factor. The statistical significance of the results was analysed by the Student–Newman–Keuls test at the 5% level.

Results

Growth parameters

Drought drastically decreased the rate of leaf appearance in the rosette throughout the stress period: the mean duration required for the macroscopic appearance of a new leaf was always higher in stressed plants than in controls (Fig. 2A). The total leaf surface was lower in stressed plants than in controls (Fig. 2B), as a result of not only the lower number of leaves but also a strong decrease in the mean leaf area (Fig. 2C). The total leaf surface decreased from week 13 to week 19 due to stress-induced senescence leading to leaf abscission (Fig. 2D). The specific leaf area was significantly lower in stressed plants [148.9±14.8 and 170.4±7.5 $cm^2\ g^{-1}$ dry weight (DW) after 19 and 25 weeks, respectively] than in controls (257.8 ±10.6 and 222.6±17.2 $cm^2\ g^{-1}$ DW after 19 and 25 weeks, respectively). The mean root FW was also lower in the stressed plants, with a weight decrease of more than 50% at the end of the stress period compared with controls (Fig. 2E). The shoot-to-root ratio decreased from week 7 to week 19 and remained constant thereafter for the controls. At the end of the experiment, this ratio was 2-fold higher in the control plants compared with the stressed plants (Fig. 2F), suggesting that shoot growth was more inhibited than root growth under water shortage conditions. The ratio of the total leaf area/root DW was more than 2-fold lower in stressed plants than in controls after 19 weeks (8.6±1.1 in controls versus 3.5±0.5 in stressed plants) and after 25 weeks (7.1±1.6 in controls versus 2.3±0.3 in stressed plants).

Plant water status

The water content (Fig. 3A) was lower in the roots than in the leaves and remained higher in the control plants than in the stressed plants. Among treatments, no significant difference was recorded for the leaf water potential (ψ_w) (Fig. 3B). In contrast, both leaf and root osmotic potentials (ψ_s) were lower in the stressed plants than in the controls (Fig. 3C, 3D). As far as root ψ_s is concerned, the difference was not significant at the end of the treatment. Drought had a strong deleterious impact on the global stomatal conductance (g_s) of chicory leaves, which was lower in the drought-stressed plants than in the controls (Table 1).

Gas exchange and photosynthesis-related parameters

Notably, neither the instantaneous transpiration (*E*; Table 1) nor the net photosynthesis (*A*; Table 1) of young leaves were affected in response to drought. At week 19, net photosynthesis was even higher in the stressed plants than in the controls, while the difference between treatments remained statistically non-significant for other periods of stress. Chlorophyll concentrations (Fig. 4A, 4B) decreased in the stressed plants, but the ratio Chl a/Chl b remained unchanged (Fig. 4C). A similar stress-induced decrease was observed for carotenoids (Fig. 4D). Despite the recorded changes in the concentrations of photosynthetic pigments, photosystem II efficiency was slightly higher in the drought-stressed plants (Fig. 5A) than in the controls. Non-photochemical and photochemical quenchings (Fig. 5B, 5C) were lower and higher, respectively, in the stressed plants compared with the controls, although the differences among treatments were only significant at week 19. Rubisco initial and total activities were affected by drought at weeks 13 and 19 (Table 2). The activation state of Rubisco remained similar in the control and drought-treated plants.

MDA, soluble sugars, and inulin concentrations

The leaf MDA concentration (Fig. 6A) increased in response to water shortage after 19 and 25 weeks of culture. The root MDA concentration was similar in the control and drought-treated plants, except at week 19 when it was significantly higher in the latter than in the former. Water stress also drastically increased the leaf total soluble sugar concentration (Fig. 6B), which culminated at the beginning of the stress exposure at week 13 and then gradually decreased until the end of the treatment.

Drought increased the endogenous concentrations of glucose, fructose, sucrose, galactose, and *myo*-inositol in leaves at weeks 13 and 19, while sucrose also accumulated in response to drought at week 25 (Table 3). Mannose and raffinose were also detected in our leaf samples but remained unaffected in response to drought (detailed data not shown). The soluble sugar (glucose, fructose, sucrose, kestose, and nystose) concentrations were not affected by water stress in the roots (detailed data not shown).

Root inulin concentration, which was quantified at the different harvests (Table 4), remained unaffected when expressed on a DW basis. This result, however, implies that the inulin concentration expressed on a FW basis was higher in the stressed plants than in the controls at the end of the treatment period (Table 4). Considering the previously mentioned root growth inhibition, the total amount of inulin produced per plant was 54.3 and 28.5 g $plant^{-1}$ for control and stressed plants, respectively: assuming a plant density of 150 000 plant ha^{-1} in field conditions, such water stress would imply a loss of inulin yield corresponding to 3.8 T ha^{-1}. The mean DP remained unaffected in the water-stressed plants compared with the controls (Table 4). Because the columns could be considered to be closed systems and because the volume of solution supplied to each column was registered during the time course of the experiment, the water-use efficiency could be estimated. The synthesis of 1 g of root dry matter required 405±62 ml of solution in control plants and only 244±32 ml in stressed

