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RESEARCH ARTICLE

Phenolic acids and flavonoids classes in *Acacia arabica* (Lam) Willd. Seedling during water stress and subsequent re-hydration

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Abstract

Background and aims A greenhouse experiment was conducted to analyze the physiological behavior of *Acacia arabica* in relation to concentration of phenolic compounds during drought and subsequent recovery.

Methods Seedlings were exposed to water shortage during 32 days and then rehydrated and allowed to recover during 15 days. Parameters related to plant water status, photosynthesis and oxidative stress were quantified after 7, 15 and 32 days of stress, and after 7 and 15 days of recovery. Phenolic acids, flavonoids and enzyme activities involved in their synthesis were quantified.

Results 88% of plants remained alive during the stress period and 10% died during recovery. Drought reduced water and osmotic potentials, stomatal conductance, net photosynthesis, instantaneous transpiration, non-photochemical quenching and flavanols concentration and increased proline, malondialdehyde, phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) activities, DPPH scavenging, hydroxycinnamic acids, hydroxybenzoic acids and flavonols concentration. Genes coding for PAL and chalcone synthase (CHS) were slightly downregulated at the end of drought while gene coding for C4H was upregulated at the beginning of drought. Parameters related to oxidative stress as well as hydroxybenzoic acids and flavonols remained at high values throughout the recovery period. Genes coding for C4H and CHS were up-regulated during recovery.

Conclusions *Acacia arabica* is resistant to water stress at the seedling stage and able to recover after stress relief. Recovery induces a specific physiological status in terms of transpiration rates and management of oxidative stress in relation to flavonols and hydroxybenzoic acids concentrations.

Keywords *Acacia arabica* · Drought · Flavonoids · Phenolic compounds · Water stress

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Introduction

In many parts of the world, plants are exposed to environmental stresses that limit their growth and compromise their survival. Water stress induced by a limitation of the plant's water supply is responsible for a drastic reduction in crop productivity and also compromises the stability of natural ecosystems (Liu et al. 2021). In the future, droughts will become more frequent in relation to global climate change and many regions that are not currently exposed to droughts will have to cope with them in the future (Parmesan and Hanley 2015; Wang et al. 2017a, b). Recent models for future climate change, such as CMIP6, also predict a significant change in precipitation patterns (Chatterjee et al. 2023). It is likely that episodes of more or less severe drought will be interspersed with irregular rainfalls (Yeo et al. 2022).

Under field conditions, plants are not always exposed to permanent drought. The ability of a plant to grow and complete its cycle depends not only on its ability to survive during the stress, but also on its ability to recover an optimal physiological activity after the stress relief. For a plant previously exposed to water stress, the recovery period coincides with a sudden increase in water availability, a hypoosmotic shock, and possibly a massive entry of water into partially dehydrated tissue (Bernacchia et al. 1996). These changes occurring over a short period of time are themselves stress factors that the plant must be able to manage by implementing specific mechanisms in relation to management of oxidative stress and protection of cellular structures (Bernacchia et al. 1996; Bajji et al. 2000; Huang et al. 2022; Sun et al. 2022). Indeed, it has been suggested that the physiological and biochemical processes that take place during the rehydration phase are not simply the reverse of what happened during the stress period, but that processes specific to the rehydration phase also occur (Bernacchia et al. 1996; Morales-Sánchez et al. 2022). This is especially true for perennial plant species experiencing seasonal drought. Numerous data on the modalities of rehydration processes are available for bryophytes (Morales-Sánchez et al. 2022), ferns (Prats and Brodersen 2021) and intertidal algea (Wang et al. 2022). As far as angiosperm are concerned, studies frequently consider poikilohydric « resurrection » plants able to lose more than 90% of their water content, and to resume full physiological activity after complete rehydration (Bernacchia et al. 1996; Veljovic-Jovanovic et al. 2008). However, these species are exceptions and their behavior cannot be considered fully representative of the plant kingdom as a whole.

Plants experiencing drought events in their environment are suffering from a wide range of physiological disorders and display various strategies as an attempt to cope with water shortage. However, each strategy has both advantages and disadvantages. Stomatal closure limits transpiration and thus avoids excessive water losses, but also reduces photosynthesis and compromises leaf temperature regulation (Ben Hassine and Lutts 2010; Wu et al. 2022). Osmotic adjustment implies the accumulation of a high concentration of organic soluble compounds and allows the maintenance of a favorable water potential for water uptake, but it has a metabolic cost which could not be devoted to growth purposes (Bajji et al. 2000; Wu et al. 2022). From an energetical point of view, these strategies may no longer be useful during the rehydration phase, or may even become counterproductive (Munns et al. 2020; Huang et al. 2022). The plant must then be able to change its strategy quickly. This is particularly valid if we consider a kinetic aspect, as substrate dehydration during dry spells is gradual, whereas rehydration during heavy rainfall is abrupt.

All abiotic stresses including drought induce overproduction of reactive oxygen species such as superoxide ion ($O_2.^-$), hydroxyl radicals (OH.) and hydrogen peroxide (H_2O_2) (Chavoushi et al. 2019; Saidi et al. 2021; Farouk et al. 2023). These compounds have numerous deleterious impacts on cells since they are able to react with lipids, proteins, and nucleic acids leading to membrane desorganization, enzyme modifications and mutations (Farooq et al. 2019; Zulfiqar and Ashraf 2023). The ability to survive these cellular toxins depends on the metabolic responsiveness of detoxification mechanisms consisting in antioxidative enzymes and nonenzymatic antioxidants. Beside reduced glutathion (GSH), reduced ascorbic acid (AsA) and carotenoids, phenolic acids and flavonoids assume key functions in cell protection against oxidative stress (Cheng et al. 2018; Marchiosi et al. 2020; Parvin et al. 2020; Xiong et al. 2022). As detailed in Fig. S1, synthesis of these bioactive secondary compounds is initiated by the phenylpropanoid pathway. Phenylalanine ammonia-lyase

(PAL; EC 4.3.1.5) is a crucial enzyme in phenylpropanoid metabolism, catalyzing the formation of *trans*-cinnamic acid via the deamination of phenylalanine while cinnamate-4-hydroxylase (C4H; EC1.14.13.11) converts cinnamic acid to *p*-coumaric acid (Gharibi et al. 2019; Hodaei et al. 2018). Chlacone synthase (CHS; EC 2.3.1.74) catalyzes the conversion of *p*-coumaroyl-CoA to chalcone, which is the precursor of flavonoids such as flavanols, flavonols, flavones and anthocyanins.

Some authors reported that oxidative stress occurs not only during the dehydration phase of water stress, but also at specific states during rehydration phase (Sgherri et al. 2004; Morales-Sánchez et al. 2022). The maintenance of antioxidative protection is thus justified after the stress relief. However, as far as phenolic acids and flavonoids are concerned, it is not clear which compounds are involved in protection during the dehydration and rehydration processes. Therefore, analyzing the individual fates of phenolic compounds, enzyme activities involved in their synthesis and expression of genes coding for these enzymes may afford valuable information in this respect.

The *Acacia* genus comprises perennial trees belonging to the Mimosaceae family. Most of these species are able to withstand drought conditions, although they are especially sensitive to water shortage at the seedling stage (Bajlan et al. 2020; Cory et al. 2022). Numerous *Acacia* are rich in phenolic compounds and these molecules are offering new opportunities due to their pharmacological properties which explain their use for medical or alimentary purposes (Ziani et al. 2020; Elnour et al. 2022; Pedro et al. 2022; Jyoti 2023). *Acacia arabica* is native to Africa and thrives in dry environments where rainfall is erratic. It has irregular distribution and presents agroforestry potentials in semi-arid regions of Africa. This species has great ecological value because it helps in controlling erosion and improving soil fertility (Lansky et al. 2023). Lassouane et al. (2013) demonstrated that total phenolic acids and total flavonoids compounds accumulate when young plants are exposed to water stress. However, neither the precise identity of individual compounds nor their behavior during rehydration processes were considered.

The present study involved a kinetic approach and was conducted in order to provide information on the evolution of physiological and biochemical parameters during progressive drought and during subsequent rehydration in *Acacia arabica*. The aims were (i) to assess the physiological impact of water stress and quantify the recovering capacities after the stress relief, (ii) identify and quantify the various phenolic compounds during and after water stress, (iii) assess the activities of PAL and C4H during water stress and during recovery and (iv) follow the pattern of expression for genes coding for PAL, C4H and CHS at different period during stress and during recovery.

Materials and methods

Plant material and growth conditions

Acacia arabica seeds were provided by Institut National de Recherche Forestière, Tamanrasset, Algeria. Seeds were allowed to germinate at 28 °C in the dark on filter paper moistened with deionised water for 4 days. The seedlings were then transplanted (one in each pot) in square pots (11 cm x 12 cm) filled with loam as substrate. Soil was analyzed according to Page et al. (1982) and its main chemical and physical properties are provided in Table S1. The potted seedlings were maintained in a greenhouse under 16 h photoperiod (mean light intensity of 230 µmol m^{-2} s^{-1}). Light was supplied by Philips HPIT 400-W lamps (Philips Lighting S.A., Brussels, Belgium) with a day/night temperature of 29/25°C. Plants were irrigated to 80% field capacity (FC). The soil water content (θ) was defined as (W_w/DW_s) x 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 (Lassouane et al. 2013). The soil water content was measured using a ThetaProbe soil moisture sensor type ML1 (Delta-T Devices Ltd., UK) based on changes in apparent dielectric constant, and converted to volumetric water content based on calibration of the considered substrate. After 6 weeks, irrigation was suppressed to 50% of the pots (hereafter designed as "stressed") while daily irrigation was maintained to the remaining controls to maintain soil water content at 80 ± 2% of FC. For this purpose, θ values were measured at 6.00 p.m. on 6 randomly-chosen control pots: the difference between the θ values corresponding to 80% FC and the actual measured θ allowed to measure the volume of water to afford for irrigation of control pots. The θ value was again estimated after the night at following morning (7.00 a.m.) to ensure that afforded water reached the deeper layer of the soil when plant transpiration was strongly reduced during darkness. After 32 days of stress, water was applied to all pots in order to reach the initial soil water content (80% field capacity). The control and stressed groups of plants comprised 60 plants per treatment, and plants were weekly randomly rearranged in the greenhouse.

Plant growth and water status

Plant growth was assessed on 6 living plants per treatment through measurement of leaf number and shoot dry weight after 7, 15 and 32 days of stress and after 7 and 15 days of recovery after the water stress relief. The shoot elongation rate was calculated as a percentage of the initial shoot height at the time of stress exposure. Plant survival was estimated throughout the experiment considering as dead a plant with all leaves fully necrosed. Only living plants were considered for subsequent physiological measurements.

Shoot water potential (Ψ_w) and leaf osmotic potential (Ψ_s) were determined between 9.00 and 10.00 a.m. on 6 plants per treatment. Shoot Ψ_w was evaluated immediately after sampling by the pressure chamber method (PMS Instrument Co., Orlando, USA) using the main stem on each plant. For Ψ_s determination, the three youngest fully developed leaves were pooled. Tissues were quickly collected, cut into small segments, placed in Eppendorf tubes perforated with 4 small holes, and immediately frozen in liquid nitrogen. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at 15,000 *g* for 15 min at 4 °C. Tissue sap was analyzed for Ψ_s estimation. Osmolarity (c) was assessed with a vapor pressure osmometer (Wescor 5520) and converted from mosmoles.Kg^{-1} to MPa using the formula:

$$\Psi s\ (\text{MPa}) = -c\ (\text{mosmoles.kg}^{-1}) \times 2.58 \times 10^{-3}$$

according to the Van't Hoff equation.

