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Research paper

Metabolic plasticity in the hygrophyte *Moringa oleifera* exposed to water stress

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Over the past decades, introduction of many fast-growing hygrophilic, and economically valuable plants into xeric environments has occurred. However, production and even survival of these species may be threatened by harsh climatic conditions unless an effective physiological and metabolic plasticity is available. *Moringa oleifera* Lam., a multipurpose tree originating from humid sub-tropical regions of India, is widely cultivated in many arid countries because of its multiple uses. We tested whether *M. oleifera* can adjust primary and secondary metabolism to efficiently cope with increasing water stress. It is shown that *M. oleifera* possesses an effective isohydric behavior. Water stress induced a quick and strong stomatal closure, driven by abscisic acid (ABA) accumulation, and leading to photosynthesis inhibition with consequent negative effects on biomass production. However, photochemistry was not impaired and maximal fluorescence and saturating photosynthesis remained unaffected in stressed leaves. We report for the first time that *M. oleifera* produces isoprene, and show that isoprene emission increased three-fold during stress progression. It is proposed that higher isoprene biosynthesis helps leaves cope with water stress through its antioxidant or membrane stabilizing action, and also indicates a general MEP (methylerythritol 4-phosphate) pathway activation that further helps protect photosynthesis under water stress. Increased concentrations of antioxidant flavonoids were also observed in water stressed leaves, and probably cooperate in limiting irreversible effects of the stress in *M. oleifera* leaves. The observed metabolic and phenotypic plasticity may facilitate the establishment of *M. oleifera* in xeric environments, sustaining the economic and environmental value of this plant.

Keywords: abscisic acid, flavonoids, isohydry, isoprene, MEP (methylerythritol 4-phosphate) pathway, violaxanthin-cycle pigments, water stress.

Introduction

There is increasing interest in understanding how plants cope with the severe challenges imposed by climate change. Recurrent droughts and heat waves will likely be amplified in the near future, particularly in mid-latitude and sub-tropical dry regions (Dai 2013). 'Drought tolerant' plants that are adapted to arid environments (Kozlowsky and Pallardy 2002) invest a large portion of assimilated carbon to increase leaf density and thickness and on the biosynthesis of carbon-based secondary compounds, rather than promoting new growth (Niinemets 2001, Rivas-Ubach et al. 2012). Adverse climate conditions may threaten the survival of fast-growing hygrophilic species which are largely cultivated in xeric environments for ecological restoration and profitable biomass production. This is the case of *Moringa oleifera* Lam., a fast-growing tree native to sub-Himalayan northwest India (Pandey et al. 2011), where mean annual precipitations exceed 1100 mm (Singh and Mal 2014), mainly concentrated during the monsoon season. *Moringa oleifera* is a multipurpose tree crop utilized for human food and livestock forage because of its high vitamin content (Fuglie 2001, Anwar et al. 2007, Verma et al. 2009, Nouman et al. 2014). This species is also used for many medicinal purposes and is considered a life-saving resource (Fahey 2005, Kasolo et al. 2010, Mbikay 2012, El Sohaimy et al. 2015), while its oleic acid-rich seeds can be used to produce biodiesel (Rashid et al. 2008, Da Silva et al. 2010). Because of these multiple applications, *M. oleifera* has been called a 'miracle tree' and its cultivation range has rapidly expanded into sub-tropical dry regions across Africa, South America and Asia, characterized by recurrent droughts combined with both high air temperatures and solar irradiance (Leone et al. 2015). However, if climatic constraints become harsher and more frequent under the influence of climate change, they may threaten the survival and profitable production of *M. oleifera*, which apparently does not possess any conservative functional trait of adaptation to drought (Valladares et al. 2007).

Other adaptive traits related to secondary metabolism could play a determinant role in the process of plant acclimation to harsh environments, which are not explored in *M. oleifera*. There is overwhelming evidence that secondary metabolites derived from both the methylerythritol 4-phosphate (MEP) and the phenylpropanoid pathway play a key role in the acclimation of 'mesic' species to low water availability (Tattini et al. 2015, Velikova et al. 2016; Zandalinas et al. 2018). For instance, the emission of isoprene is more widespread in hygrophytes than in xerophytes (Loreto et al. 2014), and isoprene is believed to ameliorate the response of fast-growing species to drought stress episodes (Loreto and Fineschi 2015; for reviews, see also: Sharkey et al. 2007, Fini et al. 2017). Isoprene preserves the integrity of thylakoid membranes (Velikova et al. 2011) and scavenges reactive oxygen species (ROS), particularly singlet oxygen ($^1O_2$) (Velikova et al. 2004, Vickers et al. 2009*a*, Zeinali et al. 2016), which are generated at considerable rates in drought-stressed leaves. The benefits of isoprene biosynthesis on chloroplast membrane-associated processes may improve the use of radiant energy for carbon fixation under stressful conditions (Pollastri et al. 2014, Vanzo et al. 2015), thus reducing the risk of photo-oxidative damage (Vickers et al. 2009*b*).

In drought-stressed leaves, the enhancement of carbon flow through the MEP pathway also promotes the synthesis of isoprene and non-volatile isoprenoids such as carotenoids and abscisic acid (ABA) (Tattini et al. 2014, Marino et al. 2017). Carotenoids are known to protect photosynthesis under drought stress (Beckett et al. 2012, Tattini et al. 2015). The photoprotective functions of carotenoids include: quenching of triple state chlorophyll ($^3Chl^*$); thermal dissipation of excess energy through de-epoxidation of xanthophylls (non-photochemical quenching, NPQ) (Brunetti et al. 2015); and an antioxidant function of zeaxanthin (Zea) in the chloroplasts by strengthening thylakoid membranes under heat stress (Havaux et al. 2007, Dall'Osto et al. 2010, Esteban et al. 2015). Notably, biosynthesis of Zea throughout β-hydroxylation of β-carotene (β-car) may also enhance drought resistance (Davison et al. 2002, Du et al. 2010), possibly because Zea interacts with light harvesting complex b (LHCb), thus reducing the production of $^1O_2$ and sustaining NPQ in high light conditions (Johnson et al. 2007, Dall'Osto et al. 2010). In turn, β-car (like isoprene, see Velikova et al. 2004) is an effective chemical quencher of $^1O_2$ (Ramel et al. 2012).

A relationship between isoprene and foliar ABA has been repeatedly observed (Barta and Loreto 2006, Tattini et al. 2014, Marino et al. 2017). Abscisic acid plays a major role in the regulation of stomatal movements in plants capable of maintaining leaf water potential and relative water content unchanged under drought stress conditions (isohydric behavior) (Brodribb and McAdam 2013, McAdam and Brodribb 2014, Coupel-Ledru et al. 2017).

Phenylpropanoid metabolism is another complex 'metabolic grid' highly modulated by environmental constraints (Laursen et al. 2015). Strong evidence has been provided that enhanced biosynthesis of dihydroxy B-ring-substituted flavonoids is induced under drought, when the use of light for photosynthesis is reduced (Tattini et al. 2004, Treutter 2006, Agati et al. 2012, Ma et al. 2014). Alterations in ROS homeostasis and/or in the electron transport chain are main drivers for flavonoid biosynthesis (Taylor and Grotewold 2005, Fini et al. 2012, 2014). Flavonoids accumulate to a large extent in the mesophyll cells of sun-exposed leaves and may complement the functions of primary antioxidants in plants, both acting at different places and at different times (Brunetti et al. 2015, Tattini et al. 2015). In fact, flavonoids are found in sub-cellular compartments, such as the nucleus, vacuole and outer chloroplast membrane, where other antioxidants do not effectively operate (Agati et al. 2007, Ferreres et al. 2011).