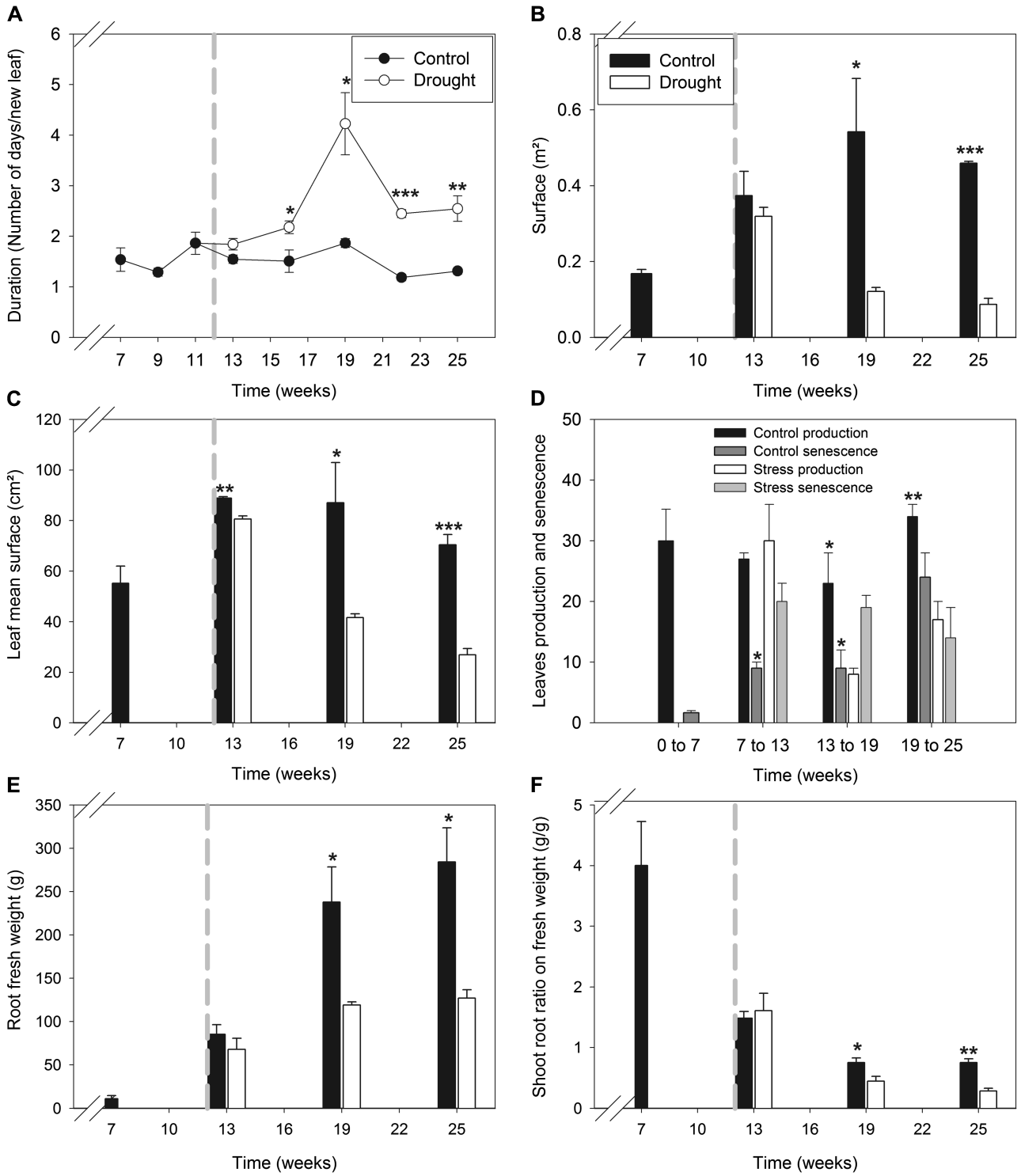

Fig. 2. Evolution of the duration required for the macroscopic appearance of a new leaf (A), total leaf surface (B), leaf mean area (C), leaf abscission and senescence (D), root FW (E), and shoot-to-root ratio (F) in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing (grey dashed line), and plants were harvested for analysis 7, 13, 19, and 25 weeks after sowing. Non-destructive measurements for the duration required for the macroscopic appearance of a new leaf were made 7, 9, 11, 13, 16, 19, 22, and 25 weeks after sowing. Filled symbols indicate control plants, and open symbols indicate drought-stressed plants. Each value represents the mean of three replicates, and vertical bars represent the standard errors. The asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