Photosynthetic activities and proline concentrations

Chlorophyll fluorescence-related parameters were measured on five randomly chosen plants within each plot and analyzed by a Fluorescence Monitoring System II (Hansatech Instruments, Norfolk UK). Leaf n°4 numbered from the top of the plant was considered for analysis. Leaf portions located in the middle part of the blade were acclimated to darkness for 30 min using pliers fitted with a sliding clip. After removal of the leaf clip, the minimal fluorescence level (F_0) was measured using modulated light (0.1 µmol m^{-2} s^{-1}). Maximal fluorescence with all PSII reaction centers closed was assessed by applying a 0.8 s saturating pulse of 8,000 µmol m^{-2} s^{-1}. The leaf was then illuminated with white actinic light (1,200 µmol m^{-2} s^{-1}) during 4 min. F_s corresponding to the steady state fluorescence was recorded and a second saturating pulse of 8,000 µmol m^{-2} s^{-1} was applied to determine the maximal fluorescence in the light-adapted state (F_m'). After removing the actinic light, minimal fluorescence level at the light adapted state was determined by illuminating the leaf with a 3 s pulse of far-red light. Maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m), photochemical quenching (qP), non-photochemical quenching (NPQ) and operational efficiency of PSII photochemistry (Φ_{PSII}) were calculated based on the above-mentioned parameters according to Swoczyna et al. (2022).

Gaz exchange measurements were performed on the same leaves using an Infrared Gaz Analyzer (LCA4 8.7., ADC Biocientific) with a Parkinson leaf cuvette, under an air flow of 300 mL min^{-1} during 1 min (20 records) at 23 ± 2 °C and on a leaf segment of 6.25 cm^2 at a light intensity of 725 µmol m^{-2} s^{-1}. The net CO$_2$ assimilation rate (A) and instantaneous transpiration (E) were measured. The instantaneous water use efficiency ($\text{WUE} = A/E$) was then calculated. The stomatal conductance (g_s) was measured with an AP4 porometer system (Delta-T Devices). All measurements were performed between 11.00 a.m. and 2.00 p.m. in a sunny day with no clouds. The leaves used for photosynthetic measurements were then collected and cut in small segments (3 × 2 cm). Photosynthetic pigments (*Chla* + *Chlb*) and total carotenoids (xanthophylls and β carotene) were quantified after extraction with cold acetone 80% and spectrophotometric analysis according to Lichtenthaler (1987).

Leaf proline was quantified using the ninhydrin method according to Bates et al. (1973) on the three pooled youngest fully developed leaves on five plants per treatment. For each sample, ca. 200 mg FW of leaves were ground in liquid nitrogen in a mortar containing 10 mL of 3% sulfosalicylic acid. Samples were then centrifuged at 1,000 *g* for 5 min and 2 mL of the supernatant were incubated at 100 °C in the presence of 2 mL ninhydrin and 2 mL acetic acid. After extraction with toluene (2 mL), proline was quantified at 520 nm with a Beckman DU640 spectrophotometer using proline standards (SigmaAldrich).

Malondialdehyde, total antioxidant activity and electrolyte leakage

Malondialdehyde (MDA) was quantified on leaves of five plants per treatment, following the method of Heath and Packer (1968), with slight modifications. Frozen samples (0.25 g fresh weight) were homogenized in a prechilled mortar with 10 mL of ice-cold 5% (w/v) trichloroacetic acid and centrifuged at 12,000 *g* for 10 min at 4 °C. An assay mixture containing a 2 mL aliquot of supernatant and 2 mL of 0.67% (w/v) thiobarbituric acid was heated to 100 °C for 30 min and then rapidly cooled to 4 °C in an ice bath. After centrifugation at 12,000 *g* for 1 min at 4 °C, the supernatant absorbance was recorded (532 nm), and values corresponding to nonspecific absorption (600 nm) were subtracted. Malondialdehyde concentration was calculated using its molar extinction coefficient (155 mM. cm^{-1}).

The estimation of the antioxidant capacity of the samples was based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Chen et al. (1999) with some adaptations. For each sample, 100 µM of the methanolic extract was mixed with 3 mL of 0.06 mM DPPH in methanol. After incubation in darkness (30 min), the absorbance at 517 nm was measured against methanol blank. The scavenging percentage of DPPH radical was calculated according to Maisuthisakul et al. (2007): DPPH radical scavenging capacity (%) = $[A_0-(A_1-A_S)]/A_0\times100$ where A_0 is the absorbance of the control solution (containing only DPPH), A_1 is the absorbance for the plant extract in the presence the DPPH solution, and A_S is the absorbance of the sample extract solution without DPPH, which is used for correcting the unequal color of the sample extracts. Each sample was analyzed by triplicate.

Electrolyte leakage was determined according to Bajji et al. (2002) through measurements of electrical conductivity of PEG solution containing leaf segments for 4 h, and a second time after autoclaving.

Total phenolic compounds and individual analysis through *UPLC/ESI-Q TRAP-MS/MS*

Polyphenols were extracted from frozen leaf samples (0.3 g) with 2 mL of 80% methanol. The mixture was centrifuged at 10,000 *g* for 10 min at 4 °C.

Total phenolic concentration was assayed using the Folin-Ciocalteu reagent, following Singleton's method (Singleton and Rossi 1965) with slight modifications. An aliquot (20 µL) of sample was added to 1.58 mL of deionized water and 100 µL of the raw Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 300 µL of 2% sodium carbonate (Na_2CO_3) solution. After incubation for 2 h at room temperature in dark, the absorbance was measured at 765 nm. Total phenolic contents were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg of GAE g^{-1} FW) through the calibration curve with gallic acid.

Total flavonoid concentration was measured using a colorimetric assay developed by Dewanto et al. (2002). An aliquot (250 µL) of the sample appropriately diluted was mixed with 1.25 mL of deionized water and 75 µL of 5% $NaNO_2$. After 6 min, 150 µL of 10% $AlCl_3$ and 500 µL of 1 M NaOH were added to the mixture. Finally, the mixture was adjusted to 2.5 mL with deonised water. The absorbance of the mixture was determined at 510 nm. Standard curve was established using 0 to 450 µg mL^{-1} catechin as standard. Total flavonoid content was expressed as mg (+)-catechin equivalent per gram of fresh weight (mg catechins. 10^{-2} g^{-1} of FW).

For individual identification of phenolic compounds, sample were vacuum freeze-dried for 20 h and ground in a mixer mill at 25 Hz for 150 s. Each powdered sample (100 mg) was then extracted with 1.2 mL of 70% aqueous methanol and stored at 4 °C overnight. After vortexing, the samples were centrifuged at 14,000 *g* for 15 min and supernatant was filtered with a 0.22 millipore filter. Analysis was performed with an UPLC-ESI-MS/MS system (SHIMADZU Nexera X2). Column temperature (Agilent SB-C18 (pore size 1.8 µm, length 100 mm x 2.1 mm) was set at 40 °C with the following parameters: mobile phase, eluent A (0.1% formic acid), eluent B (acetonitrile containing 0.1% formic acid); sample injection volume: 2 µL; flow rate: 0.3 mL.min^{-1}. Gradient was established according to Qian et al. (2023). Linear trap and triple quadrupole scans were acquired on 4500Q TRAP LC/MS/MS system coupled with an ESI Turbo Ionspray interface; mass spectrometry analysis was performed as detailed by Chu et al. (2019). Individual phenolic compounds identification was performed on 3 biological replicates.

Enzyme activities

Phenylalanine ammonia-lyase (PAL; EC4.3.1.5) activity was measured as previously described by Solecka and Kacperska (2003) with slight modification. Briefly 2 g of frozen fresh leaves were homogenized with 12 mL of extraction buffer containing 50 mM Tris-HCl buffer (pH 8.9), 15 mM β-mercaptoethanol, 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM ascorbic acid, 10 mM leupeptin,1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was filtered through 4 layers of cheesecloth (# Grade 80 µm) and centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was then used as a source of crude enzymes for assaying PAL activity. The reaction mixture (3 mL) contained 16 mM L-phenylalanine, 50 mM Tris-HCl buffer (pH 8.9), 3.6 mM NaCl and 0.5 mL crude enzyme. The substrate was pre-incubated at 37 °C for 15 min before mixing with the enzyme extract. The reaction was performed at 37 °C for 1 h and was stopped by adding 500 µL 6 M HCl. The tubes were then centrifuged for 10 min at 12,000 *g* to pellet the denatured protein. The absorbance was measured at 290 nm against a blank without substrate. PAL activity equaled the amount of PAL that produced 1 µmol of cinnamic acid in 1 h, and was expressed as µmol CA. h^{-1}. g^{-1} FW.

Cinnamate-4-hydroxylase (C4H; EC1.14.13.11) was extracted as described by Lamb and Rubery (1975) with slight modification. Briefly 2 g of frozen fresh leaves were homogenized with 12 mL of extraction buffer containing 50 mM Tris-HCl buffer, pH 8.9, 15 mM β-mercaptoethanol, 4 mM $MgCl_2$, 2.5 mM ascorbic acid, 10 mM leupeptin, 1 mM PMSF, 10% glycerol, and 0.15% w/v PVP. After the homogenate was centrifuged at 12,000 *g* for 20 min at 4 °C, the supernatant was used as a source of crude enzymes for assaying C4H activity. The reaction mixture (3 mL) contained 2 mM *trans*-cinnamic acid, 50 mM Tris-HCl buffer (pH 8.9), 2 mM NADP-Na_2, 5 mM G-6-PNa_2 (glucose-6- phosphate disodium salt) and 0.5 mL crude enzyme. Reaction mixture was incubated in shaking condition in water bath set at 30 °C for 30 min and the reaction was stopped by addition of 100 µL 6 M HCl. The denaturated protein was pelleted by centrifuging for 10 min at 12,000 *g*. The supernatant was alkalified to 11.0 by addition of 5 N NaOH and absorbance was measured at 340 nm before and after incubation. One unit of C4H activity was equal to a change of 0.01 in absorbance per 30 min, and expressed as µmol min^{-1} g^{-1} FW.

Gene expression

Total RNA was isolated from *Acacia arabica* (Lam.) Willd. leaves according to Reid et al. (2006) with slight modifications. All steps were performed at 4 °C. Approximatively 500 mg of leaf tissues were grounded in liquid nitrogen and transferred into 7 mL extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, 2% (v/v) β-mercaptoethanol, pH 8) previously preheated at 65 °C. The mixture was incubated at 65 °C for 10 min in a water bath and agitated every 2 min. After centrifugation at 4,400 *g* for 15 min at 4 °C, 7 mL of chloroform / isoamylalcohol (24:1) were added to the supernatant and the mixture was centrifuged at 4,400 *g* for 15 min. This operation was repeated 3 times before transfer of the aqueous phase in a new tube and addition of 0.1 volume of Na-acetate 3 M pH 5.2 and 0.6 volume of isopropanol. After precipitation at -80 °C for 30 min and centrifugation at 8,400 *g* for 30 min, the pellet was dried and then re-suspended in 1 mL of Tris-EDTA buffer pH 7.5. Total RNA was then precipitated overnight after addition of 300 µL of 10 M LiCl. After centrifugation (18,000 *g* during 30 min), the pellet was washed in 500 µL 70% ethanol and re-suspended in DEPC-water. RNA concentration was quantified using the NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands) by reading absorbance at 260 nm and purity was evaluated with 260/280 and 230/260 ratios.