In plants exposed to drought, the modulation of secondary metabolism may be mostly intended to reduce excess of ROS by increasing the production of metabolites with antioxidant properties, including isoprenoids and phenylpropanoids (Nakabayashi et al. 2014, Loreto and Fineschi 2015, Tattini et al. 2015). We hypothesize that both enhanced production and profound readjustment in the isoprenoid and phenylpropanoid pool (i.e., metabolic plasticity) may occur in hygrophilic and fast-growing plants such as *M. oleifera* when facing drought conditions. To test this hypothesis and explore physiological and biochemical mechanisms linked to drought resistance in hygrophilic plants, we exposed *M. oleifera* plants to a water stress treatment of increasing severity.

Materials and methods

Plant material and experimental conditions

Two-month-old seedlings of *Moringa oleifera* Lam. were planted in 50 l pots with a sand/peat substrate (9/1, v/v), and were grown outside in Florence, Italy (43° 49′ N, 11° 37′). The experiment was conducted during summer 2014, under minimum/maximum temperatures of 17.7 ± 2.4/30.8 ± 3.2 °C (mean ± standard deviation, SD) and midday irradiance (measured over the 200–3000 nm range of solar wavebands) of 780 ± 85 W m^{-2} (mean ± SD). Saplings were irrigated to pot capacity before the onset of water stress treatment, that was applied to plants on average ~190 cm tall and with stem diameter of ~2.0 cm. Water stress was imposed by withholding water for 30 days (WS, water-stressed plants), whereas control plants (C) were irrigated daily to pot capacity. A total of thirty plants were grown under these two experimental conditions (12 assigned to well-watered treatment and 18 assigned to water-stressed treatment). Plants were assigned on the basis of preliminary leaf gas exchange measurements to exclude significant differences in photosynthesis (A_N) and stomatal conductance (g_s) among plants ($t < 0.05$, data not shown). The fraction of transpirable soil water (FTSW) and g_s were used as water stress indicators (Sinclair and Ludlow 1986, Brilli et al. 2013). Measurements were conducted in water-stressed plants at increasing stress severity. The three stress levels corresponded to decreasing FTSW from 100% (in control plants, $FTSW_{100}$), to 60% ($FTSW_{60}$), 40% ($FTSW_{40}$) and 25% ($FTSW_{25}$), corresponding to 10, 20 and 30 days after withholding water, respectively. Control plants were also sampled at the same days as water-stressed plants, to make sure that control conditions were maintained across the experimental period. The physiological lower limit of available soil water, corresponding to the FTSW endpoint, was calculated prolonging water stress, until stomatal conductance approached zero, on some additional plants.

As *M. oleifera* has bipinnate compound leaves, water relations, gas exchange, chlorophyll fluorescence, isoprene and *n*-hexanal measurements were conducted on the two medial leaflets in the secondary pinna (hereafter denoted as leaf), on four replicate plants per treatment, at each sampling date. The adjacent leaf was collected for biochemical measurements, between 12:00 and 14:00 h.

Growth, water relations, gas exchange and chlorophyll fluorescence

Biomass was measured at the end of the water stress period (30 d) on 10 replicate plants per treatment. Plants were divided into shoots and roots and oven dried at 70 °C until a constant weight was reached (after about 72 h). Biomass allocation was calculated on a dry mass (DM) basis, using as parameters the ratio of shoot dry mass to total dry mass (BAS) and the ratio of root dry mass to total dry mass (BAR). Predawn measurements of relative water content (RWC), water (ψ_w) and osmotic (ψ_π) potentials were made on well-watered and water-stressed leaves (two leaves for each selected replicate).

Gas exchange was measured on intact leaves using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Measurements were performed at a photosynthetic photon flux density (PPFD) of 1000 μmol photons m^{-2} s^{-1}, a CO_2 concentration of 400 μmol mol^{-1} and ambient temperature. This system was utilized also to measure leaf temperature. Photosynthesis and g_s were calculated using the LI-6400 software. Chlorophyll (Chl) fluorescence was measured using a modulated PAM-2000 fluorometer (Heinz Walz, Effeltrich, Germany). Minimum fluorescence (F_O) was measured with a 0.8 μmol m^{-2} s^{-1} measuring light beam on leaves that were dark-adapted for 20 min. Maximum fluorescence in the dark-adapted state (F_m) was determined using a saturating pulse (0.5 s) of red light (8000 μmol m^{-2} s^{-1}), thus allowing calculation of $F_v/F_m = (F_m - F_O)/F_m$. Actinic red continuous light (1000 μmol m^{-2} s^{-1}) was then switched on, and steady-state fluorescence was recorded (F_s). Saturating pulses were then applied to record the maximum fluorescence under actinic light (F'_m). These data were used to calculate non-photochemical quenching ($NPQ = (F_m - F'_m)/F'_m$) (Schreiber et al., 1986), actual quantum yield of PSII ($\Phi_{PSII} = (F'_m - F_s)/ F'_m$) (Genty et al. 1989), and electron transport rate (ETR = 0.5 · ΦPSII · PAR · 0.84), where 0.5 and 0.84 are coefficients indicating an equal distribution of photons between PSI and PSII and leaf absorptance, respectively.

Isoprene, abscisic acid and photosynthetic pigments

To measure isoprene emission, the outlet of the cuvette was disconnected from the LI-6400 system and the flow was diverted into a silcosteel cartridge packed with 200 mg of Tenax (Agilent, Cernusco sul Naviglio, Italy). A volume of 4.5 dm^3 of air was pumped through the trap at a rate of 200 cm^3 min^{-1}. The cartridge was analyzed using a Perkin Elmer Clarus 580 gas chromatograph coupled with a Clarus 560 Mass-Selective-Detector and a thermal desorber TurboMatrix (Perkin Elmer Inc., Waltham, MA, USA). The desorbed compounds were separated in a 30-m Elite-5-MS capillary column. The column oven temperature was kept at 40 °C for the first 5 min, then increased by 5 °C min^{-1} to 250 °C, and maintained at 250 °C for 2 min. Helium was used as carrier gas. Compounds were identified using the NIST library provided with the GC/MS Turbomass software. Quantification of isoprene was conducted using authentic standards of isoprene (Rivoira, Milan, Italy) to prepare a calibration curve as well as to compare the peak retention time and the peak fragmentation of isoprene found in the samples.

Abscisic acid, both in its free (free-ABA) and conjugated form (ABA glucoside ester, ABA-GE), was extracted and quantified as reported in Tattini et al. (2017). In detail, 200 mg of lyophilized leaf tissue were ground in liquid nitrogen and combined with 50 ng of d^6-ABA and d^5-ABA-GE (National Research Council of Canada), then extracted with 3 × 1 cm^3 pH 2.5 CH_3OH/H_2O (50:50; v:v), at 4 °C for 30 min. The supernatant, after defatting with 3 × 3 cm^3 of *n*-hexane, was purified using Sep-Pak C18 cartdriges (Waters, Milford, MA, USA) and eluted with 1 cm^3 of ethylacetate. The eluate, dried under nitrogen, and rinsed with 500 µl pH 2.5 CH_3OH/H_2O (50:50), was injected (3 µl aliquots) in a LC–DAD-MS/MS system, consisting of a Shimadzu Nexera HPLC and a Shimadzu LCMS-8030 quadrupole mass spectrometer, operating in electrospray ionization (ESI) mode (Kyoto, Japan). The eluting solvents consisted of H_2O (added with 0.1% of HCOOH, solvent A) and CH_3CN/CH_3OH (1:1, v:v, added with 0.1% of HCOOH, solvent B). The analysis was performed in negative ion mode, using a 3 × 100 mm Poroshell 120 SB C18 column (2.7 µm, 100 × 4.6 mm, Agilent Technologies) and eluting a 18 min-run from 95% solvent A to 100% solvent B at a flow rate of 0.3 cm^3 min^{-1}. Quantification was conducted in multiple reaction mode (MRM), as reported by López-Carbonell et al. (2009).