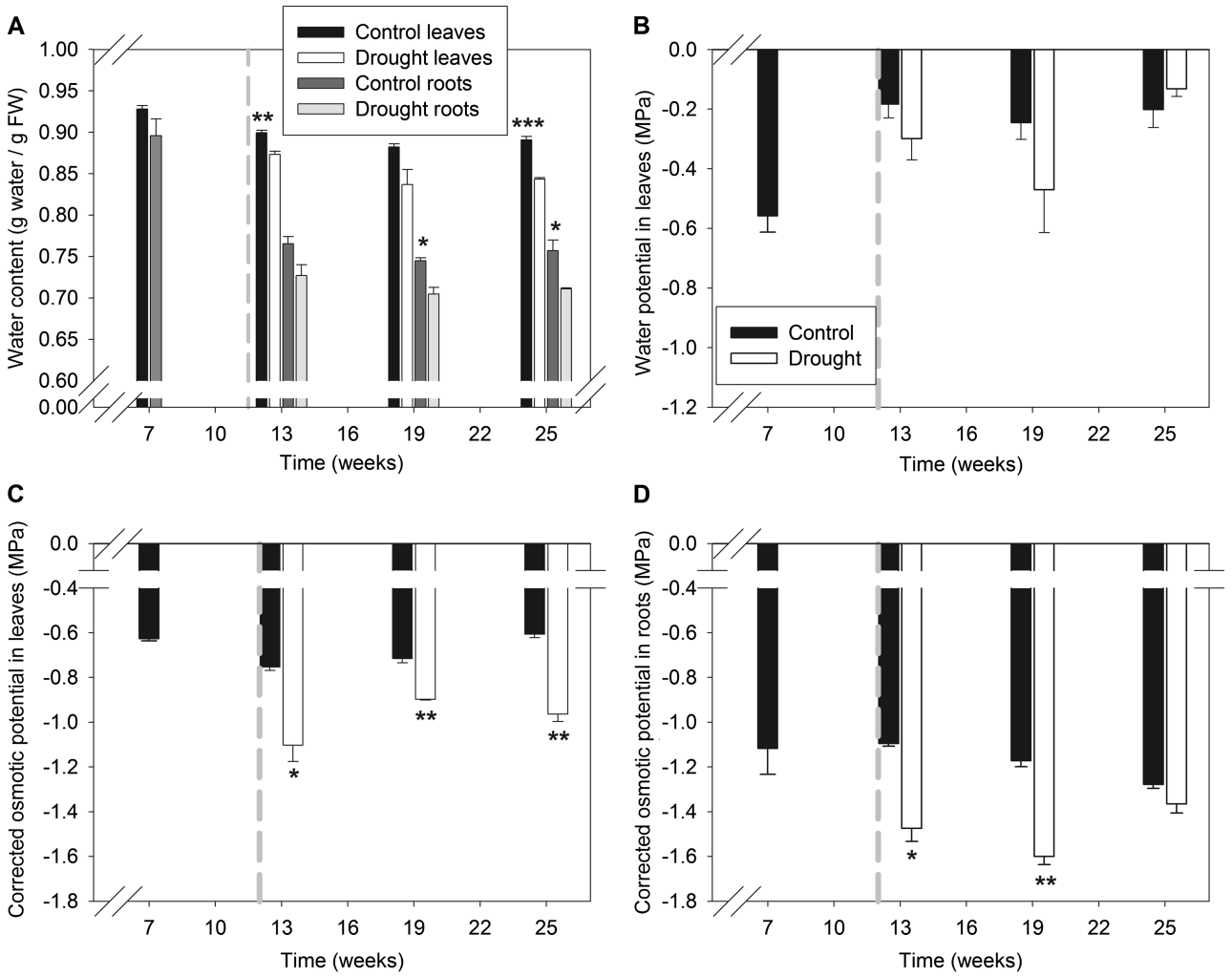

Fig. 3. Evolution of leaf and root water content (A), leaf water potential (B), corrected leaf osmotic potential (C), and corrected root osmotic potential (D) in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. The osmotic potentials were adjusted to the water content of the control plants. All other details are as for Fig. 2.

ones. Similarly, the synthesis of 1 g of inulin required 551±30 ml of nutrient solution in controls for only 354±62 ml in stressed plants.

Sugar-metabolizing enzymes

Water stress had no impact on the protein concentration, whatever the considered duration of stress (detailed data not shown). Water stress increased leaf SPS activity (Fig. 7A). In contrast, water stress had no significant impact on leaf SuSy activity (Fig. 7B). The acid invertase activity was higher in stressed leaves than in controls (Fig. 7C) at week 13. This enzyme activity exhibited an obvious burst at week 25, although no difference was recorded between the control and stressed plants at this time. The only difference between treatments for leaf neutral invertase was recorded at the end of the experiment, when the drought-stressed plants exhibited a lower activity than the controls (Fig. 7D). SPS, SuSy, and invertase activities were also measured in the roots: the activities of these enzymes decreased with root age, but water stress had no impact on these activities compared with controls (detailed data not shown).

The activities of inulin-metabolizing enzymes were quantified in the roots (Table 5). A high 1-SST activity was recorded by week 7; it progressively decreased with plant age and to a higher extent in water-stressed plants than in controls. Such a development-induced decrease was not recorded for 1-FFT activity, which remained lower in the drought-stressed plants than in the controls at weeks 19 and 25. An opposite trend was observed for 1-FEH activity, which was higher at weeks 19 and 25 in the water-stressed plants than in the controls.

Discussion

Drought tolerance is a major concern in agronomy and plant research. In numerous studies performed under laboratory conditions, drought tolerance is scored based on an improvement in the survival rate under lethal conditions that are not necessarily relevant to field conditions. Growth

Table 1. Evolution of the stomatal conductance (g_s) and gas exchange (*E* and *A*) in plants of *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing, and measurements were made 11, 13, 16, 19, 22, and 25 weeks after sowing. Each value represents the mean of five (g_s) or 10 (*E* and *A*) replicates and is given with its standard error. Asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Week	Stomatal conductance, g_s ($mmol_{H2O}$ m^{-2} s^{-1})		Instantaneous transpiration, *E* ($mmol_{H2O}$ m^{-2} s^{-1})		Net photosynthesis, *A* (μmol_{CO2} m^{-2} s^{-1})	
	Control	Drought	Control	Drought	Control	Drought
11	71.8±7.5		0.8±0.1		1.8±0.3	
13	53.5±7.7	31.3±1.8***	0.8±0.1	0.6±0.1*	1.7±0.1	1.9±0.2*
16	62.8±7.3*	31.4±2.9***	0.9±0.1	0.6±0.1*	1.8±0.2	1.5±0.2*
19	51.5±6.2*	36.2±3.2***	1.1±0.1	1.1±0.1*	1.5±0.2	2.2±0.2*
22	55.3±6.8*	37.3±4.3***	0.8±0.1	0.8±0.1*	1.9±0.3	2.3±0.5*
25	65.0±10.2	29.3±4.8***	0.7±0.1	0.5±0.1*	2.1±0.4	2.0±0.4*

inhibition may help the plant save and redistribute resources that become limited under stress, but it can also be considered counter-productive in terms of yield in agriculture (Skirycz and Inzé, 2010; Muller et al., 2011; Skirycz et al., 2011). Carbohydrates generated by photosynthesis are major building blocks and energy sources for biomass production and maintenance. Thus, in cultivated plants, determining whether growth inhibition is a consequence of a stress-induced decrease in photosynthesis appears important.

As far as root chicory is concerned, the present work clearly showed that growth was strongly affected by drought conditions, while photosynthesis exhibited resilience to those environmental constraints. Growth inhibition occurred in both the shoots and roots, although the former was more affected than the latter after 19 and 25 weeks of culture. Drought reduced the number of leaves and the mean leaf area. The proliferation and subsequent expansion of founder cells recruited from the shoot apical meristem to form new leaves may have thus been altered in response to water stress (Beemster et al., 2005).