DNase treatments were realized using RQ1 RNase-free DNase (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. Reverse Transcription was performed with 1 µg of total RNA using the GoScriptTM Resverse Transcription Mix, Oligo (dT) Protocol Kit (Promega Benelux bv., Leiden, The Netherlands) by following the manufacturer's instructions.

Amplifications were conducted using GoTaq DNA polymerase (Promega Benelux b.v., Leiden, The Netherlands) with 500 ng of cDNA and specific primers for *Actin* and target genes *PAL*, *C4H* and *CHS* (Table 1). After an initial denaturation step at 95 °C for 2 min, each cycle consisted of 30 s at 95 °C, 45 s at an annealing temperature depending on the primer combination and 15 s extension at 72 °C, followed by a final extension of 10 min at 72 °C. The PCR products were resolved on agarose gels. At least three independent PCR amplifications were conducted for each gene using the primer pairs, annealing temperatures, and number of cycles presented in Table 1. Expression differences were analyzed by gel densitometry using ImageJ software and expressed as relative values compared to *Actin* expression [Average (peak size of target gene/peak size of *Actin*]. Gene expression analyses were repeated for three independent cultures and gave similar results.

Statistical treatment of the data

The Statistical analysis was carried out using Statistica v.5.0 (Statsoft. Inc.). Normality of the data was estimated using Shapiro-Wilk tests, and homoscedasticity using the Levene test. Analysis of variance (ANOVA) was used to determine the main effects of the duration of treatment and of the stress on the physiological and biochemical parameters on the studied plants of *Acacia arabica*. Percentage data were arc-sin transformed before analysis. The Tukey HSD test at the 0.05, 0.01 and 0.001 probability level, was used in order to determine the significance of the differences. The experiment was repeated three times and gave similar trends.

Results

Plant survival and growing properties

At the end of the drought period, more than 88% of the plants from *Acacia arabica* remained alive (Table 2). However, 10% of surviving stressed plants died after rehydration of the substrate during the recovery period. Among the plants that remained alive, no difference between control and stressed plants was recorded after 7 days of stress for the mean shoot dry weight or leaf number (Table 2) while significant differences were recorded for all considered parameters after 15 and 32 days of stress. At the end of the stress period, percentages of inhibition induced by drought was 25.7%, 26.9% and 45.5% for shoot dry weight, leaf number and shoot elongation rate, respectively. During the recovery period, all surviving plants were able to resume growth. Although significant differences between control and stressed plants were still noticed at the end of the recovery period ($p = 0.0082$), percentage of inhibition was reduced to 15.1% for the shoot dry weight, 16.2% for the leaf number and 31.2% for the shoot elongation rate.

Soil water content and plant water status

The soil water content (Fig. 1A) progressively decreased during water shortage ($F_{5.30}$ = 240.44, $p < 0.001$), already after 7 days of treatment. It reached a minimal value of 34.7% of field capacity

Table 1 Primer sequence of the phenylpropanoid and flavonoid genes and amplification conditions used for semi-quantitative RT-PCR expression analysis

Gene name	GenBank accession no	Primers F/R (5' → 3')	Size of PCR product (bp)	T*m* (° C)	No of cycles
PAL	DQ377806.1	AAGAACACGAGCACATCA GCTCTTCCCTCACAAACT	157	55	31
C4H	JN204274.1	CATTGCTGGTCCCACACA CCTGAAGTCGTTCCCGTT	200	55	31
CHS	JN812063.1	CCCGACTACTACTTCCGCAT CTGACGAGCATCCAAAGACG	177	55	31
Actin	GR482442.1	GATCCACCGATCCAGACACT CTGTTCCCACCATCCTTCAT	249	55	31

Table 2 Survival rate (in %), shoot dry weight (in g), leaf number and shoot elongation rate in plants of *Acacia arabica* (Lam.) Willd maintained under control conditions or exposed to water stress

Duration	Survival (in %)		Shoot dry weight (g)		leaf number		Shoot elongation rate	
	Control	Stress	Control	Stress	Control	Stress	Control	Stress
Drought								
7 days	100	96.67	4.73 ± 0.37	4.56 ± 0.28	16.1 ± 0.8	15.8 ± 1.1	15.1 ± 0.2	13.2 ± 0.1*
15 days	98.34	91.67	5.64 ± 0.31	5.07 ± 0.42*	18.9 ± 0.9	17.1 ± 0.4**	39.6 ± 2.9	30.3 ± 0.8**
32 days	98.34	88.33	8.12 ± 0.74	6.03 ± 0.29***	25.2 ± 1.3	18.4 ± 0.9***	74.9 ± 5.1	40.8 ± 2.3***
Recovery								
7 days	96.67	80.00	10.36 ± 0.88	8.23 ± 1.01**	28.4 ± 1.2	23.9 ± 1.1**	107.8 ± 9.7	70.3 ± 10.5**
15 days	96.67	78.33	12.27 ± 0.74	10.41 ± 0.32*	32.6 ± 2.1	27.3 ± 1.9 *	123.5 ± 6.8	84.9 ± 12.2

Stressed plants encountered a progressive dehydration of the substrate during 32 days and were then allowed to recover for 15 days after rehydration of the substrate to 80% field capacity. For shoot dry weight, leaf number and shoot elongation rate, values are means of 6 replicates ± S.E. Asterisks indicated significant difference with control at *$p < 0.05$. **$p < 0.01$ or ***$p < 0.001$

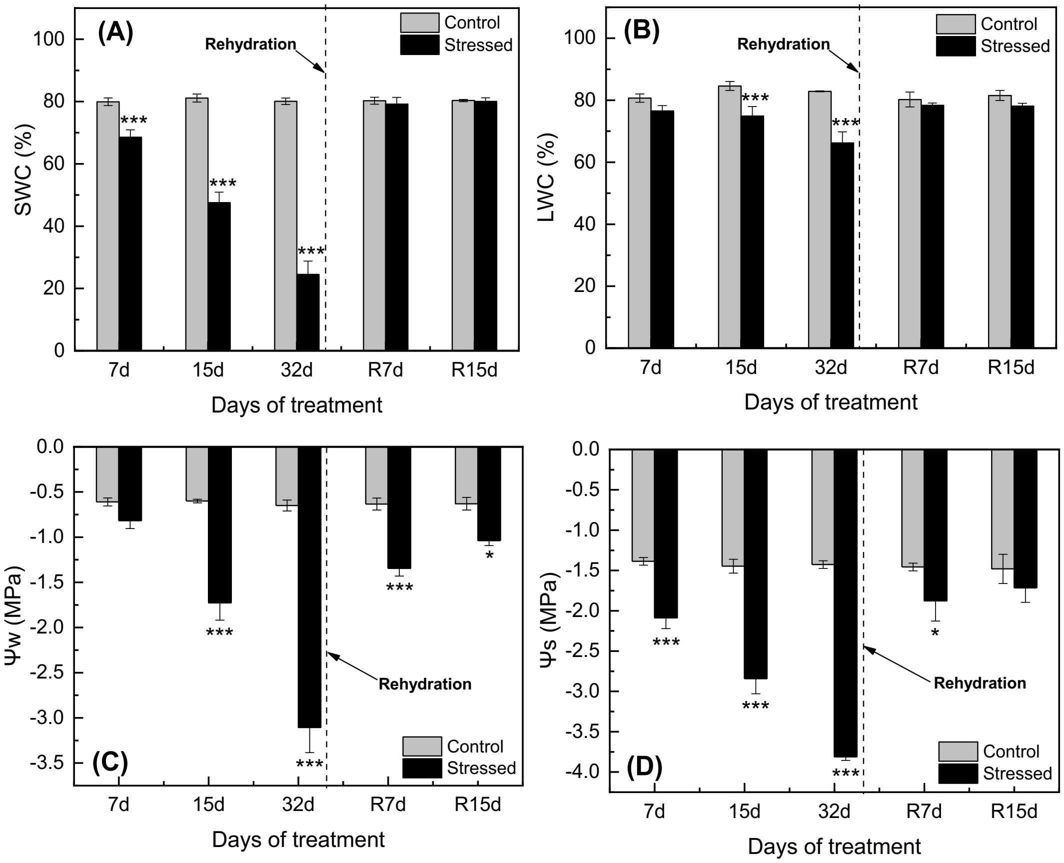

Fig. 1 Soil water content (SWC) expressed as percentage of field capacity (**A**) leaf water content (**B**) shoot water potential (Ψ_w; **C**) and leaf osmotic potential (Ψ_w; **D**) in control (*grey square*), and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean ($n = 6$). Asterisks indicated significant difference with control at *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ by Tukey's HSD Post Hoc test

after 32 days while it increased up to 80% after rehydration and then remained similar to control. The leaf water content (Fig. 1B) was lower for stressed plants than for control after 15 ($F_{1.10} = 25.45$, $p<0.001$) and 32 ($F_{1.10} = 63.84$, $p<0.001$) days of treatment but re-increased to reach similar values than in controls after rehydration ($F_{3.20} = 3.25$, $p=0.0809$). Both Ψ_w (Fig. 1C; $F_{5.30} = 142.82$, $p<0.001$) and Ψ_s (Fig. 1D; $F_{5.30} = 255.45$, $p<0.001$) decreased in response to drought, Ψ_s decreasing earlier than Ψ_w. For both parameters, minimal values were recorded after 32 days of stress. These parameters increased during rehydration: Ψ_w was still lower for stressed plants than for controls after 15 days of recovering ($F_{1.10} = 8.925$, $p=0.0014$) while this was not the case anymore for Ψ_s ($F_{1.10} = 2.004$, $p=0.187$).

Photosynthesis-related parameters

Stomatal conductance (g_s, Fig. 2A; $F_{5.30} = 22.123$, $p<0.001$), net photosynthesis (A, Fig. 2B; $F_{5.30} = 35.33$, $p<0.001$) and instantaneous transpiration (E; Fig. 2C; $F_{5.30} = 405.80$, $p<0.001$) drastically decreased already after 7 days of stress and remained to constant low values throughout the stress period. No significant differences were recorded for g_s between stressed plants and controls during the recovery period ($F_{3.20} = 3.100$, $p=0.05$). Unexpectedly, during the recovery period A values were slightly higher for preliminary stressed plants comparatively to controls after 7 days ($F_{1.10} = 4.996$, $p=0.049$) while values were similar after 15 days ($F_{1.10} = 0.465$, $p=0.511$). In contrast, although E values increased in preliminary stressed plants during the recovery period, the recorded values remained clearly lower than those recorded for control plants ($F_{3.20} = 72.62$, $p<0.001$).