Chlorophyll *a* and *b*, and individual carotenoids were identified and quantified as reported by Beckett et al. (2012). Briefly, lyophilized leaf tissue (0.2 g) was extracted with 3 × 5 cm^3 acetone (added with 0.5 g cm^{-3} of $CaCO_3$) and injected (15 µl) in a Flexar high performance liquid chromatography (HPLC) system equipped with a quaternary 200Q/410 pump and a LC 200 diode array detector (DAD) (all from Perkin Elmer Bradford, CT, USA). Photosynthetic pigments were separated in a 250 × 4.6 mm Agilent Zorbax SB-C18 (5 µm) column operating at 30 °C, eluted for 18 min with a linear gradient solvent system, at a flow rate of 1 cm^3 min^{-1}, from 100% CH_3CN/MeOH (95/5 with 0.05% of triethylamine) to 100% MeOH/ethylacetate (6.8/3.2). Violaxanthin cycle pigments [violaxanthin (Vio), antheraxanthin (Ant), zeaxanthin (Zea), collectively named (VAZ)], neoxanthin (Neo), lutein (Lut), β-carotene (β-car), chlorophyll *a* and chlorophyll *b* were identified using visible spectral characteristics and retention times. Carotenoids and chlorophylls were calibrated using authentic standards from Extrasynthese (Lyon-Nord, Genay, France) and from Sigma Aldrich (Milan, Italy), respectively. The de-epoxidation state of VAZ (DES) was calculated as:

$$DES = (0.5A + Z)/(V + A + Z)$$

Flavonoids

Individual flavonoids were extracted and quantified as previously reported in Tattini et al. (2015). Briefly, lyophilized leaf tissue (0.2 g) was extracted with 3 × 5 cm^3 of 75% $EtOH/H_2O$ adjusted to pH 2.5 with formic acid, and the supernatant partitioned with 4 × 5 cm^3 *n*-hexane, reduced to dryness and finally rinsed with 2 cm^3 CH_3OH/H_2O (8:2, v:v). Aliquots of 10 µl were injected into the Perkin Elmer liquid chromatography system reported above, and compounds separated in a 150 × 4.6 mm Sun Fire column (5 µm) (Waters Italia, Milan, Italy) operating at 30 °C and eluted at a flow rate of 1 cm^3 min^{-1}. The mobile phases were (A) H_2O pH 4.3 (CH_3COONH_4/CH_3COOH)/ CH_3CN (90/10, v/v) and (B) CH_3CN/H_2O pH 4.3 (CH_3COONH_4/CH_3COOH) (90/10, v/v). Flavonoids were separated using a linear gradient elution from A to B over a 46 min-run. Flavonoids were identified by comparison of their retention times and UV spectral characteristics with those of authentic standards (Extrasynthese, Lyon-Nord, Genay, France) and quantified at 350 nm using five-point calibration curves of authentic standards.

Lipid peroxidation indicator (n-hexanal)

n-Hexanal is one of the lipid peroxide-derived carbonyl compounds (oxylipin carbonyls) that reveals abiotic stress-induced damage of plants, and in particular of cellular membranes (Mano 2012). Analysis of *n*-hexanal was done using the same procedure as for isoprene (see above). Quantification of *n*-hexanal was conducted using an authentic standard (Sigma Aldrich, Milan, Italy) to prepare a calibration curve, as well as comparing the peak retention time and the peak fragmentation in all samples.

Experimental design and statistics

The experiment was performed using a completely randomized design. Biomass was measured on 10 replicates for both well-watered and water-stressed plants at the end of the experiment. Physiological and biochemical measurements were conducted on four replicate plants, both in well-watered plants and in plants exposed to water stress of increasing severity. Data were analyzed using repeated-measures ANOVA, with water treatment as between-subjects effect and sampling date as within-subjects effect (SPSS v.20; IBM, Chicago IL, USA). Significant differences among means were estimated at the 5% ($P < 0.05$) level, using Tukey's test.

Results

Water stress effects on water relations, photosynthesis and biomass production

Predawn leaf water potential (Ψ_w, Figure 1A) declined in water-stressed plants compared to control plants, though differences became significant only at $FTSW_{40}$ and $FTSW_{25}$. It is noteworthy that at the end of the water stress cycle, when g_s of $FTSW_{25}$ plants was on average about 15% of the control values, predawn Ψ_w was still rather high (i.e., −0.60 MPa). Significant differences in leaf bulk osmotic potential (Ψ_π, Figure 1B) were recorded only at the end of the experiment ($FTSW_{25}$), whereas RWC did not significantly vary between water-stressed and control plants (Figure 1C).

As FTSW declined, A_N, g_s and C_i (Figure 2; see Table SM1 available as Supplementary Data at *Tree Physiology* Online) were progressively reduced. A strong reduction of A_N (−30%, Figure 2A) and g_s (−43%, Figure 2B) was observed already under mild water stress ($FTSW_{60}$). Under a more severe water

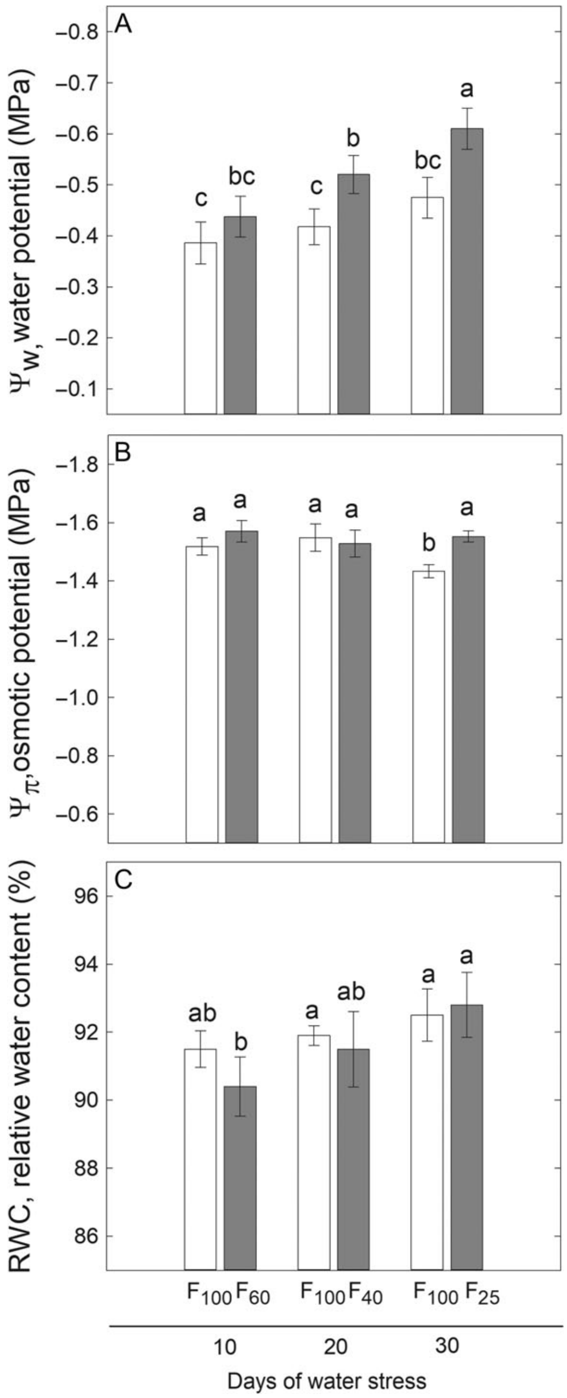

Figure 1. Predawn leaf water (Ψ_w, A) and osmotic (Ψ_π, B) potentials, and relative water content (RWC, C) in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*, corresponding to 10, 20 and 30 days after withholding water, respectively. Data (means ± SD, $n = 4$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

stress ($FTSW_{25}$), A_N and g_s declined by 71% and 85% respectively, compared with control leaves ($FTSW_{100}$) (see Table SM1 available as Supplementary Data at *Tree Physiology* Online). Similarly, C_i (Figure 2C) was reduced by about 55% in $FTSW_{25}$ plants relative to $FTSW_{100}$ plants. The maximum quantum yield of PSII (F_v/F_m, Figure 2D) did not vary between control and water-stressed plants, irrespective of the severity of the stress. In contrast, the actual efficiency of PSII photochemistry (Φ_{PSII}), significantly declined already at $FTSW_{60}$ and was further impaired at $FTSW_{40}$ and $FTSW_{25}$, relative to $FTSW_{100}$ plants (Figure 2E). Water stress reductions in Φ_{PSII} were paralleled by corresponding increases in the non-photochemical quenching of fluorescence (NPQ, Figure 2F and see Figure SM1 available as Supplementary Data at *Tree Physiology* Online).