The net leaf photosynthesis in root chicory is poorly influenced by the water regime when expressed on a surface

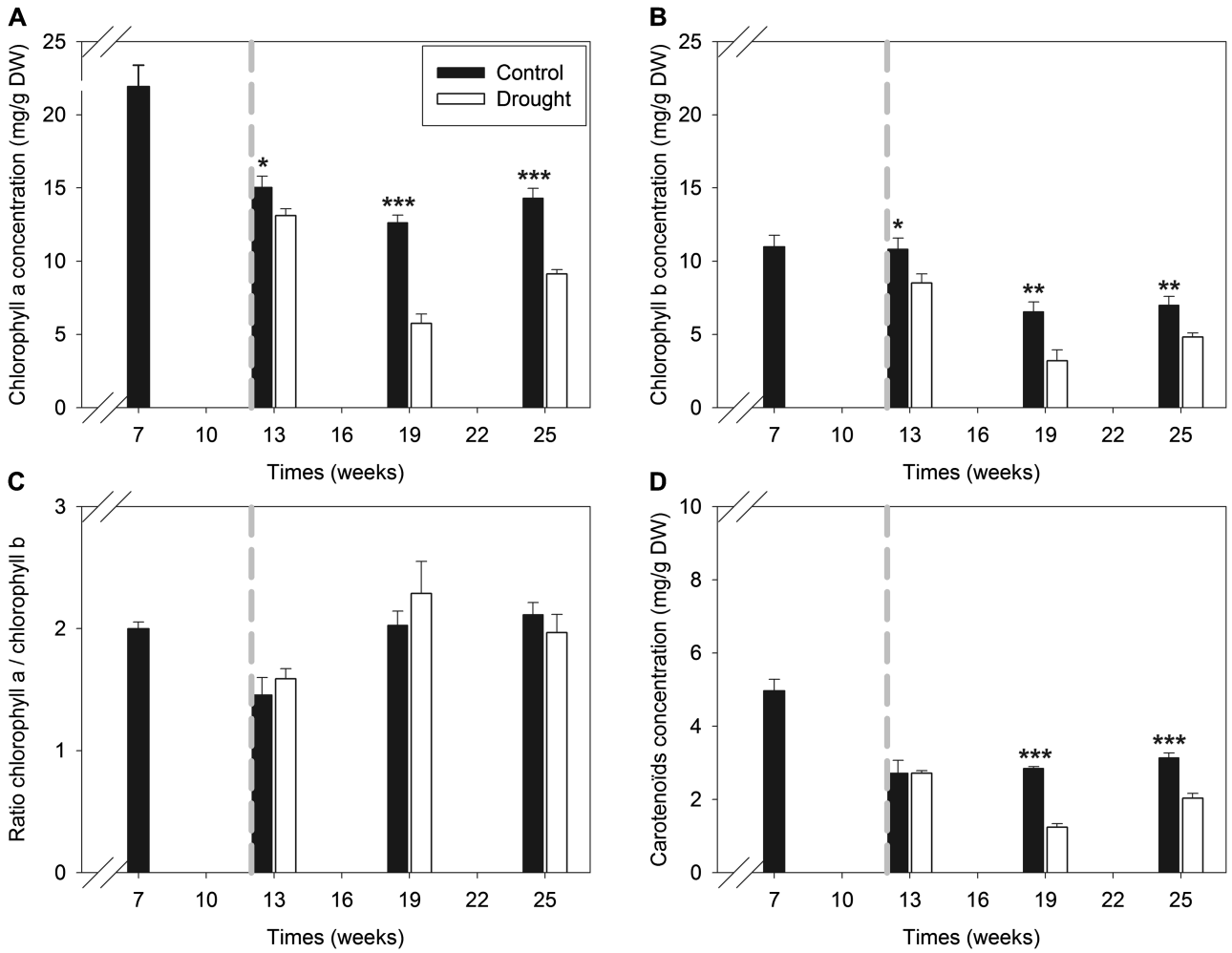

Fig. 4. Evolution of chlorophyll a (A), chlorophyll b (B), the Chl a/Chl b ratio (C), and carotenoid concentration (D) in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. All other details are as for Fig. 2.