Neither Φ_{PSII} (Fig. 3A; $F_{5.24} = 1.34$, $p=0.313$) nor qP (Fig. 3B; $F_{5.24} = 0.67$, $p=0.651$) were affected in stressed plants, whatever the considered period. In contrast F_v/F_m decreased after 15 days ($F_{1.8} = 6.09$, $p=0.039$) and 32 days ($F_{1.8} = 31.55$, $p<0.001$) of drought treatment (Fig. 3C). Stressed plants did not recover for this parameter which remained lower after the stress relief for previously stressed plants than for controls. Non-photochemical quenching (Fig. 3D) increased in stressed plants during the drought period ($F_{5.24} = 124.31$, $p<0.001$), the difference with control plants being proportional to the duration of stress

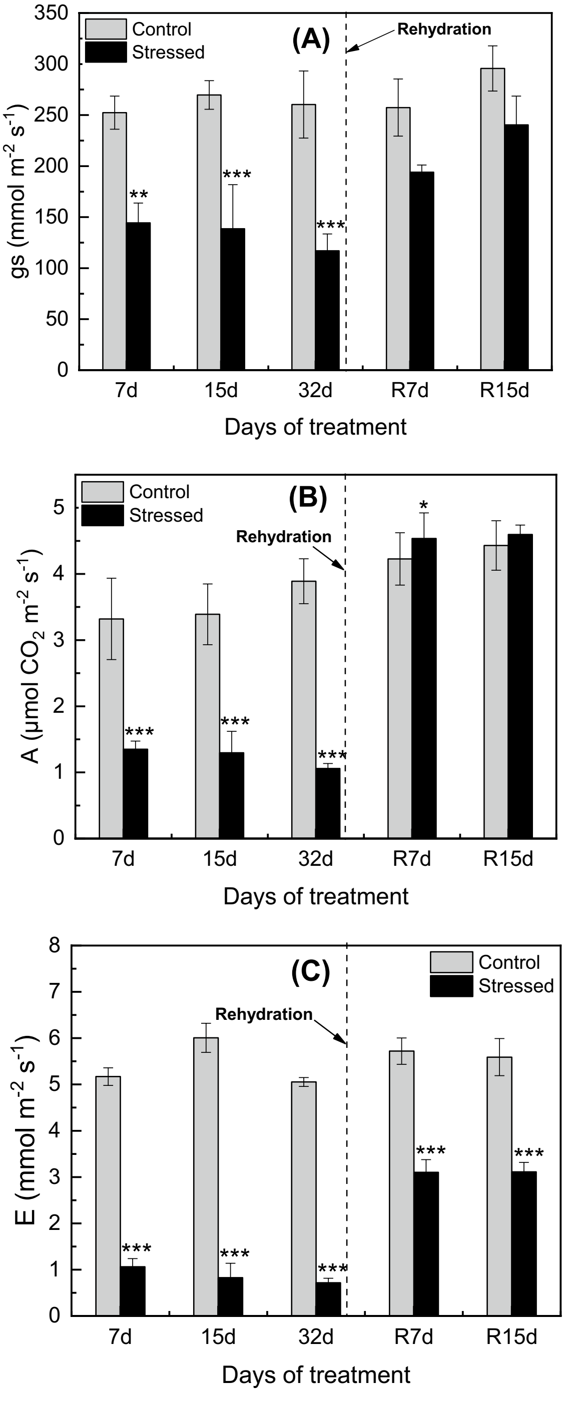

Fig. 2 Stomatal conductance (g_s) (**A**) net photosynthesis (A) (**B**) and instantaneous transpiration (E) in control (*grey square*), and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean ($n=6$). Asterisks indicated significant difference with control at $*p<0.05$, $**p<0.01$ or $***p<0.001$ by Tukey's HSD Post Hoc test

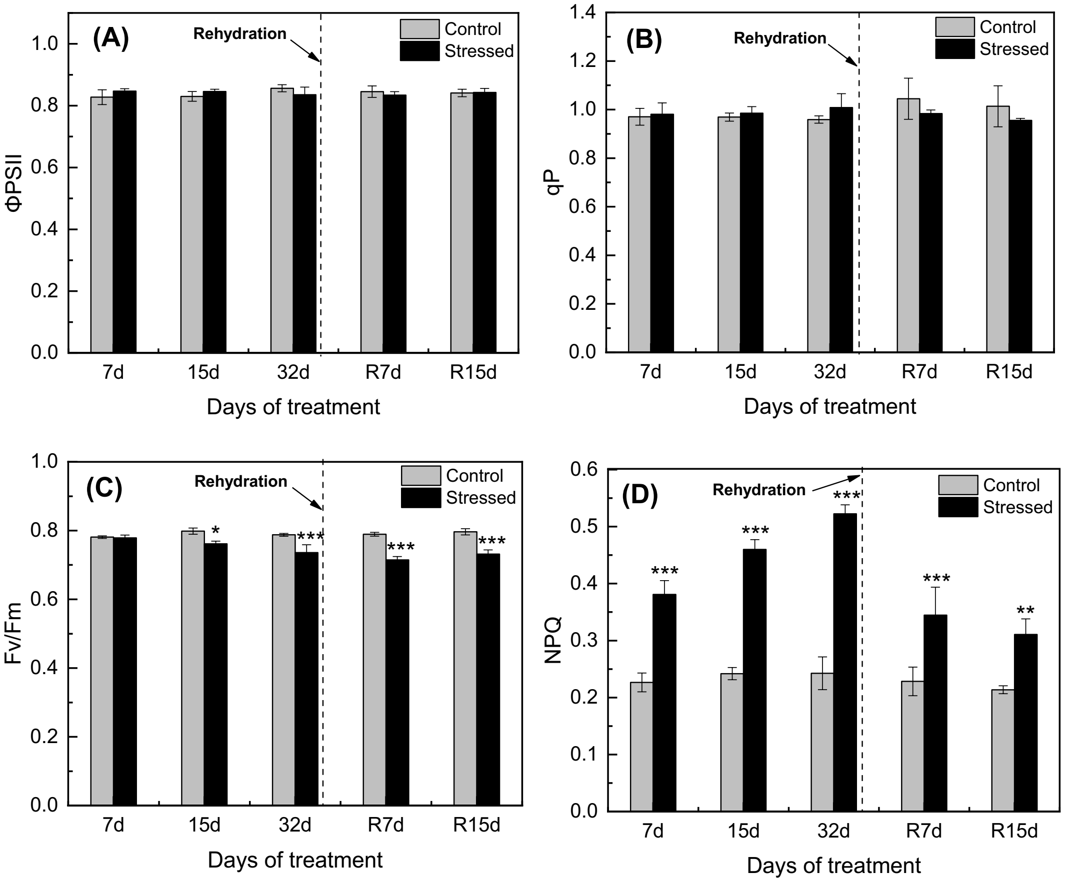

Fig. 3 Operational efficiency of PSII photochemistry (Φ_{PSII}) (**A**) photochemical quenching (qP) (**B**) maximal efficiency of PSII photochemistry in the dark-adapted state (F_v/F_m) (**C**) and non-photochemical quenching (**D**) in control (*grey square*), and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean ($n=5$). Asterisks indicated significant difference with control at $*p<0.05$, $**p<0.01$ or $***p<0.001$ by Tukey's HSD Post Hoc test

exposure. After rehydration, NPQ values progressively decreased but remained higher for previously-stressed plants than for controls ($F_{3.16}$ = 12.610, $p=0.002$).

The total chlorophyll concentration (Fig. 4A) was lower in stressed plants than in controls after 15 days ($F_{1.8}$ = 10.901, $p=0.011$) and 32 days ($F_{1.8}$ = 31.719, $p<0.001$) of treatment. Plants were however able to recover after rehydration and no difference was recorded anymore between control and stressed plants ($F_{3.16}$ = 3.625, $p=0.064$). The plants exposed to drought during 7 days encountered a burst in carotenoid concentration (Fig. 4B; $F_{1.8}$ = 142.19, $p<0.001$). After 15 days of treatment, the same carotenoid concentration was recorded for the two types of plants ($F_{1.8}$ = 0.345, $p=0.573$) while concentration was lower in stressed plants than in controls after 32 days of treatment ($F_{1.8}$ = 32.076, $p<0.001$). During recovery, carotenoid concentrations were similar in control and previously-stressed plants ($F_{3.16}$ = 5.210, $p=0.052$).

Proline concentration and oxidative-related parameters

Proline concentration (Fig. 4C) increased already after 7 days of stress exposure ($F_{5.24}$ = 5.68, $p=0.001$): concentration of accumulated proline was proportional to the stress duration. Just after rehydration, proline concentration in stressed plants

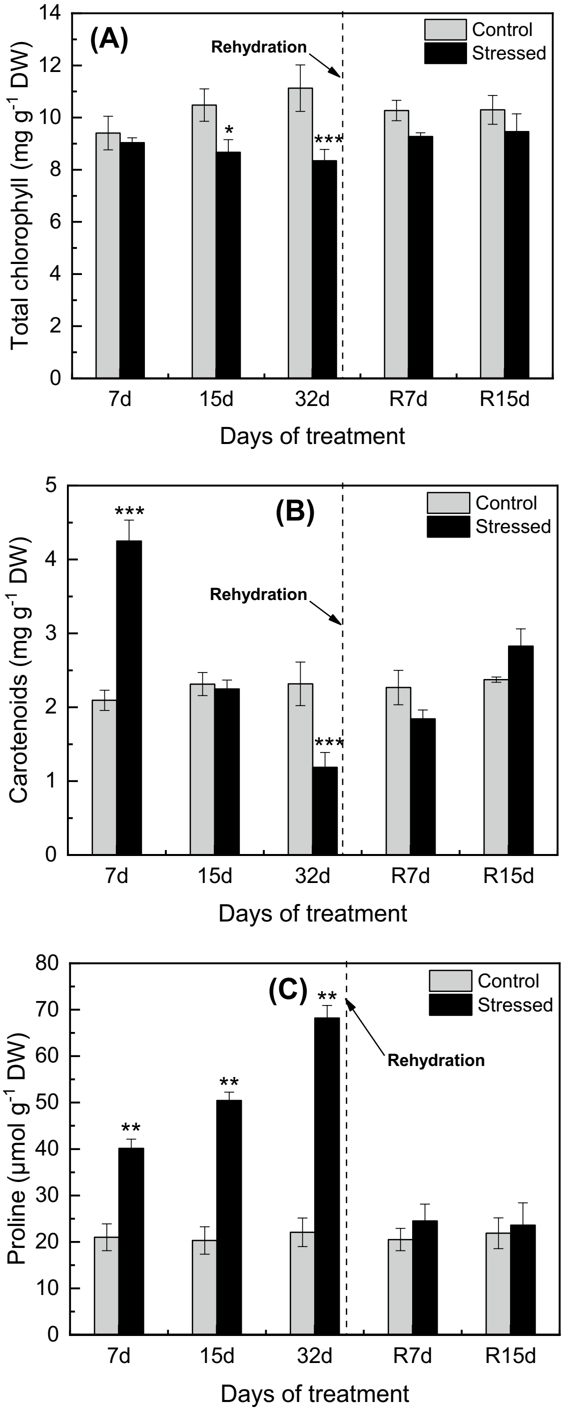

Fig. 4 Total chlorophyll (**A**) carotenoids (**B**) and proline concentration (**C**) in control (*grey square*), and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean ($n=5$). Asterisks indicated significant difference with control at *$p<0.05$, **$p<0.01$ or ***$p<0.001$ by Tukey's HSD Post Hoc test

decreased and reached similar values than for control plants ($F_{3.16}=0.731$, $p=0.561$).

Malondialdehyde (MDA; Fig. 5A) increased in stressed plants after 15 days ($F_{1.8}=46.821$, $p<0.001$) and thereafter remained higher than in control plants; it only slightly decreased during the recovery period ($F_{3.16}$ = 43.59, $p<0.001$). A similar pattern was observed for the rate of electrolyte leakage (Fig. 5B), which remained higher in stressed plants than in controls ($F_{5.24}$ = 33.25, $p<0.001$), including during the rehydration period ($F_{3.16}=58.85$, $p<0.001$).