Plant biomass was significantly reduced in $FTSW_{25}$ compared to $FTSW_{100}$ plants (Figure 3A). At the end of the experiment, the root to shoot ratio was also significantly higher in $FTSW_{25}$ than in $FTSW_{100}$ plants, whereas the shoot to total dry mass ratio was significantly reduced in water-stressed plants (Figure 3B).

Water stress effects on isoprene, non-volatile isoprenoids, pigments, flavonoids and membrane lipid peroxidation

Isoprene emission increased significantly in $FTSW_{100}$ leaves during the experiment (Figure 4A), likely because of the prolonged exposure to elevated temperatures during the summer season. Isoprene emission strongly and significantly increased in response to water stress. This increment was particularly relevant at $FTSW_{25}$ (+86% compared to $FTSW_{40}$). The carbon lost as isoprene (C_{iso}%), also increased largely in $FTSW_{25}$ plants, due to the simultaneous increase of isoprene emission and reduction of A_n (Figure 4B). The surging emission of isoprene was positively correlated to both the decline of C_i (Figure 5A) and the increase of the ETR/A_N ratio (Figure 5B) in water-stressed leaves.

The content of free-ABA and ABA-GE increased in water-stressed compared to control leaves (Figure 6A and B), and the effect was particularly strong in $FTSW_{25}$ plants where free-ABA and ABA-GE contents were about seven and two folds higher than in $FTSW_{100}$, respectively. A strong negative linear relationship ($R^2 = 0.915$) was found between foliar free-ABA levels and g_s (inset of Figure 6A). Whereas, free and conjugated ABA contents were both positively related to isoprene emission rates (inset of Figure 6B).

Total chlorophyll (Chl_{tot}) declined significantly in $FTSW_{40}$ (−14%) and $FTSW_{25}$ (−23%) leaves in comparison to $FTSW_{100}$ leaves (Figure 7A). In contrast, total carotenoid (Car_{tot}) content did not vary between control and water-stressed leaves, and increased during the experiment irrespective of water treatments (Figure 7B). However, water stress markedly altered the composition of the carotenoid pool. The content of Lut (Figure 7C) increased, whereas the content of β-car (Figure 7D) declined significantly under severe water deficit ($FTSW_{25}$). Among xanthophylls, Vio (Figure 7E) declined and Zea (Figure 7F) increased significantly in water-stressed plants. Vio reduction was particularly strong at $FTSW_{60}$ and $FTSW_{40}$,

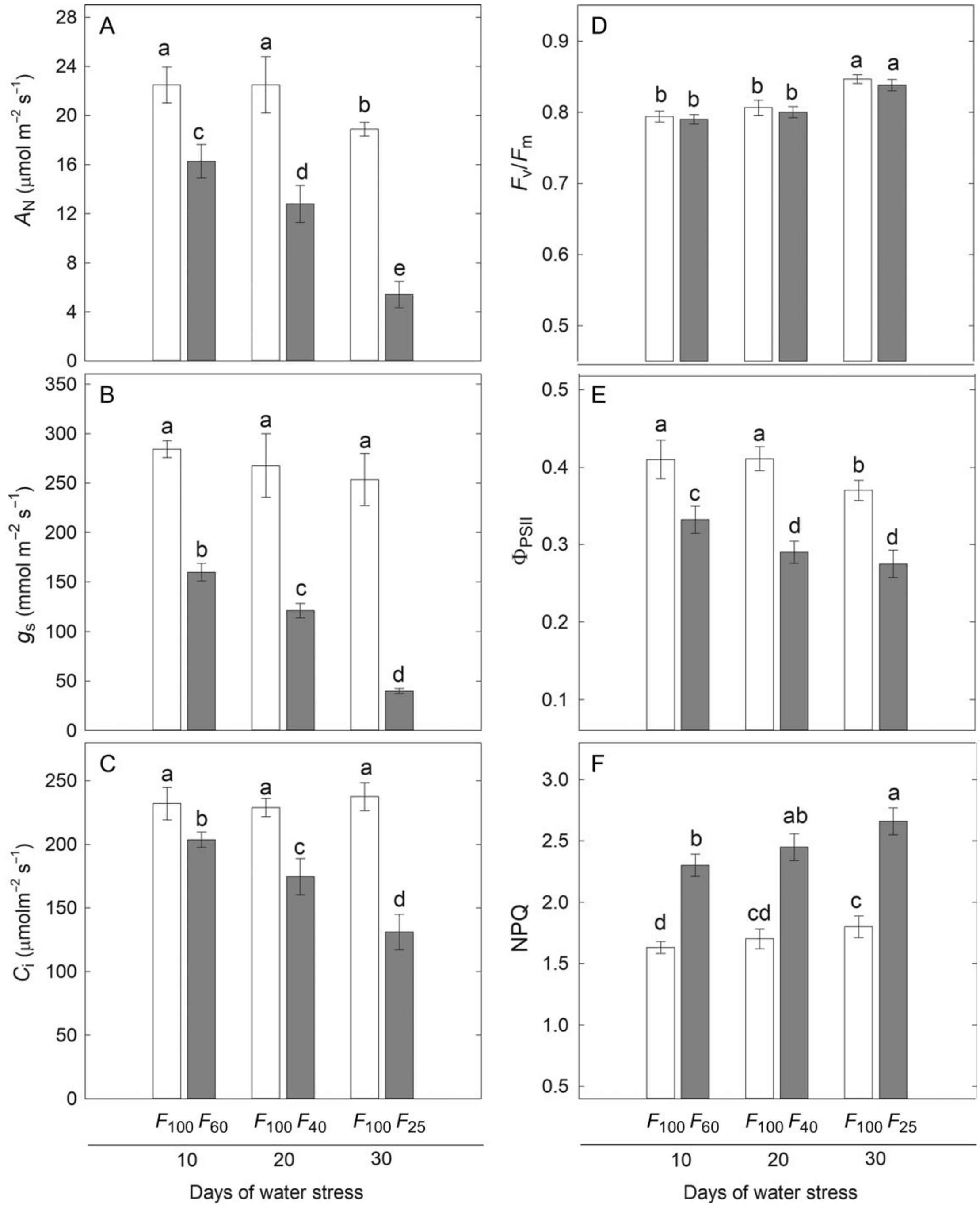

Figure 2. Photosynthesis (A_N, A), stomatal conductance (g_s, B), intercellular CO_2 concentration (C_i, C), maximum (F_v/F_m, D) and actual (Φ_{PSII}, E) efficiency of PSII photochemistry and non-photochemical quenching (NPQ, *F*) in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, $n = 4$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

and partially recovered under severe stress conditions ($FTSW_{25}$). The contents of Ant (Figure 7G) and Neo (Figure 7H) were not affected by water stress. However, Neo increased during the experimental period in both well-watered and water-stressed plants. The content of violaxanthin-cycle pigments (VAZ) relative to Chl_{tot} increased significantly as water stress progressed, and the effect was particularly high (+35%) at $FTSW_{25}$ compared to FTSW100 after 30 days of water stress (Figure 7I). In addition, DES increased in water-stressed compared to control leaves, but the difference was already noticeable under mild water stress conditions ($FTSW_{60}$) (Figure 7J).