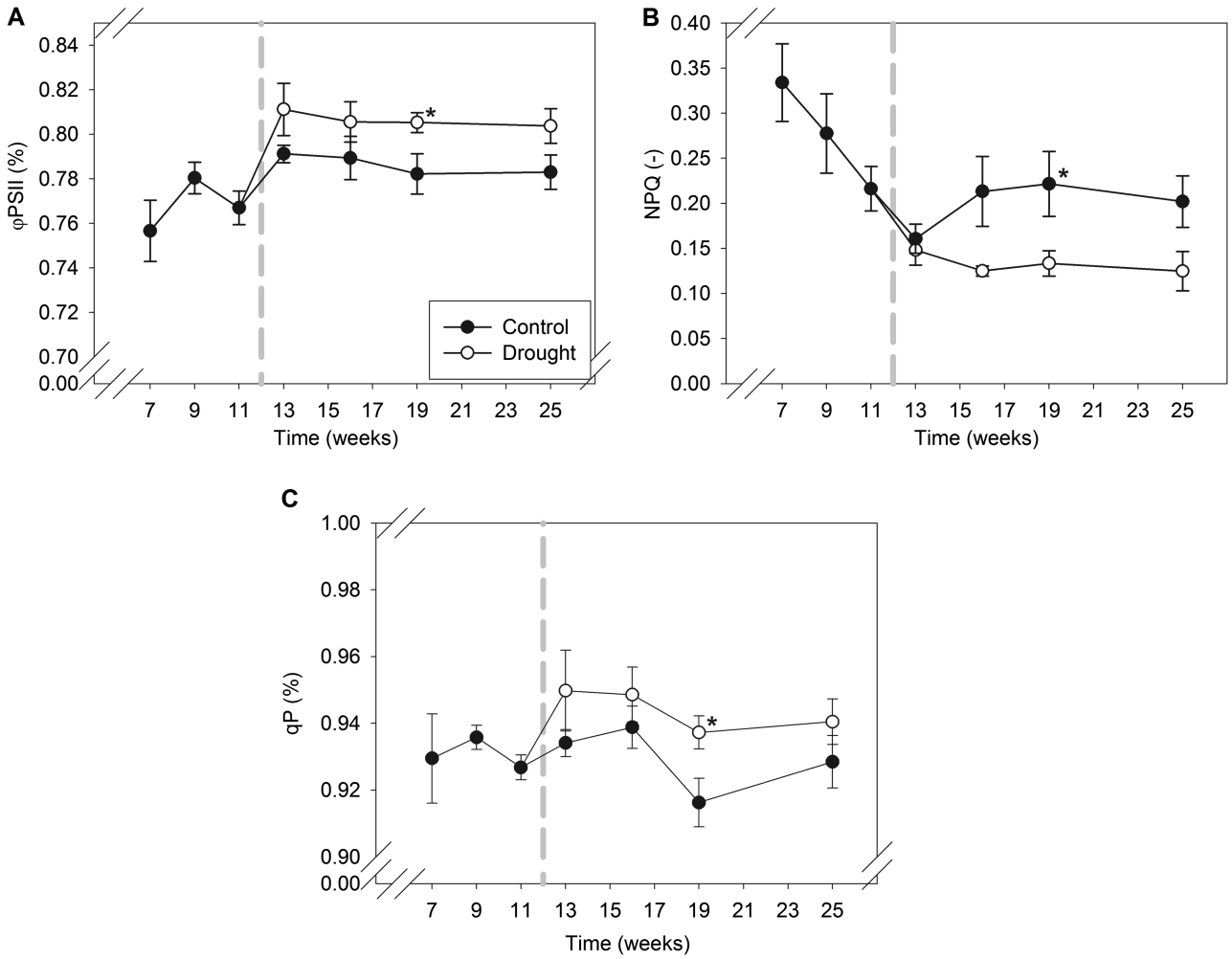

Fig. 5. Evolution of φ_{PSII} (A), NPQ (B), and q_p (C) in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. Each value represents the mean of five replicates and vertical bars represent the standard errors. All other details are as for Fig. 2.

Table 2. Evolution of the initial and total Rubisco activities (nmol mg prot^{-1} min^{-1}) and activation state (%) in plants of *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing, and plants were harvested for analysis 7, 13, 19, and 25 weeks after sowing. Each value represents the mean of six replicates and is given with its standard error. Asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Week	Initial activity (nmol mg prot^{-1} min^{-1})		Total activity (nmol mg prot^{-1} min^{-1})		Activation state (%)	
	Control	Drought	Control	Drought	Control	Drought
7	420.0±21.7		646.4±38.8		65.6±3.3	
13	423.0±46.5	254.2±48.3*	637.5±64.71	396.4±67.2*	59.8±4.7	62.3±5.4
19	377.7±83.3	171.3±22.6*	621.1±119.7	317.7±36.7*	58.4±1.7	53.4±2.5
25	146.8±24.3	136.2±17.5*	241.2±42.21	245.9±40.0*	63.8±5.9	57.2±2.8

basis, although a decrease in the specific leaf area may contribute to the decreased photosynthesis on a DW basis. Stomatal conductance exhibited a weak correlation with net photosynthesis, as also demonstrated by Monti et al. (2005). According to these authors, the impact of water stress on root chicory photosynthesis was mainly due to non-stomatal causes. Our work demonstrates, for the first time in this species, that the efficiency of the light phase was maintained or even increased in stressed tissues, as indicated by a stress-induced decrease in NPQ and an increase in q_P and φ_{PSII} (Fig. 5). A similar observation was reported in the sunflower (Cechin et al., 2008), but the underlying physiological reasons remain poorly documented. Several osmoprotectants are known to protect photophosphorylation and electron transport of chloroplast membranes against desiccation, particularly for galactinol, which is produced from galactose and *myo*-inositol (Nishizawa et al., 2008). These two compounds were increased in our stressed leaves

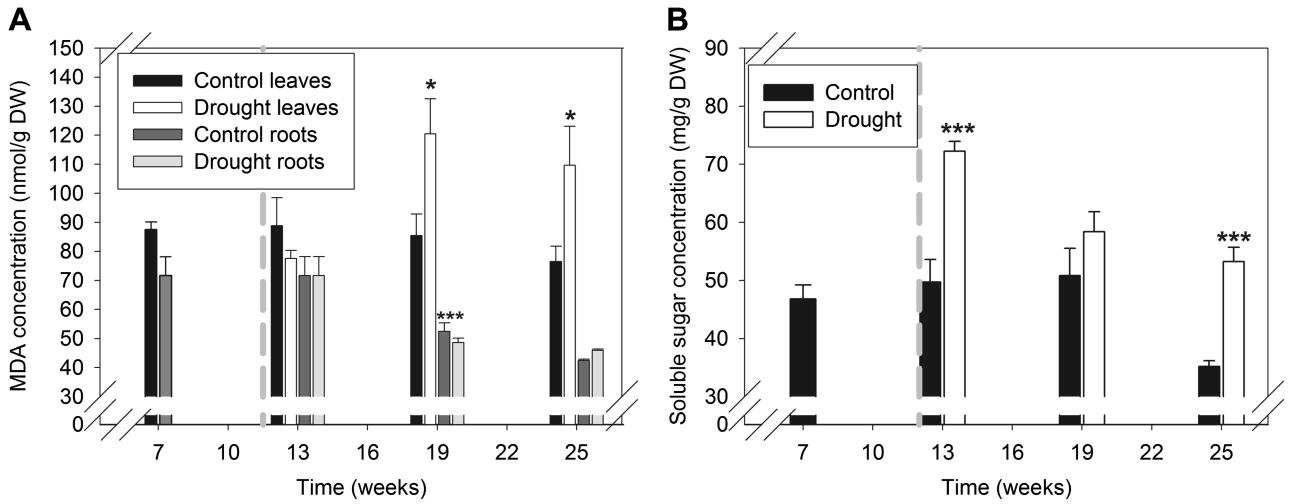

Fig. 6. Evolution of root and shoot MDA concentration (A) and leaf total soluble sugars (B) in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. All other details are as for Fig. 2.