The total antioxidant capacity is provided in Fig. 5C and was estimated through the DPPH scavenging activity. The antioxidant capacity increased slightly, although significantly, after 7 days of treatment in stressed plants ($p<0.05$); a stronger increase was observed after 15 days ($p<0.001$) and even after 32 days ($p<0.001$) of drought. The DPPH scavenging percentage only slightly decrease after rehydration and remained higher than in controls throughout the recovery period ($p<0.001$).

Concentrations in phenolic compounds

The concentration of total phenolic compounds (Fig. 6) was not affected in stressed plants comparatively to controls after 7 days of treatment ($F_{1.8}$ = 1.016, $p=0.343$) but it strongly increased after 15 days ($F_{1.8}$ = 33.437, $p<0.001$) and even more after 32 days of drought ($F_{1.8}$ = 68.805, $p<0.001$). It only slightly decreased after rehydration and remained higher than in controls ($F_{3.16}=79.18$, $p<0.001$).

Hydroxycinnamic acids progressively increased during drought (Fig. 7A; $F_{5.12}$ = 406.22, $p<0.001$) and then decreased after rehydration, reaching similar values than in controls at the end of the recovery period ($F_{1.4}$ = 4.877, $p=0.091$). As far as various hydroxycinnamic acids are concerned (Table 3), *p*-coumaric acid, caffeic acid, and sinapic acid already increased after only 7 days of stress. For all compounds, maximal concentration was observed after 32 days of treatment, except for *trans*-cinnamic acid which exhibited its highest concentration after 15 days of stress. In all cases, however, concentration decreased after rehydration and only chlorogenic acid ($F_{3.8}$ = 7.483, $p=0.010$) was still higher in stressed plants than in controls at the end of the recovery period.

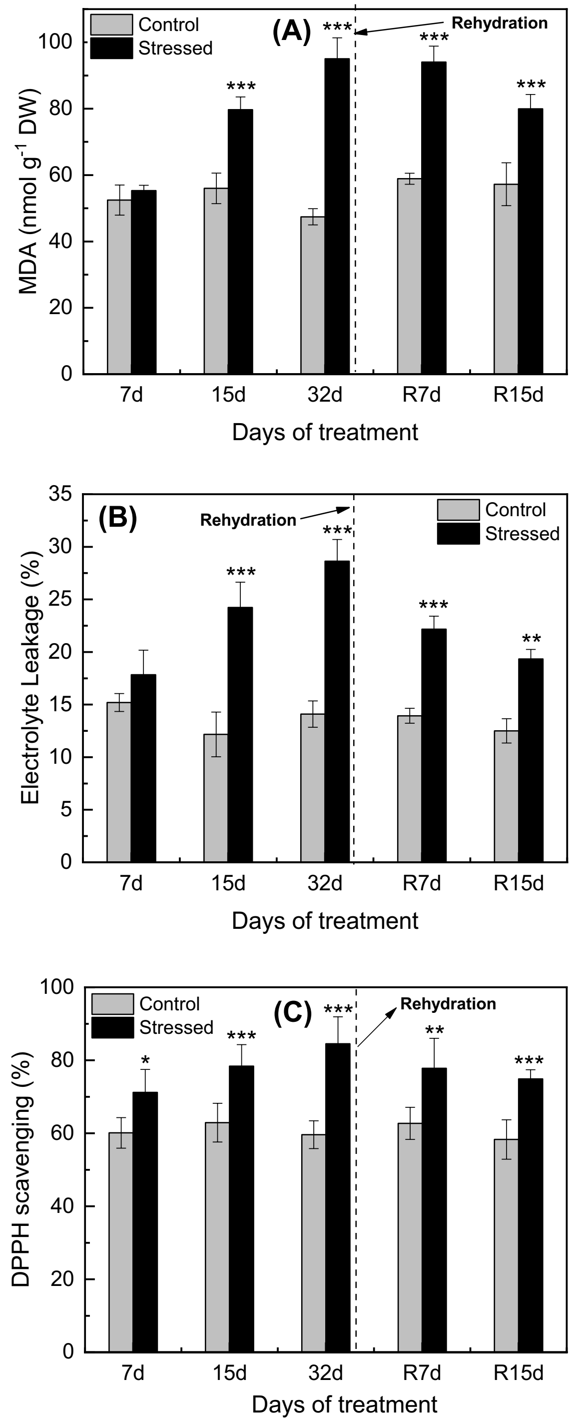

Fig. 5 Malondialdehyde concentration (**A**) electrolyte leakage (**B**) and DPPH scavenging (**C**) in control (*grey square*) and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean ($n = 5$). Asterisks indicated significant difference with control at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test

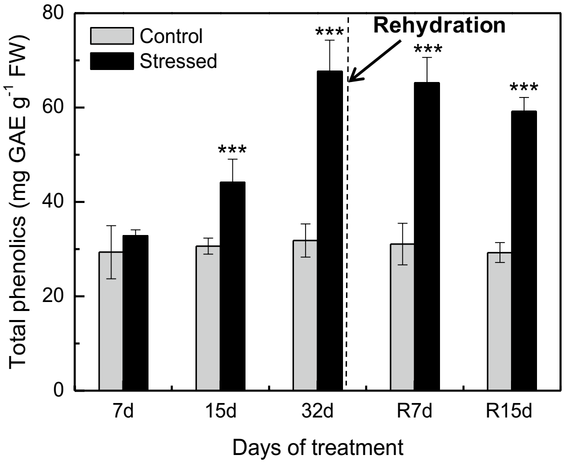

Fig. 6 Total phenolics content in control (*grey square*) and drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Bars represent standard error of mean ($n = 5$). Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days.Asterisks indicated significant difference with control at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test

Hydroxybenzoic acids (Fig. 7B; $F_{5.12} = 32.135$, $p < 0.001$) slightly, but significantly increased during the stress period. After rehydration, this class of compounds did not decrease during recovery and remained higher in stressed than in control plants ($F_{3.8} = 39.378$, $p < 0.001$). As shown in Table 4, all identified compounds (benzoic acid, *p*-hydroxy-benzoic acid, vanillic acid, salicylic acid, gallic acid and protocatechuic acid) behaved in the same way, the major compounds being benzoic acid and gallic acid. As a result, the total concentration of phenolic acids (Fig. 7C) increased during drought ($F_{5.12} = 109.52$, $p < 0.001$) and decreased during recovery ($F_{3.8} = 42.865$, $p < 0.001$) but remained higher in stressed plants than in controls at the end of the experiment.

Flavanols (Fig. 8A) concentrations decreased in response to water stress, already after 7 days of stress ($F_{1.4} = 8.626$, $p = 0.043$), but then slightly increased after rehydration to reach values like those observed in control plants after 7 days of recovery ($F_{1.4} = 0.221$, $p = 0.662$). Such a trend was observed for catechin-gallate, epicatechin and gallocatechin-gallate (Table 5) while catechin was still lower in stressed plants than in controls. The only identified flavone is acacetin which was detected only in stressed plants. Moreover, at the end of the recovery period,

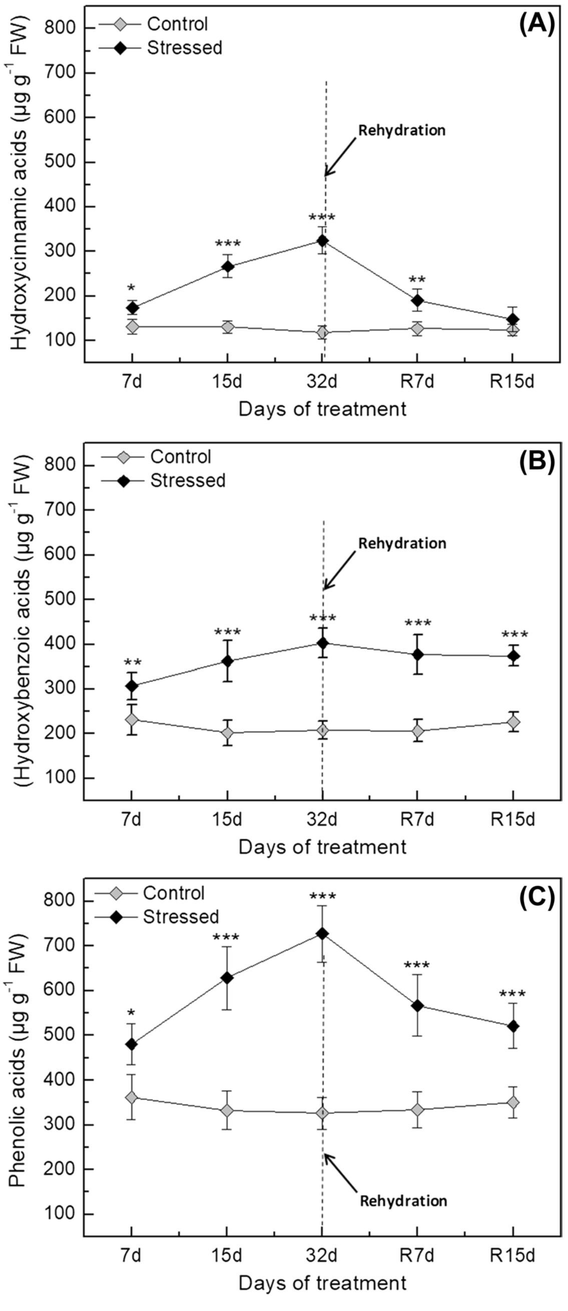

Fig. 7 Hydroxycinnamic acids (**A**), hydroxybenzoic acids (**B**) and phenolic acids (**C**) contents in control (*grey diamond*) and drought stressed plants (*black diamond*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard error of mean ($n = 3$). Asterisks indicated significant difference with control at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test

gallocatechin was higher in previously-stressed plants than in controls. In contrast to flavanols, the flavonols concentration (Fig. 8B; $F_{5.12} = 72.361$, $p < 0.001$) linearly increased during the stress period but also during the recovery period, reaching the highest concentration at the end of the experiment ($F_{1.4} = 346.47$, $p < 0.001$) (Table 6). Once again, a uniform trend was observed for all identified compounds belonging to this category (Table 5): quercetin and quercetin-3-0-rutoside exhibited the highest concentration comparatively to other compounds. As an overall consequence, the concentration of total flavonoids (Fig. 8C) increased with time during both drought ($F_{5.12} = 10.579$, $p < 0.001$) and recovery ($F_{3.8} = 100.38$, $p < 0.001$) periods.

Enzyme activities and gene expression

Activities of phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) are presented in Fig. 9A and B, respectively. Both enzyme activities exhibited similar increase during the drought period and increased already after 7 days of treatment (PAL : $F_{1.10} = 76.289$, $p < 0.001$; C4H : $F_{1.10} = 12.089$, $p = 0.025$). Both activities decreased in stressed plants during the recovery period. After 15 days of rehydration, PAL activity was still higher in stressed than in control plants ($F_{1.10} = 32.333$, $p < 0.001$) while C4H activity was similar for the two types of plants ($F_{1.10} = 2.183$, $p = 0.199$).