Water stress also considerably altered the content and composition of the flavonoid pool (Figure 8A–C). Quercetin-3-*O*-glycoside and its derivatives were the most responsive compounds to water

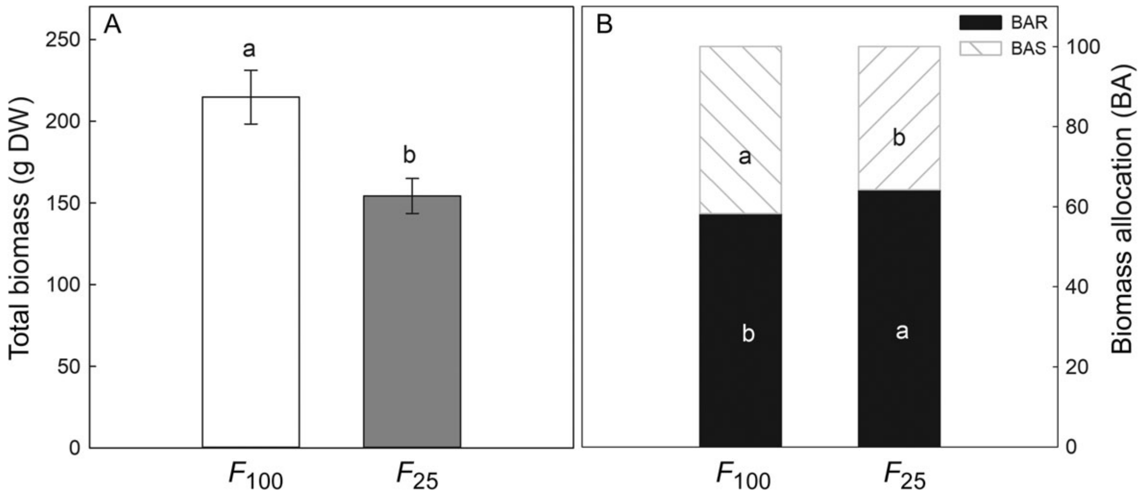

Figure 3. Total biomass (A) and biomass allocation (B) in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. The percentage of biomass allocation (BA) was calculated considering the ratio of shoot dry mass to total dry mass (BAS) and the ratio of root dry mass to total dry mass (BAR). Data (means ± SD, $n = 10$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

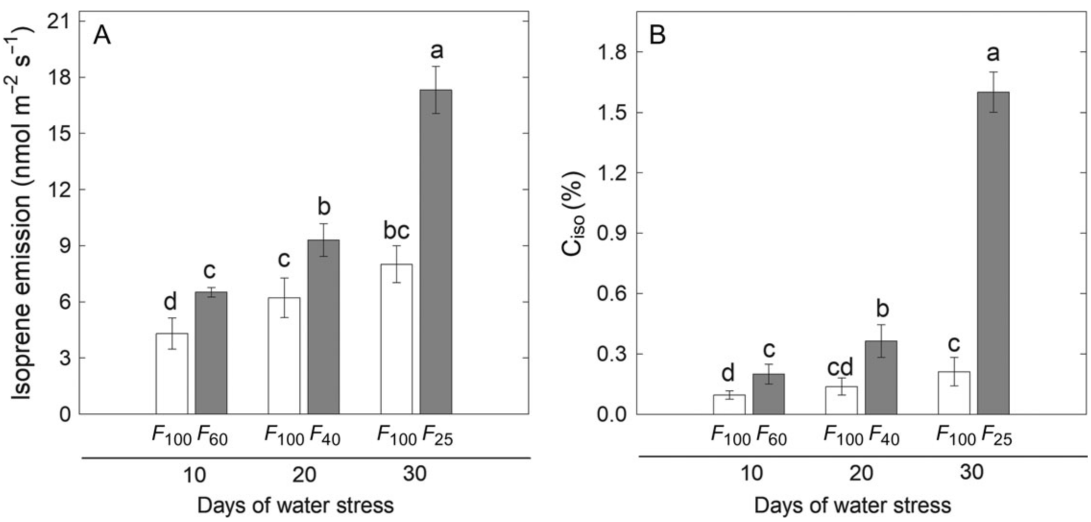

Figure 4. Rates of isoprene emission (A) and carbon lost as isoprene (C_{iso}, B) in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, $n = 4$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

stress, as their content significantly and consistently increased with the intensity of the stress (Figure 8A). In addition, the content of Kaempferol-3-*O*-glycoside derivatives also significantly increased in response to stress, but the difference between water-stressed and control leaves remained constant as water stress progressed (Figure 9B). In contrast, the content of Apigenin-7-*O*-glycoside and its derivatives significantly decreased in $FTSW_{40}$ and $FTSW_{25}$ leaves (Figure 8C).

Compared to control leaves, the emission of *n*-hexanal did not significantly vary at both $FTSW_{60}$ and $FTSW_{40}$, whereas it significantly increased in $FTSW_{25}$ plants (Figure 9).

Discussion

Understanding the impact of water stress on the physiology of the isohydric plant M. oleifera

Moringa oleifera is a fast-growing species able to produce large quantities of biomass (Sánchez et al. 2006). However, whether *M. oleifera* is able to acclimate and produce at satisfactory rates in arid conditions is yet not known. Our study offers novel insights on the physiological and biochemical strategies adopted by this species to cope with extended periods of soil water stress.

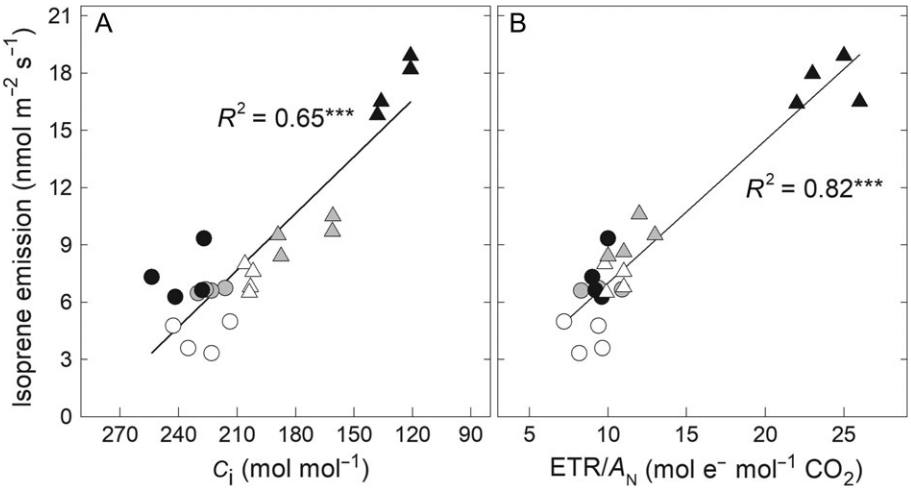

Figure 5. Linear relationships between isoprene emission rate and (A) internal CO_2 concentration (C_i) or (B) the ratio of electron transport rate to photosynthesis (ETR/A_N) in *Moringa oleifera* plants. Measurements were made at $FTSW_{60}$ (10 d, open symbols), $FTSW_{40}$ (20 d, gray symbols), and $FTSW_{25}$ (30 d, closed symbols) both in well-watered plants ($FTSW_{100}$) (triangles) and water-stressed (circles) plants. Coefficient of determination (R^2) of each relationship are reported; *** indicate $P < 0.0001$.

Our results show that *M. oleifera* possesses an effective avoidance mechanism (i.e., isohydry, Tardieu and Simonneau 1998, Nardini et al. 2014) when subjected to water stress. This involved a rapid reduction of g_s in stressed leaves, that possibly contributed to the maintenance of ψ_w and RWC even in conditions of severe water stress ($FTSW_{25}$), when only a moderate (8%) reduction of the osmotic component ψ_π became significant (Figures 1 and 2B). The response of g_s of *M. oleifera* to soil drying (see Table SM1 available as Supplementary Data at *Tree Physiology* Online) is remarkably different from that observed in other fast-growing trees species such as *Eucalyptus citriodora* (Brilli et al. 2013) and Populus spp. (Marron et al. 2002, Yin et al. 2005, Brilli et al. 2007, Centritto et al. 2011) that showed no or very little decline in g_s under moderate water stress conditions. Isohydry is a crucial adaptive trait for the survival of deciduous woody plants exposed to high evaporative demand and low soil water availability, as an early and tight control of stomatal aperture may prevent xylem embolism (Franks et al. 2007, Yi et al. 2017). While stomatal closure increased intrinsic water use efficiency (iWUE, determined as the ratio of A_n to g_s) during water stress progression, it also constrained photosynthesis due to increased diffusional limitations to CO_2 entry, with consequent reduction of C_i (Figure 2C) (Lawlor and Cornic 2002, Centritto et al. 2011, Lauteri et al. 2014, Fini et al. 2016). The observed drop in photosynthesis under water stress caused a biomass reduction (Figures 2 and 3), probably inducing a redistribution of the assimilated carbon between shoots and roots (Peuke et al. 2006). These results suggest a high degree of plasticity of *M. oleifera* in biomass allocation in response to water stress (Figure 3).