Table 3. Evolution of the free glucose, fructose, sucrose, galactose, and *myo*-inositol concentrations in the leaves of *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing, and plants were harvested for analysis 7, 13, 19, and 25 weeks after sowing. Each value represents the mean of three replicates and is given with its standard error. Asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Week	Glucose [μmol (g DW)$^{-1}$]		Fructose [μmol (g DW)$^{-1}$]		Sucrose [μmol (g DW)$^{-1}$]	
	Control	Drought	Control	Drought	Control	Drought
7	21.9±9.8		5.4±1.3		44.8±3.2	
13	9.7±3.3	48.9±8.4***	5.1±2.1	20.0±5.6*	47.7±7.7*	81.5±10.6*
19	16.9±7.6*	59.7±10.1**	7.0±3.2	21.4±5.4*	42.4±10.1	76.0±6.7**
25	218.4±6.6*	31.3±8.2***	5.1±2.4	8.4±3.8	32.7±1.3*	67.3±9.2**
	Galactose [μmol (g DW)$^{-1}$]			*Myo*-inositol [μmol (g DW)$^{-1}$]		
	Control	Drought				
7	0.44±0.06			12.9±1.3		
13	0.47±0.11	3.54±1.02**		10.1±1.4*	29.4±6.1*	
19	1.06±0.24	2.31±0.16**		5.2±1.3	12.2±2.8*	
25	0.56±0.15	1.09±0.51**		4.4±0.2	8.1±3.5	

(Table 3). Both the initial and total activities of Rubisco and pigment concentrations were reduced in response to water stress. Such decreases, however, only had a marginal impact on net photosynthesis, suggesting that both Rubisco and chlorophylls are present in excess in the control leaves. Wang et al. (2008) also reported that in addition to a decrease in the total chlorophyll content, the maintenance of the Chl a/Chl b ratio is an important parameter involved in the stability of the light phase of photosynthesis under stress. The response of Rubisco to water stress varies greatly among plant species (Parry et al., 2002). The similarity of the activation states in the control and stressed plants suggests that water stress did not reduce the *in vivo* activity of Rubisco, expressed as a percentage of the carboxylation potential. Nevertheless, this carboxylation potential was clearly reduced by drought (Table 2), which could be related to the binding of inhibitors within the catalytic site of the enzyme.

Plant growth inhibition associated with the maintenance of photosynthesis activity leads to the accumulation of soluble sugars within plant cells. This accumulation may have numerous impacts on plant metabolism because sugars play key roles in plants, not only as nutrients but also as signalling and protecting molecules (Bolouri-Moghaddam et al., 2010; Pagter et al., 2011). The total soluble sugars in the leaves peaked 1 week after the onset of stress and then decreased progressively until week 25. The sugar levels, however, remained higher in the stressed plants than in the controls. The decrease in sugar concentration recorded in the control plants is a developmentally regulated process corresponding to a general decrease in the physiological activity that occurs at the end of the first year of growth in this biannual plant species, the time at which the plants start to cope with the late autumn/winter period. Although sugar accumulation in stressed plants correlated with the decrease in osmotic potential, a rough quantitative analysis based on the mean water content and the use of the Van't Hoff equation suggests that the soluble sugars did not contribute more than 8% to the total lowering of the osmotic

Table 4. Evolution of the inulin content expressed on a DW and FW basis, mean DP of the inulin chains, and quantity of inulin in the roots of *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing, and plants were harvested for analysis 7, 13, 19, and 25 weeks after sowing. Each value represents the mean of three replicates and is given with its standard error. Asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Week	Inulin content (g inulin 100 g DW^{-1})		Mean DP		Quantity of inulin in roots (g)	
	Control	Drought	Control	Drought	Control	Drought
7	75.9±7.4	8.0±0.6	0.99±0.54			
13	81.1±2.8	77.6±2.1	11.9±0.4	11.2±0.6	16.2±2.2	14.3±2.7*
19	80.6±3.3	80.6±4.8	12.9±0.3	13.6±0.5	48.7±7.8	28.5±2.7*
25	79.1±4.8	77.3±1.9	13.4±0.1	14.4±0.6	54.3±7.3	28.5±3.0*
Week	**Inulin content (g inulin 100 g FW^{-1})**					
	Control	**Drought**				
7	7.0±1.6					
13	17.5±0.1	19.4±1.4**				
19	19.0±1.0	22.1±2.0**				
25	17.6±0.2	20.6±0.5**				