Semi-quantitative expression of the gene coding for PAL (Fig. 10A) provides evidence that stressed and control plants did not differ after 7 days ($F_{1.10} = 0.023$, $p = 0.881$) and 15 days ($F_{1.10} = 1.756$, $p = 0.191$) of stress, and that *PAL* expression was even slightly lower in stressed plants than in controls after 32 days of stress ($F_{1.10} = 27.102$, $p < 0.001$). During the recovery period, *PAL* gene expression was similar after 7 days of rehydration and was again slightly lower in previously-stressed plants than in controls 15 days after rehydration ($F_{1.10} = 25.684$, $p < 0.001$). As far as gene coding for C4H is concerned (Fig. 10B), its expression was stimulated in stressed plants after 7 days ($F_{1.10} = 77.789$, $p < 0.001$) and 15 days ($F_{1.10} = 103.61$, $p < 0.001$) of stress, while an inverse trend was recorded at the end of the stress period, gene expression being higher for control plants than for stressed ones ($F_{1.10} = 164.69$, $p < 0.001$). During the recovery period *C4H* gene expression was higher

Table 3 Cinnamic acid and derivatives content in leaves extracts of *Acacia arabica* (Lam.) Willd (C: control). under water stress treatment (S) and after recovery (R) during growth

	Days of treatment						
Cinnamic acid and derivatives ($\mu g\ g^{-1}$ FW)			*7d*	*15d*	*32d*	*R7d*	*R15d*
	t-Cinnamic acid	*C*	10.9 ± 3.5	12.3 ± 1.7	10.5 ± 2.1	12.5 ± 3.3	12.6 ± 2.2
		S	15.2 ± 4.1	21.8 ± 3.4*	19.8 ± 6.8*	21.0 ± 4.7*	12.8 ± 4.0
	p-Coumaric acid	*C*	13.2 ± 2.9	12.7 ± 1.8	14.2 ± 1.9	12.6 ± 2.3	12.4 ± 2.6
		S	28.1 ± 1.8***	32.8 ± 2.4***	40.8 ± 4.2***	23.0 ± 4.1***	14.9 ± 2.7
	Caffeic acid	*C*	37.2 ± 1.2	35.8 ± 2.4	30.1 ± 1.2	39.6 ± 4.3	35.1 ± 2.1
		S	45.7 ± 4.7*	69.3 ± 5.6***	75.3 ± 7.0***	51.8 ± 2.6**	34.5 ± 4.8
	Chlorogenic acid	*C*	48.6 ± 5.8	54.1 ± 5.5	43.9 ± 7.8	44.5 ± 4.8	44.7 ± 3.2
		S	63.8 ± 5.2	113.4 ± 12.1***	139.6 ± 13.1***	71.9 ± 7.9**	61.7 ± 13.9*
	Ferulic acid	*C*	23.3 ± 4.7	20.5 ± 3.7	23.1 ± 3.8	23.9 ± 2.4	22.9 ± 4.2
		S	22.3 ± 2.4	33.9 ± 3.7**	49.4 ± 3.9***	34.9 ± 8.8*	25.5 ± 4.2
	Sinapic acid	*C*	8.5 ± 1.7	7.4 ± 0.6	6.6 ± 0.6	6.4 ± 1.5	9.2 ± 1.1
		S	14.2 ± 1.6***	17.0 ± 1.6***	19.9 ± 2.4***	8.9 ± 1.3	10.9 ± 2.0

(ND: Not detected). Each value is the mean of three replicates ± S.E. Asterisks indicated significant difference with control at $*p < 0.05$. $**p < 0.01$ or $***p < 0.001$

Table 4 Benzoic acid and derivatives content in leaves extracts of *Acacia arabica* (Lam.) Willd. (C: control) under water stress treatment (S) and after recovery (R) during growth

	Days of treatment						
Benzoic acid and derivatives ($\mu g\ g^{-1}$ FW)			*7d*	*15d*	*32d*	*R7d*	*R15d*
	Benzoic acid	*C*	91.7±8.6	76.4±5.4	73.0±8.2	83.3±9.0	73.2±9.0
		S	130.2±21.9**	124.5±12.8***	144.4±8.1***	131.9±13.5***	137.2±13.9***
	p-Hydroxy-benzoic acid	*C*	4.7±0.6	4.8±0.3	4.6±0.5	4.6±0.7	4.6±0.6
		S	6.2±0.3*	7.86±1.13***	9.6±0.5***	8.9±0.8***	9.4±0.8***
	Vanillic acid	*C*	10.2±1.1	9.7±0.5	9.2±1.1	7.7±0.3	7.7±0.3
		S	14.2±1.4***	15.4±1.0***	16.6±0.6***	16.1±0.6***	15.7±0.8***
	Salicylic acid	*C*	25.9±8.9	24.7±4.7	22.3±4.5	26.4±5.6	25.7±3.6
		S	51.5±3.4***	45.7±1.7***	43.1±4.3***	38.7±7.9*	36.4±3.1*
	Gallic acid	*C*	177.3±21.1	153.7±22.8	163.0±14.0	159.7±16.8	181.3±16.5
		S	228.0±24.0*	278.0±39.3***	315.7±27.2***	296.0±32.1***	295.0±16.5***
	Protocatechuic acid	*C*	13.00±2.16	8.8±0.5	9.2±0.5	8.4±1.5	6.5±1.1
		S	6.8±1.3***	14.9±2.0***	17.5±0.6***	17.0±2.2***	17.3±1.7***

(ND : Not detected). Each value is the mean of three replicates ± S.E. Asterisks indicated significant difference with control at $*p < 0.05$. $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test

after 7 days ($F_{1.10}$ = 178.32, $p < 0.001$) of rehydration in stressed plants comparatively to controls, while values were similar after 15 days of recovery ($F_{1.10}$ = 1.859, $p = 0.203$).

The activity of chalcone synthase (CHS) could not have been accurately quantified. The expression of gene coding for CHS revealed that it was down-regulated at the end of the stress period (Fig. 10C; $F_{1.10}$= 69.705, $p < 0.001$). In contrast, during the recovery period, expression of the gene coding for CHS was clearly higher in stressed plants than in controls ($F_{3.20}$ = 98.692, $p < 0.001$).

Discussion

The present work confirms that *Acacia arabica* is tolerant to water stress even at the seedling stage.

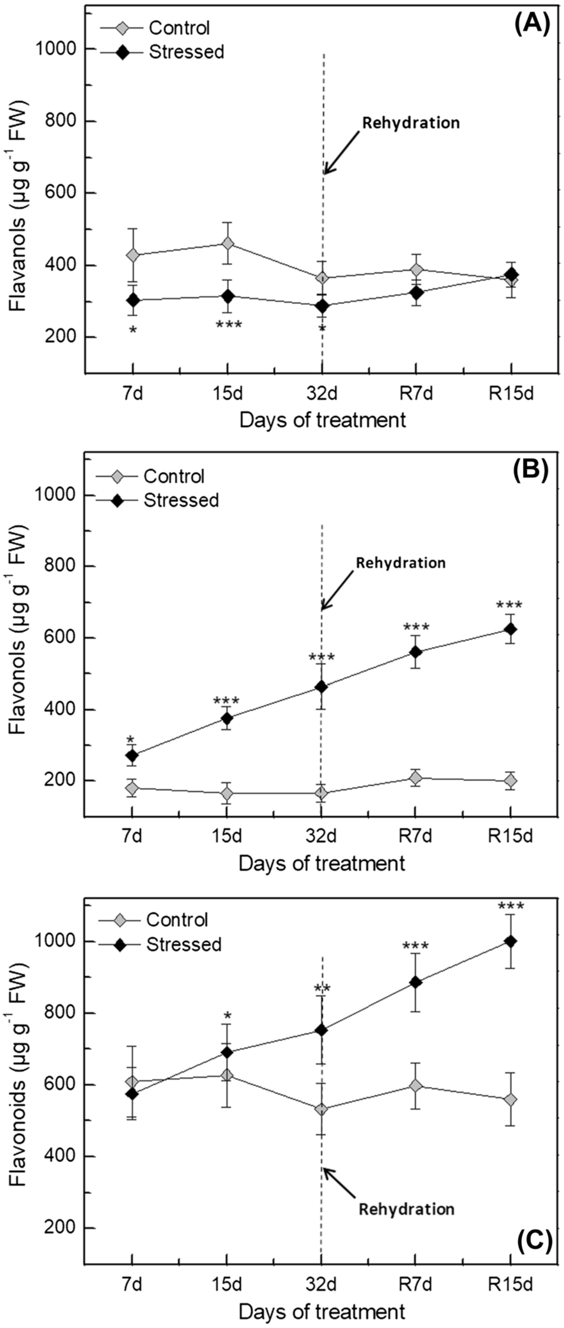

Fig. 8 Flavanols (**A**), flavonols (**B**) and flavonoids (**C**) contents in control (*grey diamond*) and drought stressed plants (*black diamond*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard error of mean ($n = 3$). Asterisks indicated significant difference with control at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ by Tukey's HSD Post Hoc test

Indeed, 88% of the stressed plants were able to survive to a progressive drought of 32 days which lowered the soil water content from 80% to less than 35% of field capacity. During the stress period, *A. arabica* triggered adaptative mechanisms allowing the plants to cope with water shortage. The decrease in leaf number could be regarded as an attempt to reduce the total transpiring surface (Nadal-Sala et al. 2021; Huang et al. 2022; Wu et al. 2022). The plants also reduced their osmotic potential to maintain a favorable water gradient and closed their stomata to reduce water losses. Cory et al. (2022) demonstrated that first year seedling in *A. tortilis* and *A. robusta* typically behave as water spending anisohydric plants, but the rapid stomatal closure observed with *A. arabica* associated with a rapid osmotic adjustment suggests that this species is able to find a good compromise between isohydric and anisohydric strategies. While the soil water content strongly dropped during the stress period, the mean leaf water content decreased after 15 days but the recorded decrease was rather low comparatively to the decrease in the soil WC and it remained always compatible with physiological activities. Moreover, *A. arabica* seedlings appeared quite responsive to water stress since numerous parameters (Ψ_s, g_s, A, E, NPQ, carotenoids, proline, DPPH activity, phenolic acids, PAL and C4H activities) were already modified after 7 days of treatment while SWC was only slightly decreased and LWC was unaffected.