Water stress did not cause permanent damages to the photosynthetic apparatus. In fact, maximal PSII photochemical efficiency (F_v/F_m) did not decline even under severe water stress ($FTSW_{25}$), suggesting stability of photochemical reactions and structures (Figure 2) (Flexas et al., 2006). However, PSII quantum yield in the light (Φ_{PSII}) was reduced as compared to $FTSW_{100}$ leaves. While this mirrored A_n reduction at mild ($FTSW_{60}$) and moderate ($FTSW_{40}$) stress level, Φ_{PSII} did not drop further in severely water-stressed leaves (Havaux 1992, Lu and Zhang 1999) revealing a likely increase of photorespiratory electron transport, or alternative electron sinks (see discussion below about ETR driving isoprene emission). Furthermore, changes in NPQ and Φ_{PSII} were strongly correlated throughout the experiment ($\Phi_{PSII} = -0.13$ NPQ $+ 0.62$, $R^2 = 0.844$, linear relation shown in Figure SM1 available as Supplementary Data at *Tree Physiology* Online). Large excess of light energy not used by photosynthesis, as revealed by the fluorescence parameter NPQ (Figure 2F), may directly photoreduce O_2, thus causing large ROS generation in water-stressed leaves, with consequent damage to PSII. To explain why this was not observed in this experiment, we hypothesize a potential contribution of isoprenoids and phenylpropanoids as antioxidant compounds, as discussed below.

Exploring the significance of enhanced isoprene emission during water stress and its relationship with foliar ABA

Our study revealed that *M. oleifera* is an isoprene emitting species (Figure 4). Isoprene emission is typical of hygrophytes that are fast-growing in temperate areas of the world (Loreto et al. 2014, Loreto and Fineschi 2015), where isoprene serves important defensive (antioxidant and thermo-protective) properties (Loreto and Schnitzler 2010, Velikova et al. 2011, Pollastri et al. 2014). We also show that water stress promoted I_e, particularly when the stress became severe. Isoprene biosynthesis is generally resistant to water stress (Brilli et al. 2007, 2013, Centritto et al. 2011), and the emission of isoprene is enhanced when isoprene-

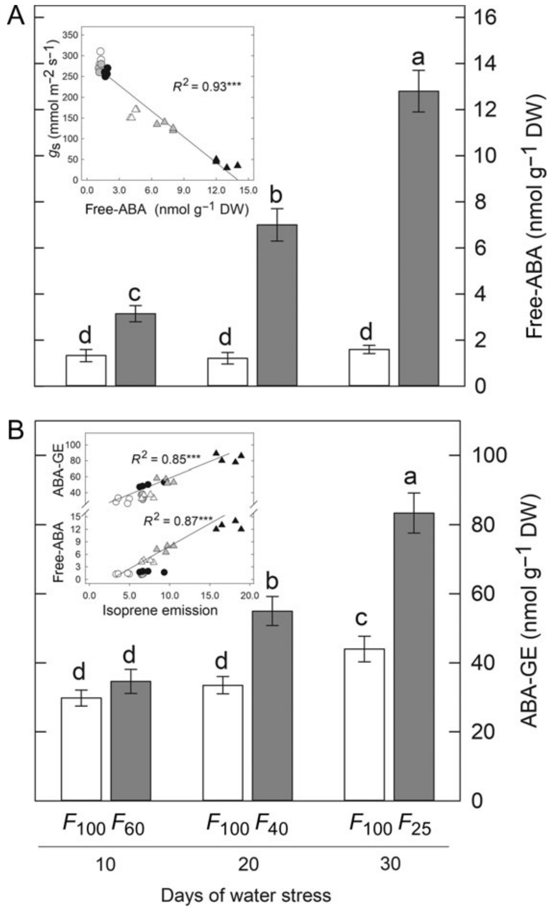

Figure 6. Contents of free-ABA (A) and ABA-GE (B) in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, $n = 4$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test. Inset in Figure 6A shows the inverse relationship between foliar free-ABA content and stomatal conductance (g_s). Inset in Figure 6B shows the linear relationships between isoprene emission rates (nmol m^{-2} s^{-1}) and free-ABA and its glucoside ester (ABA-GE) contents in $FTSW_{100}$ plants (circles) and in water-stressed (triangles) plants at $FTSW_{60}$ (white symbols), $FTSW_{40}$ (gray symbols), and $FTSW_{25}$ (dark symbols), respectively. Coefficient of determination (R^2) of each relationship are reported; *** indicate $P < 0.0001$.

emitters recover from water stress (Sharkey and Loreto 1993, Fortunati et al. 2008). Stimulation of isoprene biosynthesis 'during' water stress episodes is less reported (Haworth et al. 2017, Marino et al. 2017). *Moringa oleifera* is a typical isoprene emitting species, since it is a fast-growing species with high photosynthetic rates which thrives wild in secondary tropical deciduous forests of the sub-Himalayan area (Loreto and Fineschi 2015). Our data suggest that declines in internal CO_2 concentration (C_i) and the increasing electron flux generated by Photosystem II not used for carbon assimilation (ETR/A_N) are two important physiological drivers of isoprene biosynthesis under water stress conditions (Figure 5) (Guidolotti et al. 2011, Harrison et al. 2013, Morfopoulos et al. 2014, Marino et al. 2017). Reduced photosynthesis due to CO_2 starvation may indeed increase the fraction of ETR available for alternative biosyntheses, including isoprenoids. In addition, the increase in leaf temperature induced by stomatal closure under water stress (from 31.2 ± 0.7 °C in $FTSW_{100}$ leaves to 34.4 ± 0.6 °C in $FTSW_{25}$ leaves, mean ± SD) might have contributed to further enhance the rate of isoprene emission (Singsaas and Sharkey 1998, Fares et al. 2011, Brilli et al. 2013, Arab et al. 2016). Indeed, the activity of isoprene synthase is known to be stimulated by high temperatures (Monson et al. 1992, Li et al. 2011). Increasing isoprene synthase activity may also help explain the increase in I_e and C_{iso}% observed in well-watered leaves, along rising summer temperatures during the course of our study (Rasulov et al. 2015).

We hypothesize that the rising investment of newly assimilated carbon for isoprene biosynthesis helped leaves tolerate water stress because: (a) isoprene protects the photosynthetic apparatus from heat and oxidative damage by preserving the integrity of thylakoid membranes (Siwko et al. 2007, Velikova et al. 2011, 2014, 2015) or by scavenging singlet oxygen ($^1O_2$), a highly reactive ROS in chloroplasts (Velikova et al. 2004, Zeinali et al. 2016); (b) isoprene makes faster and smoother the electron transport flow (Pollastri et al. 2014), especially under water stress conditions (Marino et al. 2017). We found that NPQ did not vary between $FTSW_{40}$ and $FTSW_{25}$ leaves. Lower NPQ values in isoprene emitters compared to non-emitters were reported both in stressful (Behnke et al. 2007, 2010) and physiological conditions (Pollastri et al. 2014). We, therefore, hypothesize a relationship between the reduction of NPQ and the increase I_e along with the severity of water stress. A downregulation of chloroplastic ATP-synthase and the consequent reduction in the flexible heat dissipation component (qE) of NPQ (Demmig-Adams and Adams 2006) was reported in isoprene emitting species by Velikova et al. (2014).