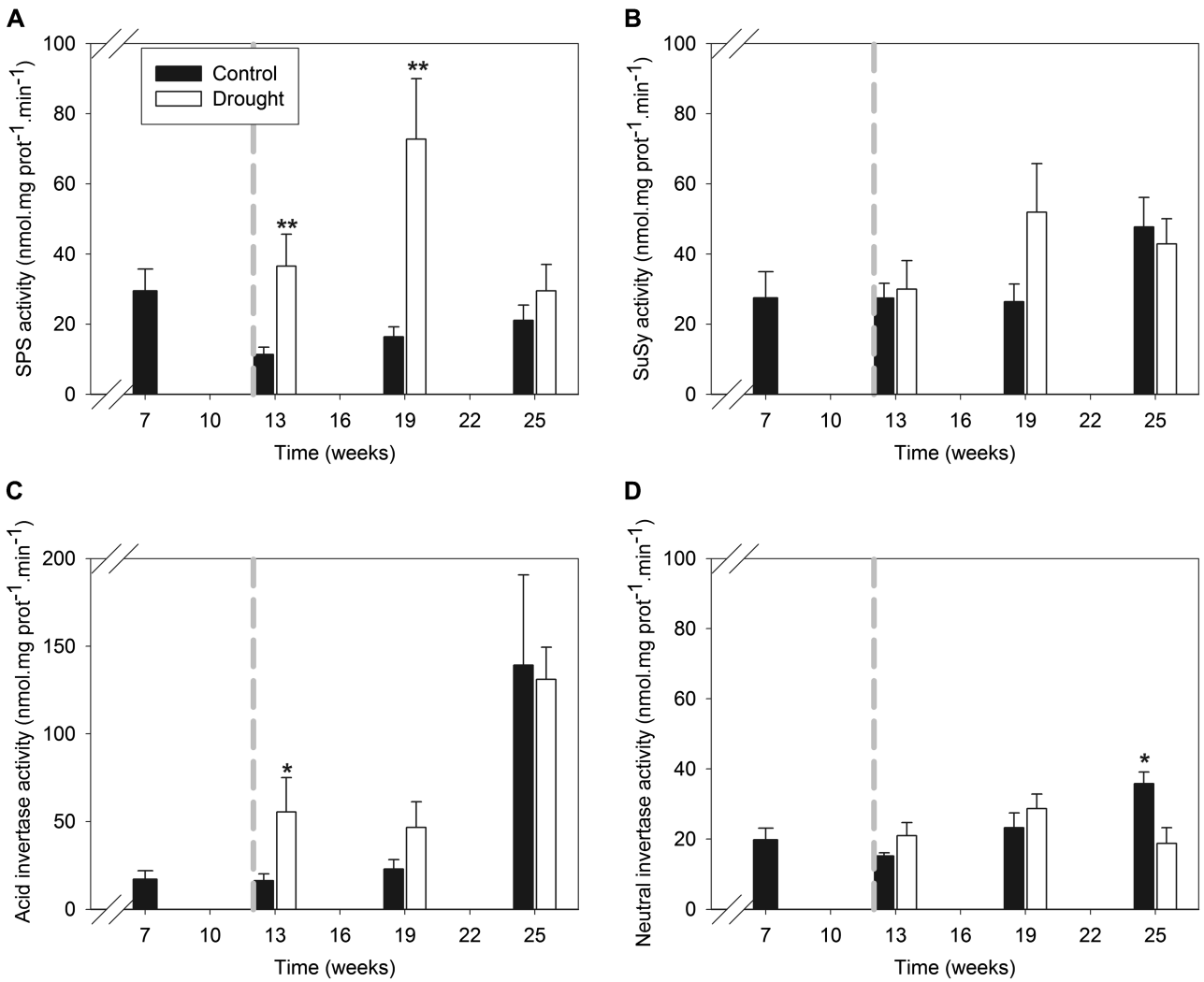

Fig. 7. Evolution of leaf SPS (A), SuSy (B), acid invertase (C), and neutral invertase (D) activities in *C. intybus* plants exposed to well-watered (control) and drought (drought) conditions. All other details are as for Fig. 2.

Table 5. Evolution of the 1-SST, 1-FFT, and 1-FEH activities in the roots of *C. intybus* exposed to well-watered (control) and drought (drought) conditions. Water stress was imposed 12 weeks after sowing, and plants were harvested for analysis 7, 13, 19, and 25 weeks after sowing. Each value represents the mean of three replicates and is given with its standard error. Asterisks indicate the presence of a significant difference between the treatments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Week	1-SST (nmol mg prot^{-1} min^{-1})		1-FFT (nmol mg prot^{-1} min^{-1})		1-FEH (nmol mg prot^{-1} min^{-1})	
	Control	Drought	Control	Drought	Control	Drought
7	157.3±10.1	31.7±4.4	11.4±0.9			
13	98.4±20.7	80.2±6.2	92.2±2.11	86.2±6.03	9.3±2.7	8.7±1.6
19	35.3±9.0	10.0±2.1***	105.1±4.1	76.1±5.8***	5.5±0.5	10.2±1.2***
25	23.8±13.9	5.7±0.8*	84.1±16.0	63.3±5.7*	5.9±0.6	7.6±1.4*

potential. Other compounds, such as organic acids and proline, that may assume key roles in osmotic adjustment (Hummel et al., 2010), were not quantified in the present study.

The accumulation of the non-reducing sucrose did not only result from a passive growth inhibition but also from an active stimulation of SPS activity (Fig. 6). Moreover, the fact that inulin concentration was not reduced by water stress (see below) indicates that sucrose transport from source leaves to sink roots was not severely hampered under stress. Not only sucrose but also glucose and fructose accumulated at weeks 13 and 19 in the leaves, at a time when the activities of acid invertase and, to a lesser extent, SuSy increased in the stressed leaves compared with the control leaves. Hexokinase is a glucose sensor with separate catalytic and signalling activities (Moore et al., 2003), and the produced glucose may act as a signalling molecule (Rolland et al., 2006). Hummel et al. (2010) recently reported that in *Arabidopsis thaliana* exposed to water deficit the rosette relative expansion rate was decreased more than photosynthesis, leading to a more positive carbon balance, and that water deficit has a limited impact, often stimulating enzyme activities. In that study, however, root growth was promoted, while it was clearly inhibited in *C. intybus*. Such a discrepancy may be related to the pattern of root development, which involves organ enlargement after initiation of the tap root, a process that is especially sensitive to water shortages but does not occur in *A. thaliana*. The absence of a drought effect on the root sugar content confirms the view of Hummel et al. (2010) that there is no global down-regulation of carbon metabolism under conditions of soil water deficit, in contrast to data obtained from drastic drought protocols involving drying out of the plant or immersion in osmotica (Verslues et al., 2006).