Contrasting with the low level of mortality at the end of the stress, it is noteworthy that 10% of the previously stressed surviving plants died after the stress relief. This, however, is not a proof that brutal rehydration of the substrate is toxic for the plant since it might be hypothesized that some stressed plants were exhausted (although still alive) at the end of the stress period and that their death occurring after rehydration was just an ultimate consequence of disorders occurring during the stress period. Alternatively, some authors reported that a sudden rehydration of tissue is inducing an internal constraint since numerous cellular structures could be transiently disorganized as a result of a massive water flow (Morales-Sánchez et al. 2022; Prats and Brodersen 2021; Wang et al. 2022). Most of these studies, however, are based on desiccation-tolerant poikilohydric plant species able to lose almost all their water content, arrest all physiological activities, remain in a quiescent dehydrated state and then

Table 5 Flavanols content in leaves extracts of *Acacia arabica* (Lam.) Willd. (C: control) under water stress treatment (S) and after recovery (R) during growth

	Days of treatment						
Flavanols (μg g^{-1} FW)			*7d*	*15d*	*32d*	*R7d*	*R15d*
	Catechin	*C*	211.9±32.4	234.5±32.3	186.1±10.1	186.6±11.0	183.4±34.1
		S	131.0±21.7***	131.9±14.4***	104.6±7.5***	131.8±13.8**	158.0±14.8
	Catechin-gallate	*C*	11.3±00.0	ND	14.4±00.0	12.7±00.0	ND
		S	13.4±1.0	18.5±3.3***	21.8±3.1***	15.2±0.3	10.2±00.0***
	Epicathechin	*C*	62.5±16.2	58.2±6.2	56.7±7.5	60.1±11.3	64.2±3.6
		S	46.1±5.4*	38.2±7.8*	29.2±8.3**	44.0±4.9	53.8±5.6
	Gallocatechin	*C*	111.8±15.4	140.6±15.5	86.9±24.3	97.4±14.7	89.3±7.7
		S	85.9±8.8*	78.2±4.7***	67.1±6.9	90.70±9.02	128.9±8.2**
	Gallocatechin-gallate	*C*	31.5±9.9	28.3±3.9	21.4±5.3	31.8±4.4	22.9±2.8
		S	27.8±5.1	47.7±15.1**	65.7±6.1***	43.0±6.6	24.4±5.4
Flavone (μg g^{-1} FW)	*Acacetin*	*C*	ND	ND	ND	ND	12.3±00.0
		S	ND	15.8±0.7***	16.1±3.3***	18.9±3.5***	17.3±2.2**

(ND : Not detected). Each value is the mean of three replicates ± S.E. Asterisks indicated significant difference with control at *$p<0.05$. **$p<0.01$ or ***$p<0.001$ by Tukey's HSD Post Hoc test

Table 6 Flavanols content in leaves extracts of *Acacia arabica* (Lam.) Willd. (C: control) under water stress treatment (S) and after recovery (R) during growth

	Days of treatment						
Flavonols and glycosides (μg g^{-1} FW)			*7d*	*15d*	*32d*	*R7d*	*R15d*
	Quercetin	*C*	64.50±6.22	57.70±3.08	52.70±6.63	65.47±5.85	69.33±5.90
		S	68.43±5.00	96.23±6.58***	119.73±7.10***	143.60±10.83***	172.70±9.56***
	Quercetin-3-O-rutinoside (= rutin)	*C*	54.87±5.40	40.57±12.89	46.63±6.20	59.43±4.22	50.70±3.65
		S	103.17±8.85**	134.87±11.54***	172.07±38.01***	215.33±18.38***	232.27±9.71***
	Kaempferol	*C*	26.43±4.34	28.67±5.37	23.80±4.71	28.30±6.17	26.27±1.93
		S	41.07±4.92**	48.70±3.70**	59.10±3.80**	70.53±5.10**	76.33±3.80**
	Myricetin	*C*	22.27±8.84	25.37±5.65	24.43±6.65	24.57±4.54	25.07±4.29
		S	25.50±5.25	36.53±4.41*	48.33±1.98***	56.97±3.09***	56.03±4.21***
	Isorhamnetin	*C*	12.00±00.00	13.85±3.89	19.20±00.00	13.55±1.77	17.67±8.88
		S	19.05±6.15	28.07±2.51*	30.23±7.08*	33.93±6.39**	43.43±6.13**
	Isorhamnetin-3-O-rutinoside	*C*	ND	ND	ND	18.05±0.35	11.70±0.57
		S	14.90±00.00***	31.80±3.76***	35.27±4.34***	40.93±3.27***	44.67±7.29***

(ND : Not detected). Each value is the mean of three replicates ± S.E. Asterisks indicated significant difference with control at *$p<0.05$. **$p<0.01$ or ***$p<0.001$ by Tukey's HSD Post Hoc test

resume full physiological activities and metabolism after rehydration. Situation may be somewhat different in "classical" plants such as *A. arabica* because desiccation was limited and the plants still maintained physiological activities during the whole stress period. However, the behavior of stressed plants is not only a function of stress intensity, it is also a direct function of the kinetics of stress application (Huang et al. 2022). It is commonly assumed that a progressive stress imposition allows the plant to acclimate while a sudden stress application is quite deleterious, even if final stress intensities are similar in both cases (Almansouri et al. 1999). In the present work, soil dehydration occurred progressively while soil rehydration occurred suddenly, as it is the case after heavy rain in field conditions. It is therefore interesting to compare the kinetics of physiological modifications occurring at the plant

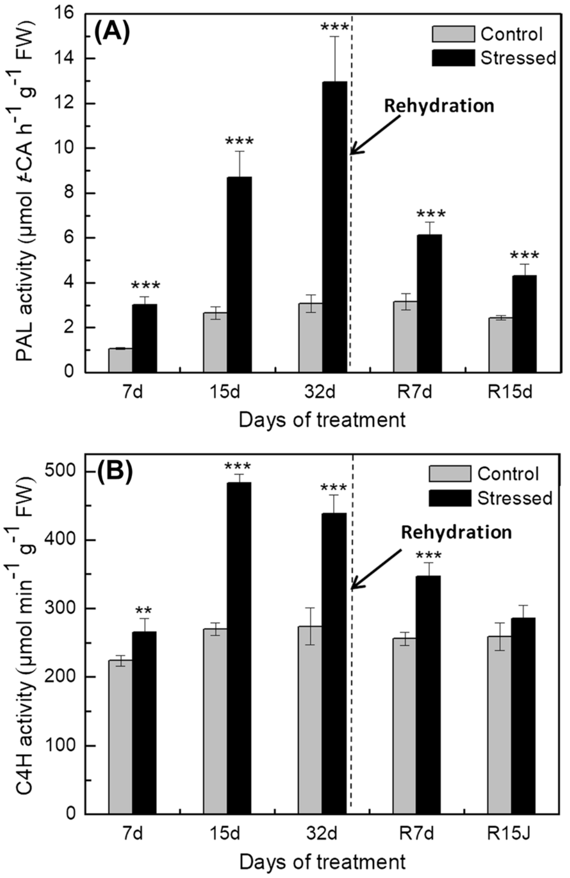

Fig. 9 Phenylanine ammonia-lyase (PAL) (**A**) and cinnamate-4-hydroxylase (C4H) (**B**) activities in control (*grey square*), drought stressed plants (*black square*) of *Acacia arabica* (Lam.) Willd. Plants were exposed to progressive drought during 32 days and allowed to recover for 15 days. Bars represent standard deviation of mean (n = 6). Asterisks indicated significant difference with control at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test

level during a progressive soil dehydration on the one hand, and a steep soil rehydration on the other hand.

Among the recorded physiological parameters, a first category comprises those parameters that fully and quickly recovered after rehydration. This was the case for leaf WC, Ψ_s, g_s, A, total chlorophyll, carotenoids, proline, hydroxycinnamic acids, flavonols and C4H activities. For all these parameters, values recorded after 15 days of rehydration were similar to those recorded for non-stressed controls. Stomatal movement are controlled by the phytohormone abscisic acid (ABA): it sometimes occurs that the persistence of ABA in previously stressed plants induces a delay in stomatal opening and gas exchange recovery after rehydration (Hasan et al. 2021). According to Schley et al. (2022), specific aquaporins may be involved in the recovery of stomatal opening during rehydration. After drought relief in the present work, gas exchanges were measured in leaves appearing during the recovery period and this could explain the high g_s values recorded for those leaves. Changes in stomatal patterning may also occur on emerging leaves and could have an impact on gas exchanges (Huang et al. 2022; Xiong et al. 2022). Although net photosynthesis (A) fully recovered after drought relief, instantaneous transpiration (E) did not and remained quite lower in the previously stressed plants than in the control ones. The consequence was that water use efficiency (A/E) was by far higher in the previously stressed plants (1.54 ± 0.2 µmol CO_2.mmol H_2O^{-1}) than in the control ones (0.83 ± 0.09 µmol CO_2.mmol H_2O^{-1}). It could not be excluded that embolism processes occurred during the drought period and compromised water flow during rehydration. However, according to the hydraulic stomatal limitation model (Hasan et al. 2021), the rapid drop of g_s recorded in the present study after only 7 days of water shortage should have prevented embolism process. A low transpiration rate during recovery despite full water availability did not compromise growth resumption after drought relief, at least for surviving plants as clearly stated in Table 2, but it could explain that NPQ values remained higher than in controls in order to contribute to leaf protection against an excess of light energy absorption.

A specific attention should be paid to phenolic compounds. Indeed, the total phenolics increased in response to drought and remained high throughout the recovery period (Fig. 6). Numerous studies demonstrated that phenolic compounds act as free radical scavengers and assume key functions in the antioxidative status of stressed plants (Sgherri et al. 1994, 2004; Grace and Logan 2000; Cheng et al. 2018; Chu et al. 2019; Saidi et al. 2021). Malondialdehyde concentration (Fig. 5A) and rate of electrolyte leakage (Fig. 1B) remained high during recovery, confirming that oxidative stress still occurred after drought relief. Beside phenolic compounds, proline is also considered as a powerful antioxidant in stressed plants (Zulfiqar and Ashraf 2023) but its concentration quickly decreased after drought relief suggesting that

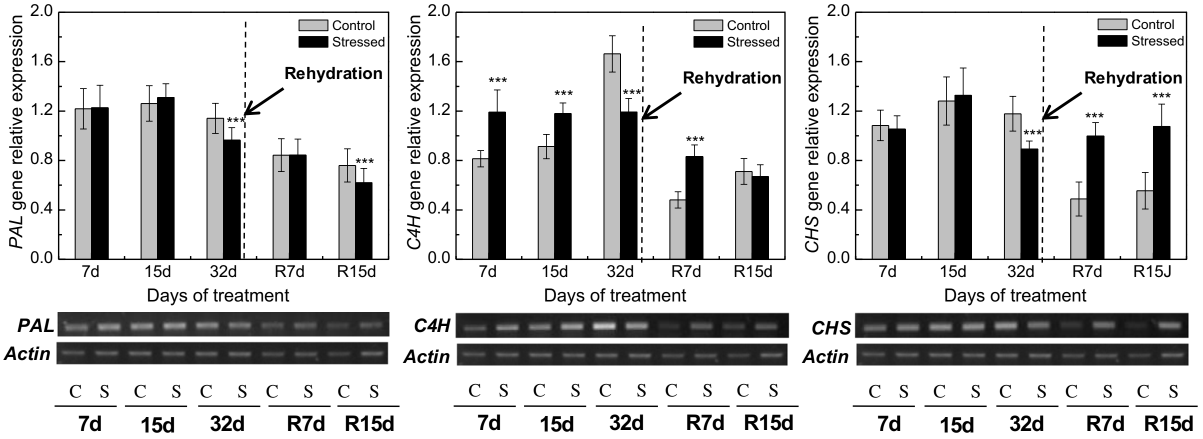

Fig. 10 *PAL* (**A**), *C4H* (**B**) and *CHS* (**C**) gene expression pattern by using semi-quantitative RT-PCR. Bars represent standard deviation of mean (n = 6). Asterisks indicated significant difference with control at $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ by Tukey's HSD Post Hoc test. Semi quantitative-reverse transcriptase PCR analysis of *Phenylalanine ammonia-lyase (PAL)* (DQ377806.1), *Cinnamate-4-hydroxylase (C4H)* (JN204274.1) and *Chalcone synthase (CHS)* (JN812063.1) implicated in control, drought stressed and rehydrated plants of *Acacia arabica* (Lam.) Willd. *Actin* (GR482442.1) transcripts were used as PCR control

it assumes osmotic rather than antioxidant functions in *A. arabica* exposed to water stress. It is therefore temping to speculate that the maintenance of the phenolic compounds may account, at least partly, for the maintenance of the total antioxidant properties during recovery, as quantified by DPPH scavenging percentage (Fig. 5C), although other important endogenous antioxidant such as glutathione and ascorbic acid which were not quantified in the present study may also contribute to the protection process.