The observed strong linear relationships between I_e and foliar contents of free-ABA and ABA-GE (Figure 6B), suggest that increased isoprene formation in water stressed plants indicates enhanced carbon flow through the MEP pathway, leading to higher foliar biosynthesis of abscisic acid (Figure 6A) (Marino et al. 2017). A relationship between isoprene and foliar ABA was first reported by Barta and Loreto (2006) in well-watered *Populus alba* and by Tattini et al. (2014) in drought stressed transgenic tobacco plants. Our results also show a strong linear correlation between free-ABA and g_s (Figure 6A), despite limited variations of water relations in *M. oleifera* leaves. It is unclear whether isoprene is simply of proxy of carbon flux through the MEP pathway, or has a regulatory role. Sustained isoprene emission in water-stressed plants may reduce the accumulation of dimethylallyl pyrophosphate (DMAPP) in the chloroplast, and may prevent DMAPP-induced feedback inhibition of the entire MEP pathway (Banerjee et al. 2013). Taken together our results suggest that: (a) increased isoprene formation indicates and

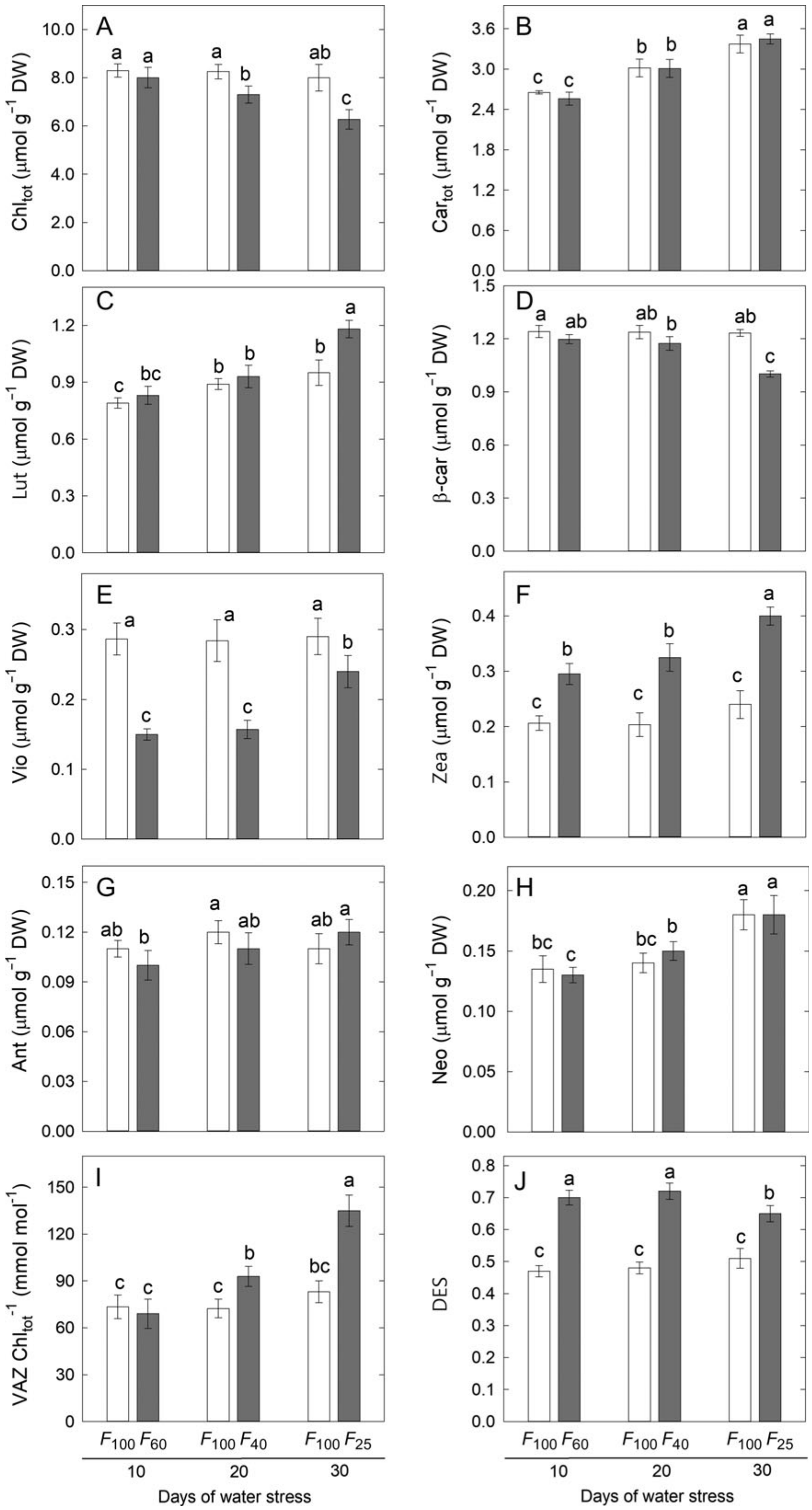

Figure 7. Effects of water stress on the contents of photosynthetic pigments (A–I), on the ratio of violaxanthin cycle pigment content to total chlorophyll content (VAZ Chltot^{-1}, I) and on the de-epoxidation state of VAZ [DES = (0.5A + Z) (V + A + Z)$^{-1}$, J] in FTSW$_{100}$ (F_{100}) plants (open bars) and in FTSW$_{60}$ (F_{60}), FTSW$_{40}$ (F_{40}) and FTSW$_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, n = 4) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

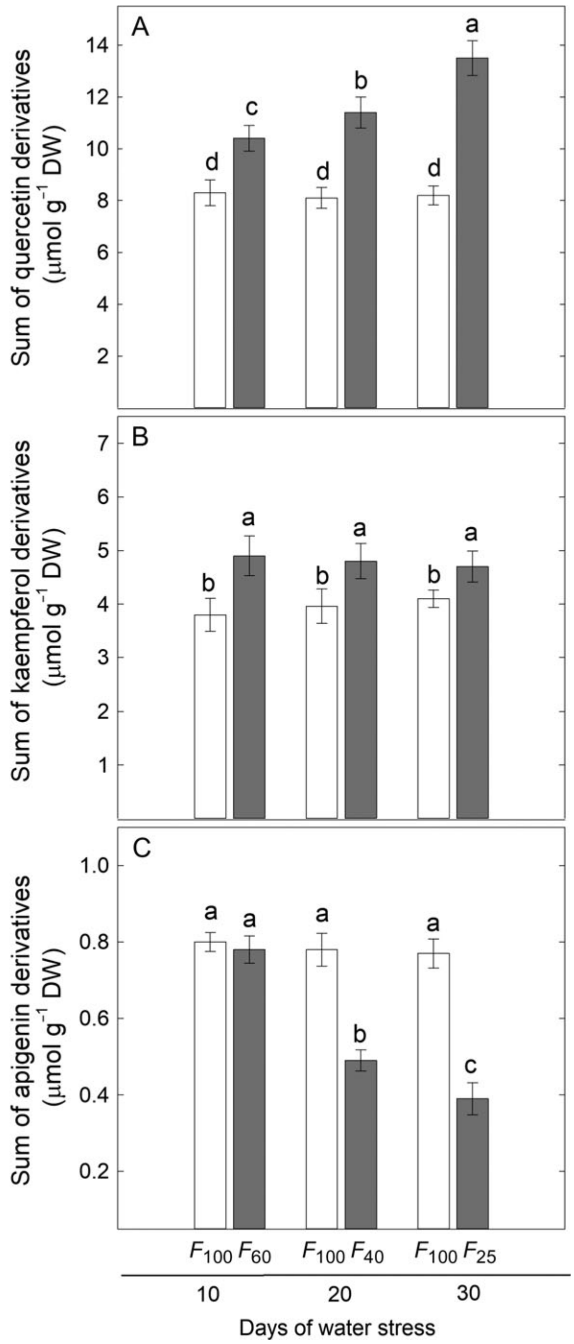

Figure 8. Contents of quercetin (A), kaempferol (B) and apigenin (C) derivatives in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, $n = 4$) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

perhaps regulates free-ABA synthesis in stressed leaves, and (b) free-ABA has a major role in the regulation of stomatal closure compared to hydraulic signals (Chaves et al. 2016, McAdam et al. 2016*b*). These results are in line with recent studies showing that, in strict isohydric plants such as *M. oleifera*, high levels of free-ABA could be responsible for stomatal closure and could promote a higher root to shoot ratio/carbon allocation (McAdam et al. 2016*a*, Nolan et al. 2017).