Water stress hastened the leaf senescence process, at least between weeks 7 and 19 (Fig. 2). Photosynthesis can be argued to have remained active in only a small proportion of the foliage, which, in turn, may influence the global plant carbon budget. Stress-induced ethylene oversynthesis is frequently reported to hasten senescence (Lim and Nam, 2005) but may also influence sugar metabolism and leaf development (Kendrick and Chang, 2008; Cho and Yoo, 2009). A slight burst in ethylene synthesis has been noticed in drought-treated root chicory (S. Lutts, unpublished results). In this species, however, stress-induced senescence mainly involves the basal part of the rosette and not the most photosynthetically active young leaves. Although the ratio of the total leaf area/root DW decreased in response to the soil water deficit, a simultaneous decrease in specific leaf area mitigated the impact of stress on the shoot/root ratio. Despite senescence processes, modification of the leaf structure associated with a more efficient photosynthesis light phase is suggested to allow the plant to maintain sugar synthesis to fulfil requirements for the accumulation of reserves in the roots.

Oxidative stress may also be responsible for hastening senescence. Small soluble sugars and the enzymes associated with their metabolic pathways are connected to oxidative stress and reactive oxygen species signalling. Although excess sugar production in source leaves may result in the generation of excess cytosolic H_2O_2, endogenous sugar availability may also feed the oxidative pentose phosphate pathway, creating reducing power for glutathione production (Bolouri-Moghaddam et al., 2010). In the present case, the highest MDA concentration was observed at weeks 19 and 25 (Fig. 6A), at a time when soluble sugars had already decreased in the stressed leaves. In contrast, a low level of MDA was recorded at week 13, when the total soluble sugar concentration was the highest, suggesting that sugars may be involved in quenching reactive oxygen species (Couée et al., 2006). Similarly, a low level of MDA in the root of *C. intybus* could be, at least partly, related to the presence of inulin because fructans are efficient in membrane protection against drought and low temperatures (Van Laere and Van den Ende, 2002; Valluru and Van den Ende, 2008).

According to Schittenhelm (1999), water stress is a major factor affecting yield in root chicory. The present work shows that root growth, but not inulin synthesis, was the main parameter involved in the deleterious impact of the water shortage. Under our experimental conditions, inulin concentration remained similar in the control and stressed plants. Although the *in vitro* activities of the enzymes involved in inulin synthesis (1-SST and 1-FFT) were significantly reduced in stressed plants at weeks 19 and 25 (Table 5), this change had no impact on the final inulin concentration (Table 4).

1-SST is involved in the initiation of inulin chain synthesis, and its activity regularly decreased from week 7 to the end of the culture. Such a decrease was already

mentioned by De Roover et al. (2000) and corresponds to a precise pattern of root development. According to Druart et al. (2001), 1-SST is initiated only after the first phase of structural root growth, which ends 42 days after seeding. The corresponding enzyme activity, which is involved in sink strength regulation (Améziane et al., 1995), culminated 9 weeks after seeding. It thereafter decreased progressively until the end of the culture. Water stress effects on 1-SST may have started at a time when the enzyme activities were already decreased and almost all of the inulin chains were initiated. De Roover et al. (2000) noticed that, at the seedling stage, the drought-induced stimulation of 1-SST could lead to a short-term accumulation of fructan in the roots, as recorded 2 weeks after an abrupt stress imposition. The present work shows, however, that a progressive decline in water availability maintained on a long-term basis had no detrimental impact on inulin concentration expressed on a DW basis. This observation suggested that stress-induced stimulation at the seedling stage should be regarded as transient.

Under our experimental conditions, water stress significantly reduced 1-FFT activity, which is involved in inulin chain elongation. The drought conditions also slightly increased 1-FEH activity, which catalyses inulin chain depolymerization. Although a decrease in the mean DP of the inulin chain was expected, water stress had no significant impact on this parameter (Table 4). The recorded FEH activities in the present study (Table 5) remained rather low compared with a previously reported cold-induced FEH increase (Van Laere and Van den Ende, 2002). Thus, FEH likely does not significantly contribute to depolymerization *in vivo*. Soluble sugars, particularly sucrose, fructose, and glucose, are frequently reported in the literature as being among the main parameters influencing the regulation of the activities of inulin-metabolizing enzymes (Van Laere and Van den Ende, 2002; Van den Ende et al., 2004, and references in these studies). The current work was nevertheless unable to detect any significant impact of water stress on the concentration of these compounds within the roots. Above a certain inulin concentration, a phase transition to a gel-like state can be hypothesized to occur in the vacuole, which may sequester and inactivate fructan enzymes *in planta* but not *in vitro*.

In addition to post-translational regulation, transcriptional regulation has also been reported for inulin-metabolizing enzymes. Kusch et al. (2009) recently demonstrated that numerous biochemical factors (including Ca^{2+} signalling, protein kinases, and phosphatases) are directly involved in the modulation of the expression of the gene encoding 1-FFT. Similarly, the FEH enzyme activity depends on three distinct isoforms (1-FEHI, IIa, and IIb) that may exhibit various patterns of regulation (Van Laere and Van den Ende, 2002; Van den Ende et al., 2004; Le Roy et al., 2007; Kusch et al., 2009).

In conclusion, *C. intybus* displays a good level of water stress resistance, which could be at least partly due to its Mediterranean origin. Plant growth inhibition could be involved in this resistance strategy but leads to a significant decrease in the total inulin production. Nevertheless, the effect of water stress on Rubisco and sugar-metabolizing activities did not lead to modifications of the inulin concentration expressed on a DW basis. Further experiments are therefore required to identify the major causes of root growth inhibition in this species.

Acknowledgements

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