Exogenous applications of numerous phenolic acids were shown to improve drought and salt tolerance in various plant species (Sun et al. 2012; Ozfidan-Konakci et al. 2015; Saidi et al. 2021; Gharbi et al. 2016; Cheng et al. 2018; Chavoushi et al. 2019; Quan and Xuan 2018; Parvin et al. 2020; Zafar-ul-Hye et al. 2021). In most cases, the protective impact was due to a better antioxidant ability of treated plants: phenolic acids act either directly as antioxidant or indirectly through the stimulation of antioxidant enzymes (Sun et al. 2012; Ozfidan-Konakci et al. 2015; Cheng et al. 2018). Although all phenolic acids increased during drought period, a clear distinction should be established between hydroxycinnamic acid which decreased during the recovery period to reach control level, and hydroxybenzoic acids whose concentrations did not decrease and remained constant during the recovery period (Fig. S1). It is noteworthy that within each class of compounds, all phenolics exhibited a uniform trend. This suggests that for each class of compounds, pathways were coordinately regulated but also that hydroxycinnamic acids and hydroxybenzoic acids assume distinct functions, at least during recovery.

The precursor of hydroxycinnamic acids *trans*-cinnamic acid (Fig. S1) accumulated during the drought period but nevertheless remained at low concentrations in stressed plants (less than 22 $\mu g.g^{-1}$ FW). *Trans*-cinnamic acid may have a dual impact in stressed plants. It has been reported to dissipate the proton gradient across membranes and interferes with plant ion uptake (Marchiosi et al. 2020). In contrast, Sun et al. (2012) reported that *trans*-cinnamic pretreatment of sorghum increased antioxidative enzyme activities during water stress. It could however be argued that cinnamic acid in this latter work was applied as a short pretreatment before stress imposition and that it might have been considered by the plant as an indicator of stress which triggered antioxidative defense.

Cinnamic acid is also the precursor of hydroxycinnamic acids (Fig. S1) whose protective roles are clearly established. Caffeic acid plays important roles in several physiological processes in plants including regulation of turgor pressure and cell expansion (Zafar-ul-Hye et al. 2021). It also acts as a precursor of lignin and in some plant species such as *Ocimum basilicum*, caffeic acid is the major antioxidant under drought conditions (Zare et al. 2021). Caffeic acid is converted into ferulic acid by an O-methyltransferase (Fig. S1). Ferulic acid is an important compound in plant metabolism since it could inhibit auxin decarboxylation, contribute to the growth and development of root system and reverse the growth inhibition induced by ABA (Marchiosi et al. 2020). Hura et al. (2009) reported that under drought conditions, an important proportion of ferulic acid is present in the apoplasm where it is bound to cell wall polysaccharides by ester links, inducing a decrease in cell wall extensibility and organ elongation. Such a growth inhibition may somewhat be regarded as an attempt of stress adaptation rather than a symptom of injury since osmotic adjustment required to maintain leaf water content consumes metabolites and energy that are then not available for growing purposes. When water was fully available after drought relief, osmotic adjustment was not required anymore and ferulic acid should then decrease in newly-formed leaves to allow growth resumption. From a quantitative point of view, chlorogenic acid was the major hydroxycinnamic acid detected in *A. arabica* (Table 3). Desiccation-tolerant resurrection plants often contain high constitutive concentrations of chlorogenic acid and its poly-hydroxy nature makes it a more active antioxidant than monohydroxy-derivatives of benzoic acid (Sgherri et al. 2004; Hodaei et al. 2018).

The first step of hydroxycinnamic acid synthesis is catalyzed by phenylalanine ammonia-lyase (PAL EC 4.3.1.5) (Fig. S1). It is an important regulation point in the phenylpropanoid pathway and induces deamination of phenylalanine to form *trans*-cinnamic acid (Marchiosi et al. 2020; Liu et al. 2023). PAL activity strongly increased in response to water stress (Fig. 9A) in agreement with data obtained by other authors who demonstrated a positive correlation between phenolic content and PAL activity (Zare et al. 2021). However, genes coding for PAL were not up-regulated by water stress (Fig. 10A) suggesting that PAL activation in *A. arabica* mainly occurred as a result of post-transcriptional activation processes. It has however to be mentioned that several PAL isoforms encoded by distinct genes exist in plant species (Olsen et al. 2008; Liu et al. 2023). The determination of total PAL activity and the use of one single consensus sequence for RT-PCR analysis did not allow us to discriminate among isoforms and it could be therefore not excluded that some of them were transcriptionally regulated as demonstrated by Olsen et al. (2008). The second step of the phenylpropanoid pathway is regulated by the cytochrome P450 monooxygenase cinnamate 4-hydroxylase (C4H) which catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid (Fig. S1). As previously reported for PAL, a positive correlation is commonly found between C4H activity and phenolic compounds (Wang et al. 2017; Marchiosi et al. 2020). However, in contrast to PAL, our data suggest that C4H may be transcriptionally regulated in *A. arabica* in response to water stress. C4H activity indeed increased during the drought period and was still higher than in controls 7 days after rehydration (Fig. 9B). Similarly, gene expression was upregulated during drought period (except at day 32 where control displayed an unexpectedly high gene expression). The gene was still overexpressed comparatively to controls after 7 days of recovery, but not anymore after 15 days when C4H activity was also similar to control. Wang et al. (2017a, b) also found a drought-induced overexpression of *IbC4H* in sweet potato.

C4H activity is not involved in hydroxybenzoic acids synthesis which are directly produced from *trans*-cinnamic acid (Fig. S1) by shortening the propanoid side chain with Co-A-independent or CoA-dependent oxidative pathways. Hydroxybenzoic acids may also be produced through CoA-dependent non-oxidative pathways (Marchiosi et al. 2020). Hydroxybenzoic acids increased during drought period, and then remained constant during the recovery phase. The most important from a quantitative point of view is gallic acid which reached 300 $\mu g.g^{-1}$ FW at the end of the drought period (Table 5). Gallic acid was reported to increase the proline content in rice exposed to osmotic stress (Ozfidan-Konakci et al. 2015). Although this might have occurred in *A. arabica* during the drought period when gallic acid and proline accumulated concomitantly, this was not the case anymore during recovery since proline decreased very quickly while gallic acid remained at high concentrations. Gallic acid enhances antioxidant capacity reducing ROS production and preventing lipid peroxidation through the enhancement of catalase, ascorbate peroxidase and glutathione reductase activities (Saidi et al. 2021) and these properties support our hypothesis that hydroxybenzoic contribute to regulate oxidative status during rehydration. Benzoic acid also accumulated at high concentrations in the stressed tissues and remained higher than 130 µg.g^{-1} FW throughout the recovery phase. Benzoic acid was reported to assume positive functions in response to stress such as inhibition of ethylene synthesis (Marchiosi et al. 2020) and increase in chlorophyll content, photosynthetic rate and stomatal conductance (Anjum et al. 2013). Concentration of salicylic acid was lower than concentrations recorded for benzoic or gallic acids but follow the same trend during drought/recovery cycle. Nevertheless, considering its status of phytohormone, salicylic acid controls plethora of physiological properties in plants. It also plays a role in stomatal regulation and interferes with ethylene (Chavoushi et al. 2019; Bajlan et al. 2020; Gharbi et al. 2016; Mimouni et al. 2016). Since recovery of net photosynthesis and decrease of senescing ethylene produced during water stress (Ben Hassine and Lutts 2010) are of paramount importance for plants experiencing recovery, it is not surprising that hydroxybenzoic compounds were maintained after water stress relief.

Chalcone synthase (CHS) catalyzes the conversion of 4-coumaroy-CoA to chalcone and is the entry point for flavonoid biosynthesis (Fig. S1). Total flavonoids increased in *A. arabica* exposed to water stress both during and after the stress relief. A similar trend was reported by Dias et al. (2021) for olive tree. In the present work, gene coding for CHS was however not upregulated by water stress and was even downregulated after 32 days of stress exposure. This contrasts with the results obtained by Ma et al. (2014) in wheat and by Gharibi et al. (2019) in *Achillea pachycephala*. In contrast, *CHS* gene expression was upregulated in *A. arabica* comparatively to control during the recovery period (Fig. 10C), supporting the view of Sun et al. (2022) who performed a transcriptomic analysis in *Tamarix taklamakanensis* and demonstrated that some genes involved in flavonoid biosynthesis may be regulated in opposite ways during and after drought events.

It has to be mentioned that the linear increase recorded for flavonoids was mainly due to flavonols accumulation since flavanols decreased as a consequence of water stress (Fig. 8A). Such a decrease was recorded for all flavanols, except catechin-gallate and gallocathecin-gallate (Table 5). Flavanols, especially catechin, may complex with DNA or RNA and are consequently found in nuclei. Drought was reported to decrease the flavanols content in *Taxus baccata* thus inducing several changes in the chromatin architecture which hamper the mitotic process and induce epigenetic modification of histone structures (Feucht et al. 2013). The recorded decrease in flavonols may thus be related to drought-induced growth inhibition in *A. arabica* while both growth and flavonols re-increased during the recovery period.

Quercetin and kaempferol are major flavonols in plants. Both compounds increased linearly during the time course of the experiment, including during the recovery period. The proportions of these flavonols vary depending on the plant species. According to Gharibi et al. (2019) flavonoids with an ortho-dihydroxy pattern in the B-ring of flavonoid skeleton are more efficient radical scavengers than compounds mono-hydroxylated in the B-ring. Accordingly, quercetin should be regarded as one of the best electron donors among flavonoids. Quercetin concentration consistently increased in stressed plants of *A. arabica* and reached higher values than kaempferol. Flavonoids are synthesized at the cytoplasmic surface of endoplasmic reticulum but may be distributed in different cell compartments. The protective role of quercetin as ROS scavenger explains why this flavonoid is present in chloroplast and co-located with photosynthetic machinery to scavenge singlet oxygen and superoxide (Pirie et al. 2013). Beside its role as free radical scavenger, quercetin may interfere with auxin translocation blocking the polar transport and thus modifying the plant architecture. However, no modification of plant architecture was noticed in *A. arabica* accumulating quercetin in our experiment. Rutin (quercetin-3-*O*-rutinoside) is another major flavonoid detected in our samples; it is even the most important flavonoids in stressed tissues from a quantitative point of view. In plant leaves, rutin is mainly located in the epidermis and plays a protective role mainly against UV. The precise functions of rutin in response to water stress remain poorly documented but according to Gharibi et al. (2019), rutin should also be involved in the management of secondary oxidative stress. As a whole, flavonols thus assume protective functions even during the rehydration phase. However, the fact that such an accumulation was maintained during two weeks without saturation suggests that beside the rehydration step itself, recovery requires long lasting physiological adaptations to be efficient.

Conclusion

The present study demonstrates that seedlings of *Acacia arabica* were able to cope with drought and to fully recover after the stress relief. Different classes of phenolic acids and flavonoids displayed contrasting behavior during drought and recovery. Phenolic acids were higher in stressed plants than in controls: hydroxycinnamic acids decreased during recovery while hydroxybenzoic acids remained constant. Flavanols were lower in stressed plants than in controls while flavonols exhibited an opposite trend and still increased during recovery. PAL and C4H activities increased in response to water stress while gene coding for CHS was up-regulated during recovery. It is suggested that metabolism of phenolic compounds assume key functions in the management of oxidative stress occurring during both the stress and the recovery periods. Additional experiments considering reactive oxygen species synthesis and other endogenous antioxidants are required to test this hypothesis.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nassima Lassouane, Muriel Quinet and Stanley Lutts. The first draft of the manuscript was written by Stanley Lutts and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests 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.

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