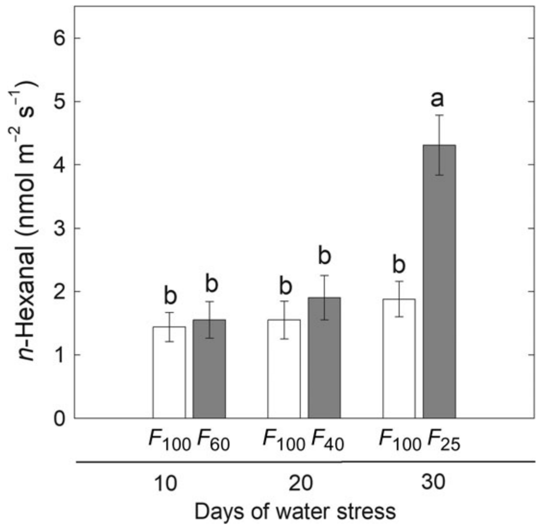

Figure 9. Rates of *n*-hexanal emission in $FTSW_{100}$ (F_{100}) plants (open bars) and in $FTSW_{60}$ (F_{60}), $FTSW_{40}$ (F_{40}) and $FTSW_{25}$ (F_{25}) water-stressed plants (gray bars) of *Moringa oleifera*. Data (means ± SD, $n =$ 4) were subjected to repeated measures with ANOVA, and bars not accompanied by the same letter significantly differ at the 5% level, using Tukey's test.

Plasticity of secondary metabolism in M. oleifera during water stress progression

We observed several changes in carotenoids and phenylpropanoids in response to increasing water stress that can be interpreted as a photoprotective trait to limit water stress induced damage. The content of total carotenoids on a leaf mass basis also increased over the course of the experiment in both well-watered and water-stressed leaves. While this shows a general upregulation of the MEP pathway (see previous section) over the season, we argue that the investment in carotenoids was much stronger in water-stressed leaves mirroring the depression in carbon assimilation. The blend of carotenoids also changed along stress progression, perhaps favouring compounds active in stress protection (Figure 7). The increase in lutein in severely water-stressed plants might have enhanced the capacity of leaves to quench $^3Chl^*$, that was likely generated during stress exposure (Dall'Osto et al. 2006, Jahns and Holzwarth 2012). In addition, compared to photosynthesis, Chl_{tot} content was less affected by severe water stress, indicating a successful mechanism of protection. We also note that a large switch in the composition of xanthophylls occurred in water-stressed plants. The increase in Zea content was accompanied by a parallel decrease in Vio content under mild and moderate water stress, showing the classic mechanism of de-epoxidation that is a major element of photoprotection in plants (Demmig-Adams and Adams 2006). However, when plants experienced the most severe water stress the content in Zea and in Vio both increased. We suggest that the large increase in Zea biosynthesis might have been originated from hydroxylation of β-car (Davison et al. 2002, Du et al. 2010). This is consistent with the reduction of β-car concentration observed in leaves at $FTWS_{25}$. β-car might have been also used as a chemical quencher of $^1O_2$ (Ramel et al. 2012), thus explaining the relatively stronger decline of β-car (−0.18 μmol g^{-1} DW) as compared to the increase in Zea (+0.07 μmol g^{-1} DW) when the stress became severe. The content of VAZ relative to Chl_{tot} was on average >70 mmol mol^{-1} in both well-watered and water-stressed plants throughout the whole experiment, as commonly observed in leaves long acclimated to full solar irradiance (Fini et al. 2014, Esteban et al. 2015). This implies that only a fraction of the VAZ pool was bound to antenna systems and, hence, involved in NPQ (Figure 7I and J). In addition, the VAZ to Chl_{tot} ratio increased linearly during the water stress cycle. This increasing 'unbound' VAZ pool might have served specific antioxidant functions in water-stressed leaves, increasing membrane thermo-stability hence limiting lipid peroxidation (Havaux et al. 2007, Esteban et al. 2015). This is an action similar to that suggested for isoprene (Velikova et al. 2011), and cooperation between volatile and non-volatile isoprenoids was surmised by Beckett et al. (2012). Indeed, the rate of *n*-hexanal emission, a marker of lipid peroxidation (Beckett et al. 2012, Mano 2012), was only affected when a sever water stress was imposed ($FTWS_{25}$, Figure 9), and was not accompanied by irreversible degradation of membrane-bound photosynthetic machineries, namely PSII photochemistry (as shown earlier).

The biosynthesis of antioxidant flavonoids, here constituted mainly by quercetin derivatives, was stimulated in water-stressed leaves of *M. oleifera* (Figure 8), similarly to what has been observed in other plants (Tattini et al. 2004, Velikova et al. 2016, Ahrar et al. 2017). These high levels of foliar flavonoids, commonly found in leaves grown under full sunlight, are not compatible with their exclusive distribution in epidermal cells (Jaakola et al. 2004, Tattini et al. 2005, Agati et al. 2009, Majer et al. 2014). Therefore, we suggest that water stress induced the accumulation of quercetin derivatives mainly in mesophyll cells (Tattini et al. 2015), likely conferring increasing protection against enhanced ROS generation (Agati and Tattini 2010, Agati et al. 2012, Nakabayashi et al. 2014), while reducing the risk of permanent photodamage to PSII, by additionally acting as UV-B filters in the chloroplast (Mierziak et al. 2014, Zavafer et al. 2017). The finding that water stress induced profound changes in the composition of the flavonoid pool, with major increases in the biosynthesis of 'effective antioxidant' quercetin derivatives (on average +46%), further supports our hypothesis. In contrast, the content of less effective antioxidant' flavonoids either increased little (kaempferol glycosides, +15%) or largely declined (apigenin glycosides −35%) in response to water stress. This significant changes in the composition of flavonoids may also have contributed to reduce lipid peroxidation, as previously discussed.

Conclusions

Despite being originated in hygrophylic habitats, *M. oleifera* possesses multiple biochemical and physiological mechanisms that allow this species to successfully tolerate water stress episodes. These mechanisms include a strict isohydric behavior in response to water deprivation that is typical of hygrophytes. The fast stomatal closure driven by high contents of foliar-ABA, however, caused an early and strong depression in carbon assimilation with negative consequences for biomass production. More interestingly, this study revealed that *M. oleifera* is an isoprene emitting species. Increasing isoprene emission during progressive water stress was a valuable indicator for the general activation of the MEP-pathway. The simultaneous increment of volatile and non-volatile isoprenoids and of flavonoids, is suggested to be the key mechanism that allows *M. oleifera* to limit lipid peroxidation and prevent severe photoinhibitory processes under water stress. This may allow a prompt recovery of photosynthesis and growth rates when water is newly available to the roots. While the observed high plasticity of stomatal conductance and secondary metabolites production may take its toll on primary productivity of *M. oleifera*, it possibly also facilitates the establishment of this plant to xeric environments. The extent to which the trade-off between primary and secondary metabolism affects the resistance and whole-plant performance of a fast-growing plant such as *M. oleifera*, remains to be determined in presence of recurrent periods of water stress.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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Authors' contributions

C.B., F.L., F.F. and M.T. planned the experiment. C.B. conducted the study, collected samples, analyzed the data and prepared the draft. A.G., L.G. and D.R. helped in performing physiological and chemical analyses. C.B. and M.T. interpreted the results and drafted the manuscript. F.L., A.F. and M.C. reviewed the manuscript.

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